# Laboratory Procedures Used for the Third National Health and Nutrition Examination Survey (NHANES III), 1988-1994

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# CONTENTS

# 1. INTRODUCTION

## a. Overview

This manual was designed to document the full scope of the laboratory component of the third National Health and Nutrition Examination Survey (NHANES III), which was conducted by the Division of Health Examination Statistics (DHES), National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention (CDC), from 1988 to 1994. It is a complete, working laboratory manual for nutritional biochemistry, immunology, hematology, and toxicology analyses as they were performed for this multi year national survey. NHANES III, which gathered data on 30,000 survey participants (or "SPs") in 89 different geographic locations across the United States, was divided into two phases, each of which provided nationally representative subset distributions of the U.S. population: Phase I (1988-1991) and Phase II (1992-1994). A complete description of the survey, including all questionnaires used and descriptions of all survey procedures may be found in the *Plan and Operation of the Third National Health and Nutrition Examination Survey 1988-1994* (National Center for Health Statistics. Vital Health Stat 1(32).1994.)

Sections II - V of this introduction describe the procedures used by the Mobile Examination Center (MEC) field laboratory staff (who were employed by the Westat Corporation, Rockville, MD) to collect and process specimens from examinees for the laboratory analyses in NHANES III. Analytical methods used by each of the participating laboratories are described in their entirety in Section VII, in a format consistent with the requirements of the Clinical Laboratory Improvement Act of 1988 (CLIA '88). The quality control protocols are included in each method description, and the summary data will be presented as an addendum to this publication.

Long-term survey work is inherently difficult, and quite different from the usual smaller studies or conventional hospital analyses. Rigid adherence to defined protocols governing all aspects of the specimen collection, processing, shipment, storage (whether short- or long-term), and analysis is absolutely critical. Unlike fixed-site surveys, the National Health and Nutrition Examination Surveys are conducted in a mobile examination center, each of which consists of four interconnected trailers. During NHANES III, three sets of trailers were identically outfitted so as to provide a standardized environment for the survey duration; two MECs were in operation at any given time, and the third was in transit to the next most distant location. All equipment, including National Institute of Standards and Technology (NIST)-calibrated scales, x-ray machines, and hematology instruments had to be specially designed and tested to withstand the rigors of travel over 6 years, and attention to seemingly minor details such as what pesticide or deodorizer sprays could be used in the MECs (to avoid background contamination for the pesticide and blood volatiles assays) was essential.

Each laboratory participating in the survey was required to comply with CLIA '88 requirements, which included participation in external proficiency-testing programs for all analytes, but survey operational requirements are even more extensive. Overall quality assurance procedures were followed in order to ensure the validity of laboratory results. Such procedures included rigorous method validation, the development of large quantities of multiple concentration levels of stable quality control materials in the same matrix as the specimen for each analyte, the incorporation of blinded quality control materials, the use, wherever possible, of primary standards and reference materials (such as standard reference materials from NIST), scrupulous pre-screening of all collection materials associated with the collection, storage, and analysis of trace elements, external review of analytical methods by expert panels before the methods were implemented, and extensive documentation of all phases of the survey work.

Such attention to all details of the analytical work is required when data from these analyses are used to help define public health policy for the United States. If these protocols are followed, analysts can confidently differentiate between apparent laboratory analytical error and a genuine trend represented by continuously increasing or declining values of an analyte. Statistical analysis of blood lead data from NHANES II (1976-1980) revealed such a trend, in which the blood lead measurements for the U.S. population were shown to be declining steadily, a phenomenon later attributed to the removal of lead from gasoline (1). Analysts were able to confirm the comparability of analytical methods which changed from one NHANES survey to the subsequent one with extensive comparison studies involving a large number of specimens and external reference materials that they analyzed simultaneously over multiple days by both methods. Such studies comparing the results of original methods and with the results from newer methods with improved limits of detection, accuracy, and precision, as well as the use of the same rigid specimen collection protocols, also allowed analysts to confirm the continuing downward trend in blood lead levels from NHANES II to NHANES III (2,3). Similarly, the use of additional or different instruments to perform the same methods so as to increase analytical throughput was allowed only when no statistically significant difference in results was demonstrated. On the other hand, some methods have been used for the entire 25 years of the surveys, thus ensuring the comparability of results. For example, the continued use of the Abell-Kendall reference method for

cholesterol has helped analysts document the success of the National Cholesterol Education Program by demonstrating declining U.S. cholesterol levels (4).

Once a method has been validated and adopted, NHANES policy generally requires that the same method be used for the duration of the survey. Occasionally, however, changes in the assay may be inevitable for valid scientific reasons or because an unanticipated change by the manufacturer of a particular assay kit changes the assay. For example, an adjustment of the serum and red cell folate values determined during previous analysis of specimens when the calibration materials for that assay were later shown to be erroneously high, was verified with simultaneous assays of 2000 serum and RBC specimens, external standards, and quality control materials with “old” and “new” calibrators over 20 runs (6). Similarly, when the original manufacturer of the insulin kit initially chosen for NHANES III was bought out by another company, significant changes also occurred in that assay. A third kit (which had been used for the Diabetes Control and Complications Trial) was chosen and validated, and analytical data generated during use of the first two kits were adjusted to data generated by this kit after a method comparison study had been completed and externally reviewed.

Iron subset data from NHANES II and III, vitamin A data from NHANES I and II and HHANES, zinc data from NHANES II, and folate data from NHANES II and III have all been the subjects of review by various expert committees of the Life Sciences Review Office of the Federation of American Societies for Experimental Biology (5-9). Long-term quality assurance protocols and extensive documentation have facilitated these review processes.

**b. References**

(1) Miller DT, Paschal DC, Gunter EW, Stroud PE, D'Angelo J. Determination of blood lead with electrothermal atomic absorption using a L'vov platform and matrix modifier. Analyst 1987;112:1701-4.

(2) Brody DJ, Pirkle JL, Kramer RA, et al. Blood lead levels in the US population: Phase 1 of the Third National Health and Nutrition Examination Survey (NHANES III, 1988 to 1991). JAMA 1994; 272(4):277-83.

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(4) Johnson CL, Rifkind BM, Sempos CT, et al. Declining serum cholesterol levels among U.S. adults. JAMA 1993;269:3002-8.

(5) Senti FR, Pilch SM. Assessment of the folate nutritional status of the U.S. population based on data collected in the Second National Health and Nutrition Examination Survey, 1976-1980. Prepared for the Food and Drug Administration under Contract No. FDA 223-83-2384 by Life Sciences Research Office, Federation of American Societies for Experimental Biology (FASEB). Bethesda, MD: Special Publications Office, FASEB, 1984.

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(9) Pilch SM, Senti FR. Assessment of the iron nutritional status of the U.S. population based on data collected in the Second National Health and Nutrition Examination Survey, 1976-1980. Prepared for the Food and Drug

Administration under Contract No. FDA 223-83-2384 by Life Sciences Research Office, Federation of American Societies for Experimental Biology (FASEB). Bethesda, MD: Special Publications Office, FASEB, 1984.

**c. Participating Laboratories**

The NHANES Laboratory, Nutritional Biochemistry Branch, Division of Environmental Health Laboratory Sciences (EHLS), National Center for Environmental Health (NCEH), CDC, was established in 1971 as the central laboratory for NHANES I. It has continued to serve in that capacity for all NHANES projects as the coordinating laboratory for biochemistry analyses. In addition to performing biochemical analyses for erythrocyte protoporphyrin, serum iron, total iron-binding capacity, serum ferritin, serum and red cell folate, serum vitamins A, B12, C, D, and E, carotenes and retinyl esters profile, blood lead, urinary cadmium, serum selenium, and total and ionized calcium, the NHANES Laboratory also develops and validates clinical and nutritional biochemistry methods for future implementation. The chief of the NHANES Laboratory serves as a consultant to the Survey Planning Branch, DHES, NCHS, and helps coordinate the selection of the contract laboratories and the oversight of their analytical and quality control data.

For NHANES III, other biochemistry and toxicological analyses were performed by additional laboratories in the Division of Environmental Health Laboratory Sciences. In the 1000-specimen Priority Pollutants Reference Range subset of the survey, which was sponsored by the Agency for Toxic Substances and Disease Registry, a second phlebotomy was performed on 45 adult volunteers from each stand (or geographic location of the Survey) to obtain blood and urine specimens. Analyses of these specimens for urinary phenols and blood volatile organic compounds were performed in the Toxicology Branch of EHLS. Both EIA screening and liquid chromatography-mass spectrometry confirmational measurements for serum cotinine were performed in the Clinical Biochemistry Branch. Urinary creatinine analyses (for correction of the urinary phenols data only) and white blood cell transformation/DNA extraction were performed in the Molecular Biology Branch.

The Serum Bank Branch, Scientific Resources Program (SRP), National Center for Infectious Diseases (NCID), received and distributed all the blood and urine specimens for the various CDC laboratories as well as for one outside contractor laboratory. They also sub-divided all reserve serum into 0.5-mL aliquots and archived these aliquots (which constitute a priceless nationally representative collection) for long-term storage in liquid nitrogen. In all, they processed almost a million vials for NHANES III. The Serum Bank Branch also received the MEC-generated hematology data and reviewed it for accuracy and completeness. Results of abnormal automated differential smear as well as a subset of normal smears were confirmed manually in the Epidemiologic Response Laboratory, SRP. The verified hematologic data for each completed stand (or geographic location) were transmitted to NCHS to become the vital initial component of all laboratory records for each SP. The overall hematology database and the specimen receipt and storage databases were also maintained by the Serum Bank Branch.

Four other laboratories within CDC also performed analyses:

(1) Hepatitis Branch, Division of Viral and Rickettsial Diseases, NCID
Harold Margolis, M.D., chief
**hepatitis A, B, C, delta, and core antibodies; and hepatitis B surface antigen**

(2) Serology Diagnostic Laboratory, Laboratory Investigations Branch, Division of HIV/AIDS, NCID
Charles Schable, M.S., chief
**HIV-I and HIV-II by enzyme immunoassay and Western blot confirmation**

(3) Epidemiological Response Laboratory, Serum Bank Branch, Scientific Resources Program, NCID
Suzette Bartley, chief
**hematology differential smears, Coulter complete blood count (CBC) data verification**

(4) Toxoplasmosis Laboratory, Biology and Diagnostics Branch, Parasitic Diseases Division, NCID
Mark Eberhard, Ph.D., chief
**toxoplasmosis antibody**

The following 14 laboratories contracted with NCHS to provide analytical services for the survey. These laboratories followed quality control procedures outlined by the NHANES Laboratory to ensure long-term continuity of analytical protocols.

(1) University of Missouri-Columbia School of Medicine Department of Child Health, Diabetes Reference Laboratory, Columbia, MO. David Goldstein, M.D., director
**glucose, c-peptide, and insulin**

(2) Johns Hopkins University Hospital Lipoprotein Analytical Laboratory
Baltimore, MD. Paul Bachorik, Ph.D., director
**cholesterol, triglycerides, HDL-cholesterol**, **and apolipoprotein a (Lpa)**

(3) University of Washington Department of Laboratory Medicine, Immunology Division
Seattle, WA. Mark Wener, Ph.D., director
**c-reactive protein, and rheumatoid factor**

(4) University of Massachusetts Medical Center, Department of Endocrinology
Worcester, MA. Lewis Braverman, M.D., director
**FSH and LH**

(5) University of Minnesota School of Medicine, Department of Pediatrics
Minneapolis, MN. Blanche Chavers, M.D., director
**urinary microalbumin and creatinine**

(6) Medical University of South Carolina, Department of Microbiology and Immunology
Charleston, SC. Gabriel Virella, M.D., director
**tetanus antibody**

(7) University of Southern California Endocrine Services Laboratory
Los Angeles, CA. Carol Spencer, Ph.D., director
**T4, TSH, anti-microsomal antibodies, and antithyroglobulin antibodies**

(8) White Sands Research Center
Alamogordo, NM. Jack Jones, M.D., director
**Biochemistry panel, including sodium, potassium, chloride, CO2, glucose, blood urea nitrogen, creatinine, calcium, phosphorus, uric acid, γ-glutamyl transpeptidase (GGT), alaninine aminotransferase (SGPT), aspartate aminotransferase (SGOT), lactate dehydrogenase (LDH), alkaline phosphatase, cholesterol, triglycerides, iron, albumin, globulin, osmolality, and fibrinogen. (NOTE: Glucose, iron, and lipids generated on this instrument were not used for making national estimates.)**

(9) Environmental Protection Agency
Research Triangle Park, NC Richard Everson, M.D., director
**hemoglobin and DNA adducts (NOTE: These methods and the resulting data will be reported at a later time.)**

(10) California State Department of Health Services, Viral and Rickettsial Disease Laboratory
Berkeley, CA Richard Emmons, M.D., director
**rubella and varicella antibodies**

(11) Emory University School of Medicine , Department of Pediatrics at Grady Hospital
Atlanta, GA. Francis Lee, Ph.D., director
**herpes-1 and herpes-2 antibodies**

(12) USDA Human Nutrition Research Laboratory on Aging at Tufts University
Boston, MA. Irwin Rosenberg, Ph.D., director
**homocysteine, methylmalonic acid (Phase II only)**

(13) CompuChem Laboratory (later Roche Biomedical)
Research Triangle Park, NC. Paula Childs, PhD, director
**urine drug screening panel, which includes cocaine, opiates, phencyclidine, amphetamines, and marijuana ( Phase II only)**

(14) SouthWest Regional Biotechnology Laboratory (Contract for the Food and Drug Administration, Center for Biologics Evaluation and Research), Dallas, TX. Sylvia Yetts, M.S., Director
**IgE (Phase II only)**

(NOTE: Methods for methylmalonic acid, hemoglobin and DNA adducts, urine drug screening, and IgE are not included because these analyses were not completed at the time of publication of this document. They may be included in a future appendix.)

All excess serum samples remaining after analyses at the contract laboratories were stored on-site for at least 1 year after analysis. These serum samples were returned to NCHS's contract repository, Ogden Biosciences (Rockville, MD, Steve Lindenfelser, project director). Several times during the survey, Ogden was asked to locate aliquots of a number of NHANES III specimens and send them to various laboratories to allow method comparisons and to complete missing data records where analytes were stable. Several laboratories were also given access to nationally representative subsets (where possible) from excess specimens for special proposals submitted after each survey phase ended, so that they might evaluate the prevalence of newly identified biomarkers, such as the Hantavirus antibody.

## 2. SPECIMEN COLLECTION

Unlike previous NHANES, no attempt was made in NHANES III to collect blood specimens from small children by fingerstick collection techniques. Even though explicit protocols for proper collection had been followed in previous surveys, the hemodilution of fingerstick samples caused by excessive milking, inadequate sample volumes, breakage of containers during shipment or possible contamination of the blood lead specimen invariably led to the deletion of these data from the database. Therefore, NCHS officials decided that no attempt would be made to collect blood from children less than 1 year of age. Furthermore, MEC teams received extensive training in pediatric phlebotomy techniques, including instruction by a pediatric nurse practitioner, which they used successfully in collecting blood from young children more than 1 year old.

Volumes of blood collected from NHANES participants ranged from 7 mL from children 1-3 years old to 100+ mL in some adults 20-59 years old. The collection tubes, volumes, and subsets are described in Table 1. As part of the overall quality assurance process for the survey, all collection materials, Vacutainer tubes, and storage containers to be used for analyzing trace elements were initially prescreened by the NHANES Laboratory for background contamination levels of lead, cadmium, and selenium. (Lead and cadmium are fairly ubiquitous contaminants; selenium contamination is less of a problem, and for selenium analysis, blood may be collected in ordinary red-top tubes after the acceptability of the test tubes has been confirmed. Special lead-free tubes are not required, however; ordinary EDTA tubes may similarly be used after prescreening has confirmed no contamination.)

Randomly collected or “spot” urine specimens were collected by clean-catch technique into sterile 250-mL polyethylene containers (from a large production lot, previously prescreened for trace elements contamination). Although the collection of 24-hour urine specimens is scientifically desirable, it simply is not feasible for survey purposes; thus, the use of spot urine collections has been a necessity. Urine creatinine concentrations from these specimens have been used (instead of a 24-hour volume correction) to correct for the degree of dilution or concentration of some of the urine analytes such as the phenols or microalbumin.

Collect samples in evacuated specimen tubes (Becton-Dickinson Co., Rutherford, NJ) as shown in Table 1. **Use only those lots that have been prescreened by CDC**.

Table 1
Venipuncture Blood Collection Protocol

| | A | B | C | D | E |
|---|---|---|---|---|---|
| Age (years) | 1-3 | 4-5 | 6-11 | 12-19 | 20+ |
| Label Color Code | Red | Green | Yellow | Blue | Orange |
| Tube Type | | | | | |
| **4-mL SST**, gel barrier B-D #6514 | 1 | 0 | 0 | 1 | 1/1* |
| **2-mL lavender-top** (0.04 mL $K_3$2EDTA) B-D #6384 | 0 | 0 | 0 | 1 | 1 |
| **3-mL lavender-top** (0.06 mL 7.5% K3EDTA) B-D #6385 | 1 | 1 | 1 | 1 | 1 |
| **3-mL gray-top** (6mg potassium oxalate)(7.5 mg sodium fluoride) B-D #6383 | 0 | 0 | 0 | 0 | 1/1* |
| **10-mL red-top** B-D #6430 | 0 | 1 | 1 | 1 | 0 |
| **15-mL red-top** B-D #6432 | 0 | 1 | 2 | 3 | 5 |
| **1.8-mL light blue-top** (6.6 mg sodium citrate)(0.84 mg citric acid) B-D #6394 | 0 | 0 | 0 | 0 | 1** |
| **8-mL red/green-top** (1.0 mL sodium citrate)(2.0 mL Ficoll-hypaque)(3.0 gm polyester gel) B-D #2597 | 0 | 0 | 0 | 1 | 1 |
| **10-mL gray-top** (20.0 mg potassium oxalate)(25.0 mg sodium fluoride) B-D #6428 | 0 | 0 | 0 | 0 | 1*** |
| **10-mL red-top**[#] B-D #6440 | 0 | 0 | 0 | 0 | 1*** |

* Drawn at the time of the second venipuncture for GTT if SP is age 40-74.
** Drawn if SP is ≥40 years old.
*** Drawn if SP is 20-59 years old and volunteers for this component for which she or he will be paid extra.
[#] Not silicone-coated; to be used only for this special subset.

## 3. SPECIMEN PROCESSING PROCEDURES

### a. Centrifugation

Perform all processing work under the laminar-flow biological safety cabinet, using only those materials that CDC has prescreened for contamination. Do not centrifuge the 10-mL gray-top tubes used to collect blood volatiles (vials 28 and 29) and the light blue-top tube for fibrinogen (vial 23). Keep these tubes refrigerated until shipment.

Allow blood in each red-top tube to clot for 30 to 40 min at room temperature. Centrifuge ALL tubes unopened. Centrifuge and separate plasma aliquots from the 3-mL gray-top tubes as soon as possible.

Place all serum tubes in balanced carriers of appropriate size, and centrifuge them at 2,900 RPM (relative centrifugal force (RCF) = 1115 x g) for 15 min. Centrifuge all 3-mL gray-top tubes at 2,400 RPM (RCF=1115) for 15 min.

The 8-mL red/green-top cell preparation tube (CPT) (vial 31) is used for blood drawn from participants > 11 years old for genetic testing. This special tube contains Ficoll-Hypaque density gradient gels, and when filled, it is centrifuged at 1800 x g for 20 min to separate peripheral blood mononuclear cells from erythrocytes and granulocytes. When the separation is complete, the tube is refrigerated until it is shipped, according to instructions given in Exhibits 4-14 and 4-15. Do NOT open this tube after centrifugation.

### b. Separation and Pooling of Serum

Do NOT open the centrifuged 4-mL SST tubes for ionized calcium (vial 15). It is critical that these tubes be carefully refrigerated for 60 min, then frozen in a horizontal position before shipment to maintain the specimen pH, which is required for normalization of the ionized calcium assay.

For all other red-top tubes (except the semivolatiles tube), remove the serum from contact with the clot as soon as possible after centrifugation has been completed. Using a serum separator, gently push down into the centrifuged red-top tubes until the end filter of the serum separator is just above, but not touching the surface of the clot, and carefully decant serum yields from all red-top tubes **except the 10-mL tube for semivolatiles** and pool the serum into a 50-mL polypropylene centrifuge tube, carefully avoiding hemolysis or the introduction of any cellular debris that could falsely elevate the results for some analytes (such as folate, zinc, or homocysteine) that are present in much higher levels in the red blood cells than in serum. Process the 10-mL red-top tube for semivolatile toxicants (vial 33, identified with a red band of tape) separately from all other red-top vials because it is a special nonsiliconized tube and its serum yield is intended for use in special toxicological analyses only.

If the serum in any individual red-top tube is grossly hemolyzed, do not pool it with clear serum from the remaining tubes. If all of the serum from one SP is turbid, lipemic, or icteric, pool and allocate it as usual. (Not all biochemical tests can be performed on such specimens without compromising analytical accuracy, however.) Cap the 50-mL tube and mix its contents gently by inversion. Aliquot pooled serum immediately or refrigerate it at 4 °C no longer than 4 hours after separation.

**Table 2.**
**Blood and Urine Assessments, by Specimen Type and Age Group**

| *Age group* | | | | |
|---|---|---|---|---|
| *1–3 years* | *4–5 years* | *6–11 years* | *12–19 years* | *20 years and over* |
| Whole blood | | | | |
| CBC[1] /RDW | CBC[1] /RDW | CBC[1] /RDW | CBC[1] /RDW | CBC[1] /RDW |
| Platelets | Platelets | Platelets | Platelets | Platelets |
| 3-cell differential | 3-cell differential | 3-cell differential | 3-cell differential | 3-cell differential |
| Differential smear | Differential smear | Differential smear | Differential smear | Differential smear |
| Lead[5] | Lead[5] | Lead[5] | Lead[5] | Lead[5] |
| Protoporphyrin[5] | Protoporphyrin[5] | Protoporphyrin[5] | Protoporphyrin[5] | Protoporphyrin[5] |
| | Red blood cell folate | Red blood cell folate | Red blood cell folate | Red blood cell folate |
| | Glycated hemoglobin[5] | Glycated hemoglobin[5] | Glycated hemoglobin[5] | Glycated hemoglobin[5] |
| Serum | | | | |
| Iron[5] | Iron[5] | Iron[5] | Iron[5] | Iron[5] |
| Total iron binding capacity[5] | Total iron binding capacity[5] | Total iron binding capacity[5] | Total iron binding capacity[5] | Total iron binding capacity[5] |
| Ferritin[5] | Ferritin[5] | Ferritin[5] | Ferritin[5] | Ferritin[5] |
| | Folate[5] | Folate[5] | Folate[5] | Folate[5] |
| | Apolipoprotein $A_1$, B[4,5] | Apolipoprotein $A_1$, B[4,5] | Apolipoprotein $A_1$, B[4,5] | Apolipoprotein $A_1$, B[4,5] |
| | Total cholesterol[5] | Total cholesterol[5] | Total cholesterol[5] | Total cholesterol[5] |
| | HDL/LDL[5] | HDL/LDL[5] | HDL/LDL[5] | HDL/LDL[5] |
| | Triglycerides[5] | Triglycerides[5] | Triglycerides[5] | Triglycerides[5] |
| | Lp(a)[2,5] | Lp(a)[2,5] | Lp(a)[2,5] | Lp(a)[2,5] |
| | Cotinine | Cotinine | Cotinine | Cotinine |
| | C-reactive protein[5] | C-reactive protein[5] | C-reactive protein[5] | C-reactive protein[5] |
| | Rheumatoid factor | Rheumatoid factor | Rheumatoid factor | Rheumatoid factor |
| | Vitamin A (retinol)[5] | Vitamin A (retinol)[5] | Vitamin A (retinol)[5] | Vitamin A (retinol)[5] |
| | Carotenes [5] | Carotenes [5] | Carotenes [5] | Carotenes [5] |
| | Retinyl esters[5] | Retinyl esters[5] | Retinyl esters[5] | Retinyl esters[5] |
| | Vitamin E[5] | Vitamin E[5] | Vitamin E[5] | Vitamin E[5] |
| | Tetanus | Tetanus | Tetanus | Tetanus |
| | | Vitamin C | Vitamin C | Vitamin C |
| | | Hepatitis A | Hepatitis A | Hepatitis A |
| | | Hepatitis B/delta | Hepatitis B/delta | Hepatitis B/delta |
| | | Hepatitis C | Hepatitis C | Hepatitis C |
| | | Hepatitis E | Hepatitis E | Hepatitis E |
| | | Rubella[5] | Rubella[5] | Rubella[5] |
| | | Varicella[5] | Varicella[5] | Varicella[5] |
| | | | Herpes simplex I and II | Herpes simplex I and II |
| | | | HIV 1 (ages 18+)[3,5] | HIV 1[3,5] |
| | | | Toxoplasmosis[5] | Toxoplasmosis[5] |
| | | | Vitamin D (OH D) | Vitamin D (OH D) |
| | | | Total/ionized calcium | Total/ionized calcium |
| | | | Selenium[5] | Selenium[5] |
| | | | Thyroxine ($T_4$) | Thyroxine ($T_4$) |
| | | | Thyroid-stimulating hormone (TSH) | Thyroid-stimulating hormone (TSH |
| | | | Antithyroglobulin antibodies | Antithyroglobulin antibodies |
| | | | Antimicrosomal antibodies | Antimicrosomal antibodies |
| | | | | FSH/LH (females ages 35–60 yea |
| | | | | Insulin |
| | | | | C-peptide |
| | | | Biochemistry profile[5] | Biochemistry profile[5] |
| | | | Total carbon dioxide | Total carbon dioxide |
| | | | Blood urea nitrogen | Blood urea nitrogen |
| | | | Total bilirubin | Total bilirubin |
| | | | Alkaline phosphatase | Alkaline phosphatase |
| | | | Total cholesterol | Total cholesterol |
| | | | AST (SGOT) | AST (SGOT) |
| | | | ALT (SGPT) | ALT (SGPT) |
| | | | LDH | LDH |

**Table 2.**
**Blood and Urine Assessments, by Specimen Type and Age Group—Con.**

| *Age group* | | | | |
|---|---|---|---|---|
| *1–3 years* | *4–5 years* | *6–11 years* | *12–19 years* | *20 years and over* |
| Serum—Con. | | | | |
| | | | GGT<br>Total protein<br>Albumin<br>Creatinine<br>Glucose<br>Calcium<br>Chloride<br>Uric acid<br>Phosphorus<br>Sodium<br>Potassium | GGT<br>Total protein<br>Albumin<br>Creatinine<br>Glucose<br>Calcium<br>Chloride<br>Uric acid<br>Phosphorus<br>Sodium<br>Potassium |
| Plasma | | | | |
| | | | | Glucose (ages 20–39 years, 75 years and over)<br>OGTT (ages 40–74 years)<br>Fibrinogen (ages 40 years and over)[5] |
| Urine | | | | |
| | | Cadmium<br>Creatinine<br>Microalbumin<br>Iodine | Cadmium<br>Creatinine<br>Microalbumin<br>Iodine<br>Cocaine[2,3] (ages 18 years and over)<br>Opiates[2,3]<br>Phencyclidine[2,3] (ages 18 years and over)<br>Amphetamines[2,3] (ages 18 years and over)<br>Marijuana[2,3] (ages 18 years and over) | Cadmium<br>Creatinine<br>Microalbumin<br>Iodine<br>Cocaine[2,3]<br>Opiates[2,3]<br>Phencyclidine[2,3]<br>Amphetamines[2,3]<br>Marijuana[2,3]<br>Pregnancy test (females ages 20–59 years) |
| White cells | | | | |
| | | | Storage/banking[5] | Storage/banking[5] |

[1] Includes hematocrit, hemoglobin, red and white cell counts, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.
[2] Phase 2 only.
[3] Anonymous.
[4] Phase 1 only.
[5] Home examination also.

## 4. SPECIMEN ALLOCATION PROCEDURES

As each specimen vial is processed, label it with the bar-coded identification labels for SP and analyte number, and verify that all aliquots are correctly labeled when finished.

### a. Serum

Using clear pooled serum from the 50-mL tube only, fill as many vials as possible in the order of priority and with the required volumes indicated in Table 3(NHANES III Processing Protocol for Biochemistry Specimens). Prepare the serum extract in vial 14 for **vitamin C** analysis by diluting 500 µL serum with 2.0 mL freshly prepared 6 gm/dL metaphosphoric acid diluent, and thoroughly mix the resulting solution of clear liquid and white precipitated proteins. Aliquot serum from the **nonsiliconized** red-top tube directly into a 6-mL, solvent-rinsed, glass Wheaton vial (vial 33) for the semivolatiles analysis. After initially cooling the centrifuged 4-mL serum separator (SST) tubes for **ionized calcium/vitamin D** (vial 15), freeze them in a horizontal position to avoid tube breakage. These tubes must be shipped frozen and must not be thawed until the day of analysis at CDC, in order to maintain proper pH of the serum so that ionized calcium results may be normalized correctly.

When 4-mL SST tubes are used on second phlebotomies for the **insulin** (vial 6) analyses from the 2-hour glucose tolerance test, centrifuge and process these specimens separately. The 4-mL SST tubes are also used to collect serum for the iron, TIBC, and ferritin analyses for children 1-3 years old. This is the only serum processed for this age group and should be placed in **vial # 7 (iron/TIBC) -- for this age group only.**

Continue to fill as many vials as possible and as appropriate for the SP's age group. After priority analyte vials have been properly filled, aliquot all remaining serum into storage vials 1-4 (vials 24-27), which will be maintained in the CDC Specimen Repository under vapor-phase liquid nitrogen pending requests by future investigators as new biomarkers as identified.

### b. Plasma

Using a plastic transfer pipette, remove the plasma from each timed **glucose** specimen in a 3-mL gray-top tube or **fibrinogen** specimen in a 2-mL blue-top tube, and place it in the appropriately labeled vials (glucose or GTT vials 5, 5A, or 5B), which are frozen after processing. Refrigerate the red-green and 10-mL gray-top tubes and ship them unopened to the appropriate laboratories.

### c. Whole Blood

For all age groups except <12 years old, for which only one EDTA tube is filled, use the first lavender-top tube for hematology determinations (**CBC** and **differential smear**) and the second tube for certain biochemical tests. First, aliquot 0.5 mL of the well-mixed EDTA-whole blood into a prescreened 2.0-mL Nalge vial for the **blood lead** specimen (vial 1), working under the laminar flow hood of the biological safety cabinet to prevent external contamination of the specimens. Prepare the whole blood hemolysate in vial 3 for the r**ed blood cell folate** analysis by diluting 100 µL of EDTA-whole blood with 1.0 mL of freshly prepared 1 gm/dL ascorbic acid solution and gently mixing the resulting reddish-brown solution of hemolyzed whole blood. Aliquot 0.5 to 1.0 mL of the remaining whole blood from this EDTA tube into a Nalge vial for **erythrocyte protoporphyrin** analysis (vial 2). Freeze these 3 vials after processing.

After the CBC has been completed and verified, save and refrigerate the remainder of this EDTA tube (vial 4) to ship to the University of Missouri for **glycosylated hemoglobin** analysis.

### d. Urine

Mix the contents of the urine specimen container well, and decant a 10-mL aliquot into a 15-mL polystyrene centrifuge tube (which has also been prescreened) for **urine cadmium** analysis. Cap this tube securely and label it "U1" for urine cadmium.

Remove another aliquot to perform a **pregnancy test** on specimens from all women > 18 years old to ensure that they are not pregnant, and that it is safe to proceed with special subset MEC examinations such as bone density measurements of the hip and spine for osteoporosis and x-rays of the wrists and knees for arthritis, which are omitted for pregnant SPs.

Place 2.5 mL urine in a 4.5-mL plastic vial for urine **creatinine/microalbumin** analysis, and label this tube “U2." (For Phase II of the survey only, prepare a 10-mL aliquot of urine for the urinary **iodine** analysis in a vial labeled “U3", and a 15-mL aliquot for **drug screening** in a vial labeled “UD".) Decant the remaining urine into a 2.0-mL Nalge vial (“U6") and a solvent-rinsed 60-mL glass Wheaton bottle (“U5") for the Priority Pollutant Reference Range **urine creatinine and phenols\phenoxy herbicides** assays.

**e. Blood Clots**

Carefully transfer the residual clots from the 10- and 15-mL red-top tubes to 8-mL Sarstedt vials for **DNA** and **hemoglobin adduct** analyses, and label them “C1" and “C2."

## 5. SPECIMEN SHIPPING PROCEDURES

Detailed shipping instructions for the MEC laboratory technicians are discussed in their entirety in *Laboratory Technician's Manual, the Third National Health and Nutrition Examination Survey (NHANES III)* (prepared by WESTAT, Rockville, MD) , as well as in *Plan and Operation of the Third National Health and Nutrition Examination Survey, 1988-1994*. General instructions are summarized below. All SP identification labels, whether for paperwork or for specimen vials, were bar-coded for greater data integrity, and all specimen shipments could be coded by specimen, box, shipper, or shipment levels. Each shipment contained transmittal sheets and a high-density diskette containing the transmittal file listing the identification number, age, and sex of each SP for whom vials were sent. (No other personal identifiers are oermitted to be used in the laboratory component, to maintain confidentiality of the SP's identity.) An extensive database of tubes collected and vials processed on all SPs was maintained in the MEC VAX computer system.

### a. CDC Shipments

Twice weekly, prepare shipments of frozen specimens for overnight delivery using a large shipper containing the specimens and 12-15 lb dry ice. Include a specimen log transmittal form and a high-density diskette containing the identification number, age, and sex of the SP, and the dates of collection for each specimen set sent. Include the white copy of the Hematology Worksheet, one copy of the Hematology Log, and a copy of the Coulter histogram from the CBC. Include U-1 urine samples, transmittal sheets, and diskettes. Differential smears are collected in a slide box and shipped to CDC.

Ship vial 20 for HIV analysis by overnight delivery weekly on dry ice and include transmittal sheets and diskettes. Ship vial 28 for volatile organic toxicants ship by overnight delivery two-three times per week on wet ice. Ship vials 29, U4, U5, U6 for urinary phenols and urinary creatinine analyses by overnight delivery twice a week in a shipper on dry ice and include chemical exposure questionnaires. Ship vial 31 for genetic analyses by overnight delivery two-three times per week on wet ice.

Vials or tubes shipped to CDC: 1, 2, 3, 7, 8, 10, 11, 14, 15, 17, 18, 19, 24-27, TO, Ul, 20, 28, 29, U4, U5, U6, 31.

### b. Other Contract Laboratory Shipments
**(NOTE: All shipments are made in styrofoam insulated shippers.)**

(1) University of Missouri
No. 4 vials are accumulated in the refrigerator and shipped two to three times per week with coolant packs to arrive within 5 days of collection. Nos. 5, 5A, 5B, 6, 6A, 6B vials are accumulated in the freezer and shipped weekly to the University of Missouri-Columbia, Department of Child Health, Columbia, MO.

(2) Lipid Profile
No. 9 vials are accumulated in the freezer and shipped weekly on dry ice to the Johns Hopkins University Hospital, Lipoprotein Analytical Laboratory, Baltimore, MD.

(3) University of Washington
No. 12 vials are accumulated in the freezer and shipped weekly on dry ice to the University of Washington, Immunology Division, Seattle, Washington.

(4) White Sands
Nos. 13 and 23 vials are accumulated in the freezer and shipped weekly on dry ice to the White Sands Research Center, Alamogordo, NM.

(5) Medical University of South Carolina.
No. 16 vials are accumulated in the freezer and shipped weekly on dry ice to the Medical University of South Carolina, Dept. of Microbiology and Immunology, Charleston, SC.

(6) University of Southern California
No. 21 vials are accumulated in the freezer and shipped weekly on dry ice to the University of Southern California, Endocrine Services Laboratory, Los Angeles, CA.

(7) University of Massachusetts
Nos. 22 and U3 vials are accumulated in the freezer and shipped weekly on dry ice to the University of Massachusetts Medical Center, Worcester, MA.

(8) SRA
Nos. Cl and C2 vials are accumulated in the freezer and shipped weekly on dry ice to SRA Technologies, Research Triangle Park, NC.

(9) University of Southern California at Berkeley
No. RU vials are accumulated in the freezer and shipped weekly on dry ice to the University of Southern California--Berkeley, Dept. of Health Services, Berkeley, CA.

(10) Bratton (Repository contractor for the FDA Center for Biologics Evaluation and Research)
No. IgE vials are accumulated in the freezer and shipped weekly on dry ice to Bratton Biotech.

(11) University of Minnesota
No. U2 vials are accumulated in the freezer and shipped weekly on dry ice to the University of Minnesota Dept. of Pediatrics, Minneapolis, MN.

(12) CompuChem
No. UD vials are accumulated in the refrigerator and shipped weekly to CompuChem (later Roche Biomedical), Research Triangle Park, NC.

Table III - NHANES III blood processing protocol for biochemistry specimens

| Test ID Number | Test Name | Age Group | Sample Sixe, mL | Specimen Type | Collection Type | Vial Type | Analyzed By | Other Remarks |
|---|---|---|---|---|---|---|---|---|
| 1. | Lead | A-E | 0.50 | EDTA-WB | 3 mL lavender | 2.0 mL | EHLS | Aliquot first |
| 2. | Erythrocyte Protoporphyrin | A-E | 0.5/0.1 | EDTA-WB | 3 mL lavender | 2.0 mL | EHLS | Aliquot 0.5 for Group A and 1.0 for Groups B-E |
| 3. | RBC Folate | B-E | 0.1 | EDTA-WB | 3 mL lavender | 2.0 mL | EHLS | 0.1 mL EDTA plus 1 mL 1% ascorbic acid. |
| 4. | Glycosylated hemoglobin | B-E | $\geq$ 0.5 | WB | 3 mL lavender | 3 mL Lavender | U. of Mo. | Refrigerate EDTA tude. Ship twice a week on wet ice. |
| 5. | Glucose | E (20-39. 75+) | 1.0 | P | 3 mL gray | 2.0 mL | U. of Mo. | Spin and aliquot plasma. |
| 5.A/B | Glucose (GTT) | E (40-74) | 1.0 | P | 3 mL gray/3 mL gray | 2.0 mL | U. of Mo. | Spin and aliquot plasma |
| 6. | Insulin/ C-peptide | E (20-39, 75+) | 1.5 | S | 10/15 mL red | 2.0 mL | U. of Mo. | |
| 6. A/B | Insulin/C-peptide | E (40-74) | 1.5 | S | 10/15 mL red/ 4 mL SST | 2.0 mL | U. of Mo. | |
| 7. | (Iron/TIBC/Ferritin) Iron/TIBC | A<br>B-E | All<br>1.25 | S<br>S | 4 mL SST<br>10/15 mL red | 2.0 mL<br>2.0 mL | CDC/EHLS | |
| 8. | Ferritin/ Folate | B-E | 1.5 | S | 10/15 mL red | 2.0 mL | CDC/EHLS | |
| 9. | NHLBI Lipids | B-E | 2.5-5.5 | S | 10/15 mL red | 6.0 mL | Hopkins | |
| 10. | Vitamins A/E/ Carotene | B-E | 1.25 | S | 10/15 mL red | 2.0 mL | CDC/EHLS | Retinyl esters on subset |
| 11. | Cotinine | B-E | 2.0 | S | 10/15 mL red | 2.0 mL | CDC/EHLS | |
| 12. | C-Reactive Protein/RF | B-E | 0.5/1.0 | S | 10/15 mL red | 2.0 mL | U. WA | Aliquot 0.5 mL for adults less than 60 and 1.0 mL for adults 60+ |
| 13. | SMAC Profile | D-E | 1.0 | S | 10/15 mL red | 2.0 mL | White Sands | |
| 14. | Vitamin C | C-E | 0.1 | S-extr | 10/15 mL red | 2.0 mL | EHLS | 0.1 mL serum plus 0.4 mL 6% MPA. |
| 15. | Vitamin D Total/Ironized Calcium | D-E | All | S | 4 mL SST | SST | CDC/EHLS | pin down after clotted; chill 60 min; freeze on side; ship unopened, frozen. |
| 16. | Tetanus | B-E | 0.5 | S | 10/15 mL red | 2.0 mL | MUSC | |
| 17. | Hepatitis | C-E | 1.0 | S | 10/15 mL red | 2.0 mL | CDC-1 | |
| 18. | Herpes | D-E | 1.0 | S | 10/15 mL red | 2.0 mL | CCD-2 | |

Table III - NHANES III blood processing protocol for biochemistry specimens

| Test ID Number | Test Name | Age Group | Sample Size, mL | Specimen Type | Collection Type | Vial Type | Analyzed By | Other Remarks |
|---|---|---|---|---|---|---|---|---|
| 19. | Selenium | D-E | 0.5 | S | 10/15 mL red | 2.0 mL | CDC/EHLS | |
| 20. | HIV I | D-E (18+) | 0.5 | S | 10/15 mL red | 2.0 mL | CDC-3 | |
| 21. | Thyroid | D-E | 1.0 | S | 10/15 mL red | 2.0 mL | USC | |
| 22. | FSH & LH hormones | E | 0.75 | S | 15 mL red | 2.0 mL | U. Mass | Women 35-60. |
| 23. | Fibrinogen | E | All | P | 2 mL blue | 2.0 mL | White Sands | age 40+ only |
| TO. | Toxoplasmosis | D-E | 0.5 | S | 10/15 mL red | 2.0 mL | CDC/EHLS | |
| RU. | Rubella | C-E | 0.5 | S | 10/15 mL red | 2.0 mL | CDC | |
| IE. | Ige | C-E | 0.5/1.0 | S | 10/15 mL red | 2.0 mL | Bratton | 0.5 mL for group |
| C. | | | | | | | | 1.0 mL for Group D and E |
| 27. | Storage #1-4 | C-E | 1.0/2.0 | S | 10/15 mL red | Multiple 2.0 mL | CDC/EHLS | One vial has1.0 mL; others have 2.0 mL. |
| 28. | Volatile toxicants | E(20-59) | All | P | 10 mL gray | 10 mL gray | CDC/EHLS | Do not spin. Refrigerate; ship onwet ice |
| 29. | Volatile toxicants | E(20-59) | All | S | 10 mL red non-silicone coated | 5.0 mL | Wheaton | Ship on dry ice with Vol. Tox. Urines |
| C1. | DNA/HGB | C-E | Clot | Clot | 10/15 mL red | 2.0 mL | SRA | Ship on dry ice. |
| C2. | DNA/HGB Adducts | C-E | Clot | Clot | 10/15 mL red | 2.0 mL Sarstedt | SRA | ship on dry ice. |
| 31. | Genetic Analyses | D-E | All | WB | 8 mL Leukoprep | 8 mL Leukoprep | | CDC Refrigerrate ship on wet ice |
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Table 4
Special Studies

| Study | | | Participants |
|---|---|---|---|
| Priority Toxicants | Volatile Organic Compounds (VOCs) | | volunteers, ages 20-59 years |
| | Benzene | 1,1,1-Trichloroethane | |
| | Toluene | 1,1,2-Trichloroethane | |
| | Styrene | Trichloroethene | |
| | Ethylbenzene | 1,1,2,2-Tetrachloroethane | |
| | o-Xylene | Tetrachloroethene | |
| | m-Xylene | Hexachloroethane | |
| | p-Xylene | Methylene Chloride | |
| | Chlorobenzene | Chloroform | |
| | 1,2-Dichlorobenzene | Carbon Tetrachloride | |
| | 1,3-Dichlorobenzene | 1,2-Dichloropropane | |
| | 1,4-Dichlorobenzene | Bromoform | |
| | 1,1-Dichloroethane | Dibromomethane | |
| | 1,2-Dichloroethane | Bromodichloromethane | |
| | 1,1-Dichloroethene | Dibromochloromethane | |
| | cis-1,2-Dichloroethene | Acetone | |
| | trans-1,2-Dichloroethene | 2-Butanone | |
| | Pesticides or Metabolites | | |
| | 2,4-Dichlorophenol | 1-Naphthol | |
| | 2,5-Dichlorophenol | 2-Naphthol | |
| | 2,4,5-Trichlorophenol | 2-Isopropoxyphenol | |
| | 2,4,6-Trichlorophenol | Carbofuranphenol | |
| | Pentachlorophenol | 3,5,6-Trichloro-2-pyrinine | |
| | 4-Nitrophenol | 2,4-Dichlorophenoxyacetic-acid | |
| Dehydroepiandrosterone | | | 1400 persons ages 20-90 from 14 age/sex groups. |

Note: Data from these studies are not from probability samples and may not be available for public use.

Table 5
Surplus Serum Analyses

| Test Name |
|---|
| Vitamin B-12 |
| Methyl malonic acid |
| Helicobacter pylori antibody |
| Homocysteine |
| Periodontal pathogens |
| Cryptosporidia antibody |
| Diphtheria antitoxin |
| Latex allergy |
| Measles antibody |
| Serum bone alkaline phosphatase |
| Osteocalcin |
| HDL phospholipid |

Note: Data from these studies may not be available for public use.

## 6. RECEIPT, DISTRIBUTION, AND STORAGE PROCEDURES

Upon arrival at CDC or contract laboratories, the frozen specimens are sorted by vial type, and stored initially at -20 ˚C. The refrigerated samples are stored at 4-8 ˚C. Frozen specimens whose analysis may be delayed are stored at -70 ˚C or lower. The urine drug specimens may be kept in short-term storage ambient temperature.

Information about long-term storage vials 24-27 is entered into the CDC Specimen Repository database, and the vials remain under the control of the Director, DHES, NCHS.

The vials intended for analysis in the NHANES Laboratory, CDC, are sorted by analyte (or vial number), and analytical runs of 20 samples (including a randomly inserted blind quality-control pool aliquot of either low-normal or high-normal concentration, prepared as a pseudo-patient vial, with identification numbers known only to the laboratory supervisor) are prepared for distribution to the analysts. All SP vials are racked in the same order, whether or not a vial is actually present for a particular analyte. Vials for herpes analysis are grouped in boxes of 81; vials for hepatitis analysis are grouped in racks of 50 specimens.

After analysis, all excess serum specimens not required for repeat analyses are sent to the Ogden Biosciences Repository.

NOTE: In this manual, the use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Free erythrocyte protoporphyrin (FEP) is measured by a modification of the method of Sassa et al. (1). Protoporphyrin is extracted from EDTA-whole blood into a 2:1 (v/v) mixture of ethyl acetate-acetic acid, then back-extracted into diluted hydrochloric acid. The protoporphyrin in the aqueous phase is measured fluorometrically at excitation and emission wavelengths of 404 and 655 nm, respectively. Calculations are based on a processed protoporphyrin IX (free acid) standard curve. After a correction for the individual hematocrit is made, the final concentration of protoporphyrin in a specimen is expressed as micrograms per deciliter of packed red blood cells (µg/dL RBC).

## 2. SAFETY PRECAUTIONS

This method is performed under an exhaust hood (not a laminar-flow hood) because the ethyl acetate-acetic acid and hydrochloric acid fumes are very irritating. Observe Universal Precautions. Wear gloves, lab coat, and safety glasses at all times. Treat all specimens as potentially positive for HIV and Hepatitis B. Dispose of leftover acid solutions as hazardous wastes. All leftover blood specimens and materials that have been in contact with blood must be autoclaved before disposal.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation of the run and hematocrit correction are accomplished with a SAS program, "PROTOIX," which is run in ROSCOE. Protoporphyrin values in µg/dL are entered into the program; these values were previously printed out by the Hitachi F-2000. Hematocrit results for each patient are entered from the NHANES III runsheet.

   After the data are calculated and the final values are approved for release by the reviewing supervisor, the results are transcribed by the data entry clerk into the NHANES III database which is located in RBASE on the NCEH/EHLS PC network; data entry is proofed by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the protoporphyrin values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe automatically are backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special dietary instructions are given to donors.

b. Specimens for erythrocyte protoporphyrin analysis should be fresh or frozen EDTA-whole blood. Heparinized blood may be used, but it is not preferred because of the tendency of the blood to form microclots upon prolonged storage. If possible, hematocrit data should be collected in order to correct for the effects of anemia, and the final FEP concentration should be reported as µg/dL RBC.

c. The optimal amount of sample is 1 mL; the minimum is about 100 µL.

d. Specimens may be stored in glass or in plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

e. Protoporphyrin is stable for years at -20 °C and below. Quality control pools for the HANES Lab are normally stored at -70 °C for maximum stability. Several freeze-thaw cycles appear to have minimal effect on the specimen. However, after prolonged storage at 4-8 °C, blood specimens undergo necrosis, which results in formation of fluorescent compounds that can interfere with FEP analysis.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was drawn.

g. Samples that are partially clotted may not give accurate test results. In addition, care should be taken not to introduce any fluorescent artifacts into the sample during processing through the use of equipment such as wooden applicator sticks.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and in the Special Activities Branch Specimen Handling Office.) Specimen collection and transport and special equipment required are discussed in the protocol. In general, whole blood specimens should be transported and stored at no more than 4-8 °C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at ≤-20 °C. Samples thawed and refrozen several times are not compromised. If the specimen needs to be divided because it contains more than one analyte of interest, transfer the appropriate amount of blood into a sterile Nalgene cryovial labelled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Hitachi model F-2000 fluorescence spectrophotometer, with R928 photomultiplier tube, xenon lamp, and custom-made microcell (10- x 75-mm) holder positioned to allow the passage of light through the aqueous phase only (Hitachi Instruments Inc., Danbury, CT).

(2) Cary model 3E double-beam spectrophotometer (Varian Instrument Group, Sugar Land, TX).

(3) Vortex mixer (Fisher Scientific Co., Fairlawn, NJ).

(4) Mettler model PM400 balance (Mettler Instruments Corp., Hightstown, NJ).

(5) Beckman TJ-6 centrifuge (Beckman Instruments Co.).

(6) Three Digiflex automatic dispensers, (Micromedic Systems, Div. of Rohm and Haas, Horsham, PA) equipped with the following:
(a) A 10-mL dispensing syringe and a 2-mL sampling syringe.
(b) A 2-mL dispensing syringe and a 200-µL sampling syringe.
(c) A 10-mL dispensing syringe.

### b. Materials

(1) Protoporphyrin IX, dimethyl ester, 99.3% purity, grade 1 (Sigma Chemical Co., St. Louis, MO), or 99.9% purity (Porphyrin Products, Logan, UT).

*Store at ≤-20 °C over a desiccant. Purchase of one lot is recommended. If possible, prepare (and label) for storage aliquots of each lot in ampoules.*

(2) Ethyl acetate, high-performance liquid chromatography (HPLC) grade (J.T. Baker Co., Phillipsburg, NJ).

(3) Acetic acid, glacial, "Baker Analyzed" (J.T. Baker Co.).

(4) Hydrochloric acid (HCl), concentrated, "Baker Analyzed" (J.T. Baker Co.).

(5) Kimble 10- x 75-mm disposable glass culture tubes (Kimble Div., Owens-Illinois Co., Toledo, OH).

(6) Parafilm M (American Can Co., Greenwich, CT).

(7) Actinic glass volumetric flasks (Corning Glassworks, Corning, NY).

*Wash all nondisposable glassware used in this assay in 10% (v/v) hydrochloric acid and rinse them six times with deionized water.*

(8) Formic acid, 88%, reagent grade (J.T. Baker Co.).

(9) Deionized water, greater than or equal to 1.0 megaOhm-cm at 25 °C (Continental Water Co., Atlanta, GA).

c. **Reagent Preparation**

(1) 7.0 mol/L hydrochloric acid (HCl) (for hydrolysis)
Dilute 551 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.

(2) 1.62 mol/L HCl (for daily absorbance readings)
Dilute 128 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.

(3) 0.43 mol/L HCl (for analysis-extraction)
Dilute 68 mL concentrated HCl to volume with deionized water in a 2-L volumetric flask.

(4) 1.5 mol/L HCl (for blanking spectrophotometer)
Dilute 118 mL concentrated HCl to volume with deionized water in a 1-L volumetric flask.

*These dilutions assume concentrated HCl to be 12.7 mol/L. The molar concentration of different lots of HCl should be calculated by using the following formula:*

$$mol/L = \frac{\text{relative density} \times \% HCl}{36.453}$$

(5) 2:1 (v/v) ethyl acetate-acetic acid
Working under a hood, combine 200 mL ethyl acetate and 100 mL glacial acetic acid. Mix the solution well; this volume is sufficient for the standards, controls, and 80 specimens in duplicate. (Prepare the reagent daily, immediately before sampling the whole blood.)

*Ethyl acetate quality appears to be the most frequent source of problems in performing the assay. The grade of the reagent is less critical than its ability to pass the potassium iodide (KI) test for quenching agents. If a quenching problem arises, test the ethyl acetate for the presence of impurities such as peroxides. Transfer 50 mL of ethyl acetate into a glass beaker and place the beaker on a white surface (e.g., paper). Add 10 mL of 10% (w/v) KI in deionized water solution and gently swirl the contents of the beaker. The presence of a distinct yellow color indicates impurities that would result in low, out-of-control values for quality control material. Reject any bottles of ethyl acetate that test positive. Purchase 500-mL size bottles; larger bottles may pass the KI test initially, but the contents may degrade as the bottles are repeatedly opened for use.*

d. **Standards Preparation**

Prepare all standard solutions in actinic glass volumetric flasks, in very reduced light. At present there are no NIST SRM's available for FEP standardization. The standard material used for the HANES method contains the highest purity standard material available, and that purity is confirmed by TLC, HPLC, fluorescence, and spectroscopy.

(1) Protoporphyrin IX standards
Concentrations are expressed in terms of protoporphyrin IX free acid. The millimolar absorptivity of protoporphyrin IX conventionally has been determined in 1.5 mol/L HCl; thus, the weekly absorbance reading of the hydrolysate is determined at this acid concentration (2).

(2) 200 mg/L protoporphyrin IX free acid hydrolysate (stock standard)

Weigh 42.0 mg protoporphyrin IX dimethyl ester (PPIX DME). Dilute to volume in a 200-mL actinic volumetric flask with 7 mol/L HCl, washing PPIX off the weighing paper with a few drops of formic acid. Add a small stirring bar, cover the flask with aluminum foil, and mix the contents at 20-25 °C for 3 hours, using a magnetic stirrer. (Prepare when the absorbance of the standard falls below 0.5100.)(3)

(3) 10 mg/L intermediate stock
After 3 hours, dilute 25.0 mL of 200-mg/L solution with deionized water to volume in a 500-mL actinic volumetric flask, to yield a 10-mg/L solution in 0.35 mol/L HCl. This stock solution will be used to prepare the working standards for daily instrument calibration. (Store in actinic bottles at 4 °C. Allow the solution to reach consistent room temperature before using.)

(4) 1 mg/L standard for weekly absorbance readings
Dilute 10.0 mL of 10-mg/L intermediate stock (brought to ambient temperature before dilution) to volume in a 100-mL actinic volumetric flask with 1.62 mol/L HCl to yield a 1-mg/L protoporphyrin IX standard in 1.5 mol/L HCl. Use an aliquot of this standard for absorbance readings.

*The theoretical concentration of this solution with respect to protoporphyrin IX free acid (PPIX FA) is calculated as follows:*

$$\frac{42\ mg\ PPIX\ DME}{200\ mL} \times \frac{562.27\ mg\ PPIX\ FA}{590.72\ mg\ PPIX\ DME} = .1999\ mg\ PPIX\ FA/mL$$

$$\frac{.1999\ mg\ PPIX\ FA}{mL} \times \frac{25\ mL}{500\ mL} \times \frac{10\ mL}{100\ mL} = .0009995\ mg/mL\ PPIX\ FA\ (or\ 99.95\ \mu g/dL)$$

$$\frac{99.95\ \mu g}{1\ dL} \times \frac{1\ mmol}{562.27} \times \frac{10\ dL}{1\ L} \times \frac{1\ mg}{1000\ \mu g} = .00178\ mmol/L\ PPIX\ FA$$

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. 0-80 µg/dL Working Standards

(1) Using the Digiflex, prepare the following working standards daily by diluting the 10 mg/L standard with 0.43 mol/L HCl according to the dilution scheme shown in Table 1.

*Be sure to work under very subdued lights when diluting and extracting the standard materials, since they are photo-labile.*

(2) Extract the standards as described in the procedure section of this method. The Hitachi F-2000 will prompt the analyst to read the nine standards in duplicate starting with level 0.

(3) When all the standards have been read, the instrument will draw the linear standard curve (x = concentration, y = intensity). Additional statistical information may also be obtained upon request, including the correlation coefficient (r). The correction coefficient squared must be greater than or equal to 0.9900 for a standard curve to be considered valid.

Table 1
Preparation of Working Standards

| Working Standard Concentration (µg/dL) | Volume 10 mg/L Standard (µL) | Volume 0.43 mol/L Hcl (µL) | Final Volume (µL) |
|---|---|---|---|
| 80 | 400 | 4600 | 5000 |
| 70 | 350 | 4650 | 5000 |
| 60 | 300 | 4700 | 5000 |
| 50 | 250 | 4750 | 5000 |
| 40 | 200 | 4800 | 5000 |
| 30 | 150 | 4850 | 5000 |
| 20 | 100 | 4900 | 5000 |
| 10 | 50 | 4950 | 5000 |
| 0 | 0 | 5000 | 5000 |

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

*To protect hands against acids and solvents during sampling, wear nitrile gloves. To avoid evaporation and degradation of specimens, process samples as rapidly as possible. After centrifugation, samples are stable for 1-2 hours.*

a. **Preliminaries**

(1) Thaw specimens and quality control materials of frozen EDTA-whole blood at room temperature. (Control pools with elevated levels of FEP have been prepared from blood (EDTA-anticoagulated) collected from cows that have been fed lead acetate.)

(2) Once per week, using the spectrophotometer and quartz cuvettes, measure the absorbance at the wavelength maximum (approximately 407-408 nm) of the 1 mg/L standard in 1.5 mol/L HCl standard solution against a blank of 1.5 mol/L HCl, scanning from 380 to 420 nm. This measurement will be used in determining the quality of the standard. Clean cuvettes with 5% Contrad detergent solution after use, and rinse thoroughly with deionized water followed by ethanol to remove water droplets.

(3) Prepare the working standard dilutions from the 10 mg/L standard solution, using 0.43 mol/L HCl as a diluent. These dilutions are unstable; therefore, prepare them as rapidly as possible.

(4) Prepare the 2:1 ethyl acetate-acetic acid mixture, and fill a dispenser bottle of one Digiflex dilutor for delivering 1.0 mL of reagent. Fill the dispenser bottle of another Digiflex dilutor with 0.43 mol/L HCl for delivery of 1.0 mL. (Place dilutors under a hood to minimize exposure to fumes.)

b. **Sample Preparation**

(1) Before sampling, thoroughly vortex each standard dilution, quality control pool, or whole blood specimen. Using the Digiflex, transfer 10 µL of the sample, followed by 1 mL of the ethyl acetate-acetic acid mixture, to a 10-x 75-mm disposable glass tube, in duplicate.

(2) Add 1.0 mL of the 0.43 mol/L HCl to each sample using the third Digiflex. Wrap the tube with Parafilm, and vortex thoroughly for 10 sec.

(3) Sample in this order: standards, quality control pools, and whole-blood specimens in duplicate.

(4) Prepare each of two blank tubes (0 standards) with 1.0 mL of ethyl acetate-acetic acid and 0.43 mol/L HCl; use10 µL of 0.43 mol/L HCl as a sample.

(5) When all sampling is completed, centrifuge all tubes for 4 min at 1400 rpm.

c. **Hitachi F-2000 Spectrofluorometer Settings:**

Set the parameters for the F-2000 as shown in Table 2.

Table 2
Hitachi F-2000 Spectrofluorometer Settings

| Parameter | Setting |
|---|---|
| Data Mode | Conc |
| num WL | 1 |
| Sample Num | 1 |
| WL 1 (nm) | EX 404, EM 658 |
| Replicates | 2 |
| Init Delay (sec) | 0 |
| Integ Time (sec) | 5 |
| Hi Limit | 40 |
| Lo Limit | 0 |
| Unit Label | Other |
| Curve Type | 1st order |
| Num Stds | 9 |
| Curve Mode | New |
| Response (sec) | 0.1 |
| Bandpass (nm) | EX 10 EM 10 |
| PM Voltage (V) | 700 |
| Text Print | On |
| Photomultiplier Tube | R928 Hamamatsu |
| Cuvettes | 10- x 75-mm in microcell adapter |

d. **Operation**

(1) Turn on the F-2000 power source, ignite the xenon lamp, and start the computer.

(2) Allow 1 hour for the F-2000 to warm up and stabilize after the xenon lamp has been ignited.

(3) Select TEST MENU from the F-2000 main menu.

(4) Select LOAD, followed by the number corresponding to the PROTO method.

(5) Insert each tube into the sample holder, close the compartment door, and press [EX] to read. The tubes should be read starting with the standards, followed by the controls, samples, and a final set of controls. See the Hitachi F-2000 manual for further information on the operation of the instrument.

(6) For samples outside the range of the standard curve, dilute the whole blood with an equal volume of normal

saline and extract. Read the sample and multiply the results by a factor of 2 when reporting values. If necessary, a 1:5 dilution with saline may also be used.

(7) Turn off the instrument in reverse order. After the power source has been turned off and the lamp has gone out, turn the switch back to the 'ON' position and allow the fan to cool the lamp for at least 15 minutes.

e. **Recording of Data**

(1) Quality control data. Use the "HANES LABORATORY PROTOPORPHYRIN STANDARD AND QUALITY CONTROL SHEET" to record this data. This reporting sheet has self-explanatory blanks for the standard absorbance data, the linear regression information, and the quality control pool results.

(2) Analytical results. Use the "HANES III ANALYTICAL WORKSHEET" to record the specimen results. These have been prepared with a list of sample IDs for each preracked run. Record the results in µg/dL in one column and the hematocrit corrected results in µg/dL RBC in another column. The corrected values will be recorded in the HANES III database. If a sample has been diluted because the values are beyond the linearity of the instrument, make a note of the dilution next to the ID number. If a sample is missing from the rack, write "NOSAX" in the result column. If a sample is not satisfactory and cannot be analyzed, write "UNSAX" in the blank.

(3) Give both types of forms to the supervisor along with the tape from the instrument and the computer printout from the calculation program. After the supervisor checks the data, the analyst's copies and the printouts should be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets.

(4) Use the "QC" program in the HANES library of ROSCOE to enter the quality control data; update the file regularly.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Xenon lamp: A spare lamp should be available. Order another if the spare is used for replacement.

(2) Printer tape: A supply of printer tape should be on hand.

g. **Calculations**

(1) Use the linear regression program in ROSCOE ("PROTOIX") to calculate the calibration curve and the specimen concentrations. The linear regression program generates slopes, intercepts, and correlation coefficients. The correlation coefficient squared should be 0.9900 or better, and the slope should be between 0.9000 and 1.1000.

(2) The millimolar absorptivity of protoporphyrin IX free acid in 1.5 mol/L HCl has been determined in our laboratory to be 297 ± 1 (600 observations from 1976 to 1988) (4-6). The purity of our material has been confirmed by elemental analysis and high-performance liquid chromatography of the extracted protoporphyrin IX free acid. Calculate the actual concentration of the 1 mg/L (.00178 mmol/L) working standard using the following equation:

$$A = \epsilon bc$$

*Where:*

*A = absorbance reading*
*$\epsilon$ = 297, the millimolar absorptivity of protoporphyrin IX free acid in 1.5 mol/L HCl*
*b = cuvette pathlength, 1 cm*
*c = concentration, in mmol/L*

For example, if the daily absorbance reading of the 1 mg/L standard at wavelength maximum is

0.520, then:

$$c = \frac{0.520}{(297\ \text{L/mmol-cm})(1\text{cm})}$$

$$= .00175\ \text{mmol/L}$$

Then:
(.00175 mmol/L)(562.27 mg/mmol)(1000 µg/mg)(1L/10dL) = 0.9840 mg/L PPIX FA

Consider 0.9840 as a percentage of 100 µg/dL (1 mg/L), and correct the standard curve accordingly:
10 µg/dL X 0.9840 = 9.84
20 µg/dL X 0.9840 = 19.68 etc.
Perform a linear regression, with x equal to the corrected standard concentration and y equal to the fluorescent intensity reading. Using the slope of the standard curve and assuming zero intercept, calculate the concentration of protoporphyrin IX per deciliter of whole blood for each specimen.

> *When using the Hitachi F-2000, you need not perform these calculations. The instrument will create a calibration curve using the standards and calculate the concentration of the unknowns automatically. Results on the instrument printout are given in µg/dL.*

To correct for hematocrit and express results as µg/dL of RBC, use this formula:

$$\frac{\text{µg/dL whole blood}}{\text{hematocrit}} \times 100 = \text{µg/dL RBC}$$

(2) Repeat a specimen analysis when duplicate values do not check within 10%. Any sample with a value of 90 µg/dL RBC or more should be diluted with normal saline and extracted. The results should be multiplied by the dilution factor before they are reported.

h. **Special Method Notes**

Once the standards have been extracted, continue through the analysis without interruption to minimize error due to incomplete extraction. Some small variations in fluorescence values may be due to poor quality 10- x 75-mm tubes used as cuvettes. Blood specimens are very stable; standards are somewhat labile and must be processed under reduced light. Accurately weighing the PPIX DME is critical, as is avoiding prolonged hydrolysis time (i.e., >3 hours).

If hematocrit correction is not used for reporting data, the traditional cutoff level is 35 µg/dL. Although we have not found this practice necessary, both New York State and the Wisconsin State Laboratory of Hygiene recommend a 1:5 dilution of fresh whole blood with saline in microtiter plates to ensure complete lysis of cells. Both also use a concomitant dilution of standard concentration.

## 9. REPORTABLE RANGE OF RESULTS

Protoporphyrin results are reportable undiluted when the values are less than 90 µg/dL RBC. Samples with concentrations of 90 µg/dL RBC or greater should be diluted at least 1:2 (v/v) with saline and reanalyzed, with the results multiplied by the dilution factor.

## 10. QUALITY CONTROL (QC) PROCEDURES

FEP is measured by a "batch" method (i.e., all specimens, standards, and QC pools simultaneously undergo the same processes, such as extraction). On an average day, 80 specimens are analyzed in duplicate, with 3 levels of bench QC specimens (low-human, medium-bovine, and elevated-bovine) analyzed at the beginning and end of each run. In every rack of 20 specimens, one blind QC specimen will be inserted randomly. Blind QC pools are prepared in the same manner as the unknown specimens and with the same types of labels and vials. Two levels are prepared (low-normal

and high-normal) in order to verify values reported in the near abnormal concentration ranges for a given analyte.

Quality control limits are established with the programs "QCLIMIT" and "QC." Preliminary limits are established with 20 consecutive runs and updated annually thereafter.

Blind QC results are examined by using similar criteria. The supervisor also evaluates the slope, intercept, and $R_2$ values for trends. The overall coefficient of variation for this method has been 4-5% over the entire analytical range.

The system is declared "out-of-control" if any of the following events occur:

On the means chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two of the two or more pools fall either both above or both below the lower 95% limit.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

On the range chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two of the two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more quality control samples fall outside the 95% limits for mean or range of duplicate values, take the following steps:

a. Prepare fresh calibration standards, and repeat the entire curve using the freshly prepared standards.

b. Prepare fresh dilutions of all whole-blood quality-control samples and re-analyze them.

If the steps outlined above do not result in the correction of the "out of control" values for QC materials, the supervisor should be consulted for other corrective actions. No analytical results should be reported for runs not in statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Range of linearity and limits of detection were previously mentioned.

b. Interfering substances

(1) External fluorescent contamination may result from the sample coming in contact with items such as wooden applicator sticks or glassware that has residual soap from washing.

(2) Acid tends to quench fluorescence. Rinse all glassware thoroughly after it has been rinsed with acid.

## 13. REFERENCE RANGES (NORMAL VALUES)

The following ranges for FEP (7) are used in evaluating data:

Females tend to have higher average values than males; children tend to have higher average values than adults.
Children <6 years old : 36-97 µg/dL RBC
Children 6-14 years old : 37-83 µg/dL RBC
Males 15-74 years old: 33-81 µg/dL RBC
Females 15-75 years old: 37-93 µg/dL RBC

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Any value of an NHANES sample ≥90 µg/dL RBC should be FAXed immediately to NCHS. The NHANES population is the only patient population for which notification is required. All other studies are purely epidemiologic in nature, and the principal investigator is responsible for notifying subjects.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should remain at room temperature during testing. Special care must be taken to keep samples out of direct light.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods for performing this test. In case of system failure, store all whole blood specimens at -20 °C until the system is functioning.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

As stated in Section 14, NCHS should be notified by FAX of all HANES samples with values ≥90 µg/dL RBC.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

RBASE and the CDC mainframe are used to keep records and track specimens. All records, including related QA/QC data, are required to be maintained for 10 years. Only numerical identifiers (e.g., case ID numbers) should be used.

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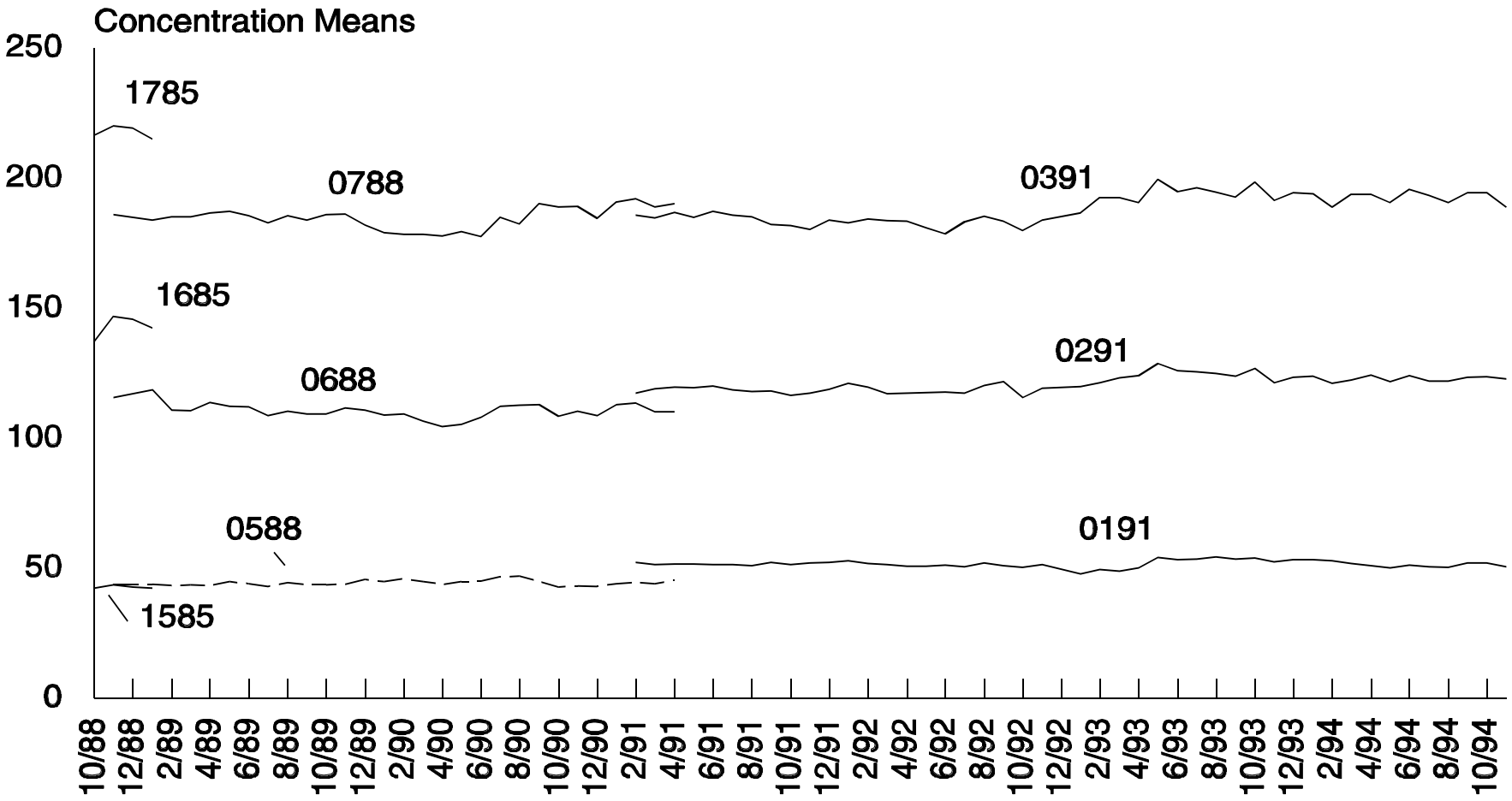
SUMMARY STATISTICS FOR
ERYTHROCYTE PROTOPORPHYRIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0191 | 02/91 - 11/94 | 51.806 | 2.31623 | 4.47101 | 2176 |
| 0291 | 02/91 - 11/94 | 121.757 | 4.66061 | 3.82779 | 2175 |
| 0391 | 02/91 - 11/94 | 189.642 | 7.73636 | 4.07947 | 2165 |
| 0588 | 11/88 - 04/91 | 44.319 | 1.84560 | 4.16436 | 979 |
| 0688 | 11/88 - 04/91 | 111.025 | 4.55681 | 4.10430 | 984 |
| 0788 | 11/88 - 04/91 | 185.538 | 6.16924 | 3.32506 | 977 |
| 1585 | 10/88 - 01/89 | 43.101 | 1.21680 | 2.82313 | 64 |
| 1685 | 10/88 - 01/89 | 144.940 | 5.32466 | 3.67370 | 68 |
| 1785 | 10/88 - 01/89 | 218.466 | 5.92560 | 2.71237 | 68 |

# Erythrocyte Protoporphyrin Monthly Means




NOTE: No specimens assayed for Pools BLHI and BLLO during 3/91-11/91.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Serum iron and total iron-binding capacity (TIBC) are measured by a modification of the automated AAII-25 colorimetric method, which is based on the procedures of Giovaniello et al. (1) and of Ramsey (2). The method has been modified further to be performed on an Alpkem RFA (rapid-flow analysis) system. Iron is quantitated by measuring the intensity of the violet complex formed in the reaction between ferrozine and $Fe^{++}$ in Ph 4.7 acetate buffer at 562 nm. Thiourea is added to complex $Cu^{++}$, which can also bind with ferrozine and yield falsely elevated iron values. In TIBC tests, serum is mixed with 400 µg/dL iron solution to saturate the iron-binding sites of the serum transferrin molecules. Magnesium carbonate is used to remove excess iron. Centrifugation is used to precipitate the magnesium carbonate, and the supernatant is measured for iron content.

Serum iron and TIBC assays can be used together with ferritin assays to aid in the diagnosis of iron deficiency or overload. In cases of iron deficiency, decreased serum iron levels and increased TIBC may be observed. Conversely, in cases of iron overload (which can be caused by hemolytic anemia, liver damage, excessive absorption of iron, and iron therapy) increased serum iron levels and decreased TIBC may be observed. In cases of infection, inflammation, and malignancy, both serum iron levels and TIBC may be decreased (3).

## 2. SAFETY PRECAUTIONS

Treat all serum specimens as potentially positive for HIV and hepatitis B. Therefore, observe Universal Precautions. Wear gloves, lab coat, and safety glasses at all times during the analysis. We recommend the hepatitis B vaccination series for all analysts working with whole blood or serum samples. When the analysis is completed, dispose of all specimens and all plastic and glassware materials coming in contact with specimens by autoclaving. Several of the reagents for this method are corrosive or caustic and should be handled appropriately. Observe proper laboratory safety guidelines with respect to pipetting, reagent preparation, etc.

Material safety data sheets (MSDSs) for L-ascorbic acid, magnesium carbonate, thiourea, sodium acetate trihydrate, and hydrochloric acid are available through the NCEH Local Area Network (LAN).

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Calculation of a given run is accomplished through the RFA (rapid flow analyzer) software installed on the PC. The original files are deleted from the hard drive when the analyst is certain that all information has been recorded and approved by the supervisor.

   When the data have been approved, they are transcribed by the data entry clerk into the NHANES III RBASE data base located on the NCEH\EHLS PC network. Data entry is verified by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor copies the values to the NCHS mainframe computer with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. For best results, a fasting sample should be obtained.

b. Specimens for iron / TIBC analysis may be fresh or frozen serum harvested from blood collected in a red-top Vacutainer brand tube by standard venipuncture procedures.

c. A 1-mL sample of serum is preferable, but a sample volume of 500 µL may be analyzed.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field are frozen and then shipped on dry ice by overnight mail. Serum iron and TIBC

are very stable and specimens may be stored at -20 to -70 °C for years. Serum iron levels are not affected by freeze-thaw cycles; after two cycles, TIBC values may tend to be reduced.

f. Specimens generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

g. Because a certain amount of diurnal variation is associated with the levels of metals in the body, the time of collection should be noted for the NHANES data analysis. Iron background contamination levels are lower in the red-top Vacutainers, whereas the royal blue-top trace metals tubes are more appropriate for zinc and copper specimen collection. Hemolyzed specimens should not be used because of the contribution of iron from hemoglobin.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the Branch laboratory and the Special Activities Specimen Handling Office). The protocol discusses the collection and transport of blood and special equipment required. In general, serum should be transported and stored at no more than 4 °C. Samples thawed and refrozen several times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or serum should be transferred into a clean Nalge cryovial labelled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Alpkem RFA or RFA 300 analyzer system (Alpkem, Inc., Clackamas, OR).

(a) Model 301 sampler.

(b) Model 302 manifold, with model 302 minipump and dialyzer with type "H" membranes and flow-rated tubing, and model 3104/314 system controller.

(c) Model 305A/510 photometer, with 15-mm flowcell, 562-nm filters, and bubble gate "ON."

(d) Data system, with RFA software, 286 computer, printer, and chart recorder.

(2) Micromedic Model 25000 automatic pipettes, with 1.0-mL dispensing and sampling pumps or Micromedic Digiflex automatic dispenser equipped with 2-mL sampling and dispensing syringes (Micromedic Systems Inc, Horsham PA).

(3) Vortex mixer (Fisher Scientific Co., Fairlawn NJ).

(4) IEC Centra 7 centrifuge (International Equipment Co., Needham Heights, MA).

b. **Materials**

(1) 0.25-mL disposable conical bottom sample cups (Baxter Scientific Products., McGaw Park, IL).

(2) Disposable filtering columns (Whale Scientific Co., Denver, CO).

(3) "Ferrozine" iron reagent [3-(2 pyridyl)-5,6 bis (4-phenylsulfonic acid)-1, 2, 4, triazine, monosodium, monohydrate], 95% purity (Hach Chemical Co., Ames, IA).

(4) L-ascorbic acid, 99.9% purity (J.T. Baker Chemical Co., Phillipsburg, NJ).

(5) Magnesium carbonate (basic), "Fisher Certified" (Fisher Chemical Co.).

(6) Thiourea, "Baker Analyzed" (J.T. Baker).

(7) Sodium acetate, trihydrate, "Baker Analyzed" (J.T. Baker).

(8) Hydrochloric acid (HCl), concentrated, reagent grade (J.T. Baker).

(9) Brij-35, 30% solution (Pierce Chemical Co, Rockford, IL).

(10) 12- x 75-mm disposable glass culture tubes (Corning Glass Works, Corning, NY) lot-tested for iron contamination.

(11) Iron wire, 99.9% purity (Mallinckrodt Chemical Works, St. Louis, MO).

(12) Deionized water, ≥1.0 megaOhm-cm at 25 °C (Continental Water Co., Atlanta, GA).

(13) Sodium chloride (NaCl), ACS certified (Fisher Scientific Co.).

(14) Kemwash detergent solution (Alpkem, Inc.).

c. **Reagent Preparation**

(1) 0.2 mol/L hydrochloric acid with 3 g/dL sodium chloride
To 250 mL of deionized water in a 2-L flask, add 34 mL concentrated HCl and 60 g NaCl. Mix well and dilute to volume with deionized water. (Prepare as needed to be used as part of the working solution; this solution is stable at 20-25 °C.)

(2) Working HCl/NaCl/ascorbic acid solution
For each 150 samples to be analyzed, add 1 g of L-ascorbic acid and 8 drops Brij to 100 mL of 0.2 mol/L HCl with 3 g/dL NaCl. Mix well. (Prepare daily.)

(3) 0.75 mol/L acetate buffer, pH 4.7 at 25 °C
Add 816.8 g sodium acetate to 4 L deionized water in an 8-L flask. Stir well and dilute to volume. Check to ensure that final pH is 4.7± 0.1, and adjust if necessary with 0.1 N NaOH or 0.1 N HCl. (Prepare weekly; the solution is stable at 20-25 °C.)

(4) 0.07 g/dL ferrozine with 1% (w/v) thiourea
Add 0.7 g Ferrozine and 10 g thiourea to 1 L of acetate buffer-thiourea solution and mix well. Filter through a 0.22-µm Millipore filter to remove any undissolved particulate matter. (Prepare weekly; the solution is stable at 20-25 °C.)

(5) 0.5 ml/L Brij-35 wash solution
Add 1.0 mL Brij-35, 30% solution, to 2 L deionized water and mix well. (Prepare weekly.)

(6) 0.1 mol/L hydrochloric acid (for standard preparation)
Add 8.3 mL concentrated HCl to 500 mL deionized water in a 1-L volumetric flask. Mix well and dilute to volume with additional water. Do not add Brij-35. (Approximately 5 L of this solution is required to prepare intermediate and working standards.)

(7) 400 mg/dL iron saturating solution for TIBC
Dilute 2.0 of the 1.0 g/dL stock iron standard to volume in a 500-mL flask with deionized water. (The solution is stable at 20-25 °C.)

d. **Standards Preparation**

(1) 1.0 g/L stock iron standard solution
Place 1.000 g iron wire in a 1-L volumetric flask. Add 12 mL concentrated HCl and dissolve the wire with slight warming. After the wire is completely dissolved, cool the flask to room temperature and dilute contents to volume with deionized water. (The solution is stable indefinitely; store in a polypropylene container at 20-25 °C.)

(2) 50.0 mg/L iron intermediate stock solution
Dilute 25 mL of the 1.0 g/dL stock iron solution to 500 mL with 0.1 mol/L HCl. (Prepare each time new working standards are required.)

(3) Working iron standards
In a series of 500-mL volumetric flasks, prepare dilutions from the intermediate standard as shown below. Dilute to 500 mL with 0.1 mol/L HCl and mix well. (Prepare as needed; the solution is stable at 20-25 °C.)

(4) NIST iron standard (SRM 937)
This standard reference material has a concentration of 1.0001 mg/mL and may be diluted at concentrations from 1 to 1000 µg/dL as a verification of the accuracy of the working standard dilutions.

Table 1
Working Iron Standards
(Dilute to 500 mL with 0.1 mol/L HCl

| 50 mg/L Intermediate Standard, mL | Final Concentration, µg/dL Iron |
|---|---|
| 3 | 30 |
| 5 | 50 |
| 8 | 80 |
| 10 | 100 |
| 15 | 150 |
| 20 | 200 |
| 25 | 250 |
| 30 | 300 |

e. **Preparation of Quality Control Material**

High-quality HIV- and hepatitis-B negative human serum from fasting subjects is used for the preparation of quality control pools. A base normal pool is prepared, and the levels of iron and TIBC are measured for reference. A portion of the base pool is diluted 25-30% with sterile physiological saline to reduce levels to low-normal. Another aliquot is subjected to filtration on a hollow-fiber column, which removes about 30% of the water content of the serum and concentrates the metals as well as the serum proteins. This technique has been used effectively to provide a high TIBC level. All three pools are filter-sterilized, and then dispensed into glass Wheaton vials, which are capped and stored at -70°C for maximum stability.

(See Section 10, Quality Control Procedures, for target values of quality control pools.)

Two levels of blind quality control pools are prepared and dispensed in a similar manner, except that vials and labels identical to those of NHANES specimen vials were used.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

The accuracy and precision of iron measurements may be verified with NIST SRM 909, multi-element serum reference material, 2.34 µg/dL or 234 µg/dL, or with NIST iron standard SRM 937, iron metal in HCl, 99.9% purity. The latter standard reference material has a concentration of 1.0001 mg/mL and may be diluted at concentrations such as 1, 5, 10, 50, 100, 500, and 1000 µg/dL in order to verify the accuracy of the working standard dilutions. Linearity of the method may also be confirmed with the same dilutions of SRM 937. NIST SRM 2124-3, iron standard solution, 10 mg/mL, may also be diluted and used for these purposes. In each case, at least three replicates of each dilution are run as unknowns against working standards, with a linear regression generated from working standards as X and NIST dilutions as Y. The correlation coefficient should be 0.98 or higher, the slope should be 1.00$\pm$ 0.05, and the y-intercept should be 0.0$\pm$1.0. This procedure should be run twice a year on each operating system used for this method, with common dilutions run on each system.

Additionally, each time new batches of working standards are prepared from stock, "new" dilutions are analyzed against "old" dilutions for accuracy and linearity, with the same criteria of acceptability required before the new batches are implemented in the assay. New standard values should be within 2% of old standard values.

The Alpkem systems are maintained under a service contract that calls for twice-yearly preventive maintenance to test calibration parameters against manufacturing specifications.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Procedure

(1) Preparation of samples for serum iron assay:
Mix serum specimens well. Filter about 0.3 mL of serum into a 0.5-mL sample cup, using a disposable plastic filtration column to remove fibrin.

(2) Preparation of samples for TIBC assay:
Using the Micromedic automatic pipette, dilute 0.2 mL well-mixed serum with 0.4 mL 400 µg/dL iron saturating solution in 12- x 75-mm tubes. Mix well by vortexing and allow the tubes to stand for at least 30 min. (We have found that results are most precise when the samples are tightly capped and kept at 4-8 $^{o}$C overnight. They may also be stored at -20 $^{o}$C for up to 3 weeks.) Add 0.1 g basic magnesium carbonate directly to each tube of diluted serum. Mix the contents of the tubes; allow the tubes to stand for 45 min, mixing at 15-min intervals. Centrifuge the tubes at 2500 x g for 10 min to pack the magnesium carbonate. Decant the supernatant into 0.25-mL sample cups and proceed as with the iron analysis.

### b. Quality Control Materials

Assay frozen serum quality control materials in the same manner as serum specimens.

NOTE: TIBC cannot be accurately determined on lyophilized, commercial, quality control serum products or materials such as the College of American Pathologists (CAP) materials.

### c. Operation

Follow standard operating protocol as outlined in the *Operations Guide for the Alpkem RFA* (4). Approximately 100 uL of serum is required for each iron analysis, and 200 mL serum is used for the TIBC dilution. The analysis rate is 72 samples per hour. Calibration is accomplished with ascending- and descending-order standard curves analyzed with every 60-80 specimens. At maximal capacity, an ascending-order standard curve, 4 racks of 20 specimens each, and a descending-order standard curve can be run in one session. Two sessions can be run in a single day, one in the morning and a second in the afternoon. Quality control materials are included with every curve and every rack of 20 specimens analyzed. With full daily use of the system, pump-tubing and type "H" membranes should be changed every 2 days. Using the strip-chart recorder as well as the data system helps analysts to monitor any unusual changes in peak size or shape, which may indicate dialysis or line clogging problems. Full scale on the recorder is 0.1A. For maximum sensitivity, the system is peaked daily with the 300 µg/dL standard.

### d. CDC Modifications

CDC has modified the Technicon AAII-25 method in the following ways (5):

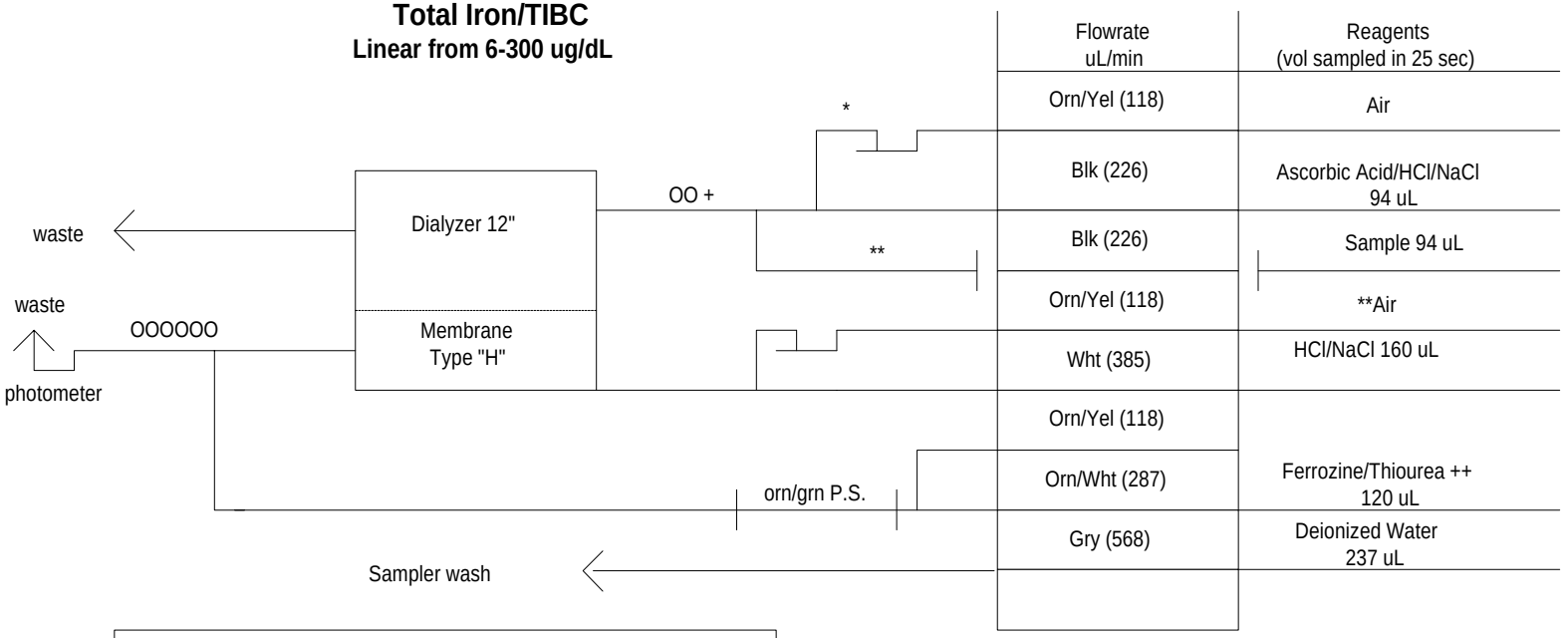
(1) The reagent concentrations used and their ratios are based on procedures developed at CDC.

(2) The use of type "H" rather than type "C" membranes was developed in conjunction with Alpkem in order to provide maximum efficiency of dialysis.

(3) Ferrozine as well as thiourea is incorporated into the acetate buffer.

(4) A 15- rather than a 10-mm stainless steel end-capped flowcell is used in the photometer to enhance sensitivity.

## Total Iron/TIBC
**Linear from 6-300 ug/dL**




| Photometer Parameters | |
|---|---|
| Filter | 570 nm |
| Flowcell | 15 mm, stainless steel endcapped |
| Absorbance range | 0.1 (AUFS) |
| Damping (RC) | 2 sec |
| **Sampler Parameters** | |
| Analysis rate | 72/hr |
| Sample time | 25 sec |
| Wash time | 25 sec |
| Pecking | off |
| Probe | Stainless steel |

| | |
|---|---|
| Manifold startup solution | Deionized water with Brij-35 (1 mL/L) |
| Cleaning solution | KemWash (ALPKEM P/N A01-5555-26) or 1 N NaOH |

| |
|---|
| + Coil, O = 5 turns |
| * Air solenoid valve |
| ** 0.015" ID Polythylene |
| ++ Ferrozine/Thiourea is delivered with 2 lines to get the proper volume at 287 uL/min flowrate |

Developed in conjunction with Alpkem Corp. 9/86
Rev 9/90

e. **Special Method Notes**

Because of the highly enhanced sensitivity of this method, developed to minimize the amount of sample required so that pediatric specimens could be used, it is very important to eliminate all sources of particulate matter or specimen fibrin that could clog the micro-sized pump tubing. We therefore recommend that the chart recorder be used at all times as a visual aid to dialysis performance. The system should be washed weekly by sampling Kemwash solution for 15 min, and then washed with water for 30 min to prevent protein buildup. The flowcell should be removed from the sample stream at this time to prevent etching of its glass interior.

## 9. REPORTABLE RANGE OF RESULTS

The serum iron concentrations of specimens and the TIBC of diluted samples are calculated from the slope and y-intercept of the 8-point regression line of the expected concentrations of the standards versus their millivolt recorder values (or absorbance values). $R^2$ for the regression line should be $\geq$.9990. The RFAC data system will generate calculations for the run. TIBC concentrations are multiplied by the dilution factor of 3. Percent saturation, which is the estimate of iron-filled available binding sites on the transferrin molecule, is expressed as iron/TIBC.

The method is linear from 0 to 1000 µg/dL, as verified by successive dilutions of NIST SRMs. Specimens with iron values <30 µg/dL or >200 µg/dL, and TIBC <250 µg/dL or >500 µg/dL are reanalyzed for confirmation. Percent recovery was established as 99.3% over the linear range by using the method of standard additions. The limit of detection is approximately 2.0 µg/dL. The average total CV over the reporting range, as demonstrated by QC pools, is approximately 3%.

## 10. QUALITY CONTROL (QC) PROCEDURES

Three levels of bench quality control pools are analyzed in duplicate with every standard curve and with every 20 specimens analyzed. In addition, one low- or high-concentration blind quality control specimen, prepared exactly as the NHANES specimens, is randomly included in every rack of 20 specimens. Quality control limits are established for new pools after 20 runs and updated annually thereafter.

The system is declared "out-of-control" if any of the following events occur:

On the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two of two or more pools fall either both above or both below the lower 95% limit.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

On the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two of the two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

Examples of the accuracy and precision of the method are demonstrated in the results of these long-term quality control pools as shown in Tables 2 and 3.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the system should be declared "out of control," take the following remedial action(s):

a. Check the system for fibrin clots in sample or metal connectors.

b. Replace the type "H" membrane.

c. Replace the pump tubing.

d. Check timing and bubble gating.

After troubleshooting procedures have been completed and the system has been verified to be "in control," reanalyze all specimens for that analytical run and report the values from the reanalysis rather than the original values.

Table 2
Serum Iron Quality Control Pool Results

| Pool | Mean | 95% limits | 99% limits | N | Total SD | Total CV |
|---|---|---|---|---|---|---|
| 0888 | 69.83 | 65.93-73.73 | 64.70-74.96 | 102 | 2.84 | 2.84 |
| 0988 | 88.12 | 84.11-92.14 | 82.84-93.41 | 102 | 3.33 | 2.93 |
| 1088 | 138.30 | 131.76-145.85 | 129.53-148.08 | 102 | 3.23 | 4.63 |
| 9114 | 73.80 | 70.75-76.81 | 67.79-77.77 | 33 | 2.11 | 2.86 |
| 9115 | 98.19 | 94.55-101.84 | 93.40-102.99 | 33 | 2.62 | 2.57 |
| 9116 | 143.31 | 139.23-147.40 | 137.94-148.69 | 33 | 2.80 | 1.95 |

Table 3
Serum Total Iron-binding Capacity Quality Control Pool Results

| Pool | Mean | 95% limits | 99%limits | N | Total SD | Total CV |
|---|---|---|---|---|---|---|
| 0888 | 285.6 | 266.02-305.09 | 259.84-311.27 | 92 | 11.97 | 4.19 |
| 0988 | 370.7 | 338.92-401.53 | 330.18-411.27 | 92 | 17.42 | 4.70 |
| 1088 | 496.6 | 465.87-527.90 | 456.06-537.71 | 92 | 18.51 | 3.73 |
| 9114 | 260.5 | 243.80-277.23 | 238.51-282.52 | 33 | 9.13 | 3.50 |
| 9115 | 369.2 | 346.55-391.85 | 339.39-399.01 | 33 | 12.87 | 3.49 |
| 9116 | 502.11 | 474.26-529.96 | 464.45-538.77 | 33 | 16.04 | 3.20 |

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

TIBC levels may tend to be reduced in samples that have undergone more than two freeze-thaw cycles. Background contamination levels of iron are lower in red-top vacutainers than in blue-top vacutainers. Hemolyzed specimens should not be used because of the contribution of iron from hemoglobin.

## 13. REFERENCE RANGES (NORMAL VALUES)

Values for adult males ( >20 years old) are generally higher than those for adult females, and children have lower values than adults. Low iron values in conjunction with elevated TIBC values, yielding <15% saturation, are generally indicative of iron deficiency anemia. Elevated iron values, in conjunction with >60% saturation and an elevated ferritin level, may be indicative of hemochromatosis, or iron overload. These are the normal ranges for the U.S. population based on the NHANES II data (6):

- Males ≥18 years old: 60-190 µg/dL iron, 300-455 µg/dL TIBC
- Females ≥18 years old: 40-175 µg/dL iron, 285-510 µg/dL TIBC
- Children 3-17 years old: 32-175 µg/dL iron, 305-490 µg/dL TIBC

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Iron values <30 µg/dL or <15% saturation are considered indicative of possible iron deficiency. Saturation >60%, in combination with a ferritin value >300 ng/mL may be indicative of possible iron overload, or hemochromatosis. No critical call results are defined for this method because of the epidemiological nature of the survey.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Allow specimens to gradually reach room temperature before sample preparation and during testing.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Since the analysis of serum for iron and TIBC is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, we recommend storing the specimens at ≤-20 °C until the analytical system is restored to functionality.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Use the "NHANES III Standard and Quality Control Sheet" to record quality control data. The entry of standard concentrations and quality control pool results on this reporting sheet is self explanatory. Prepare this form in duplicate.

Use the "NHANES III Analytical Worksheet" to record the specimen results. These have been prepared with sample IDs for each pre-racked run. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is not satisfactory (i.e. cannot be analyzed), write "UNSAX" in the blank. Prepare this form in duplicate.

Give both types of forms to the supervisor along with the chart and data printouts generated by the computer. After the supervisor checks the data, the carbon copies, computer printout, and chart will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets. The data entry clerk will transcribe the results into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network.

Use the "QCDATA6" program in ROSCOE on the CDC mainframe to record quality control data. These data should be updated regularly.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

RBASE and the CDC mainframe are used to keep records and track specimens for NHANES III. We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual serum from these analyses is transferred to the NHANES III Serum Bank in Bethesda, MD, for further long-term storage.

**REFERENCES**

1. Giovaniello TJ, Bendetto G, Palmer DW, Peters T. Fully and semiautomated methods for the determination of serum iron and total iron-binding capacity. J Lab Clin Med 1968; 71:874.

2. Ramsey WNM. The determination of the total iron-binding capacity of serum. Clin Chem Acta 1957; 2:221.

3. Gibson RS. Principles of nutritional assessment. New York:Oxford University Press,1990

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5. Gunter EW, Miller DT. Laboratory methods used by the Division of Environmental Health Laboratory Sciences, Center for Environmental Health, Centers for Disease Control for the Hispanic Health and Nutrition Examination Survey (HHANES) 1982-84. Atlanta, GA: Centers for Disease Control, 1986: 13-16.

6. Fulwood R, Johnson CL, Bryner JD, et al. Hematological and nutritional reference data for persons 6 months - 74 years of age; United States, 1976-80. Vital and Health Statistics. Series 11 - No. 232. DHHS Pub. No. (PHS) 83-1682. Washington, D.C.: Public Health Service, 1982.

**ADDITIONAL SOURCES**

Van Assendelft OW, Fairbanks VF, Gunter EW, Turner WE. Proposed standard for the determination of serum iron and total iron-binding capacity. Provisional Standard, National Committee for Clinical Laboratory Standards, 1995.

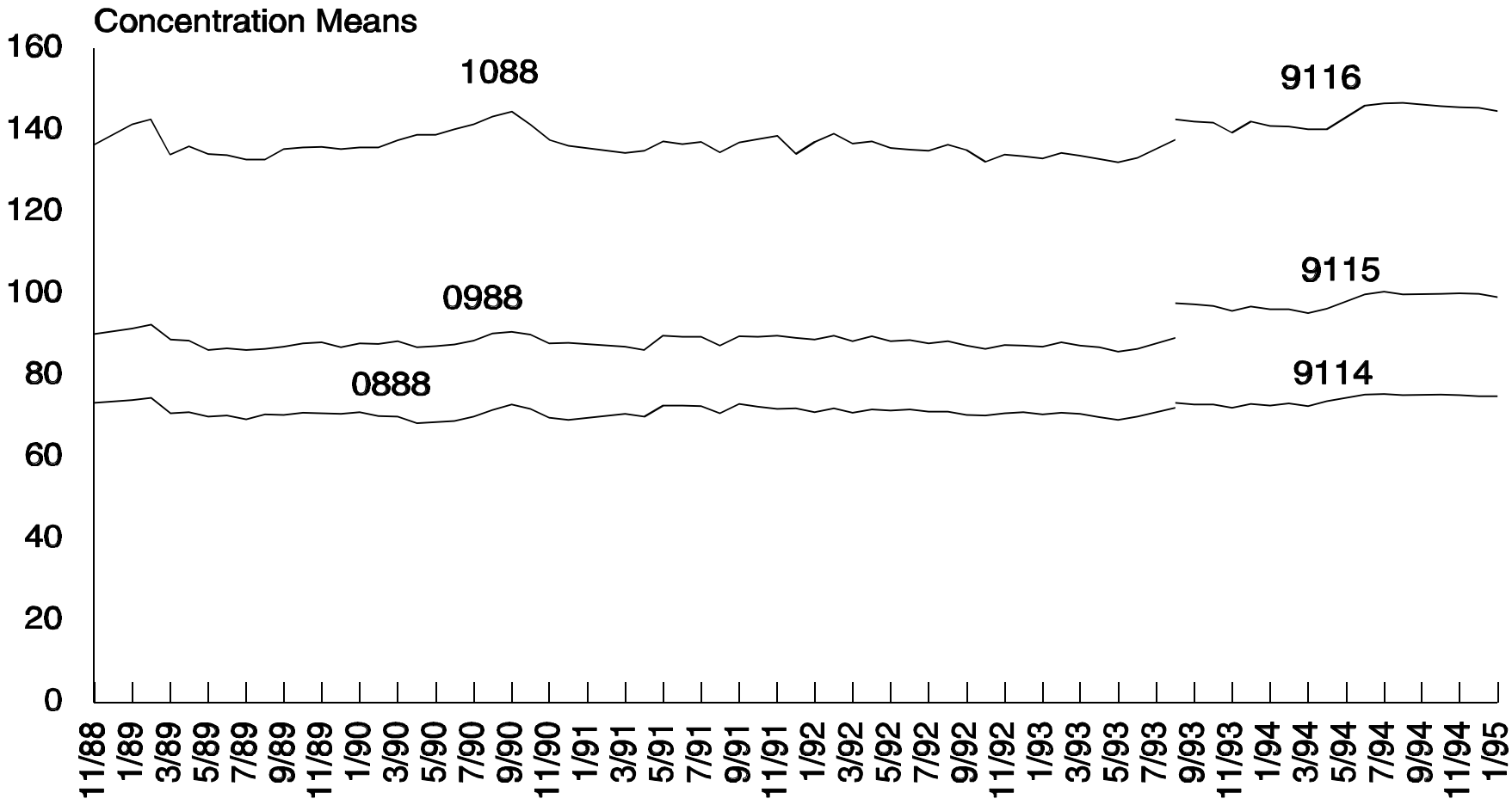
**ACKNOWLEDGMENTS**

We gratefully acknowledge the contributions of Barbara Bowman, Ph.D., Elaine Gunter, Jackie Lovingood, Dayton Miller, Ph.D., Eric Sampson, Ph.D., and Pat Yeager who assisted in developing the methodology, performing the analyses for serum iron and total iron-binding capacity in the NHANES III study, and preparing the manuscript for this chapter.

SUMMARY STATISTICS FOR
IRON
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0888 | 11/88 - 08/93 | 70.893 | 2.66175 | 3.75459 | 2183 |
| 0988 | 11/88 - 08/93 | 88.069 | 2.80611 | 3.18625 | 2179 |
| 1088 | 11/88 - 08/93 | 136.063 | 5.11601 | 3.76002 | 2193 |
| 9114 | 08/93 - 01/95 | 74.204 | 1.98707 | 2.67785 | 608 |
| 9115 | 08/93 - 01/95 | 98.501 | 2.61833 | 2.65819 | 609 |
| 9116 | 08/93 - 01/95 | 143.977 | 3.49348 | 2.42642 | 606 |

# Iron Monthly Means

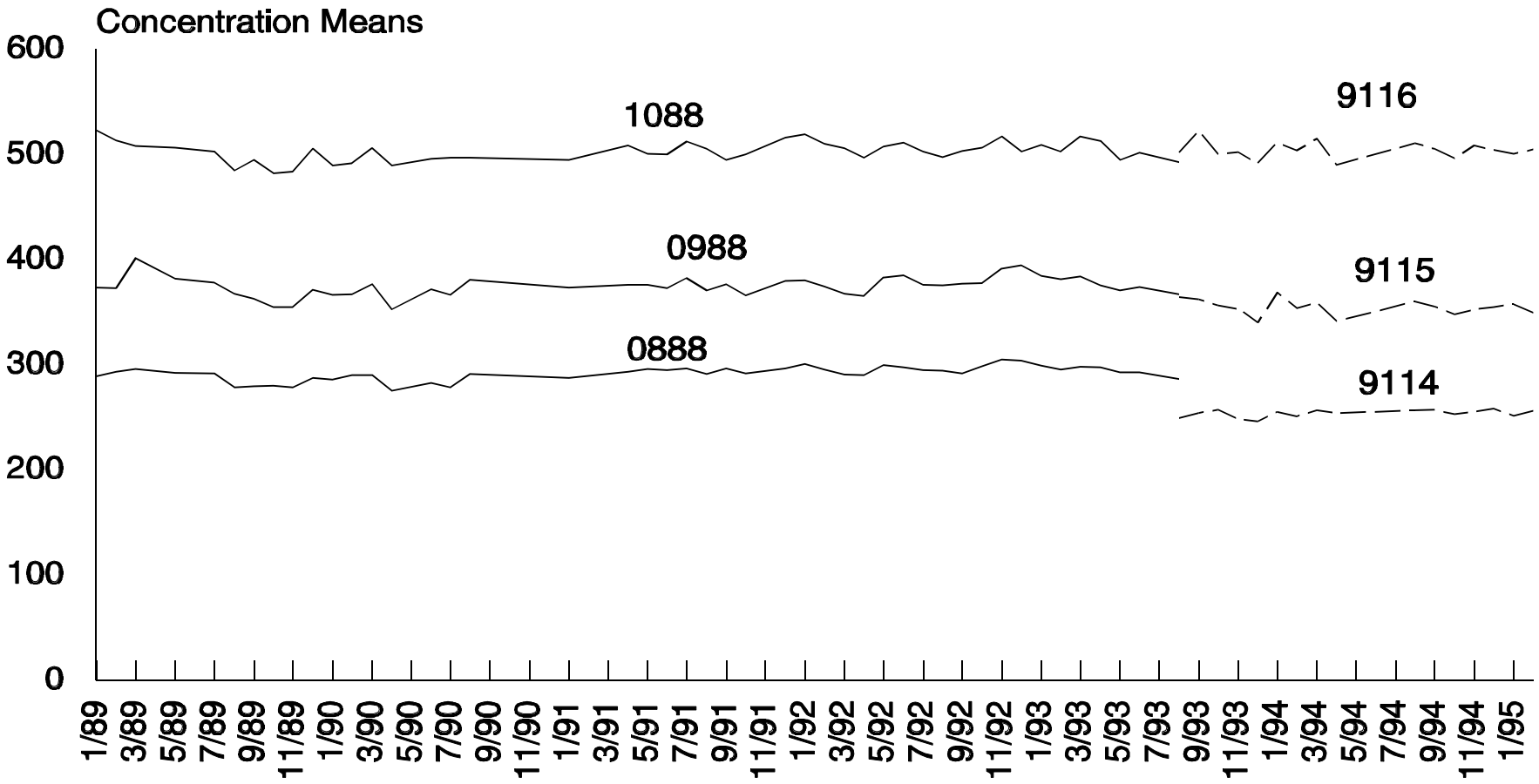



NOTE: No specimens assayed for Iron during 12/88, 1/91-2/91, 7/93, 5/94, and 9/94.
Iron and TIBC analyses were performed, alternatively, by the same analyst.

SUMMARY STATISTICS FOR
TOTAL IRON BINDING CAPACITY
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0888 | 01/89 - 08/93 | 291.876 | 11.2563 | 3.85652 | 2018 |
| 0988 | 01/89 - 08/93 | 374.207 | 14.8240 | 3.96145 | 2034 |
| 1088 | 01/89 - 08/93 | 501.890 | 17.1109 | 3.40928 | 2010 |
| 9114 | 08/93 - 02/95 | 255.249 | 6.7649 | 2.65032 | 686 |
| 9115 | 08/93 - 02/95 | 354.638 | 11.8232 | 3.33388 | 674 |
| 9116 | 08/93 - 02/95 | 504.399 | 13.2385 | 2.62461 | 685 |

# Total Iron Binding Capacity Monthly Means




NOTE: No samples assayed for Total Iron Binding Capacity during the months 4/89, 6/89, 5/90, 9/90 - 12/90, 2/91 - 3/91, 11/91, 7/93, and 5/94 - 7/94. Iron and TIBC analyses were performed, alternatively, by the same analyst.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Serum levels of vitamin A (retinol), vitamin E (α-tocopherol), retinyl esters and carotenoids are measured by isocratic high performance liquid chromatography with detection at three different wavelengths. Serum is mixed with an ethanol solution of the internal standards, retinyl butyrate and nonapreno-β-carotene. The analytes are extracted into hexane, which is removed under vacuum. The extract is redissolved in ethanol; an equal volume of acetonitrile is then added. The extract is filtered to remove insoluble material. An aliquot of the filtrate is injected onto a C18 reversed-phase column and eluted with a 50% ethanol:50% acetonitrile solution containing 100 µL of diethylamine per liter. Chromatograms at 300, 325, and 450 nm are recorded. Quantitation is accomplished by comparing the peak height of the analyte in the unknown with the peak height of a known amount of the same analyte in a standard solution. A correction based on the peak height of an internal standard is used. Retinol and the retinyl esters are compared with retinyl butyrate at 325 nm, α-tocopherol is compared with retinyl butyrate at 300 nm, and the carotenoids are compared with nonapreno-β-carotene at 450 nm.

Vitamin A deficiency is the leading cause of preventable blindness in the world and is associated with excess morbidity and mortality from infectious disease; however, it is uncommon in the United States. Excess consumption of vitamin A can cause liver damage and death. Vitamin E has extremely low toxicity; elevated serum concentrations are of concern for people receiving anticoagulant therapy. Low serum concentrations of vitamin E are of concern in neonates. A physiological requirement for the carotenoids, except as vitamin A precursors, has not been established. Excess consumption of carotenoids may cause red or orange discoloration of the skin as a result of carotenoid deposits in subcutaneous fat. Serum retinyl esters are interpretable only in specimens from fasting subjects and are used to determine if potential hepatoxicity is present in subjects with elevated serum retinol concentrations.

## 2. SAFETY PRECAUTIONS

Consider all serum samples potentially positive for infectious agents including HIV and the hepatitis B virus. Observe Universal Precautions. Wear gloves, lab coat, and protective eye-wear while handling all human blood products. We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials. Prepare aliquots of samples in a biological safety cabinet. Place disposable plastic, glass, and paper (pipet tips, autosampler cups, gloves, etc.) that contact serum in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished.

Handle solvents only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Material data safety sheets (MSDSs) for these chemicals are readily accessible through the Division LAN CD-ROM system; printed copies are filed in the Division office.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Calculation of a given run is accomplished through the Maxima software installed on the PC. After a run is complete and ready to be submitted to the supervisor, the summary table and all data files, data interchange format (DIF) files, and method files are copied to floppy disks. The original files are deleted from the hard drive when the analyst is certain that all information has been successfully copied to floppies.

   The supervisor loads the DIF files into a temporary RBASE database where the data is checked and put into the correct format and corrections are entered. When the data has been approved, it is transferred to the NHANES III RBASE data base located on the NCEH/EHLS PC network. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor copies the values to the NCHS mainframe computer with the other NHANES III data.

b. Data files are uploaded to the NCEH/EHLS PC network and then loaded on a WORM drive for storage. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. For best results, a fasting sample should be obtained, and care should be taken to avoid exposure of the serum to sunlight or other sources of ultraviolet radiation.

b. Specimens for fat-soluble vitamin analysis may be fresh or frozen serum harvested from blood collected in a red-top or royal blue-top Vacutainer brand tube by standard venipuncture procedures.

c. A 1-mL sample of serum is preferable, but a sample volume of 100 µL may be analyzed.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field are frozen, then shipped on dry ice by overnight mail. Frozen serum is stored at -70 °C. Retinol and α-tocopherol are stable for at least 5 years at -70 °C. The carotenoids are stable for 2 years at -70 °C. The stability of the retinyl esters has not been determined. Sample quality appears to degrade with successive freeze-thaw cycles, although the quantification of the analytes is unaffected.

f. Specimens generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

g. Specimens that have been through more than five freeze-thaw cycles, been refrigerated for more than 24 hours, or undergone hemolysis may give inaccurate results for one or more of the primary analytes (i.e., retinol, α-tocopherol, or β-carotene). The retinyl ester concentration of nonfasting serum is generally noninformative.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and Special Activities Branch Specimen Handling Office). The protocol discusses the collection and transport of specimens and the special equipment required. In general, serum should be transported and stored at no more than -20 °C. Samples thawed and refrozen less than five times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood or serum should be transferred into a sterile Nalge cryovial labelled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Waters HPLC system (Waters Chromatography Division, Milford, MA).
   (a) Model 590 or Model 6000 solvent delivery system.
   (b) WISP 712 auto sampler with refrigeration unit and 96-vial carriage.
   (c) TCM temperature control module with a RCM-100/Column Heater.
   (d) Model 490 absorbance detector.
   (e) Model 820 workstation (includes computer with "Maxima" software and NEC V5200 Pinwriter dot matrix printer (NEC, Boxborough, MA).

(2) Vortex mixer (American Scientific Products, McGaw Park, IL).

(3) Sorvall GLC-1 centrifuge (DuPont-Sorvall Instruments, Newton, CT).

(4) Cary 3E spectrophotometer (Varian Instruments, Palo Alto, CA).

(5) Speedvac SC200 System (Savant Instrument Co., Farmingdale, NY).

(6) Precision Model VP-190 direct drive vacuum pump (Precision Scientific Inc., Chicago, IL).

(7) Refrigerated vapor trap, model RVT-4104 (Savant Instrument Co.).

(8) Corning Model PC-351 magnetic stirrer (Corning Glassworks, Corning, NY).

(9) Waters Model U6K manual injector (Waters Chromatography Division).

(10) Digiflex automatic diluter/pipet (ICN Biomedicals, Inc., Costa Mesa, CA).

(11) SMI Digitron pipets (American SMI, Emeryville, CA).

b. **Materials**

(1) 15-cm x 4.6-mm Burdick and Jackson OD5 octadecylsilane 5-µ (C18, reversed-phase) column (Burdick and Jackson Laboratories, Muskegan, MI).

(2) Hexane, UV-grade (Burdick and Jackson Laboratories).

(3) Acetonitrile, HPLC-grade (Burdick and Jackson Laboratories).

(4) Ethanol, absolute (U.S.P.), stored in glass, (U.S. Industrial Chemical Corp., Cincinnati, OH).

(5) Chloroform, HPLC-grade (Burdick and Jackson Laboratories).

(6) Diethylamine, reagent grade (Fisher Scientific, Inc., Fairlawn, NJ).

(7) Retinol (Sigma Chemical Co., St. Louis, MO).

(8) Retinyl Palmitate (Sigma Chemical Co.).

(9) α-Tocopherol (Sigma Chemical Co.).

(10) Zeaxanthin/Lutein (Hoffman-LaRoche, Inc., Nutley, NJ).

(11) β-Cryptoxanthin (Hoffman-LaRoche, Inc.).

(12) Lycopene (Sigma Chemical Co.).

(13) α-Carotene (Sigma Chemical Co.).

(14) β-Carotene (Sigma Chemical Co.).

(15) Nonapreno-β-carotene (initially a gift of Dr. Fred Katchik, USDA, Beltsville, MD, later synthesized in-house at CDC).

(16) L-Ascorbic acid, ACS grade (Fisher Scientific, Inc.).

(17) Stearic anhydride (Sigma Chemical Co.).

(18) Oleic anhydride (Sigma Chemical Co.).

(19) Linoleic anhydride (Sigma Chemical Co.).

(20) Butyric anhydride (Sigma Chemical Co.).

(21) Triethylamine (Fisher Scientific, Inc.).

(22) Alumina, Grade III (obtained from various sources).

(23) Methanol, lab grade, (Fisher Scientific, Inc.).

(24) Argon, Ultrapure (Air Products, Inc., Chamblee, GA).

(25) 10- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY).

(26) Nitrogen (Air Products, Inc.).

(27) Red, yellow, and green pipet tips for SMI Digitron pipet (American SMI).

(28) Plastic tuberculin syringes (Becton-Dickenson, Rutherford, NJ).

(29) 0.45-µm filter, cat # SJHV004NS (Millipore Corp., Bedford, MA).

(30) 12- x 75-mm disposable glass culture tubes (Corning Glassworks).

(31) Amber autosampler vials and inserts (Sun Brokers, Wilmington, NC).

(32) Actinic glassware (obtained from various sources).

(33) 0.5-mL Nunc vials (Baxter Scientific Products, Atlanta, GA).

c. **Reagent Preparation**

(1) Mobile Phase
Pour 500 mL ethanol and 500 mL acetonitrile into a 1-L flask, add 100 µL of diethylamine (DEA) per 1 liter of solution, and stir the solution gently under vacuum for 5 min with a magnetic stirrer and stir bar.

(2) 10 g/dL Ascorbic Acid
Dissolve 5.0 g L-ascorbic acid in deionized water in a 50-mL volumetric flask and bring the volume up to 50 mL by adding water to the mark. The solution is relatively labile but normally can be used for 2 weeks. Blanket the solution with argon, seal the flask with a ground glass stopper, and store the solution at room temperature in the dark. (Solution degradation may be indicated by a brownish discoloration.)

d. **Standards Preparation**

(1) Purified Stock Solutions
Prepare stock solutions of all standards except retinyl butyrate and nonapreno-β-carotene in accordance with the following procedure:

Dissolve a small amount of a standard in chloroform that has been made alkaline by the addition of 50 µL of DEA per 250 mL of chloroform. Using a Waters U6K manual injector to minimize sample carryover, inject 2-5 µL of the chloroform solution onto a Burdick and Jackson OD5 octadecylsilane 15-cm x 4.6-mm 5-µ column and elute it with the mobile phase. Collect the central third (or "middle cut") of the peak. Dilute pooled middle cuts from several injections to approximately 10 mL with mobile phase. Measure the absorbance of the solution with the spectrophotometer, calculate the concentration, and dilute the sample with mobile phase to approximately the concentrations shown in Table 1.

Dissolve a small amount of retinyl butyrate or nonapreno-β-carotene in ethanol that has been made alkaline by the addition of 50 µL of DEA per 250 mL of ethanol. Using a Waters U6K manual injector to minimize sample carryover, inject 2-5 mL of the ethanol solution onto a Burdick and Jackson OD5 octadecylsilane 15-cm x 4.6-mm 5-µ column and elute it with ethanol containing 200 µL DEA per liter. Collect the central third of the peak. Dilute pooled middle cuts from several injections to 10 mL with ethanol. Measure the absorbance of the solution with the spectrophotometer, and dilute the solution with ethanol to obtain a solution with an absorbance of approximately 0.250 AU for retinyl butyrate at 325 nm or 0.310 AU for nonapreno-β-carotene at 450 nm.

All standard solutions are stable for at least 8 weeks when stored in 300-µL aliquots in glass vials at -70 °C.

(2) Mixed Standards
Prepare the carotene mixed standard by mixing equal volumes of zeaxanthin/lutein, β-cryptoxanthin, and

α-carotene using one volumetric pipet and rinsing the pipet with mobile phase between each standard. Prepare the retinyl ester mixed standard by mixing equal volumes of the retinyl linoleate, retinyl oleate, retinyl palmitate and retinyl stearate stock solutions using one volumetric pipet and rinsing the pipet with mobile phase between each standard. Mixed standard solutions, when stored in 300-µL aliquots in glass vials at -70 °C, are stable for at least 8 weeks. Lycopene, β-carotene, retinol, and α-tocopherol standards are single-component standards.

Prepare the internal standard solution prior to use by mixing equal volumes of C-45 and retinyl butyrate stock solutions using one volumetric pipet and rinsing the pipet with ethanol between each standard. The internal standard solution is stable for at least 6 weeks when blanketed with nitrogen and stored at -70 °C in actinic glassware.

Synthesize retinyl butyrate, retinyl linoleate, retinyl oleate, and retinyl stearate by using the following procedure:

To 0.87 mmol of retinol in a 50-mL round bottom flask, add 3 mL of triethylamine, 20 mL of n-hexane and 1.00 mmoles of the appropriate acid anhydride. Stir the reaction mixture for 3.5 hours at 60 °C, cool it, and remove the hexane and triethylamine under vacuum. Chromatograph the residue on a column of alumina grade III and isolate the ester (a colorless material with greenish-yellow fluorescence).

Table 1
Approximate Concentrations of Purified Stock Solutions

| Solution | Concentration (µg/dL) |
|---|---|
| Retinol | 50 |
| Retinyl Linoleate | 42* |
| Retinyl Oleate | 42* |
| Retinyl Palmitate | 42* |
| Retinyl Stearate | 42* |
| α-Tocopherol | 1200 |
| Lutein/Zeaxanthin | 50. |
| β-Cryptoxanthin | 50. |
| Lycopene | 20. |
| α-Carotene | 30. |
| β-Carotene | 20. |

* Concentrations of retinyl esters are reported as retinol equivalents.

e. **Preparation of Quality Control (QC) Material**

Sterile-filter all pools, aliquot them into sterile 5-mL glass Wheaton vials, blanket them with nitrogen or argon, and seal them. Store the QC pools at -70 °C.

Prepare the low QC pool by selecting and pooling serum that contains low levels of all analytes. If the levels of the analytes in the pool are above the mean level observed in normal subjects, dilute the pool with sterile saline until all of the analytes have concentrations below the mean concentrations of a normal population.

Prepare the medium quality control pool by pooling serum that contains analytes mostly at levels close to the mean levels observed in normal subjects.

Prepare the high quality control pool by pooling serum that contains higher than normal levels of most analytes. Spiking is generally successful only for retinol. (People who eat very large amounts of fresh fruits and vegetables

and have high levels of serum lipids are most likely to have high concentrations of the analytes. Type II (adult onset) diabetics without good control of the disease may have extremely high concentrations of lutein/zeaxanthin, β-cryptoxanthin, and vitamin E. Cows have serum concentrations of retinol and α-tocopherol that are within the normal range for humans, very high serum β-carotene concentrations, and virtually no other serum carotenoids. Dogs have high serum retinyl ester concentrations.)

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Before each run, prepare duplicate sets of run standards by combining 200 µL of the internal standard mix and 200 µL of a standard solution (single component or mixed) using a positive displacement pipet. After the standards are combined with an equal volume of the internal standards (IS), the true concentrations of the standards are halved. In the serum extracts, equivolume amounts of serum and IS are also combined; however, the extracts are resuspended after drying in a volume of the mobile phase equal to the original volume of the IS solution or the serum (before extraction). The ratio of standard to IS is the same as the ratio of the analyte in the serum to the IS, but the actual concentration of IS in the serum extracts is twice that of the concentration in the standard solution. To calculate the concentrations of the analytes in the serum extracts (or "unknowns"), enter the original concentration of the standard as demonstrated in Table 2 and the concentration of the IS as 1.0 for both the standards and the serum extracts.

Table 2 is an example of a worksheet used to calculate purified standard concentrations to be entered into the MAXIMA software.

Table 2.
Typical Concentrations of Standard Solutions for Various Analytes, as Well as the Wavelengths and Molar Absorptivities (in Ethanol) Used to Determine Those Concentrations.

| Analyte | Wavelength, (nm) | ϵ | Target Conc, (µmol/L) |
|---|---|---|---|
| Retinol | 325 | 53,000 | 1.750 |
| α-Tocopherol | 293 | 4,069 | 27.50 |
| Lutein/zeaxanthin | 454 | 144,482 | 0.300 |
| Cryptoxanthin | 454 | 131,012 | 0.300 |
| Lycopene | 474 | 185,185 | 0.400 |
| α-Carotene | 447 | 146,269 | 0.190 |
| β-Carotene | 454 | 137,413 | 0.350 |
| Retinyl linoleate | 325 | 53,000 | 0.330 |
| Retinyl oleate | 325 | 53,000 | 0.330 |
| Retinyl palmitate | 325 | 53,000 | 0.330 |
| Retinyl stearate | 325 | 53,000 | 0.330 |

* ϵ for the internal standards are derived from best information provided.

Beta-carotene and lycopene are prepared as separate solutions because of their tendencies to form cis-isomers as degradation products.

Read the standards as knowns (theoretical concentration) at the beginning of each run. These values are used to generate a two-point, single-concentration, linear, forced-through-zero standard curve for each analyte. Inject the standards again at the end of the run; they will be treated as unknowns by the processing software.

Transfer the standard/internal standard mixtures to microvials and place them in the WISP as shown in Table 3.

Read the standards as knowns (theoretical concentrations) at the beginning of each run. These values are used to generate a two-point, single-concentration, linear, forced-through-zero standard curve for each analyte. Inject the

standards again at the end of the run; they will be treated as unknowns by the processing software.

Reanalyze the standards as unknowns at the end of analytical runs, if space allows; they must agree within 15% of the set values.

Three times a year, NIST SRM 968a (the certificate of values is kept by the analyst) is analyzed to determine the agreement between results obtained with this method and certified values for retinol, α-tocopherol, and β-carotene. If the results by this method do not fall within the 95% confidence interval for the mean of these analytes as determined by NIST, subject specimens are not run until the system has been adjusted to produce results within the 95% confidence interval. This laboratory also participates in a proficiency testing program for retinol, α-tocopherol, and β-carotene run by NIST.

Table 3
Carousel Layout for Standards

| Standard | Vial Position |
|---|---|
| Mixed Carotenoids | 1 |
| β-Carotene | 2 |
| Lycopene | 3 |
| Vitamin A | 4 |
| Vitamin E | 5 |
| Retinyl Esters | 6 |

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Build runs on the computer as described in Section 8 Part c. Participant ID numbers may be scanned into the computer if they are barcoded.

(2) Allow frozen serum, quality control serum, and standards to reach ambient temperature.

(3) Prepare mobile phase by mixing ethanol and acetonitrile 1:1 (v/v). Add 200 µL DEA per liter of solution. De-gas under vacuum for 5 min.

(4) Turn on the HPLC system as described in the instrument set up section of this document.

b. **Sample Preparation**

(1) With the Digiflex Dilutor, dispense 10 µL of 10% L-ascorbic acid solution into a 12- x 75-mm culture tube.

(2) Using a SMI Digitron pipet with a yellow tip, add 200 µL of serum to the culture tube containing the 10% ascorbic acid solution.

(3) With the Digiflex Dilutor, add 200 µL of the internal standard solution to the tube.

(4) Seal the tube with a cork and vortex the mixture for 10-15 sec, being careful not to allow the extract to touch the cork during mixing.

(5) With the Digiflex Dilutor, add 1000 µL of hexane, reseal the tube, and vortex it for 45-60 sec, being careful not to allow the extract to touch the cork during mixing.

(6) Centrifuge the mixture for 5 min at 1500 x g.

(7) Using a Pasteur pipet, transfer the hexane (upper) layer to a 12- x 75-mm tube and dry the tube in the Speedvac without heating.

(8) Add 100 µL of ethanol to the tube containing the dried extract.

(9) Vortex the tube for 5-10 sec and let it sit for 15-30 min until the extract is dissolved.

(10) Add 100 µL of acetonitrile to the dissolved extract and vortex the tube to mix the extract.

(11) Immediately draw the extract into a tuberculin syringe, taking care to leave an air space between the plunger tip and the solution: place a 0.45-µm pore-size syringe filter on the syringe tip and filter the extract into an injection vial.

(12) Seal the vial and place it in the WISP carousel.

c. **Instrument Preparation**

(1) Turn on the WISP and refrigeration unit. Set the refrigeration unit to maintain a temperature of 4 °C.

(2) Turn on the detector and program the channels to the following settings:
Channel 1: 450 nm, 1.0 AUFS
Channel 2: 325 nm, 1.0 AUFS
Channel 3: 300 nm, 1.0 AUFS

(3) Place fresh mobile phase in the reservoir. Turn on the pump. Set the pump to external control.

(4) Turn on the temperature control module and column oven and program the module to maintain a temperature of 29 °C. (Ideally, this should be done 24 hours before the run to allow time for the column to equilibrate at the set temperature.)

(5) Turn on the SIM box.

(6) Turn on the computer and access the MAXIMA program.

(7) Press the F6 key to access the "Monitor Control Events" screen. Set the flow rate to 0.9 mL/min with a time of 0.25 min and start the routine. When the routine is concluded, exit to the main screen.

(8) Load a method that contains the following parameters:
Acquisition Parameters
Run time: 12.0 min
Sampling frequency: 2.0 points per sec
Flow rate: 0.9 ml/min
Channel 1: 450 nm
Channel 2: 325 nm
Channel 3: 300 nm
Trigger: SIM

Integration Parameters
Baseline points: 10
Filter window (in points): 9 or 5
Integration sensitivity (coarse): 5.500 microvolts/sec
Integration sensitivity (fine): 8.000 microvolts/sec$^2$
Detect negative peaks: yes
Skim ratio: 8.000
Minimum area: 500.0 microvolts-sec
Minimum height: 25.0 microvolt
Minimum width: 7.0 sec

(The integration parameters will vary with lamp age, column type and age, and other factors. The parameters

cited above are acceptable for use as a starting point from which to optimize the conditions.)

In the component table, all components are calibrated on the basis of peak height, without weighting, by using a linear curve forced through the origin. The component table should contain at least the information in Table 4. Because the retention times will vary with the age of the column, from column to column, and from instrument to instrument, the times in the table should be used only as a guide. Actual retention times for a given column/instrument combination should be determined individually and monitored on a regular basis, and the component table should be updated when necessary. Some components have very similar retention times and their order of elution may change, but, if they are on different detectors, the elution order on Table 4 is not changed. Instead, the windows are expanded to include the true retention time. Enter a manual response factor for c-BETA that is equal to the calculated response factor for β-carotene under the same chromatographic conditions.

Table 4
Component Table Information

| Component Name | Retention time | Window | Detector | Quan-titate | Internal Standard |
|---|---|---|---|---|---|
| LUZE | 2.35 | 0.20 | 1 | Yes | C-45 |
| VITA | 2.50 | 0.20 | 2 | Yes | RB325 |
| UNK1* | 2.72 | 0.20 | 1 | No | C-45 |
| RB300 | 2.95 | 0.30 | 3 | Int | -- |
| RB325 | 2.95 | 0.30 | 2 | Int | -- |
| UNK2* | 3.22 | 0.20 | 1 | No | C-45 |
| CRYP | 4.00 | 0.35 | 1 | Yes | C-45 |
| VITE | 4.37 | 0.45 | 3 | Yes | RB300 |
| RETEL | 5.16 | 0.40 | 2 | Yes | RB325 |
| LYCO | 5.17 | 0.40 | 1 | Yes | C-45 |
| ALPH | 7.53 | 0.40 | 1 | Yes | C-45 |
| RETEO | 7.86 | 0.30 | 2 | Yes | RB325 |
| BETA | 7.96 | 0.40 | 1 | Yes | C-45 |
| RETEP | 8.32 | 0.25 | 2 | Yes | RB325 |
| c-BETA | 8.47 | 0.40 | 1 | No | C-45 |
| C-45 | 9.17 | 0.50 | 1 | Int | -- |
| RETES | 10.59 | 0.35 | 2 | Yes | RB325 |

* Unknowns 1 and 2 are thought to be 2,2',3'-anhydrolutein and a carotenoid peak with the same retention time as canthaxanthin but containing additional caratenoids. These peaks are identified but are not quantitated. This method also detects γ-, δ-, and α-cryptoxanthin.

(9) Edit the sample queue so that all of the samples are correctly identified and the appropriate vial numbers entered. Enter the concentration for the internal standards as 1.00 for all samples. All samples have a status of "ACQU." Enter the correct concentrations for the components in the standards.

(10) Save the sample queue and the method.

(11) Press the F3 key to access the EXECUTE CURRENT METHOD screen. Place an "X" in the left column of the sample queue by the samples to be analyzed.

(12) Start the run by using the mouse to select the box labeled START. The relationship between peak height and concentration is linear from zero to 10 X the high-normal range for all analytes.

d. **Maintenance**

(1) Speedvac
Maintain the trap temperature (-110 °C) and check the temperature daily before turning on the vacuum pump. Empty the vapor trap of the Speedvac when full. Change the vacuum pump oil when the quality of the vacuum deteriorates or the oil becomes turbid.

(2) HPLC System
Put the WISP through a purge cycle before every run. Defrost the refrigeration unit after each run. Check the pump seals monthly and change them if necessary. Check the needle wash frit of the auto sampler monthly. Check the inlet and outlet check valves of the pump when the pump seals are changed and rebuild them if they show signs of wear or are dirty.

e. **Calculations**

The concentration of the components of the mixed standards is equal to the concentration of the stock divided by the number of components of the solution, excluding the internal standards.

Example:

In a mixed standard containing lutein/zeaxanthin, β-cryptoxanthin, and α-carotene (three components), if the concentration of lutein/zeaxanthin in the stock solution is 103.2 µg/dL, then the concentration of lutein/zeaxanthin in the mixed standard is 103.2 µg/dL ÷ 3 or 34.4 µg/dL. Enter this concentration in the concentration table for lutein/zeaxanthin even though the actual concentration of lutein/zeaxanthin in the mixed standard is half of this after the addition of the internal standards.

All calculations are performed by the computer. Calibration curves are linear, forced through zero, and calculated on the basis of a duplicate analysis of one standard concentration. For each analyte not present in a standard solution, enter a response factor and calculate the concentration using the following formula:

*Concentration = Response Factor x Peak Height*

f. **CDC Modifications**

This method is based on the method described by Sowell et al. (1). It has been modified to include the use of a bench quality control pool containing extremely low concentrations of the analytes in order to assess the performance of the assay in studies in which the subjects are at high risk for vitamin A deficiency.

## 9. REPORTABLE RANGE OF RESULTS

This method is linear for the carotenoids in the range 1-150 µg/dL, for retinol and the retinyl esters in the range 1-150 µg/dL, and for α-tocopherol in the range 100-5000 µg/dL.

## 10. QUALITY CONTROL (QC) PROCEDURES

a. **Bench Controls**
Three serum pools are normally used as bench controls. These controls represent high, medium, and low levels of the analytes in serum. For pediatric studies or studies in which vitamin A deficiency is suspected, an additional pool with a vitamin A concentration in the deficient range and extremely low concentrations of the other analytes is also included. Extract duplicates of these pools as you would patient samples and analyze them as part of each run.

With a multianalyte profile such as the fat-soluble vitamins, initial assessment of quality control acceptability is based on the results of the higher priority analytes: vitamin A, vitamin E, and β-carotene. Lowest priority is assigned to the retinyl esters other than palmitate, α-carotene, and β-cryptoxanthin because of their normally low concentrations. Check the results from the pools after each run, and repeat the run if, for either vitamin A, vitamin E, or β-carotene, two controls are outside the 95% limits or one or more controls are outside the 99% limits. Also

repeat the run if, for any combination of those three analytes, four controls are outside the 95% limits. The initial limits are established from the results of analyzing pool material in 20 consecutive runs and then are updated annually.

Repeat the run of samples whose values for vitamin A, vitamin E, or β-carotene are outside of the normal range; of any samples in which two or more of the other carotenoids are outside the reference range; and of any samples in which the retinyl palmitate concentration is zero.

b. **Blind Controls**
In every batch of 20 specimens analyzed, either one low- or one high-concentration blind quality control pool is inserted. The blind pools are prepared in a similar fashion to the bench quality control pools but are aliquoted and labelled in the same way as the NHANES III specimens. The blind QC samples are inserted into the specimen batches by Serum Bank personnel when the specimens are received from the field and racked for analysis.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. Check to make sure that the hardware is functioning properly and that the pump is operating at the appropriate pressure with steady delivery. Check the WISP to make sure the injections are being made as programmed.

b. Look for extraction errors. These can be determined by characteristic abnormalities of the carotenoid profile, such as altered peak shapes, reduced peak height, or increased concentrations of the lesser carotenoids (which indicates that the sample has been contaminated with the aqueous layer of extract).

c. Reanalyze NIST standards to determine the accuracy and precision of the system.

If the steps outlined above do not result in correction of the "out of control" values for QC materials, the supervisor should be consulted for other appropriate corrective actions. No analytical results should be reported for runs not in statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Phytofluene, a UV-absorbing carotenoid found in tomatoes, coelutes with retinyl palmitate and has been found in some serum samples. The 300-nm chromatogram of serum samples that appear to have elevated retinyl palmitate levels without elevated levels of the other retinyl esters should be examined to determine if there is coelution. If the peak maxima are at the same retention time, recalculate the concentration on the basis of the 300 nm chromatogram.

b. Under conditions in which the retention times for β-carotene and retinyl oleate are similar and the β-carotene level is elevated, the carotenoid absorption at 325 nm may cause overestimation of the retinyl oleate level.

c. The xanthophylls (lutein, zeaxanthin, and cryptoxanthin) are considerably more hydrophilic than the carotenes and therefore are more completely extracted than the later-eluting carotenoids (α- and β-carotene), including the internal standard, nonapreno-β-carotene. This can lead to overestimation of the level of xanthophylls in lipemic specimens.

d. Because large losses of serum carotenoids can occur during filtration as pools are prepared, we recommend that pools be analyzed after sterile filtration to ensure that the carotenoid concentrations remain within the desired ranges. When possible, use hydrophilic filters to minimize loss of the carotenoids and vitamin E during filtration. If the levels have become unacceptablly low and spiking needs to be done, the spiked serum must mix overnight before the final filtration step.

e. Ideally, the column oven should be allowed to warm up for 24 hours to allow the column to stabilize. The refrigeration unit needs approximately 45 min to stabilize. The lamp should have 1 hour to stabilize. The column should be under flow for at least 20 min before the first injection is made. In actual practice, the system is only turned completely off if it will be idle for more than 3 days, except for the lamp, which is turned off when not in use. The pump is left on with a flow rate of 0.0 mL/min.

f. The following substitutions may be made for the specified instrumentation:

(1) Instead of being dried with a Speedvac system, the hexane extract may be dried under a stream of nitrogen without heating. This process will be slower and less reproducible, however.

(2) Instead of the Waters 820 workstation, two Waters 730 data modules may be used by setting the detector to 0.01 AUFS at 450 nm, 0.05 AUFS at 325 nm, and 0.02 AUFS at 300 nm and connecting channel 1 to pen 1 and channel 2 to pen 2 of one data module and channel 3 to pen 1 of the second data module. When this is done, it is necessary to manually measure the peak heights and calculate the concentrations and it is not possible to measure normal levels of retinyl esters.

g. All of the HPLC equipment is attached to 2 KUA power-line conditioners to minimize the effects of fluctuations of electrical current.

**13. REFERENCE RANGES (NORMAL VALUES)**

Reference ranges have not been established for retinyl esters in serum. We are using the values in Table 5 for the reference ranges for retinol, α-tocopherol, and the carotenoids. These values are approximately based on the 1st-99th percentile ranges for 8284 specimens analyzed during the first half of NHANES III.

Table 5
A/E/Carotene Reference Ranges
(based on preliminary data from NHANES III)

| Analyte | Concentration (µg/dL) |
|---|---|
| Vitamin A | 25-115 |
| Vitamin E | 500-2650 |
| β-Carotene | 2-80 |
| Lutein/Zeaxanthin | 5-65 |
| β-Cryptoxanthin | 2-40 |
| Lycopene | 3-55 |
| α-Carotene | 1-15 |
| Retinyl Palmitate | 0-15 |

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX by the supervisor of any vitamin A result that is <10 µg/dL or of any vitamin A/retinyl ester profile that suggests hypervitaminosis A with hepatotoxicity (i.e. fasting serum with retinol elevated for the donor's age or sex and total retinyl esters ≥ 50% of serum retinoids). Copies of FAXes sent concerning abnormal results are kept in a notebook by the supervisor for the duration of the study.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, the prepared samples are placed in the WISP at 4 °C. The unused portion of the patient specimen is returned to the freezer.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Because the analysis of serum for fat-soluble vitamins is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, we recommend that the extracted specimens be stored at ≤-20 °C until the analytical system is restored to functionality.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

The collaborating agency with access to patient identifiers or the responsible medical officer is notified by FAX of any vitamin A result that is <10 µg/dL or of any vitamin A/retinyl ester profile that suggests hypervitaminosis A as determined by the supervisor. Test results that are not abnormal are reported to the collaborating agency at a frequency and by a

method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an ASCII text file, either through electronic mail or on a diskette. For NHANES III, all data are reported at the end of each stand as part of the NHANES III physician's report, as well as being reported on periodic data tapes sent to the NCHS data center. For some smaller studies, hard copies of a data report are sent.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

RBASE and the CDC mainframe are used to keep records and track specimens. Records, including related QA/QC data are maintained for 10 years after completion of a study. Only numerical identifiers (e.g., case ID numbers) should be used.

**REFERENCES**

1. Sowell AL, Huff DL, Yeager PR, Caudill SP, Gunter EW. Retinol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, trans-β-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. Clin Chem 1994; 40(3):411-6.

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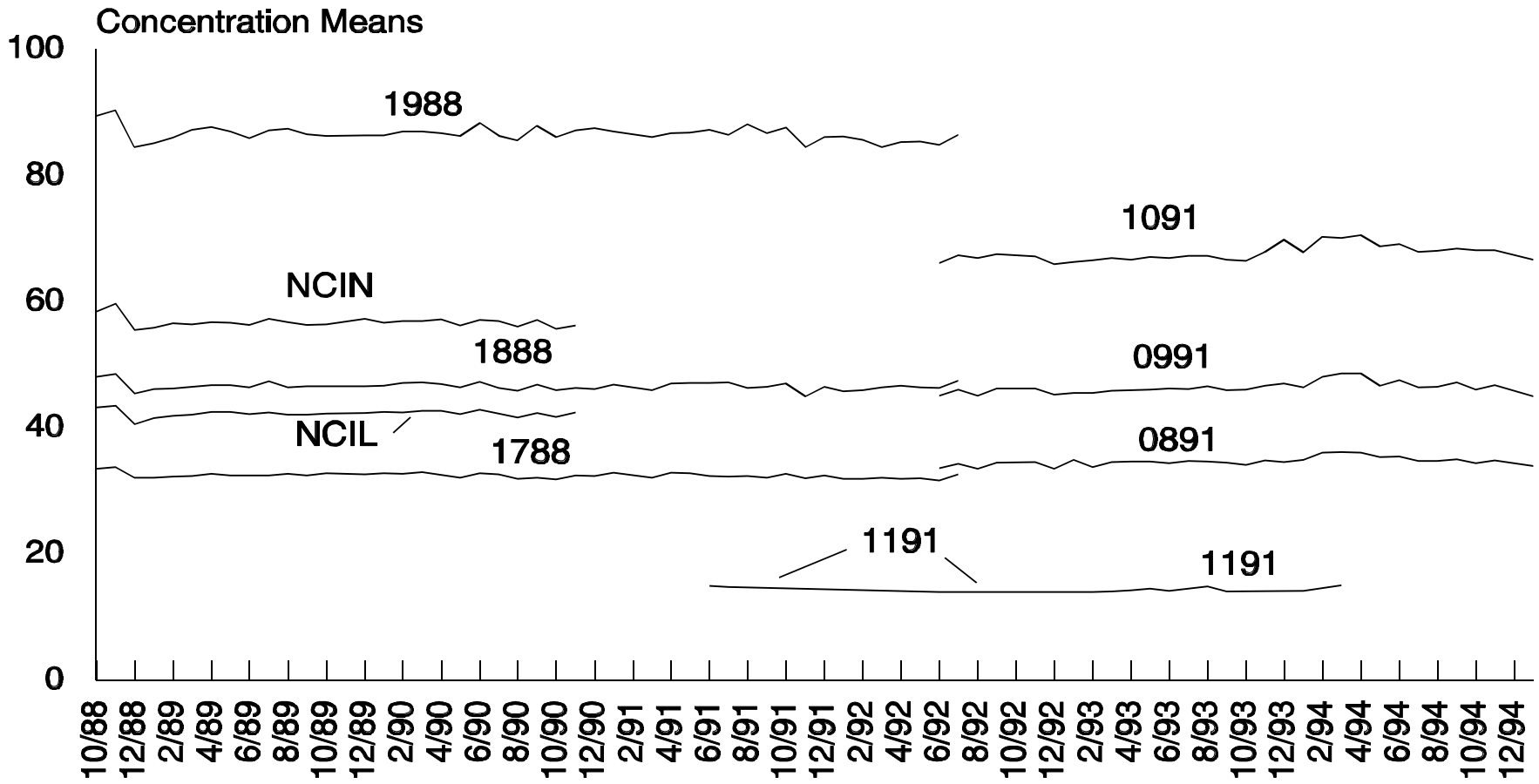
SUMMARY STATISTICS FOR
VITAMIN A
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 34.8848 | 1.00993 | 2.89505 | 420 |
| 0991 | 06/92 - 01/95 | 46.5913 | 1.41040 | 3.02717 | 424 |
| 1091 | 06/92 - 01/95 | 67.7998 | 1.98756 | 2.93151 | 424 |
| 1191 | 06/91 - 03/94 | 14.3786 | 0.50514 | 3.51316 | 56 |
| 1788 | 10/88 - 07/92 | 32.5054 | 0.82872 | 2.54948 | 1010 |
| 1888 | 10/88 - 07/92 | 46.6760 | 1.25909 | 2.69751 | 1010 |
| 1988 | 10/88 - 07/92 | 86.5847 | 2.04539 | 2.36230 | 1010 |
| NCIL | 10/88 - 11/90 | 42.3448 | 1.04390 | 2.46524 | 718 |
| NCIN | 10/88 - 11/90 | 56.7052 | 1.42766 | 2.51770 | 716 |

# Vitamin A Monthly Means

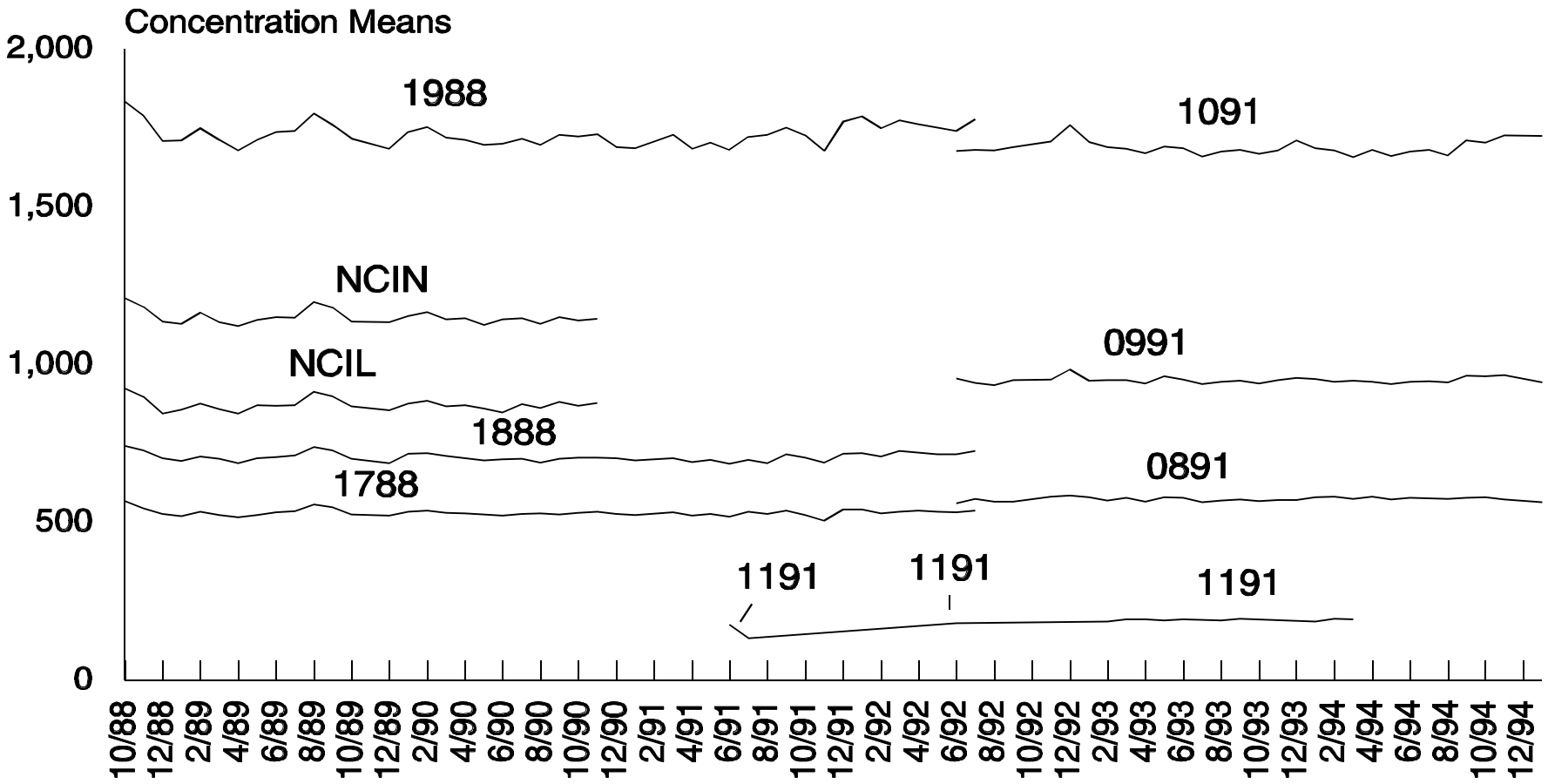



NOTE: No specimens assayed for Pool 1191 from 8/91-5/92, 7/92-1/93, and 10/93-12/93.
No specimens assayed for Vitamin A during 11/89, 2/91, 10/92, and 12/94.

SUMMARY STATISTICS FOR
VITAMIN E
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 575.75 | 15.5170 | 2.6951 | 422 |
| 0991 | 06/92 - 01/95 | 950.22 | 20.6993 | 2.1784 | 426 |
| 1091 | 06/92 - 01/95 | 1683.33 | 36.8712 | 2.1904 | 426 |
| 1191 | 06/91 - 03/94 | 189.89 | 8.0913 | 4.2610 | 55 |
| 1788 | 10/88 - 07/92 | 531.40 | 16.9183 | 3.1837 | 1008 |
| 1888 | 10/88 - 07/92 | 707.36 | 20.4822 | 2.8956 | 1008 |
| 1988 | 10/88 - 07/92 | 1725.62 | 48.5387 | 2.8128 | 998 |
| NCIL | 10/88 - 11/90 | 870.85 | 26.8473 | 3.0829 | 716 |
| NCIN | 10/88 - 11/90 | 1146.40 | 33.0420 | 2.8822 | 714 |

# Vitamin E Monthly Means

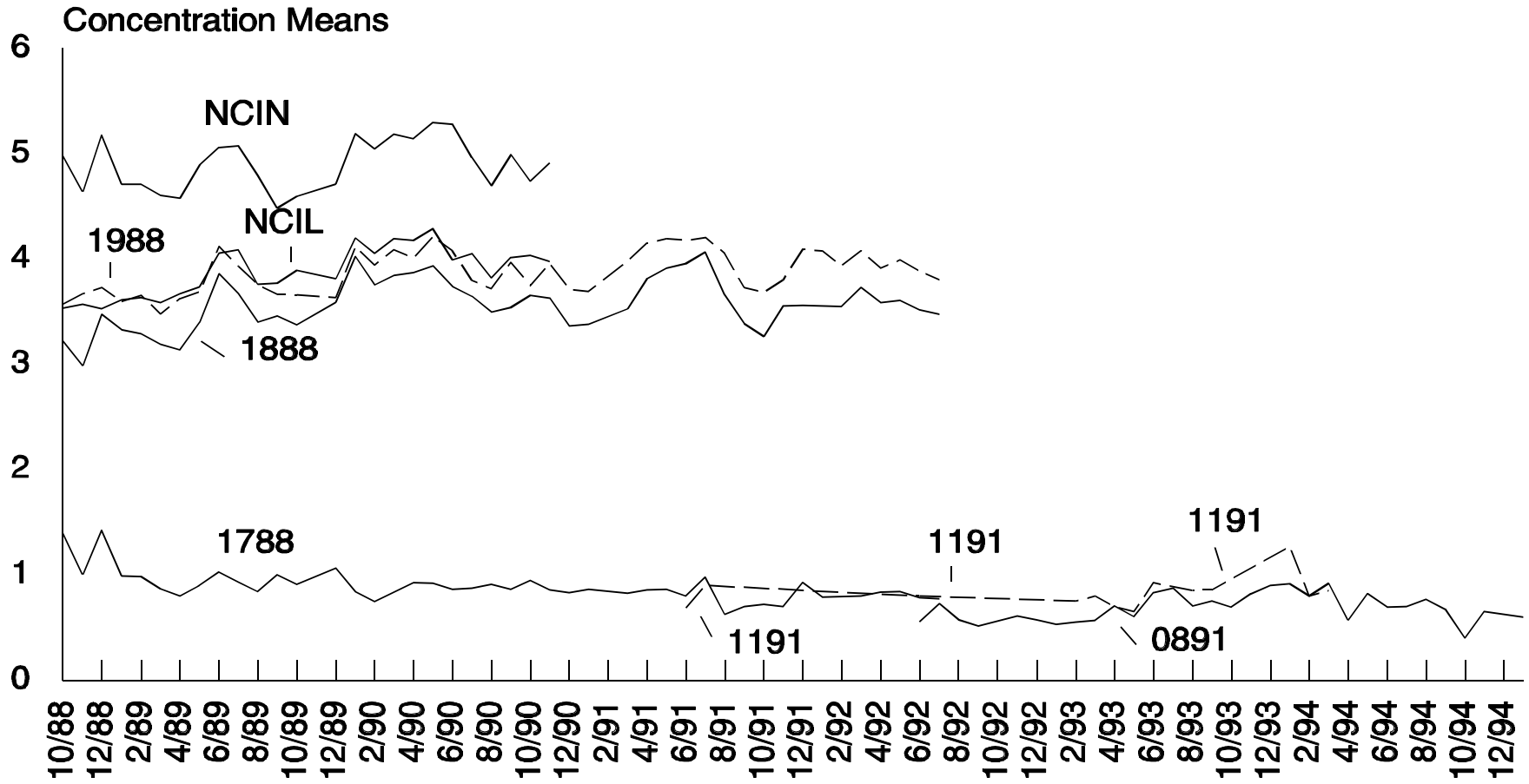



NOTE: No specimens assayed for Pool 1191 during 8/91-5/92, 7/92-1/93, and 10/93-12/93.
No specimens assayed for Vitamin E during 11/89, 2/91, 10/92, and 12/94.

SUMMARY STATISTICS FOR
ALPHA CAROTENE (ALL POOLS)
BY POOL

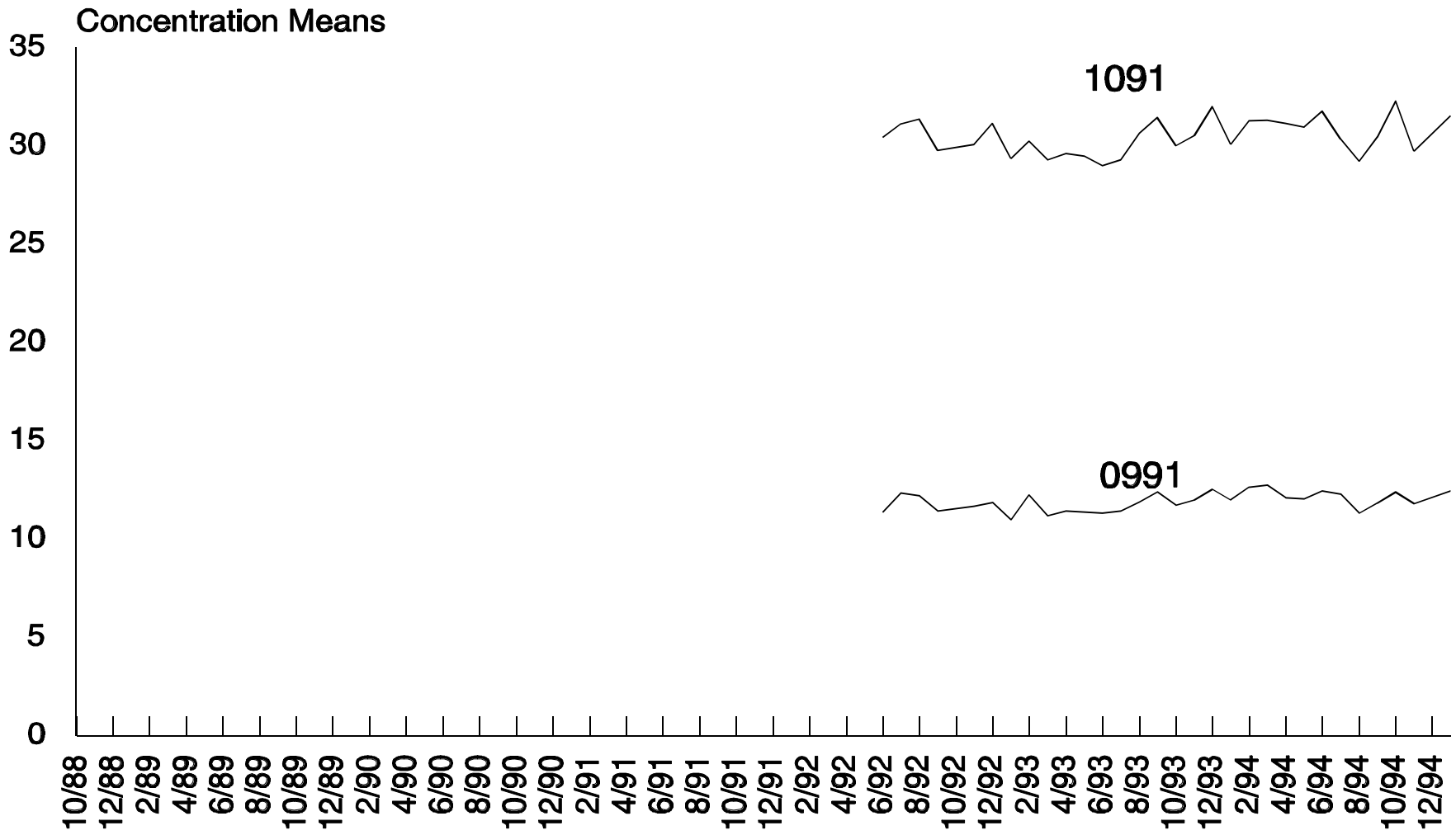
| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 0.7100 | 0.23182 | 32.6512 | 420 |
| 0991 | 06/92 - 01/95 | 11.8502 | 0.90583 | 7.6439 | 424 |
| 1091 | 06/92 - 01/95 | 30.3057 | 1.93864 | 6.3969 | 424 |
| 1191 | 06/91 - 01/95 | 0.9054 | 0.27264 | 30.1145 | 56 |
| 1788 | 10/88 - 07/92 | 0.8825 | 0.19112 | 21.6561 | 1002 |
| 1888 | 10/88 - 07/92 | 3.6226 | 0.38980 | 10.7601 | 1004 |
| 1988 | 10/88 - 07/92 | 3.9028 | 0.35713 | 9.1507 | 994 |
| NCIL | 10/88 - 11/90 | 3.9637 | 0.37488 | 9.4578 | 708 |
| NCIN | 10/88 - 11/90 | 4.9441 | 0.45683 | 9.2399 | 714 |

# Alpha Carotene Monthly Means - Low Pools




NOTE: No analysis for Pool 1191 for 8/91-5/92, 7/92-1/93, and 10/93-12/93.
No analysis for any pools during 11/89, 2/91, 10/92, and 12/94.

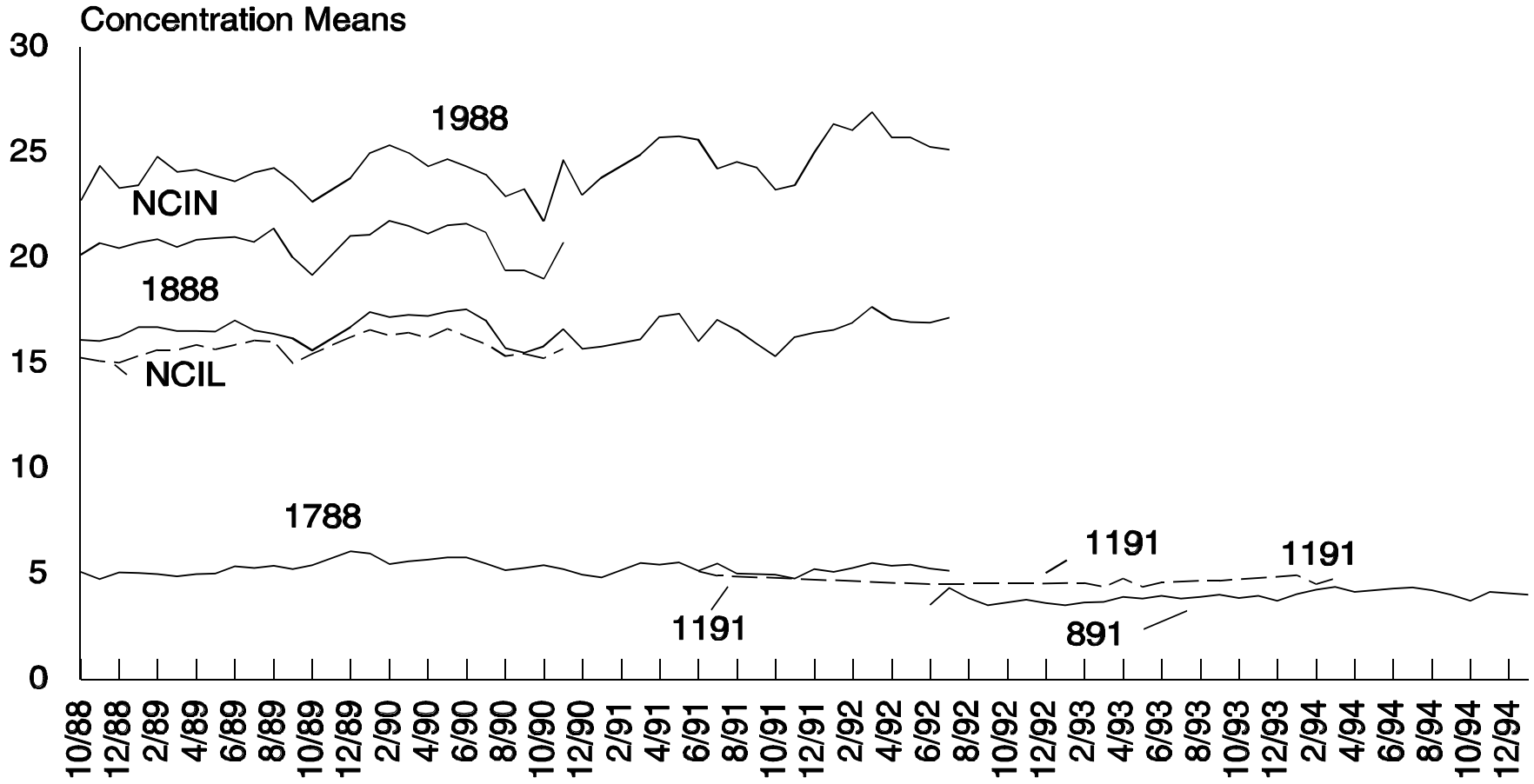
# Alpha Carotene Monthly Means - High Pools



SUMMARY STATISTICS FOR
BETA CAROTENE (ALL POOLS)
BY POOL

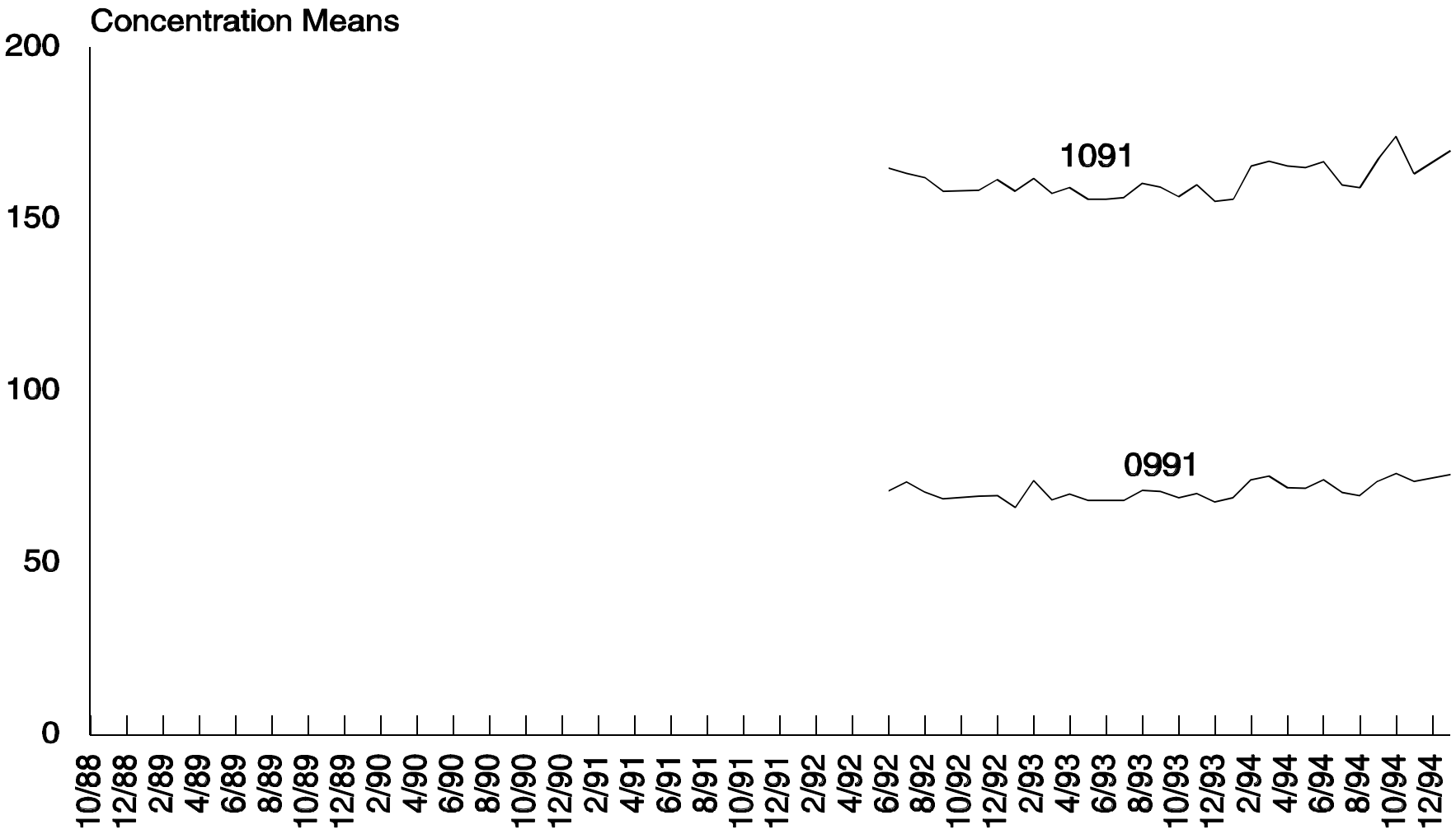
| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 4.007 | 0.40267 | 10.0489 | 420 |
| 0991 | 06/92 - 01/95 | 70.675 | 4.34409 | 6.1466 | 424 |
| 1091 | 06/92 - 01/95 | 160.560 | 9.11324 | 5.6759 | 424 |
| 1191 | 06/91 - 01/95 | 4.795 | 0.30595 | 6.3811 | 56 |
| 1788 | 10/88 - 07/92 | 5.401 | 0.47246 | 8.7472 | 1004 |
| 1888 | 10/88 - 07/92 | 16.799 | 1.08819 | 8.4779 | 1004 |
| 1988 | 10/88 - 07/92 | 24.453 | 1.70845 | 6.9867 | 994 |
| NCIL | 10/88 - 11/90 | 15.980 | 1.01280 | 6.3378 | 712 |
| NCIN | 10/88 - 11/90 | 20.926 | 1.54833 | 7.3991 | 710 |

# Beta Carotene Monthly Means - Low Pools




NOTE: No assay for pool 1191 during the periods 8/91 - 5/92, 7/92 - 1/93, and 10/93 - 12/93.
No assay for any pools during the months 11/89, 2/91, 10/92, and 12/94.

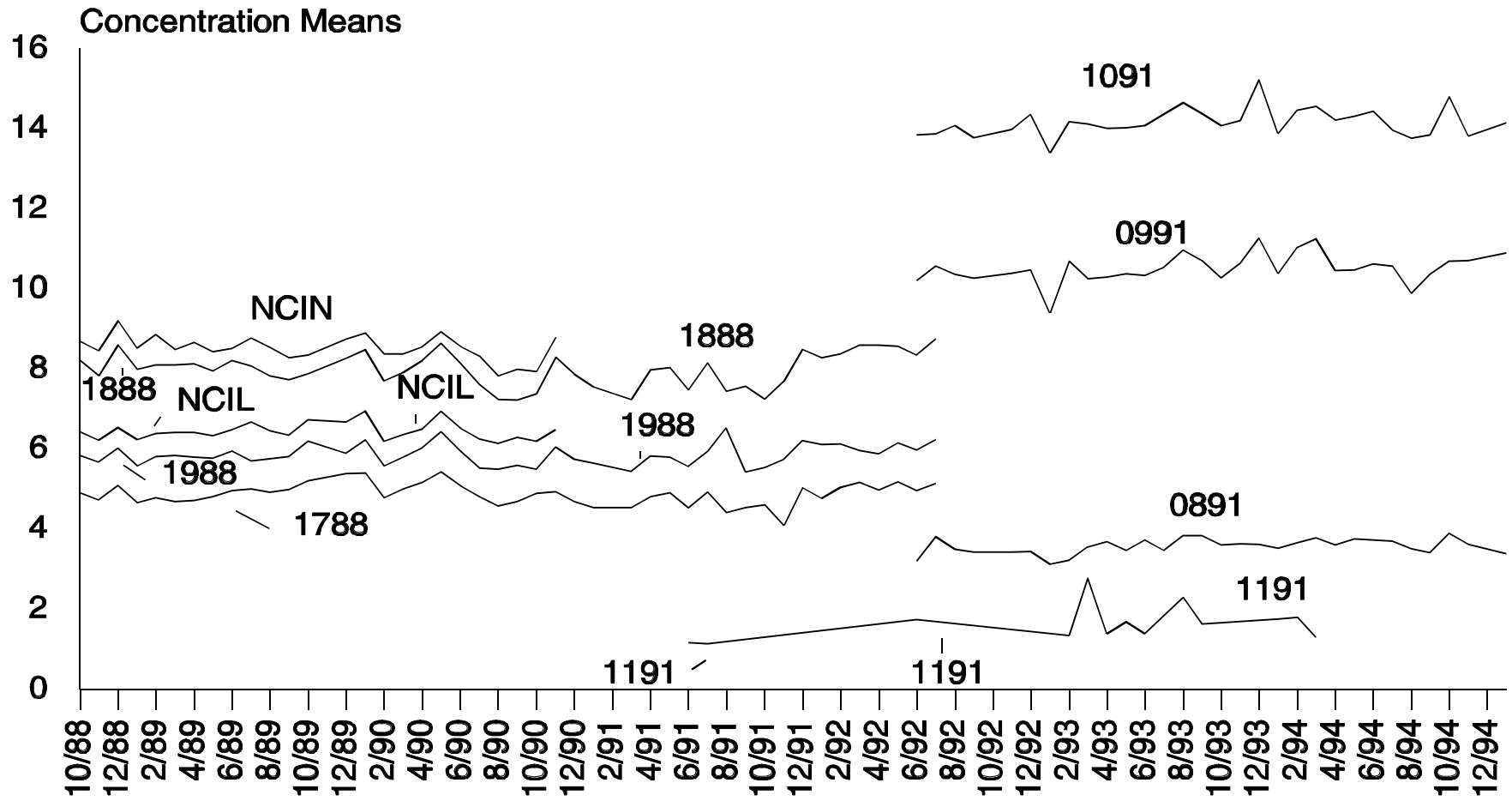
# Beta Carotene Monthly Means - High Pools



SUMMARY STATISTICS FOR
CRYPTOXANTHIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 3.6174 | 0.34601 | 9.5651 | 420 |
| 0991 | 06/92 - 01/95 | 10.4913 | 0.80395 | 7.6630 | 424 |
| 1091 | 06/92 - 01/95 | 14.1382 | 0.93197 | 6.5918 | 424 |
| 1191 | 06/91 - 01/95 | 1.6036 | 0.47212 | 29.4417 | 56 |
| 1788 | 10/88 - 07/92 | 4.9445 | 0.43361 | 8.7694 | 1008 |
| 1888 | 10/88 - 07/92 | 8.0478 | 0.70363 | 8.7431 | 1008 |
| 1988 | 10/88 - 07/92 | 5.8661 | 0.51782 | 8.8273 | 998 |
| NCIL | 10/88 - 11/90 | 6.4478 | 0.51360 | 7.9656 | 716 |
| NCIN | 10/88 - 11/90 | 8.5112 | 0.78869 | 9.2665 | 714 |

# Cryptoxanthin Monthly Means

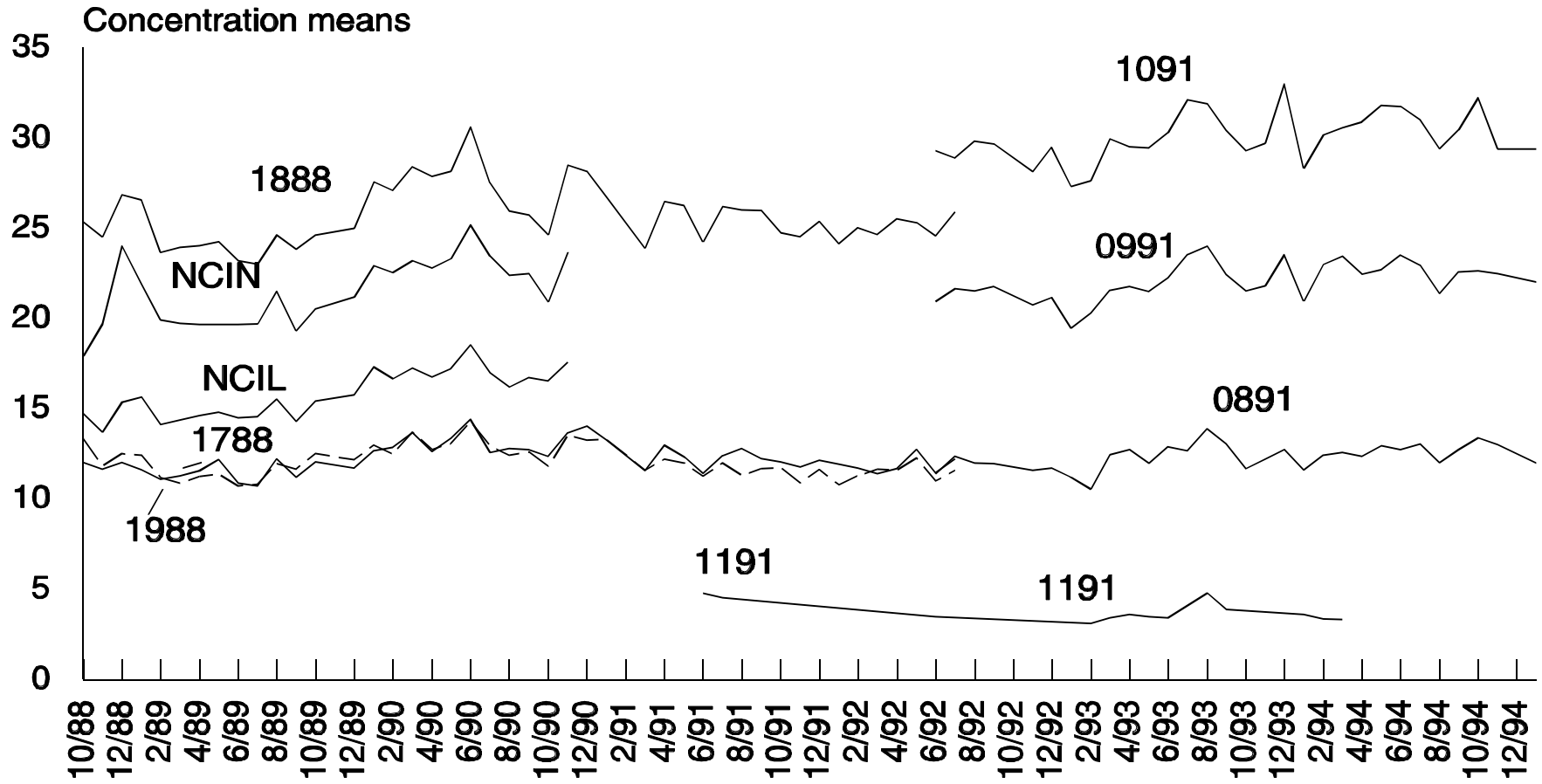



NOTE: No samples in any pools assayed for Cryptoxanthin during 11/89, 2/91, 10/92, and 12/94.
No samples assayed in pool 1191 during the periods 8/91-5/92, and 7/92-1/93.

SUMMARY STATISTICS FOR
LUTEIN/ZEAXANTHIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 12.4274 | 1.36701 | 11.0000 | 420 |
| 0991 | 06/92 - 01/95 | 22.0627 | 2.00226 | 9.0753 | 424 |
| 1091 | 06/92 - 01/95 | 29.9483 | 2.55306 | 8.5249 | 424 |
| 1191 | 06/91 - 03/94 | 3.8625 | 0.61009 | 15.7951 | 56 |
| 1788 | 10/88 - 07/92 | 12.3036 | 1.42523 | 11.5839 | 1004 |
| 1888 | 10/88 - 07/92 | 26.1859 | 2.72970 | 10.4243 | 1006 |
| 1988 | 10/88 - 07/92 | 12.4423 | 1.46179 | 11.7485 | 992 |
| NCIL | 10/88 - 11/90 | 16.1493 | 1.74717 | 10.8189 | 714 |
| NCIN | 10/88 - 11/90 | 21.8746 | 2.48995 | 11.3289 | 712 |

# Lutein/Zeaxanthin Monthly Means

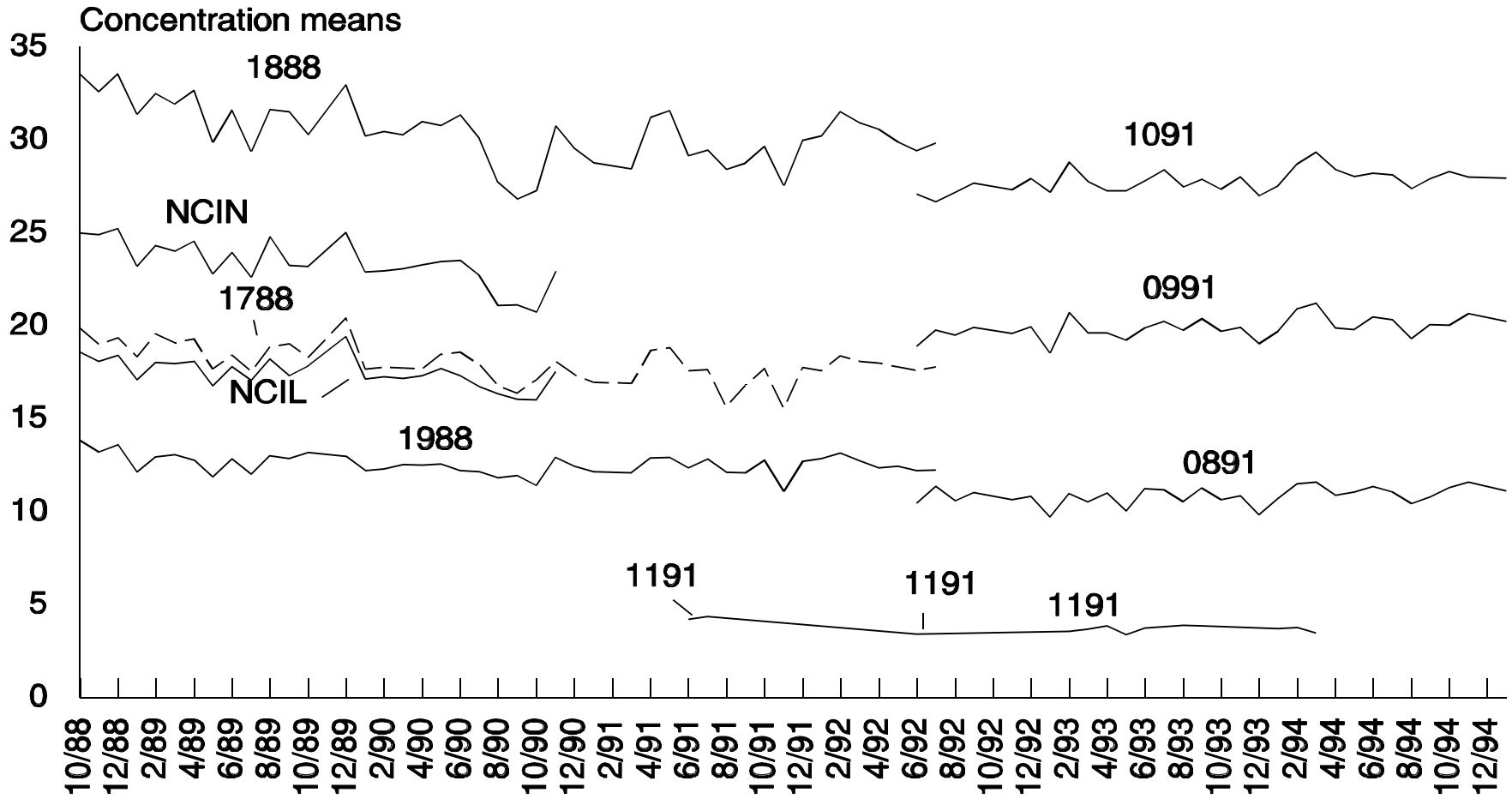



NOTE: No specimens assayed for Pool 1191 during 8/91-5/92, 7/92-1/93, and 10/93-12/93.
No specimens assayed for Lutein/Zeaxanthin during 11/89, 2/91, 10/92, and 12/94.

SUMMARY STATISTICS FOR
LYCOPENE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 10.8938 | 0.90846 | 8.3393 | 420 |
| 0991 | 06/92 - 01/95 | 19.9130 | 1.27870 | 6.4215 | 424 |
| 1091 | 06/92 - 01/95 | 27.7913 | 1.65316 | 5.9485 | 424 |
| 1191 | 06/91 - 03/94 | 3.8446 | 0.33786 | 8.7879 | 56 |
| 1788 | 10/88 - 07/92 | 18.1116 | 1.45187 | 8.0162 | 1000 |
| 1888 | 10/88 - 07/92 | 30.5718 | 2.37284 | 7.7615 | 998 |
| 1988 | 10/88 - 07/92 | 12.5280 | 0.90733 | 7.2424 | 990 |
| NCIL | 10/88 - 11/90 | 17.3663 | 1.34047 | 7.7188 | 710 |
| NCIN | 10/88 - 11/90 | 23.1910 | 1.96893 | 8.4901 | 708 |

# Lycopene Monthly Means

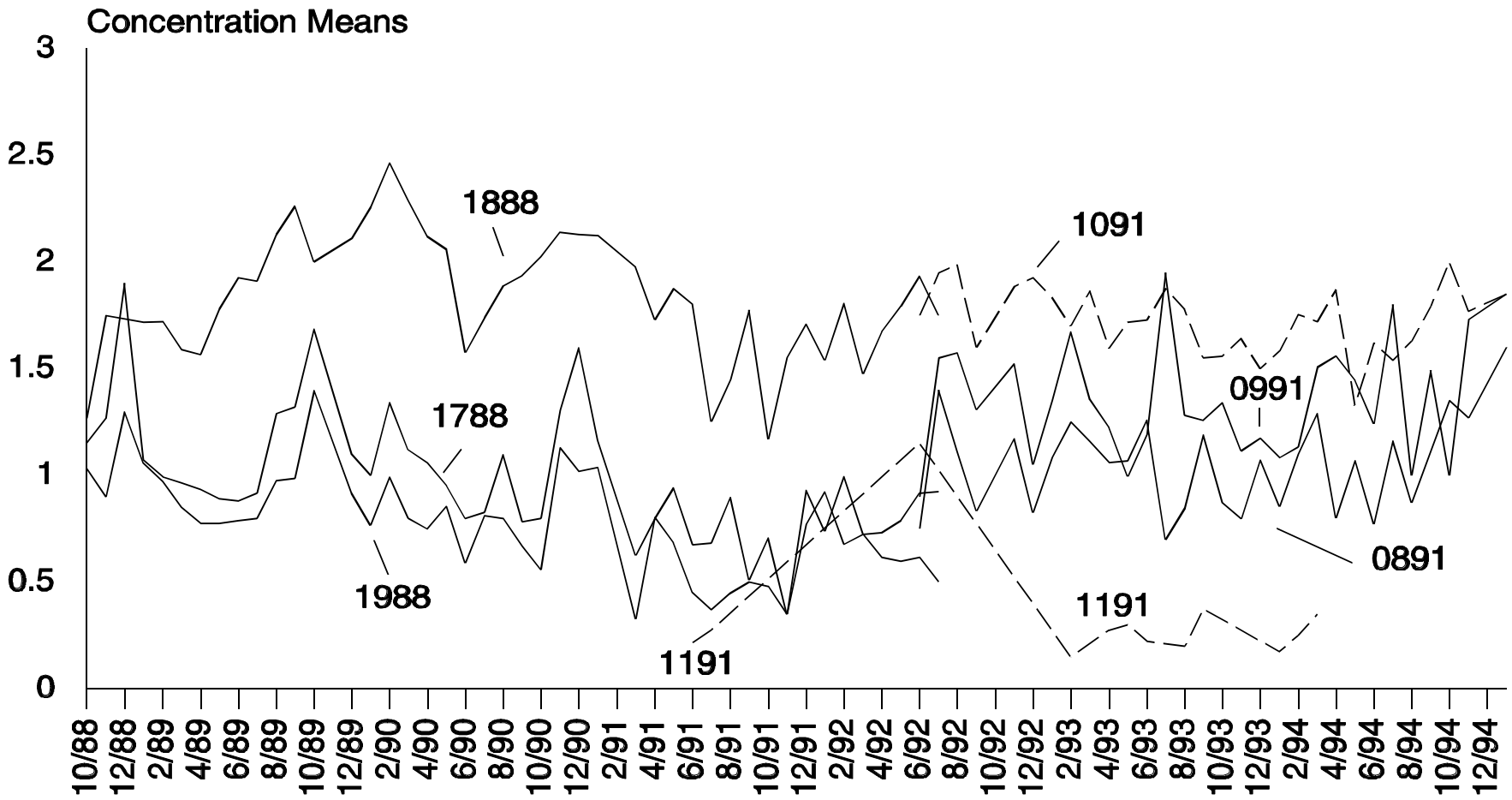



NOTE: No specimens assayed for Lycopene during the months 11/89, 2/91, 10/92, and 12/94.
No specimens assayed for Pool 1191 during 8/91-5/92, 7/92-1/93, and 10/93-12/93.

SUMMARY STATISTICS FOR
RETINYL LINOLEATE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 1.03261 | 0.57664 | 55.8434 | 414 |
| 0991 | 06/92 - 01/95 | 1.30913 | 0.61813 | 47.2167 | 416 |
| 1091 | 06/92 - 01/95 | 1.69619 | 0.59113 | 34.8505 | 420 |
| 1191 | 06/91 - 03/94 | 0.29565 | 0.22207 | 75.1111 | 46 |
| 1788 | 10/88 - 07/92 | 1.00998 | 0.43548 | 43.1174 | 962 |
| 1888 | 10/88 - 07/92 | 1.92427 | 0.54632 | 28.3912 | 956 |
| 1988 | 10/88 - 07/92 | 0.80977 | 0.36585 | 45.1798 | 962 |

# Retinyl Linoleate Monthly Means

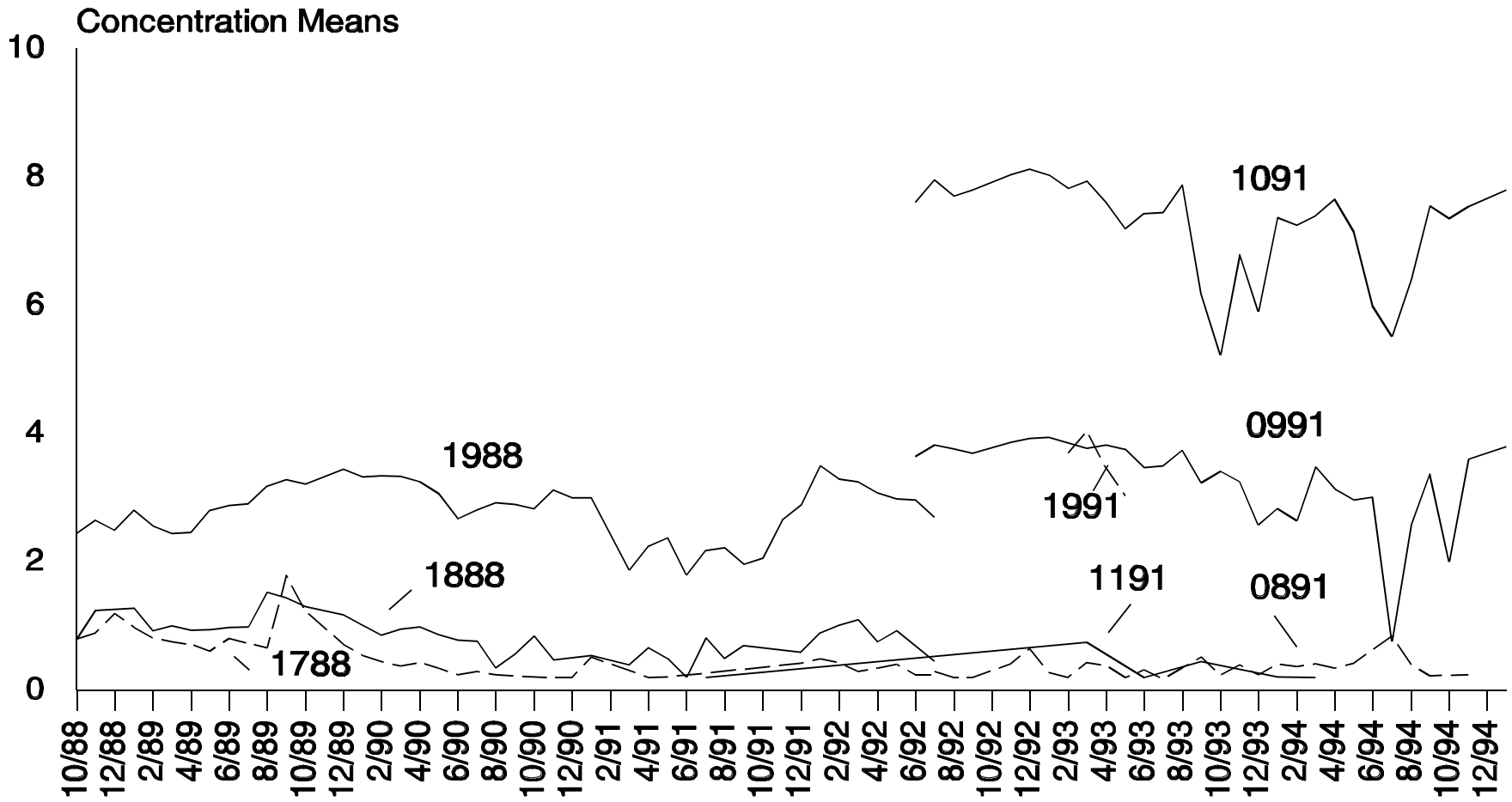



NOTE: No specimens assayed for Retinyl Linoleate during 11/89, 2/91, 10/92, amd 12/94.
No specimens assayed for Pool 1191 between 8/91-5/92 and 7/92-2/93.

SUMMARY STATISTICS FOR
RETINYL OLEATE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/91 - 01/95 | 0.37500 | 0.26994 | 71.8501 | 156 |
| 0991 | 06/91 - 01/95 | 3.25520 | 1.17975 | 36.2420 | 346 |
| 1091 | 06/91 - 01/95 | 7.25119 | 1.40301 | 19.3486 | 420 |
| 1191 | 06/91 - 03/94 | 0.32273 | 0.29428 | 91.1859 | 22 |
| 1788 | 10/88 - 07/92 | 0.53841 | 0.39647 | 73.6377 | 302 |
| 1888 | 10/88 - 07/92 | 0.92224 | 0.40064 | 43.4424 | 724 |
| 1988 | 10/88 - 07/92 | 2.94462 | 0.50367 | 17.1047 | 986 |
| 1991 | 02/93 - 05/93 | 3.60441 | 1.07070 | 29.7053 | 68 |

# Retinyl Oleate Monthly Means

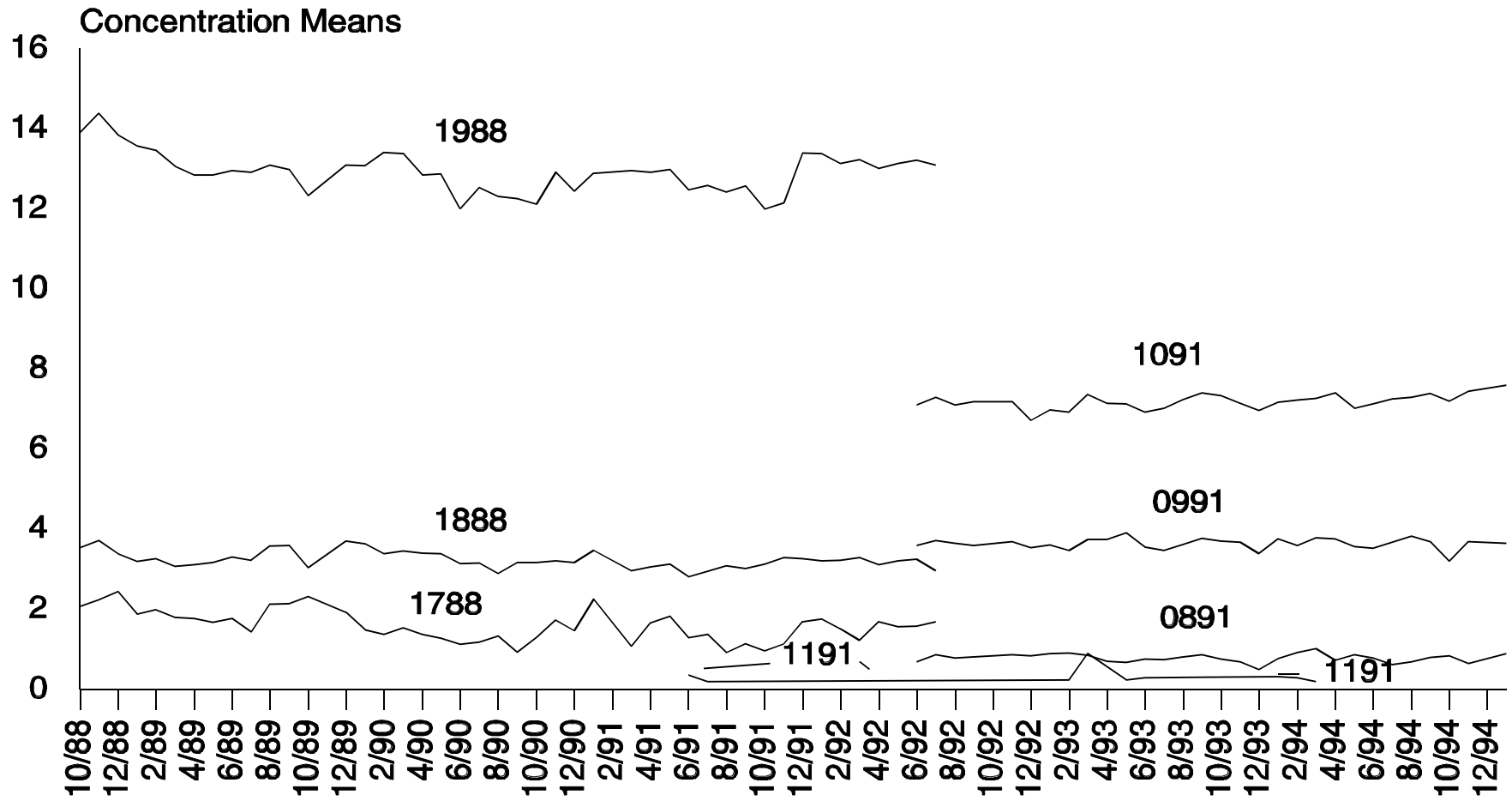



NOTE: No specimens assayed for Retinyl Oleate during 11/89. 2/91, 10/92, and 12/94.
No specimens assayed for Pool 1191 between 8/91-5/92 and 7/92-2/93.

SUMMARY STATISTICS FOR
RETINYL PALMITATE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 0.7954 | 0.33069 | 41.5730 | 416 |
| 0991 | 06/92 - 01/95 | 3.6981 | 0.39222 | 10.6061 | 420 |
| 1091 | 06/92 - 01/95 | 7.2161 | 0.54208 | 7.5121 | 422 |
| 1191 | 06/91 - 03/94 | 0.3275 | 0.20999 | 64.1193 | 40 |
| 1788 | 10/88 - 07/92 | 1.5757 | 0.52508 | 33.3223 | 1002 |
| 1888 | 10/88 - 07/92 | 3.2758 | 0.33956 | 10.3655 | 1002 |
| 1988 | 10/88 - 07/92 | 12.9701 | 0.71360 | 5.5019 | 992 |

# Retinyl Palmitate Monthly Means

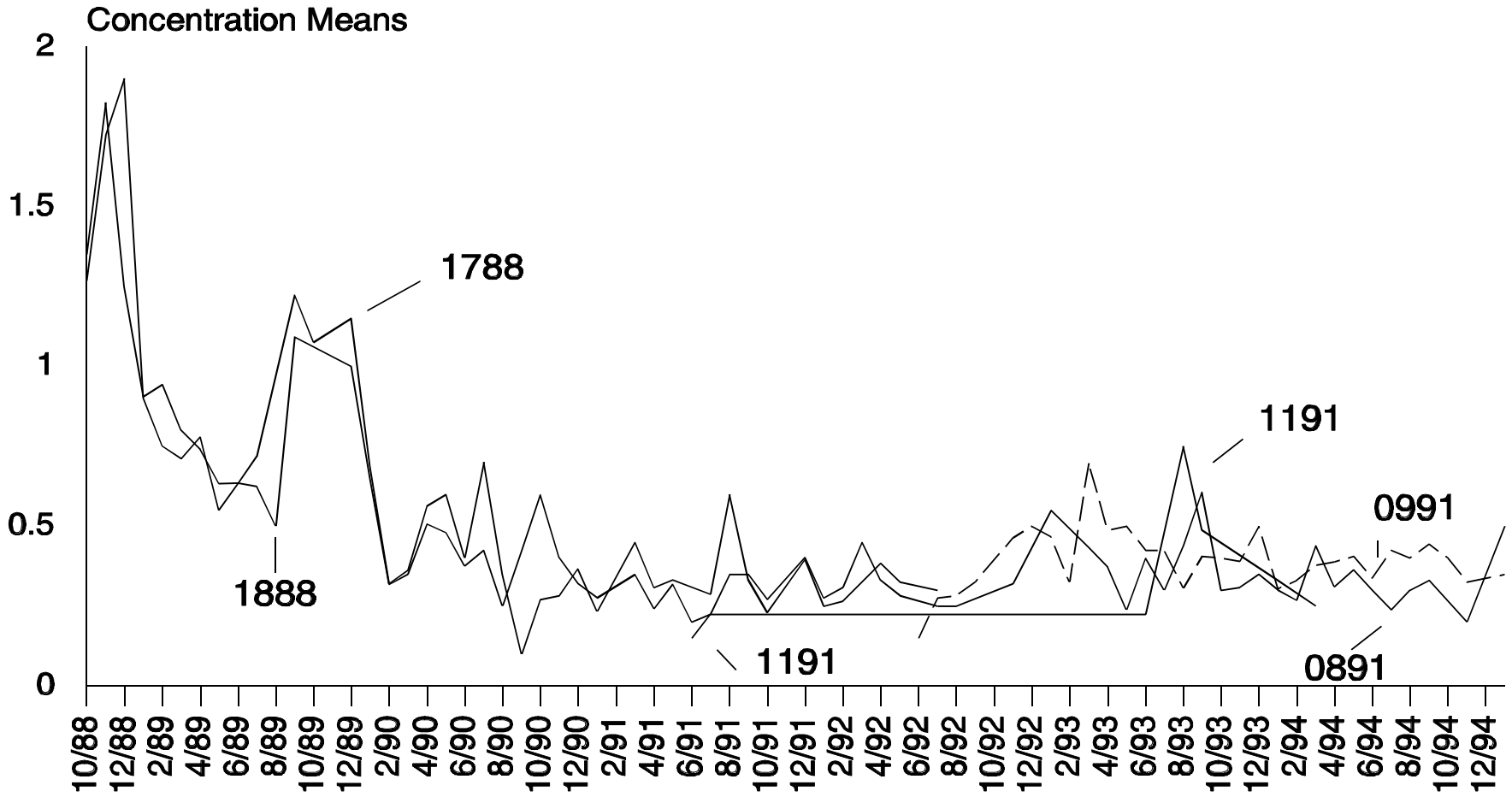



NOTE: No specimens assayed for Retinyl Palmitate during 11/89, 2/91, 10/92, and 12/94.
No specimens assayed for Pool 1191 between 8/91-5/92 and 7/92-2/93.

SUMMARY STATISTICS FOR
RETINYL STEARATE
BY POOL

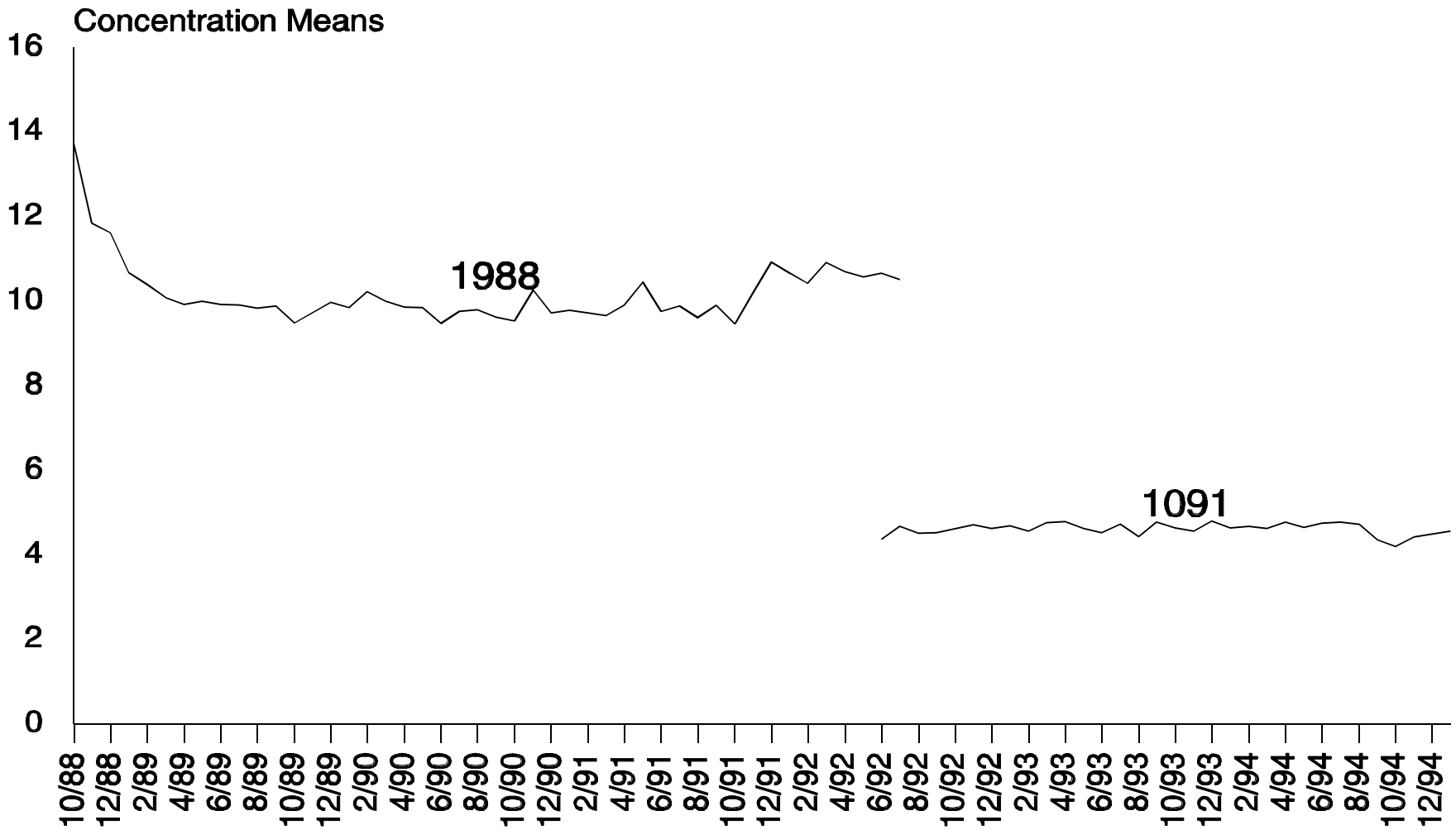
| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0891 | 06/92 - 01/95 | 0.3464 | 0.24389 | 70.4157 | 220 |
| 0991 | 06/92 - 01/95 | 0.4151 | 0.26683 | 64.2871 | 352 |
| 1091 | 06/92 - 01/95 | 4.6491 | 0.44180 | 9.5031 | 422 |
| 1191 | 06/91 - 03/94 | 0.3531 | 0.23277 | 65.9181 | 32 |
| 1788 | 10/88 - 07/92 | 0.4998 | 0.34186 | 68.4003 | 470 |
| 1888 | 10/88 - 07/92 | 0.4878 | 0.30930 | 63.4096 | 548 |
| 1988 | 10/88 - 07/92 | 10.0906 | 0.80071 | 7.9352 | 992 |

# Retinyl Stearate Monthly Mean - Low Pools




NOTE: No specimens assayed for Retinyl Stearate during 11/89, 2/91, 10/92, and 12/94.
No specimens assayed for Pool 1191 between 8/91-5/92 and 7/92-2/93.

# Retinyl Stearate Monthly Means - High Pools



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

### a. Clinical Relevance

Folic acid is required in cellular metabolism and hematopoiesis, and prolonged folic acid deficiency leads to megaloblastic anemia. Vitamin B12 is an essential cofactor in intermediary metabolism and is required for the biosynthesis of RNA and DNA. Since a deficiency of either vitamin may be the cause of megaloblastic anemia, it is essential to determine the levels of both vitamin B12 and folic acid to establish the etiology of the anemia. Untreated vitamin B12 deficiency may lead to severe anemia and potentially irreversible nervous system degeneration.

### b. Test Principle

Both vitamins are measured by using the Bio-Rad Laboratories "Quantaphase Folate" radioassay kit (1). The assay is performed by combining serum or a whole blood hemolysate sample with $^{125}$I-folate and $^{57}$Co-vitamin B12 in a solution containing dithiothreitol (DTT) and cyanide. The mixture is boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin B12 to cyanocobalamin. The reduced folate and its analogs are stabilized by DTT during the heating. The mixture is cooled and then combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins. The addition of these substances adjusts and buffers the pH of the reaction mixture to 9.2. The reaction mixture is then incubated for 1 hour at room temperature.

During incubation, the endogenous and labeled folate and B12 compete for the limited number of binding sites on the basis of their relative concentrations. The reaction mixtures are then centrifuged and decanted. Labeled and unlabeled folate and vitamin B12, binding to immobilized binding proteins, are concentrated in the bottom of the tube in the form of a pellet. The unbound folate and B12 in the supernatant are discarded, and the radioactivity associated with the pellet is counted. Standard curves are prepared by using the pre-calibrated folate/B12 standards in a human serum albumin base. The concentration of the folate and vitamin B12 in the patient serum or folate in a patient's whole blood is calculated from the standard curve.

In the erythrocyte folate procedure, the sample is first diluted 1:11 with a solution of 1 g/dL ascorbic acid in water and either incubated for 90 min prior to assay or frozen immediately for later assay. The 90-min incubation or the freeze-thaw is necessary for hemolysis of the red blood cells; either allows the endogenous folate conjugates to hydrolyze the conjugated pterylpolyglutamates prior to assay. The sample is further diluted 1:2 with a protein diluent (human serum albumin), resulting in a matrix similar to that of the standards and serum samples.

## 2. SAFETY PRECAUTIONS

The folate assay employs $^{125}$I and $^{57}$Co as tracers, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the CDC *Radiation Safety Manual*. Any laboratory using radioimmunoassay (RIA) kits must hold a current NRL Certificate of Registration. In addition, all personnel must successfully complete the CDC training course, *Radiation Safety in the Laboratory*, or demonstrate equivalent instruction. All radioactive waste and contaminated material must be disposed of according to radiation safety guidelines.

Treat all serum specimens as potentially positive for infectious agents including HIV and hepatitis B. Observe Universal Precautions; wear protective gloves, labcoat, and safety glasses during all steps of this method because of both infectious and radioactive contamination hazards. We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a labelled orange plastic autoclave bag for disposal.

> *Dithiothreitol, a primary reagent for this assay, is toxic. Avoid contact with eyes, skin, and clothing. Wash thoroughly after using.*
>
> *Wash immediately with plenty of water if exposed.*

Material safety data sheets (MSDSs) for all chemicals contained in the kit are available in the MSDS section of the

"Working Safely With Hazardous Chemicals" notebook, which is located in the laboratory. MSDSs for other chemicals are located on the CDC LAN.

**3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT**

a. Statistical evaluation and calculation of serum folate and vitamin B12 are accomplished with the ISODATA software on the Micromedic Apex gamma counter. The red cell folate calculations and statistical evaluation are performed with a SAS program, "RBCFOL," which is run in ROSCOE on the CDC mainframe.

After the data is calculated and the reviewing supervisor reviews the final values for release, the data entry clerk transcribes the results into the NHANES III database that is located in RBASE on the NCEH/EHLS PC network. Data is proofread by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. We recommend that specimen donors fast prior to specimen collection, but fasting is not required.

b. Serum folate and vitamin B12 assays are performed on fresh or frozen serum. RBC folate samples are prepared by diluting EDTA-whole blood 1:11 with 1 g/dL ascorbic acid and freezing the solution promptly, which keeps the folate in the reduced state.

c. A 400-µL serum sample is required for serum folate and vitamin B12 assays. A 400 µL solution consisting of a 100 µL whole blood specimen diluted with 1.0 mL of 1 g/dL ascorbic acid is necessary for the red cell folate assay. At assay time, 100 µL of this mixture is added to 100 µL of protein diluent in each of 2 tubes in order to provide the necessary final 1:22 dilution of the original sample for the red cell folate assay.

d. Serum specimens may be collected with regular red-top Vacutainers. Whole blood is collected with lavender-top Vacutainers that contain 1.5% $K_3$EDTA as an anticoagulant. A hematocrit measurement used for the red cell folate calculations is made at the time of collection. The appropriate amount of serum or whole blood/ascorbic acid solution is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field should be frozen and then shipped on dry ice by overnight mail. Once received, they should be stored at $\leq$-20 °C until analyzed. Serum folate and vitamin B12 are fairly stable if the serum is frozen at -20 to -70 °C before analysis. Ascorbic acid should <u>not</u> be added to the serum specimen because it will invalidate the B12 assay. Freeze-thaw cycles will cause degradation of the folate. Whole blood folate is especially sensitive to freeze-thaw degradation.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was collected. Some methods call for a 90-min incubation to hemolyze the red cells and allow the endogenous folate conjugates to hydrolyze the conjugated pteroylpolyglutamates to pteroylmonoglutamates prior to the assay for RBC folate. However, we have found that if the blood is diluted 1:11 with 1 g/dL ascorbic acid to keep the folate in the reduced state and the hemolysate is frozen promptly in the NHANES field vans, a single freeze-thaw cycle before analysis has the same effect as incubation (2).

g. Diurnal variation is not a major consideration. Hemolyzed serum specimens should not be used because they may have falsely high values. A recent article in *Clinical Chemistry* suggests that while serum vitamin B12 is light stable, serum folate specimens exposed to light for longer than 8 hours may have undergone 10-20% degradation (3). Therefore, specimens intended for folate analysis should be processed and stored frozen promptly if analysis is not to be performed within 8 hours of collection.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and the Special Activities Branch Specimen Handling Office). In the protocol, collection, transport, and special equipment required are detailed. In general, serum specimens should be

transported and stored at no more than 4 °C. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of sample should be transferred into a sterile Nalge cryovial labelled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Apex automatic gamma counter (Micromedic Division, ICN Biomedical, Costa Mesa, CA), or model 10/600 Plus gamma counter (ICN Biomedical).

(2) Model J6B centrifuge (Beckman Instruments, Inc., Palo Alto, CA), or Centra-7 centrifuge (International Equipment Co., Needham Heights, MA).

(3) BioRad Novapath SP specimen processor (BioRad Laboratories, Hercules, CA).

(4) Multitube vortexer (VWR Scientific, Marietta, GA).

(5) Gilson Pipetman pipettor 100- and 200 µL sizes (Rainin Instrument Co., Inc., Emeryville, CA).

(6) Eppendorf repeater pipettor (Brinkman Instruments, Inc, Westbury, NY).

(7) Imperial IV water bath (Lab-Line Instruments, Inc., Melrose, IL).

### b. Materials

(1) "Quantaphase II Folate or Folate/B12" radioassay kit (cat. nos. 191-1046 and 191-1041), 200-test sizes (Bio-Rad Laboratories).

(2) "Lyphochek" 3-level and "Lyphochek Anemia Control" lyophilized human serum quality control materials. Also "Lyphochek Red Cell Controls", levels I, II, and III (ECS Division, Bio-Rad Laboratories, Anaheim, CA).

(3) Disposable 12- x 75-mm polypropylene tubes (American Scientific Products, McGaw Park, IL).

(4) L-ascorbic acid, ACS certified (Fisher Scientific Co., Fairlawn, NJ).

(5) "FOAMRAC" foam rubber racks for holding tubes for decanting and blotting after centrifugation (Bio-Rad Laboratories).

(6) Red cell folate diluent: human fraction V albumin solution for diluting red cell hemolysates (Bio-Rad Laboratories).

(7) Combitips, 5.0- and 12.5-mL capacity (Brinkmann Instruments).

(8) Polypropylene test tube racks (Nalge Co., Rochester, NY).

### c. Reagent Preparation

(1) Working tracer reagent
Reconstitute the DTT with 10 mL deionized water. Agitate gently to dissolve, and let stand 5 min. Transfer the entire contents of the DTT vial into the appropriate tracer bottle. Cap and mix by inversion. Store at 2-8 °C for 30 days.

(2) Red cell folate diluent
Add 5 mL deionized water to each vial needed. Allow to stand for 30 min. The solution will be stable for 1 month at -20 °C.

Two vials are required to prepare duplicate assay tubes for every 50 hemolysates.

(3) 1 g/dL Ascorbic acid solution
Add 1.0 g L-ascorbic to 100 mL deionized water and mix well to dissolve. Prepare fresh daily when needed for red cell hemolysates.

(4) Lyphochek levels I, II, III, and anemia control
Rehydrate each vial of Levels I-III with 5.0 mL deionized water and rehydrate the anemia control with 3.0 mL water. Mix the contents gently by swirling, and let stand for 15 min. (Bio-Rad states that these quality control materials may be stored up to 10 days at 2-8 °C. Our usual practice is to rehydrate and pool multiple vials of the same lot of a level, mix them well, aliquot 0.5 mL into polypropylene vials, and store them at -70 °C to provide us with long-term quality control pools for our studies. One vial of each level is thawed for use on the day of analysis.)

During the analysis of whole blood specimens, include Lyphochek red cell controls. Add 2 mL deionized water to each vial and treat the rehydrated contents as whole blood specimens and dilute them similarly for analysis. Again, we usually prepare and pool multiple vials of each level, dispense them as 1:11 hemolysates (1 part (100 µL) EDTA-whole blood with 10 parts 1% ascorbic acid (1.0 mL)), and store the vials at -70 °C for long-term storage. The folate concentrations in the materials vary from lot to lot, but they usually include one deficient level and one normal-range level (4).

d. **Standards Preparation**

Folate/Vitamin B12 Standards
These materials (0.0, 1.0, 2.5, 5.0, 10.0 and 20.0 ng/mL folate, and 0.0, 100, 250, 500, 1000, 2000 pg/mL vitamin B12) are supplied in a liquid form as pteroylglutamic acid (PGA) and cyanocobalamin in human serum albumin, ready to be used. If the entire kit is not used in one run, store the standards at 2-8 °C until the expiration date of the kit. At pH 9.2 the binding affinities of PGA and $N_5$-methyl-tetrahydrofolate ($N_5$MeTHFA), the predominant biologically active form of monoglutamic folate in the body, are equivalent. PGA, however, is far more stable and can be used as an assay standard. It is also the standard material usually used in the traditional microbiological assay for folate in which *Lactobacillus casei* is used.

e. **Preparation of Quality Control Materials**

As outlined previously, four levels of Lyphochek serum controls and three red cell controls are analyzed in duplicate in each run as bench quality control materials. The controls are bought in bulk, rehydrated, mixed, re-aliquoted, and stored at -70 °C. Approximate values are 1.0, 5.0, 10.0, and 15.0 ng/mL in serum, and 100, 300, and 700 ng/mL in the red cell controls. Bench QC pools may also be made from filter-sterilized fasting human serum that has been lyophilized or aliquoted in appropriate quantities and stored at -70 °C.

In every 20 specimens analyzed, one low- or high-concentration blind quality control pool is included. The blind pools are prepared in exactly the same fashion as the NHANES III specimens and are inserted by the serum bank personnel when specimens are received from the field and racked for analysis.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Results of in-house recovery studies using both forms of folate showed approximately 106% recovery for various levels of vitamin B12 added externally, 93% recovery for folate added to serum as $N_5$-methyl-tetrahydrofolate, and 99% recovery for PGA. External calibration may be verified with purified PGA; there is no National Institutes of Standards and Technology (NIST) standard reference material available for folate. The National Institute for Biological Specimens and Control (UK) has prepared an international vitamin B12 reference material, 390 pg/ampoule, which will be used as an external B12 reference material. The limits of detection as determined with dilutions of purified PGA standard are 0.2 ng/mL folate and 20 pg/mL vitamin B12.

Performance checks for the assay include:

- Trace binding: The CPM for the zero standard should be >35% of the CPM of the total counts. If it is <35% a failure of the microbead reagent or a procedural error may have occured.
- Nonspecific binding: the CPM for the blank should be <6% for the CPM of the total counts.

The accuracy of the folate assay was reverified in 1994 with purified Sigma and Merck PGA folate standards diluted at 1.0, 5.0, 10.0 and 20.0 ng/mL dilutions. The overall slope of the regression line of the expected and calculated values was 0.985, the y-intercept was 0.3, and the $r^2$ was 0.9902. This procedure may be used to reverify the kit accuracy at annual intervals.

c. **Calibration of Instrument**

The Micromedic 10/600 Plus gamma counter is used for data reduction. To ensure the accuracy of test results, take the following steps:

(1) Run a background check prior to each test to determine detector contamination. The background for $I^{125}$ should be less than 90 cpm.

(2) Run an efficiency check weekly to determine the instrument's performance. The efficiency should be 75% for $I^{125}$. The % difference should be less than 5%.

(3) Run a normalization check weekly. The system reports the counts per minute from all wells. A factor is applied to each well to correct for any differences. By comparing the factors, the user can ensure that the system is normalized and working correctly. The acceptable range for the factors is 0.90 to 1.10. Multiply all counts made following normalization by the factor for each well before reporting the counts or using them in data reduction.

d. **Instructions for Calibration of Instrument**

(1) Press the ESCAPE key on the keyboard to obtain the "Isodata Main Menu."

(2) Press F2 to obtain the "Instrument Quality Control (QC) Menu." Use the arrow key to choose EFFICIENCY.

(3) When the "Instrument Quality Control Menu" comes up, highlight $I^{125}$. Highlight SELECT, and press the ENTER key.

(4) To make a background count, put an empty tube in every other well of the rack, starting with well no. 1. Place the rack on the counter so that the end having the orange dot moves forward first. Highlight COUNT, and press the ENTER key.

(5) Enter the count time for 1.0 min. Highlight COUNT, and press the ENTER key.

(6) Press the START button when you are ready to count.

(7) After the background count has been completed and falls within the accepted background limit of 90 cpm, press the PRINT SCREEN key to get a hard copy. If any of the wells have been contaminated, clean with a radioactive decontaminant before proceeding any further. Repeat the background count.

(8) Press the END key to do an efficiency check.

(9) Enter the count time for 1.0 min. Press the ENTER key.

(11) A calibrator reference set is used to check efficiency. Place a tube in every other well of the rack, starting with well no. 1. Place the rack on the counter so that the end having the orange dot moves forward first.

(12) Press the START button when ready to count.

(13) After the count is completed, note the efficiency and current disintegrations per min (DPM). Compare the current DPM with established DPM found in the $^{125}I$ reference calibrator log that comes with each calibrator

set. If a great difference is found, the correct value must be edited in the Instrument Quality Control (QC) menu. The efficiency must be 75% or greater. If not, a service representative must be called.

(14) Press the PRINT SCREEN key to get a hard copy. Then press the escape key.

(15) Enter Y to save, and press the ENTER key. The "Instrument Quality (QC) Menu" will return.

(16) Press the ESCAPE key to return to the "Isodata Main Menu."

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Table 1
Quick Reference Summary Table of Initial Assay Preparation
(All tubes in duplicate)

| Sample | Sample Size, (µL) | $^{125}I/^{57}Co$ Tracer, (mL) | Antibody Microbeads, (µL) | Blank Reagent, (µL) | RBC Prot. Diluent, (µL) |
|---|---|---|---|---|---|
| Total Counts | -- | 1 | -- | -- | -- |
| Blank NSB | 200 | 1 | -- | 100 | -- |
| 0 Standard | 200 | 1 | 100 | -- | -- |
| 5-20 ng/mL Standards | 200 | 1 | 100 | -- | -- |
| Controls | 200 | 1 | 100 | -- | -- |
| Unknown Serum | 200 | 1 | 100 | -- | -- |
| Unknown RBCs | 100 | 1 | 100 | -- | 100 |

a. **Manual Pipetting**

(1) Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts. Allow all reagents and specimens to come to room temperature before use.

(2) Add 200 µL of each standard, control, or patient serum to its replicate tubes. Add 200 µL of zero standard to the blank tubes. If assaying for red cell folate, thaw the 1:11 diluted hemolysate at room temperature and mix well. Add 100 µL of hemolysate and 100 µL of red cell diluent to replicate tubes. (RBC specimens are now at the same protein levels as serum specimens in the assay.)

(3) Thoroughly resuspend the working tracer reagent and add 1.0 mL to all tubes, including the total counts tubes.

(4) Mix by vortexing each tube. Set aside total counts tubes until step (10), next section.

b. **Using Novapath Autodilutor**

(1) Prepare reaction tube racks. Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts tube. These tubes should be arranged in the racks according to the chosen layout. The layout information is located in the Novapath instrumentation manual.

(2) Load samples and reagents as specified in the layout, and as required for either serum folate or the red cell folate procedure.

(3) Run the protocol for the desired procedure. The parameters and values for each procedure are programmed into the protocol. This information is located in the Novapath instrumentation manual and is listed in Tables 2 and 3.

(4) Mix by vortexing the entire rack of tubes on the multitube vortexer. Set motor speed on "5." Using the on/off button, turn on for 2-3 sec; repeat five times for thorough mixing of the entire rack.

Table 2
Novapath SP Format Numbers

| **Options** | Format # 25 | Format # 26 |
|---|---|---|
| Layout Type | 7 | 3 |
| Replicate Code | 2 | 2 |
| Sample Rack | 1 | 1 |
| Reaction/Dispense Rack | 2 | 2 |
| X-axis Speed | 2 | 2 |
| Y-axis Speed | 2 | 2 |
| Z-axis Speed | 3 | 3 |
| Sample Pickup Speed | 6 | 6 |
| Dispensing Speed | 5 | 5 |
| Probe Wash Speed | 1 | 1 |
| Level Option | 2 | 2 |

c. **Procedure Following Completion of Manual or Autodilutor Steps**

(1) Place the tubes in the rack, cover with aluminum foil, and place the rack in a boiling-water bath at 100 °C. Allow the bath to return to a rolling boil and incubate the tubes for a minimum of 20 min. Cool to room temp by placing the rack in a cold water bath.

(2) Thoroughly mix the bottles of microbead and blank reagents by placing them on a rocker-mixer for at least 15 min prior to use. Add 100 µL of microbead reagent to each tube except the blanks. Add 100 µL blank reagent to the blank tubes. Vortex all tubes.

(3) Incubate tubes at room temperature (about 21-30 °C) for 1 hour.

(4) Centrifuge all tubes for 10 min at 1500 x g to pack the solids at the bottom of the tubes. Proceed promptly to the next step.

(5) Place the tubes in the FOAMRACS and invert the tubes over a container designated for radioactive waste in order to discard the supernatant from each tube. (A large plastic funnel or dish pan is useful for collecting the liquid and channeling it into a large plastic bottle for proper disposal of the radioactive waste.) Remove the last drops of liquid by blotting the tube rims on plastic-backed absorbent paper.

(6) Place the tubes in racks and count for 1 min in the gamma counter. Record the counts.

d. **Calculations**

Both the Micromedic Apex and model 10/600 gamma counters have full data reduction capabilities. Logit $B/B_o$ vs $\log_{10}$ concentration is used in both counters where:

$$\text{logit } (B/B_o)=\text{Ln}((B/B_o)/(1-B/B_o))$$

and B = corrected counts/min (blank subtracted) for each tube, and $B_o$ = maximum binding.

This method results in a linearized standard curve with an inverse relationship of levels of radioactivity (measured in counts per min, or CPM) to the concentration of folate or B12 in the serum or whole blood sample. Results are expressed as nanograms of folate per milliliter of serum (ng/mL) or as picograms of vitamin B12 per milliliter of serum (pg/mL). Red cell folate values are multiplied by 22, the dilution factor of the whole blood. The serum folate values (multiplied by 1.0 minus the hematocrit expressed as a decimal) are subtracted, and the resulting value is

divided by the hematocrit to yield red cell folate in ng/mL red blood cells.

$$\text{RBC folate, ng/mL} = \frac{\text{(whole blood folate X 22) - serum folate (1-hematocrit/100)}}{\text{hematocrit/100}}$$

Table 3
Novapath SP Run File Record

| Comments | Run #1 | Run #2 | Run #3 | Run #4 | Run #5 | Run #6 | Run #7 | Run #8 | Run #9 | Run #10 | Run #11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Prime | Precision Check | Folate /B12 | Reagent | Anti-body | RBC Folate | RBC Diluent | Ferritin | Ferritin Antibody | Serum Folate Unchained | RBC & SFOL Add tracer |
| Function Number | 1 | 5 | 4 | 5 | 5 | 4 | 5 | 4 | 5 | 4 | 5 |
| Format Number | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Numbers of Dilutions | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Sample Volume | -- | -- | 200 | -- | -- | 100 | -- | 50 | -- | 200 | -- |
| Maximum Depth | -- | -- | 1050 | -- | -- | 1050 | -- | 1050 | -- | 1050 | -- |
| Sensor Depth | -- | -- | 3 | -- | -- | 0 | -- | 0 | -- | 3 | -- |
| Reagent #1 Volume | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Reagent #2 Volume | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Reagent #3 Volume | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Reagent Number | -- | 1 | -- | 1 | 1 | -- | 2 | -- | 1 | -- | 1 |
| Excess Volume | -- | 100 | -- | 10 | 100 | -- | 100 | -- | 200 | -- | 10 |
| Fill Volume | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dispense Volume | -- | 100 | -- | 1000 | 100 | -- | 100 | -- | 200 | -- | 1000 |
| Air Gap Volume | -- | 10 | 10 | 20 | 10 | 10 | 10 | 10 | 20 | 1 | 20 |
| Mix Volume | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Mix Frequency | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Surplus Volume | -- | -- | 0 | -- | -- | 0 | -- | -- | -- | 0 | -- |
| Wash Volume | -- | -- | 700 | -- | -- | 700 | -- | 700 | -- | 7 | -- |
| Number of Cycles | 5 | 0 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 2 | 0 |
| Aspirate Start Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Aspirate End Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dispense Height | -- | 815 | 795 | 645 | 630 | 815 | 795 | 837 | 790 | 7 | 645 |
| Dispense Start Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dispense End Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Mix Depth | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Mix Number | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Sample Limit 2 | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Total Volume A | -- | 210 | 920 | 1030 | 210 | 820 | 210 | 770 | 420 | 920 | 1030 |
| Total Volume B | -- | -- | 710 | -- | -- | 710 | -- | 710 | -- | 710 | -- |
| Chained Run | 0 | 0 | 4 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 |
| Time Delay | -- | -- | 0 | -- | -- | 0 | -- | -- | -- | 0 | -- |

We recommend use of the correction for serum folate level and hematocrit because it provides the most accurate reflection of folate body stores.

e. **CDC Modifications**

The CDC modifications for red cell folate specimen preparation necessitated by field lab collection have been included by Bio-Rad in the recommended kit instructions.

The folate-only, vitamin B12-only, and folate/B12 combination kits are now used exactly as outlined by the manufacturer, usually in their entirety in one analytical run.

## 9. REPORTABLE RANGE OF RESULTS

Values <2.0 ng/mL for serum folate, <2.0 ng/mL for whole blood folate, and <200 pg/mL for vitamin B12 are verified by re-assay, and values >18.0 ng/mL for serum or whole blood folate, or >1800 pg/mL for vitamin B12 are verified by reassay after the solution has been diluted 1:2 with saline. (These whole blood folate values are approximately equivalent to 75 and 600 ng/mL RBC as red cell folate concentrations.)

## 10. QUALITY CONTROL (QC) PROCEDURES

Long-term estimates of NHANES III precision in measuring serum and red cell folate during calendar year 1993 are about 3-6% coefficient of variation (CV) at 3-15 ng/mL and 5-6% at 1 ng/mL. For vitamin B12, CVs are 5-7% at 300-1500 pg/mL and 11% at 200 pg/mL.

Quality control data is entered into the mainframe ROSCOE program "QCDATA"; the plotting option of "QCPRTA" or "QCGRA" and the Division quality control programs are used to produce long term plots.

Limits for new pools are established after 20 runs. The same four levels of serum pools are used for both serum and RBC folate analyses; RBC pools are added for RBC folate analyses.

The system is declared "out of control" if any of the following events occur:

For the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two of the two or more pools fall either both above or both below the lower 95% limit.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

For the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two of the two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

Representative precision and accuracy of the method are reflected in these long-term quality control pool results:

Table 4
Vitamin B12 Pools (pg/mL)

| Pool | Mean | 95% Limits | 99% Limits | N | Total SD | Total CV |
|---|---|---|---|---|---|---|
| 8001 | 1257.7 | 1135.19-1380.11 | 1096.45- 1418.85 | 27 | 61.205 | 5.66 |
| 8002 | 797.7 | 711.10-848.36 | 689.39-870.07 | 27 | 34.33 | 5.30 |
| 8003 | 467.5 | 408.66-526.24 | 390.06-544.84 | 27 | 29.37 | 6.82 |
| 32AN | 183.1 | 146.16-220.13 | 134.46-231.83 | 27 | 18.52 | 10.50 |

Table 5
Serum and Red Cell Folate Pools (ng/mL and ng/mL RBC)

| Pool | Mean | 95% Limits | 99% Limits | N | Total SD | Total CV |
|---|---|---|---|---|---|---|
| 8301 | 11.505 | 10.06-12.978 | 9.594-13.427 | 41 | 4.90 | 7.02 |
| 8302 | 3.018 | 2.819-3.220 | 2.758-3.285 | 41 | 4.36 | 4.36 |
| 8302 | 0.949 | 0.800-1.101 | 0.750-1.142 | 41 | 4.86 | 7.98 |
| 33AN | 0.750 | 0.655-0.853 | 0.620-0.881 | 40 | 7.53 | 6.11 |
| 5111 | 33.60 | 30.17-37.03 | 29.08-388.12 | 37 | 1.75 | 5.23 |
| 5112 | 263.20 | 264.94-279.49 | 241.80-284.64 | 37 | 9.97 | 3.84 |
| 5113 | 503.06 | 454.32-551.96 | 438.90-567.14 | 37 | 26.27 | 5.25 |

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

The ISODATA software will allow the standard curve to be edited if there is a single outlying point. If the controls are still outside of the acceptable range, declare the system "out of control" and repeat the entire run. If the "out of control" condition still exists, use a new kit and evaluate the pipetting precision and accuracy of the autodiluter.

Reassay specimens for that analytical run after the system has been verified to be "in control," and report the results of the reassay rather than the original values.

If the steps outlined above do not result in the correction of the "out of control" values for QC materials, consult the supervisor for other corrective actions.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The most common causes of imprecision are intermittently inaccurate micropipettors, inadequate boiling time, and microbead reagent that was not mixed thoroughly before it was added to the solution.

Hemolyzed serum samples may give falsely elevated values. Exposure to light for more than eight hours may cause 10-20% serum folate degradation.

## 13. REFERENCE RANGES (NORMAL VALUES)

Current proposed normal ranges for serum folate are about 2.6-12.2 ng/mL for both sexes and all ages. Values are lower in females than in males. Serum levels of 1.4-2.6 ng/mL are usually termed "indeterminate" because of an overlap between "deficient" and "normal" ranges. Serum folate values <1.4 ng/mL are usually indicative of inadequate folate intake. Elevated values are caused by supplementation. (See NOTE below.)

Red cell folate values are more indicative of body stores, whereas serum levels reflect only recent dietary intake. The approximate normal range for red cell folate is 102.6-410.9 ng/mL RBC.

*Extensive evaluation by CDC, Cambridge University, and Stanford University researchers, showed PGA calibrators in original Quantaphase kits to be inaccurate by an average of 32.5% across 0-20 ng/mL range. Using freshly received NHANES III specimens, CDC performed 19 comparison runs with "old" and "new" calibrators (with spectrophotometrically verified concentrations of PGA) to establish a correction factor. The equation used for the correction was:*

$$\mathbf{Log_{10}\ (corrected\ value) = -0.1956 + 1.0199\ Log_{0}\ (uncorrected\ value)}$$

*Or, expressed in linear terms:*

$$\mathbf{corrected\ value = -0.1411 + 0.6849\ x\ (uncorrected\ value)}$$

*Evaluations were also made with $N_5$MeTHFA standards. No change occurred to affect vitamin B12 results.*

*The currently used reference ranges for serum and red cell folate are based on a mathematical correction of previously recommended values from the supporting documentation for HANES II and reflect the approximately 32.5% average difference for standards between current versions of the Quantaphase II folate kits. All of Phase I of NHANES III was performed with the original kit; hence, data from Phase I were corrected to correspond with the data from Phase II.*

The expected normal range for vitamin B12 is about 165-1600 pg/mL. Concentrations of 160-200 pg/mL are considered "indeterminate", and those less than 160 pg/mL are considered deficient. Elevated B12 levels may result from over supplementation or may reflect myeloproliferative disorders.

Values from NHANES III will be used to determine the U.S. national normal ranges. Previous data in NHANES II concerned only a subset of the population, and that from HHANES described only the Hispanic U.S. population.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Any NHANES samples with serum folate levels <2.0 ng/mL, RBC folate levels <75 ng/mL RBC, or B12 levels <200 pg/mL should be reported to NCHS for follow-up.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should be allowed to warm to and be maintained at room temperature during preparation and testing.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods of analysis for folate or vitamin B12 in the NHANES laboratory.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Samples with a serum folate value <2.0 ng/mL, an RBC folate value <75 ng/mL RBC, or a B12 value <150 pg/mL should be reported to NCHS by FAX. All folate and vitamin B12 values are reported at the end of each stand as part of the NHANES III physician's report, as well as being reported on periodic data tapes sent to the NCHS data center.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

The supervisor, or other authorized data base manager, has access to RBASE and the CDC mainframe filing systems that are used to keep records and track specimens. Records, including related QC/QA data, are maintained for 10 years after the survey's conclusion. Only numerical identifiers (e.g. case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator.

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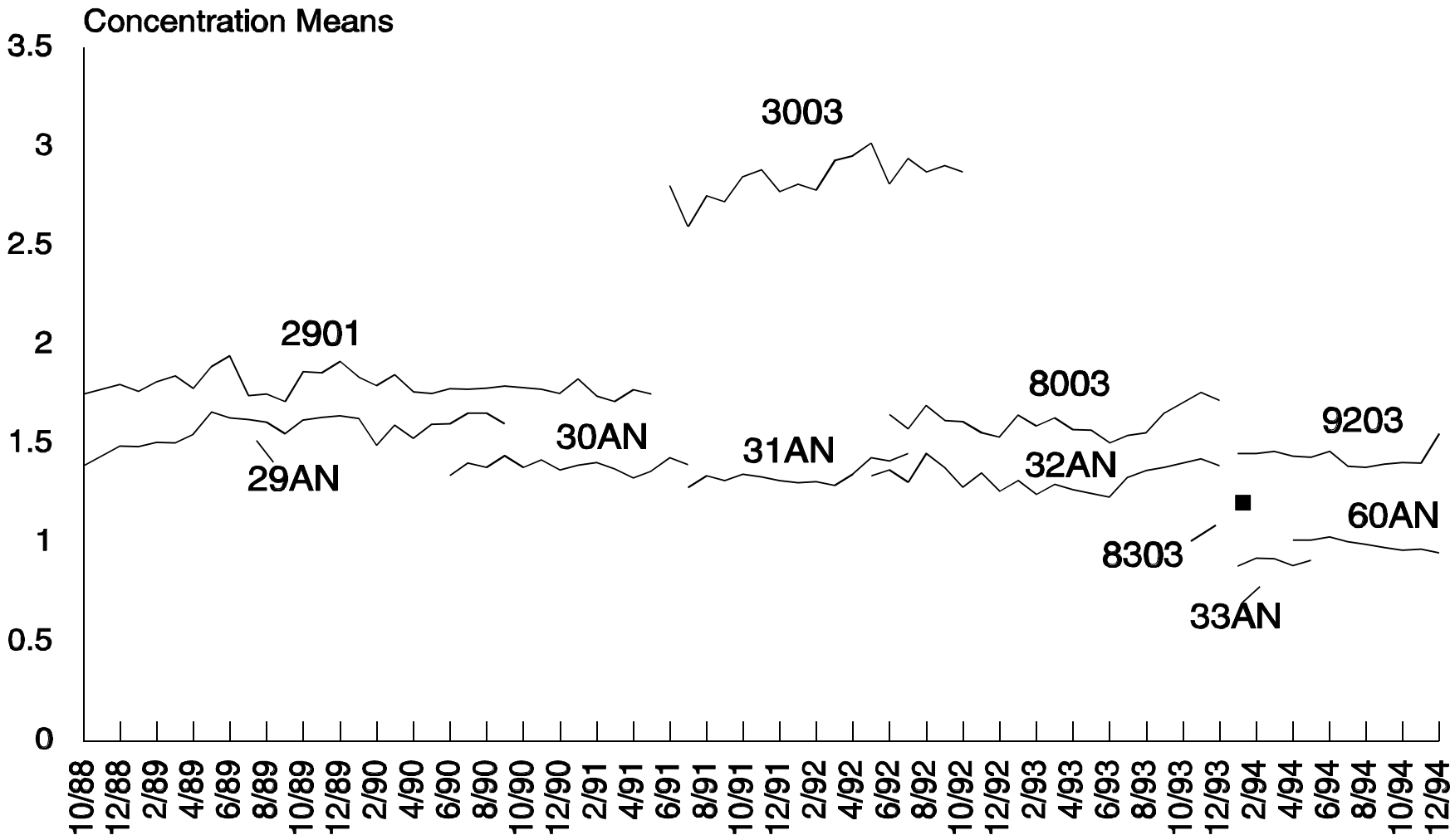
SUMMARY STATISTICS FOR
SERUM FOLATE - LOW POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 2901 | 10/88 - 05/91 | 1.7976 | 0.10714 | 5.96003 | 300 |
| 29AN | 10/88 - 09/90 | 1.5851 | 0.10047 | 6.33862 | 222 |
| 3003 | 06/91 - 10/92 | 2.8545 | 0.15812 | 5.53934 | 142 |
| 30AN | 06/90 - 07/91 | 1.3925 | 0.07986 | 5.73524 | 168 |
| 31AN | 07/91 - 07/92 | 1.3342 | 0.08105 | 6.07504 | 114 |
| 32AN | 05/92 - 12/93 | 1.3073 | 0.11134 | 8.51663 | 164 |
| 33AN | 01/94 - 05/94 | 0.9039 | 0.03901 | 4.31559 | 38 |
| 60AN | 04/94 - 12/94 | 0.9898 | 0.03553 | 3.58957 | 94 |
| 8003 | 06/92 - 12/93 | 1.6131 | 0.10553 | 6.54167 | 208 |
| 8303 | 01/94 | 1.1871 | 0.08713 | 7.33926 | 14 |
| 9203 | 01/94 - 12/94 | 1.4228 | 0.07766 | 5.45809 | 138 |

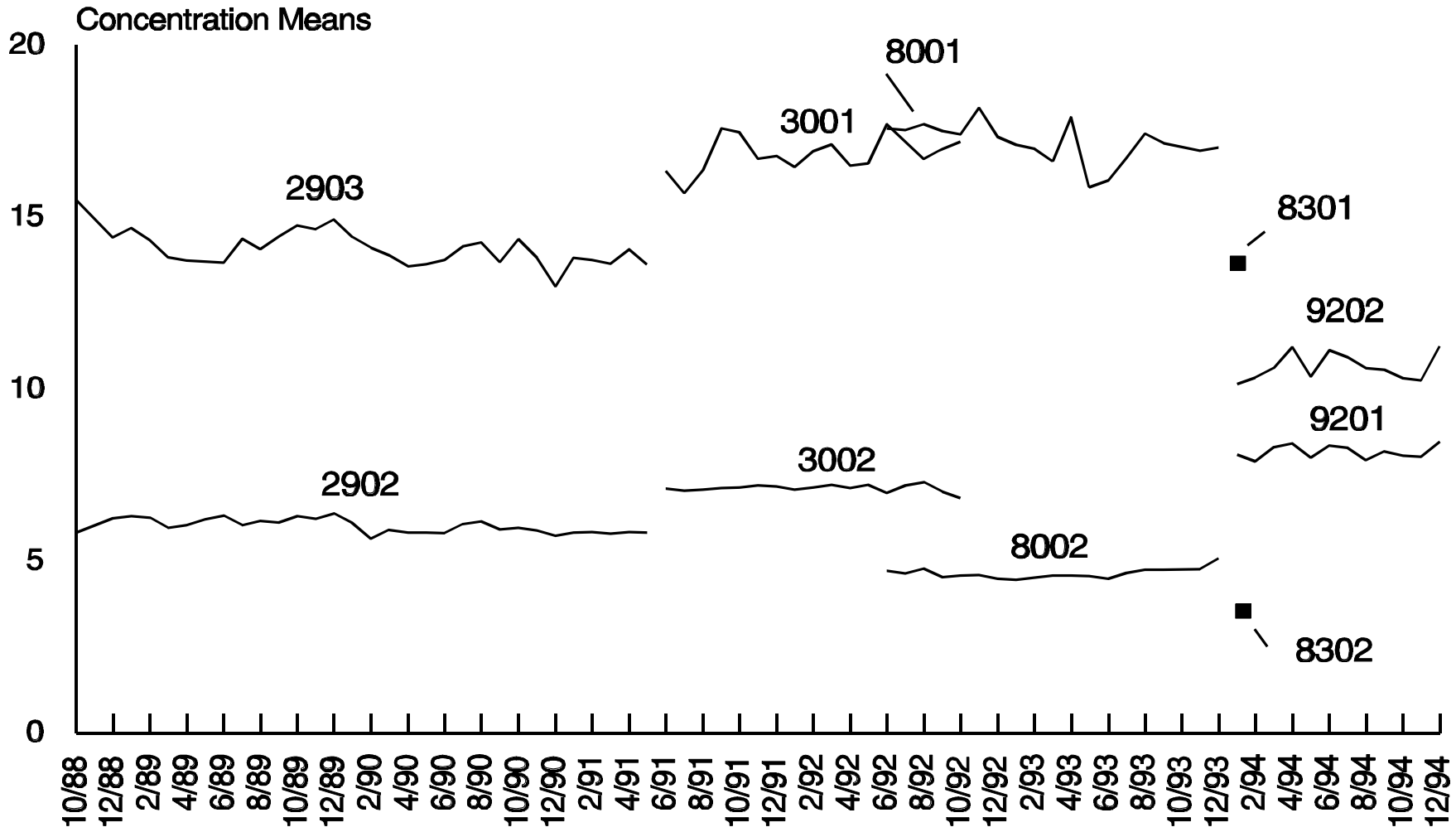
# Serum Folate Monthly Means - Low Pools



SUMMARY STATISTICS FOR
SERUM FOLATE - MEDIUM/HIGH POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 2902 | 10/88 - 05/91 | 6.0438 | 0.28784 | 4.76256 | 304 |
| 2903 | 10/88 - 05/91 | 14.0730 | 0.75488 | 5.36403 | 304 |
| 3001 | 06/91 - 10/92 | 16.8332 | 1.20871 | 7.18052 | 140 |
| 3002 | 06/91 - 10/92 | 7.1395 | 0.25792 | 3.61264 | 142 |
| 8001 | 06/92 - 12/93 | 17.2839 | 1.17701 | 6.80991 | 205 |
| 8002 | 06/92 - 12/93 | 4.6228 | 0.20550 | 4.44536 | 210 |
| 8301 | 01/94 | 13.6300 | 1.23221 | 9.04042 | 14 |
| 8302 | 01/94 | 3.5114 | 0.13393 | 3.81401 | 14 |
| 9201 | 01/94 - 12/94 | 8.1374 | 0.48342 | 5.94077 | 138 |
| 9202 | 01/94 - 12/94 | 10.5306 | 0.66183 | 6.28480 | 140 |

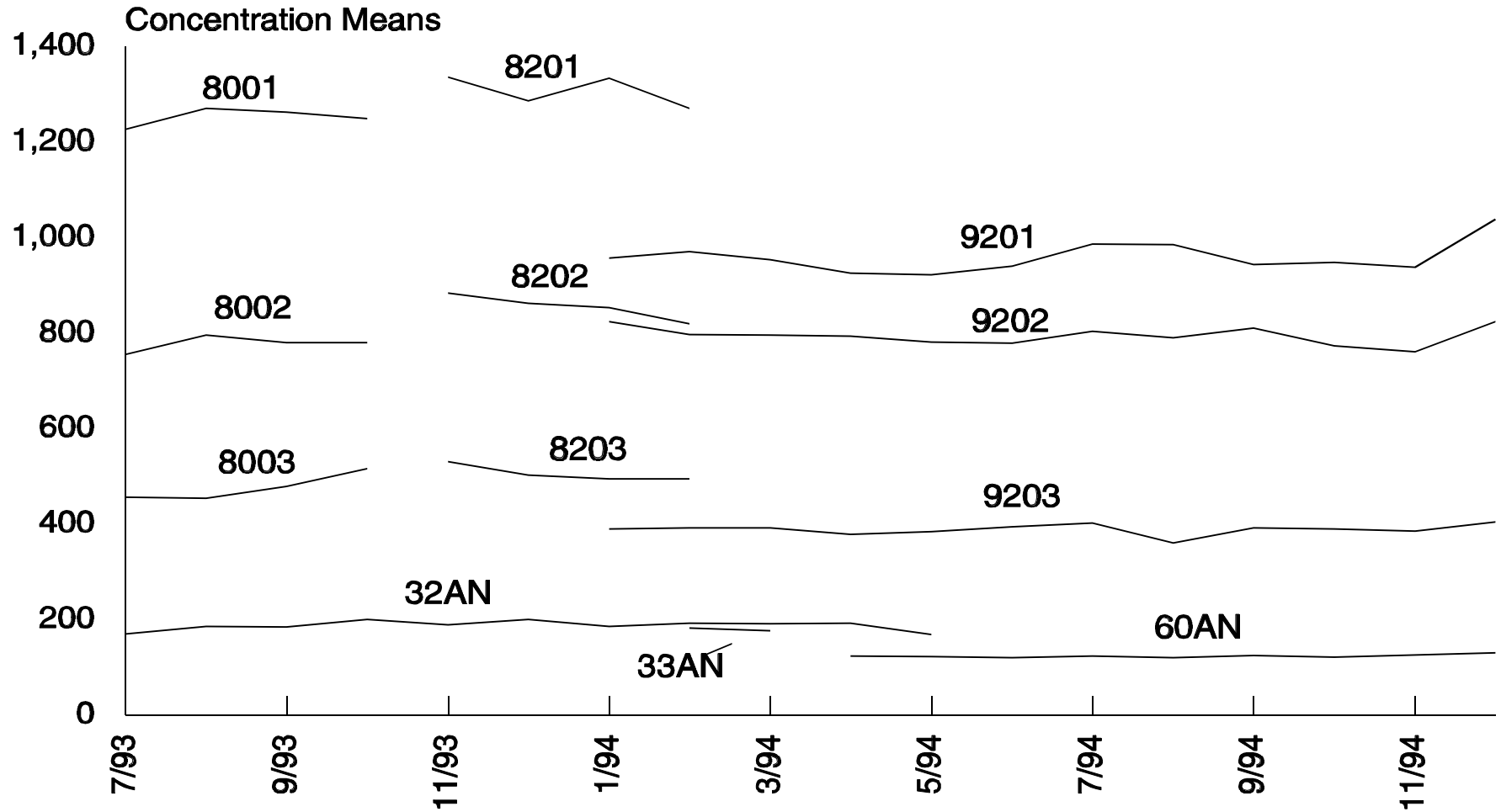
# Serum Folate Monthly Means - Medium/High Pools



SUMMARY STATISTICS FOR
VITAMIN B-12
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 32AN | 07/93 - 05/94 | 186.85 | 19.451 | 10.4098 | 178 |
| 33AN | 02/94 - 03/94 | 180.78 | 10.837 | 5.9945 | 14 |
| 60AN | 04/94 - 12/94 | 123.34 | 7.968 | 6.4596 | 86 |
| 8001 | 07/93 - 10/93 | 1235.53 | 107.786 | 8.7311 | 64 |
| 8002 | 07/93 - 10/93 | 779.91 | 37.432 | 4.7995 | 64 |
| 8003 | 07/93 - 10/93 | 466.25 | 41.188 | 8.8340 | 64 |
| 8201 | 11/93 - 02/94 | 1314.17 | 94.674 | 7.2040 | 48 |
| 8202 | 11/93 - 02/94 | 839.98 | 83.192 | 9.9040 | 50 |
| 8203 | 11/93 - 02/94 | 505.12 | 31.566 | 6.2491 | 46 |
| 9201 | 01/94 - 12/94 | 952.01 | 52.476 | 5.5122 | 173 |
| 9202 | 01/94 - 12/94 | 793.13 | 36.859 | 4.6473 | 172 |
| 9203 | 01/94 - 12/94 | 388.89 | 17.756 | 4.5659 | 171 |

# Vitamin B-12 Monthly Means




NOTE: Assay for Vitamin B-12 did not begin until July 1993.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Ferritin, like hemoglobin, is a major iron storage protein. Isoferritin moieties have been identified for liver and spleen (L isoferritin) and for heart and kidney (H isoferritin). Circulating plasma ferritin is most like the L isoferritin. A serum ferritin assay provides a much more sensitive indicator of iron body stores than a traditional serum iron assay. Serum ferritin levels increase as a result of iron overload, aging, infection, inflamation, liver disease, juvenile rheumatoid arthritis, leukemia, and Hodgkin's disease; and they decrease as a result of iron deficiency.

Ferritin is measured by using the Bio-Rad Laboratories' "QuantImune Ferritin IRMA" kit (1), which is a single-incubation two-site immunoradiometric assay (IRMA) based on the general principles of assays as described by Addison et al. (2) and Miles (3) and modified by Jeong et al.(4). In this IRMA, which measures the most basic isoferritin, the highly purified $^{125}$I-labeled antibody to ferritin is the tracer, and the ferritin antibodies are immobilized on polyacrylamide beads as the solid phase. Serum or ferritin standards (made from human liver) are mixed with the combined tracer/solid-phase antibody reagent, and the mixture is incubated. During incubation, both the immobilized and the $^{125}$I-labeled antibodies bind to the ferritin antigen in the serum or standards, thus creating a "sandwich."

After incubation, the beads are diluted with saline, centrifuged, and decanted. The level of $^{125}$I-labeled ferritin found in the pellets is measured by using a gamma counter. There is a direct relationship between the radioactive levels of the pellets and the amount of endogenous ferritin in the serum or standards, rather than the inverse relationship measured by most radioimmunoassays (RIAs).

## 2. SAFETY PRECAUTIONS

The ferritin assay employs $^{125}$I as a tracer, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the CDC Radiation Safety Manual. Any laboratory using radioimmunoassay (RIA) kits must hold a current NRC Certificate of Registration and conform to all of the storage, handling and disposal requirements. In addition, all personnel must successfully complete the CDC training course *Radiation Safety in the Laboratory* or demonstrate having received equivalent instruction. Consider all serum specimens for analysis to be potentially positive for infectious agents including HIV and hepatitis B viruses. Observe Universal Precautions; wear safety glasses, protective gloves, and labcoat during all steps of this method because of both infectious and radioactive contamination hazards. (We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials.) Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines.

Material safety data sheets (MSDSs) for all chemicals contained in the kit are available in the MSDS section of the "Working Safely With Hazardous Chemicals" notebook, which is located in the laboratory. This information is also available on the CDC LAN.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation and calculation of the run are accomplished with the ISODATA software on the Micromedic Apex gamma counter.

   After the data are calculated and the reviewing supervisor approves the final values for release, the data entry clerk transcribes the results into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network; data entry is proofed by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

b. Specimens for ferritin analysis should be fresh or frozen serum. Serum specimens may be collected by using regular red-top or serum separator Vacutainers.

c. A sample volume of 100 µL is required for the assay; 250 µL will permit repeat analysis as well.

d. Specimens may be stored in glass or plastic vials as long as the vials are tightly sealed to prevent desiccation of the sample.

e. Because ferritin is very stable, serum may be frozen at -20 °C to -70 °C for years before analysis. Several freeze-thaw cycles do not seem to adversely affect the assay.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

g. Hemolyzed specimens should not be used because red blood cells contain H isoferritin.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies are available in the NHANES laboratory in the and Special Activities Branch Specimen Handling Office.) In the protocol, collection, transport, and special equipment required are discussed. In general, whole blood specimens should be transported and stored at no more than 4 °C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at <-20 °C. Samples thawed and refrozen several times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, transfer the appropriate amount of serum into a sterile Nalge cryovial labeled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Micromedic Apex automatic gamma counter (Micromedic Division, ICN Biomedical, Costa Mesa, CA) or Micromedic model 10/600 plus gamma counter (ICN Biomedical).

(2) Beckman J6-B centrifuge, 222-tube capacity (Beckman Instruments, Inc., Palo Alto, CA), or Beckman TJ-6 centrifuge (Beckman Instruments).

(3) Gilson Pipetman pipettes, 50- and 200-µL size (Rainin Instrument Co., Woburn, MA).

(4) Eppendorf Repipettor, 1.0- to 5.0-mL size (Brinkmann Instruments, Westbury, NY).

(5) BioRad Novapath SP specimen processor (BioRad Laboratories, Hercules, CA).

(6) Multitube vortexer (VWR Scientific, Marietta, GA).

(7) Digiflex automatic pipettor (ICN Biomedical, Inc., Costa Mesa, CA).

### b. Materials

(1) Bio-Rad Laboratories' "Quantimune Ferritin IRMA" $^{125}$I-ferritin assay kit, 1000-test size (Bio-Rad Laboratories).

(2) "Lyphocheck" 3-level ferritin quality control materials and "Lyphocheck Anemia Control" lyophilized human serum materials (ECS Division, Bio-Rad Laboratories).

(3) Disposable 12- x 75-mm polypropylene tubes (American Scientific Products, McGaw Park, IL).

(4) Sodium chloride (NaCl), ACS certified (Fisher Scientific Co., Fairlawn, NJ).

(5) "FOAMRAC" foam rubber racks for holding tubes for decanting and blotting after centrifugation (Bio-Rad Laboratories, Hercules CA).

(6) Filters, 3.00-, 0.80-, 0.65, 0.45, 0.30, and 0.22-µm pore sizes (Milipore Corp., Bedford, MA).

c. **Reagent Preparation**

(1) Ferritin tracer/immunobeads
This is supplied as a single reagent, ready to use. Mix the contents to resuspend the immunobeads (the solid-phase antibody-coated matrix for the assay) prior to use. If not used in one run, store at 2-8 °C until the expiration date. This material contains $^{125}I$ and should be properly handled with gloves and disposed according to CDC radiation safety guidelines.

(2) 0.9 g/dL NaCl solution
Place 9 g NaCl in a 1-L volumetric flask and dilute to volume with deionized water.

(3) "Lyphochek" levels I, II, III, and anemia control
Rehydrate by adding 5.0 mL deionized water to each vial of levels I-III and 3.0 mL deionized water to the anemia control. (Bio-Rad says that these quality control materials may be stored up to 10 days at 2-8 °C. Our usual practice is to rehydrate multiple vials of the same lot of a level, mix them well, aliquot 0.5 mL into 2.0-mL polypropylene vials, and store them at -70 °C to provide us with homogeneous long-term quality control pools for our studies. One vial of each level is thawed for use on the day of analysis.)

d. **Standards Preparation**

Ferritin standards
These materials (0, 5, 10, 25, 100, 250, 1000 and 2500 ng/mL) are supplied in a liquid form, ready to be used. If the entire kit is not used in one run, store the standards at 2-8 °C for 10 days. These standards are prepared by BioRad and matched to the NIBSC/WHO 80/602 First International Human Liver Ferritin Standard, available from the National Institute of Biological Standards and Controls, London, UK. The NIBSC material is the only internationally recognized source of purified human liver ferritin.

e. **Preparation of Quality Control Material**

Four levels of bench quality control materials are used. QC materials may be purchased commercially (Lyphochek controls) or prepared from pooled human serum.

Lyphochek controls are bought in bulk, rehydrated with deionized water, aliquotted into 2-mL Nalge cryovials, and stored at -70 °C. Approximate values are 5, 50, 150, and 400 ng/mL. (See Section 6.c.(3) for preparation instructions.)

Two levels (low-normal and high-normal ferritin concentrations) of blind QC pools may be prepared from pooled, filter-sterilized human serum obtained from fasting donors with elevated or decreased ferritin levels. Pool serum in acid-cleaned 20-L glass carboys. Mix well on a magnetic stirrer. Clean-filter the serum through in a sequential manner using filters of the following pore sizes, each preceded by a prefilter: 3.00-µm, 1.20-µm, 0.80-µm, 0.65-µm, 0.45-µm, 0.30-µm, and 0.22-µm.

Through the use of sterile technique under a laminar-flow hood, dispense the serum in 1-mL aliquots with a Micromedic Digiflex dispenser into 2.0 mL Nalge cryovials. Cap and label the vials with NHANES barcoded labels that have been specially prepared for the QC pools. Store the pools at $\leq$-70 °C at the CDC Serum Bank in Lawrenceville where they will be inserted randomly into the NHANES runs. Select 20 vials of each level at random for characterization of the quality control limits and for testing of homogeneity.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Ferritin kit calibration standards are prepared by Bio-Rad and are matched to the NIBSC/WHO 80/602 International Human Liver Ferritin Standard, available from the National Institute of Biological Standards and Controls, London, UK. These standards are run daily.

This method results in a linearized 8-point (including zero) standard curve showing a direct relationship between

radioactivity levels measured in counts per min (cpm) and the ferritin concentration in the serum sample. Serum results are expressed as nanograms of ferritin per milliliter of serum (ng/mL).

a. **Performance Checks for the Assay**

- Nonspecific binding: The zero standard is used as the indicator of nonspecific binding. The cpm for the zero standard tube should be <6% of the cpm of the total counts tube.
- Maximum binding: The cpm of 200 µL of the 2500 ng/mL standard tube should be >45% of the cpm of the total counts tube.
- $r^2$ should be 0.9900 or greater.

In addition, dilutions of the NIBSC ferritin standard are run every 6 months to verify system calibration. This standard material consists of 9.7 µg/ampoule purified human liver isoferritin, which, when reconstituted with 1.0 mL of water, yields 9.1 µg/mL ferritin. NIBSC standards are diluted to 0.91, 9.1, 91, and 910 ng/mL. For the standard dilutions tested in June 1991, the slope of the regression line of the expected vs. calculated values was 1.018, the y-intercept was 0.8, and the $r^2$ was 0.9935. This correlation is reverified semi-annually.

b. **Calibration of Instrument**

The Micromedic 10/600 Plus gamma counter is used for data reduction. To ensure the accuracy of test results, operators must do the following:

(1) Run a background check prior to each test to determine detector contamination. The background for $I^{125}$ should be less than 90 cpm.

(2) Run an efficiency check weekly to determine the instrument's performance. The efficiency should be $\geq$75% for $^{125}I$. The difference among detectors should be less than 5%.

(3) Run a normalization check weekly. The system reports cpm from all wells. A factor is applied to each well in order to correct for any differences. By comparing the factors, the user can determine whether the system is normalized and working correctly. The acceptable range for the factors is 0.90 to 1.10. All counts made following normalization will be multiplied by the factor for each well before the counts reported or used in data reduction.

c. **Instructions for Calibration of Instrument**

(1) Press the ESCAPE key on the keyboard to obtain "Isodata Main Menu."

(2) Press F2 to obtain "Instrument Quality Control (QC) Menu." Use the arrow key to choose EFFICIENCY.

(3) When "Instrument Quality Control Menu" comes up, highlight "$^{125}I$." Highlight SELECT and press the ENTER key.

(4) To make a background count, put an empty tube in every other well of the rack, starting with well no. 1. Place the rack on the counter so that the end having the orange dot moves forward first. Highlight COUNT and press the ENTER key.

(5) Enter a count time of 1.0 minute. Highlight COUNT and press the ENTER key.

(6) Press the START button when you are ready to count.

(7) After the background count has been completed and falls within the accepted background limit of 90 cpm, press the PRINT SCREEN key to get a hard copy. If any of the wells have been contaminated, clean them with a radioactive decontaminate before proceeding any further. Repeat the background count.

(8) Press the END key to do an efficiency check.

(9) Enter a count time of 1.0 minute. Press the ENTER key.

(10) A calibrator reference set is used to check efficiency. Place a tube in every other well of the rack, starting

with well no. 1. Place the rack on the counter so that the end having the orange dot moves forward first.

(11) Press the START button when you are ready to count.

(12) After the count is completed, note the efficiency and current disintegrations per minute (DPM). Compare the current DPM with the established DPM found in the $^{125}I$ reference calibrator log that comes with each calibrator set. If the difference is great, edit the correct value in the "Instrument Quality Control (QC) menu." If the efficiency is not 75% or greater, call a service representative.

(13) Press the PRINT SCREEN key to get a hard copy, then press the ESCAPE key.

(14) Enter "Y" to save and press the ENTER key. The "Instrument Quality (QC) Menu" will return.

(15) Press the ESCAPE key to return to "Isodata Main Menu."

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Manual Pipetting

Follow steps 1-4 to prepare reaction tubes for analysis:

1) Label 12- x 75-mm tubes in duplicate for each standard, control, patient sample, and total counts.

2) Add 50 µL of standard, control, and patient sample to their replicate tubes. EXCEPTION: Add 200 µL of the 2500 ng/mL standard to each of 2 tubes to provide a maximum binding tube for the logit/log data reduction calculations.

3) Thoroughly resuspend the tracer/immunobeads and add 200 µL to all tubes including the total counts tubes.

4) Mix by shaking each rack of tubes. Set aside total counts tubes until the run is ready to be counted.

### b. Using Novapath Autodilutor

Follow steps 1-5 to prepare reaction tubes for analysis:

1) Prepare reaction tube racks. Label 12- x 75-mm tubes in duplicate for each blank, standard, control, patient sample, and total counts. These tubes should be placed in racks according to the chosen layout.

2) Load samples as specified in the layout.

3) Run the protocol for the ferritin procedure. The parameters and values are programmed into the protocol. This information is located in the Novapath instrumentation manual and shown in Tables 1 and 2.

4) Add tracer/immunobeads manually because the timing of this step is important and the autodiluter takes too long.

5) Mix by vortexing the entire rack of tubes on the multitube vortexer. The motor speed should be set on "5." Using the on and off button, turn on the vortexer for 2-3 sec. Repeat this step five times for thorough mixing.

### c. Procedure Following Completion of Manual or Autodilutor Steps

1) Incubate tubes at room temperature (about 21-30 °C) for 30 min.

2) Add 3.0 mL normal saline to all tubes (except the total counts tubes) and centrifuge 10 min at 1500 x g to pack the solids at the bottom of the tubes. Proceed promptly to the next step; mixing after the saline addition is unnecessary.

3) Place the tubes in the FOAMRACS and invert them over a container designated for radioactive waste in order to discard the supernates. (A large plastic funnel or dishpan is useful for collecting the liquid and channeling it into a plastic bottle for proper disposal of the radioactive waste.) Remove the last drops of liquid by blotting

the tube rims on plastic-backed absorbent paper.

4) Place the tubes in racks and count for 1 min in the gamma counter. Record the counts.

Table 1
Novapath Settings for Ferritin

| **Options** | Format # 25 | Format # 26 |
|---|---|---|
| Layout type | 7 | 3 |
| Replicate code | 2 | 2 |
| Sample rack | 1 | 1 |
| Reaction/dispense rack | 2 | 2 |
| X-axis speed | 2 | 2 |
| Y-axis speed | 2 | 2 |
| Z-axis speed | 3 | 3 |
| Sample pickup speed | 6 | 6 |
| Dispensing speed | 5 | 5 |
| Probe wash speed | 1 | 1 |
| Level option | 2 | 2 |

d. **Novapath SP Format Record**

Use ferritin settings as specified in Table 2. Settings may change periodically, but these are representative.

e. **Calculations**

Both the Micromedic Apex and 10/600 Plus counters have full data reduction capabilities. Calculation of serum ferritin concentration is accomplished in both counters by using logit $B/B_o$ vs $\log_{10}$ concentration, where:

$$\text{logit } (B/B_o) = \text{Ln}((B/B_o)/(1-B/B_o))$$

and B = corrected cpm (blank subtracted) for each tube, and $B_o$ = maximum binding. (If using the ISODATA software on the Apex counter, use the cubic spline option with a linear curve fit.) In the IRMA, the zero standard is used for nonspecific binding (NSB), and the maximum binding has been experimentally determined to be approximately four times the concentration of the 2500 ng/mL standard.

This method results in a linearized 8-point (including zero) standard curve showing a direct relationship between radioactivity levels (measured in cpm) and the ferritin concentration in the serum sample. Serum results are expressed as nanograms of ferritin per milliliter of serum (ng/mL).

f. **CDC Modifications**

On the basis of recommendations by CDC, the manufacturer changed the standard materials (calibrators) used in this kit from the versions originally provided and also included maximum binding tubes to permit automated data reduction with the logit-log function. The kit is now used exactly as outlined by the manufacturer, usually in its entirety in one analytical run (hence the manual pipetting of the tracer beads to increase the total number of assayable tubes per kit).

Table 2
Novapath SP Specimen Processor Run Identification and Parameter File Record

| Comments | Run #1 | Run #2 | Run #3 | Run #4 | Run #5 | Run #6 | Run #7 | Run #8 | Run #9 | Run #10 | Run #11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Prime | Precis-ion Check | Folate /B12 | Reagent | Anti-body | Folate | Diluent | Ferritin | Ferritin Antibody | Serum Folate Unchained | RBC & SFOL Add tracer |
| Function Number | 1 | 5 | 4 | 5 | 5 | 4 | 5 | 4 | 5 | 4 | 5 |
| Format Number | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 |
| Numbers of Dilutions | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Sample Volume | -- | -- | 200 | -- | -- | 100 | -- | 50 | -- | 200 | -- |
| Maximum Depth | -- | -- | 1050 | -- | -- | 1050 | -- | 1050 | -- | 1050 | -- |
| Sensor Depth | -- | -- | 3 | -- | -- | 0 | -- | 0 | -- | 3 | -- |
| Reagent #1 Volume | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Reagent #2 Volume | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Reagent #3 Volume | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Reagent Number | -- | 1 | -- | 1 | 1 | -- | 2 | -- | 1 | -- | 1 |
| Excess Volume | -- | 100 | -- | 10 | 100 | -- | 100 | -- | 200 | -- | 10 |
| Fill Volume | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dispense Volume | -- | 100 | -- | 1000 | 100 | -- | 100 | -- | 200 | -- | 1000 |
| Air Gap Volume | -- | 10 | 10 | 20 | 10 | 10 | 10 | 10 | 20 | 1 | 20 |
| Mix Volume | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Mix Frequency | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Surplus Volume | -- | -- | 0 | -- | -- | 0 | -- | -- | -- | 0 | -- |
| Wash Volume | -- | -- | 700 | -- | -- | 700 | -- | 700 | -- | 7 | -- |
| Number of Cycles | 5 | 0 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 2 | 0 |
| Aspirate Start Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Aspirate End Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dispense Height | -- | 815 | 795 | 645 | 630 | 815 | 795 | 837 | 790 | 795 | 652 |
| Dispense Start Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Dispense End Height | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Mix Depth | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Mix Number | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Sample Limit 2 | -- | -- | 0 | -- | -- | 0 | -- | 0 | -- | 0 | -- |
| Total Volume A | -- | 210 | 920 | 1030 | 210 | 820 | 210 | 770 | 420 | 920 | 1030 |
| Total Volume B | -- | -- | 710 | -- | -- | 710 | -- | 710 | -- | 710 | -- |
| Chained Run | 0 | 0 | 4 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 |
| Time Delay | -- | -- | 0 | -- | -- | 0 | -- | -- | -- | 0 | -- |

## 9. REPORTABLE RANGE OF RESULTS

The maximum range of detection possible with this method is from 0 to 2500 ng ferritin/mL undiluted serum. Although

the commonly observed range is from 0 to 500 ng ferritin/mL serum, linearity has been verified over the range of 0 to 1100 ng/mL with available NIBSC standards. In this laboratory, values less than 10 ng/mL are verified by reassay, and values greater than 2000 ng/mL are verified by reassay after the serum has been diluted 1:2 with saline. The ISODATA software reports any values lower than the 5 ng/mL lowest standard as "< 5"; other gamma counters or software may actually quantitate the value. This value is entered in the NHANES III database arbitrarily as "3." The limit of detection was previously statistically determined with dilutions of the NIBSC standard to be 1.10 ng/mL, but current software does not permit quantitation at this level.

## 10. QUALITY CONTROL (QC) PROCEDURES

Because of reliability and availability, four levels of Bio-Rad Lyphochek controls are currently used as bench quality control materials. Approximate values are 5, 50, 150, and 400 ng/mL.

Bench QC pools, as well as blind QC pools, may also be made from filter-sterilized serum from fasting human subjects as described in Section 6.e., p 6.

In every batch of 20 specimens analyzed, either one low- or one high-concentration blind quality control pool is randomly inserted. The blind pools are aliquoted and labelled in exactly the same fashion as the NHANES III specimens and are inserted in the specimen batches by the Serum Bank personnel when the specimens are received from the field and racked for analysis.

Long-term estimates of method precision from calendar years 1991 and 1992 for NHANES III (about 200 total runs) show total CVs of about 4-6% at 30-400 ng/mL and 9-10% at 5-10 ng/mL.

Quality control data is entered into the mainframe ROSCOE program "QCDATA"; the plotting option of "QCPRTA" or "QCGRA" and the Division quality control programs are used to produce long-term plots.

Limits are established for new pools after 20 runs and updated after 40 runs.

The system is declared "out-of-control" if any of the following events occur:

For the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two or more pools fall either both above or both below the 95% limit.
- Two successive run means for a single pool fall either both above or both below the 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

For the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the system should be declared "out of control," repeat the entire run. If the "out of control" condition still exists, use a new kit and evaluate the autodiluter for pipetting precision and accuracy. Reassay specimens for that analytical run after the system has been verified to be "in control."

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Sources of imprecision in the procedure may be intermittently imprecise micropipettors, an outdated tracer, and the so-called "high-dose hook effect" seen only at extremely elevated levels of ferritin (usually among renal transplant patients or those with hepatic disorders). A repeat analysis of elevated specimens at a higher dilution is performed to confirm their true levels of ferritin.

## 13. REFERENCE RANGES (NORMAL VALUES)

The normal range for serum ferritin is about 10-800 ng/mL for the overall population. Values are lower among females than among males and generally lower among children than among adult females. Values less than 10 ng/mL usually indicate iron deficiency anemia. Elevated values are caused by iron overload, aging, inflammation, malignancies, hepatic disorders, and juvenile rheumatoid arthritis (1,2,5-7).

The data from NHANES III will be used to describe normal ranges for ferritin for the entire U.S. population. Data from NHANES II describe only a certain subset of the population, and that from HHANES described the Hispanic U.S. population.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Any NHANES sample with a serum ferritin value <10 ng/mL should be reported by FAX to NCHS for follow-up by the medical officer or designee.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should remain at room temperature during preparation and testing.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods for performing this test for NHANES III. In case of system failure, store all specimens at -20 °C until the system is functioning.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

a. Use the "NHANES LAB STANDARDS AND CONTROLS SUMMARY SHEET" for ferritin to record the quality control data. This reporting sheet has self-explanatory blanks for the standard concentrations and the quality control pool results. Prepare this form in duplicate.

b. Use the "NHANES III ANALYTICAL WORKSHEET" to record the specimen results. These worksheets have been prepared with sample IDs for each preracked run. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is not satisfactory (i.e., cannot be analyzed), write "UNSAX" in the blank. Prepare these forms in duplicate.

c. Give both types of forms to the supervisor along with the hard copy of the data printout from the gamma counter computer. After the supervisor checks the data, the carbon copies and the data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets. The results are transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network.

d. Use the "QCDATA6" program in ROSCOE on the CDC mainframe to record quality control data. This program should be updated regularly.

e. The supervisor, or a designated analyst, notifies NCHS by FAX of all NHANES III samples with serum ferritin values < 10 ng/mL.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

RBASE and the CDC mainframe are used to keep records and track specimens. Records, including related QA/QC data, are maintained for 10 years beyond the duration of the survey. Only numerical identifiers (e.g., case ID numbers) should be used.

For children less than three years old, serum for the ferritin analysis is obtained from the same vial used for iron/TIBC analysis, for all older NHANES participants, serum for ferritin analysis is obtained from the serum folate/vitamin $B_{12}$ vial. Residual serum from either of these vials is retained for serum iron/TIBC repeat analyses and finally is returned to the NCHS serum repository in Bethesda, MD.

For other studies, the samples are stored at -20 °C for 1 year after analysis. At this time, the principal investigator is contacted to make a decision concerning storage or disposal of specimens.

## REFERENCES

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## ADDITIONAL SOURCES

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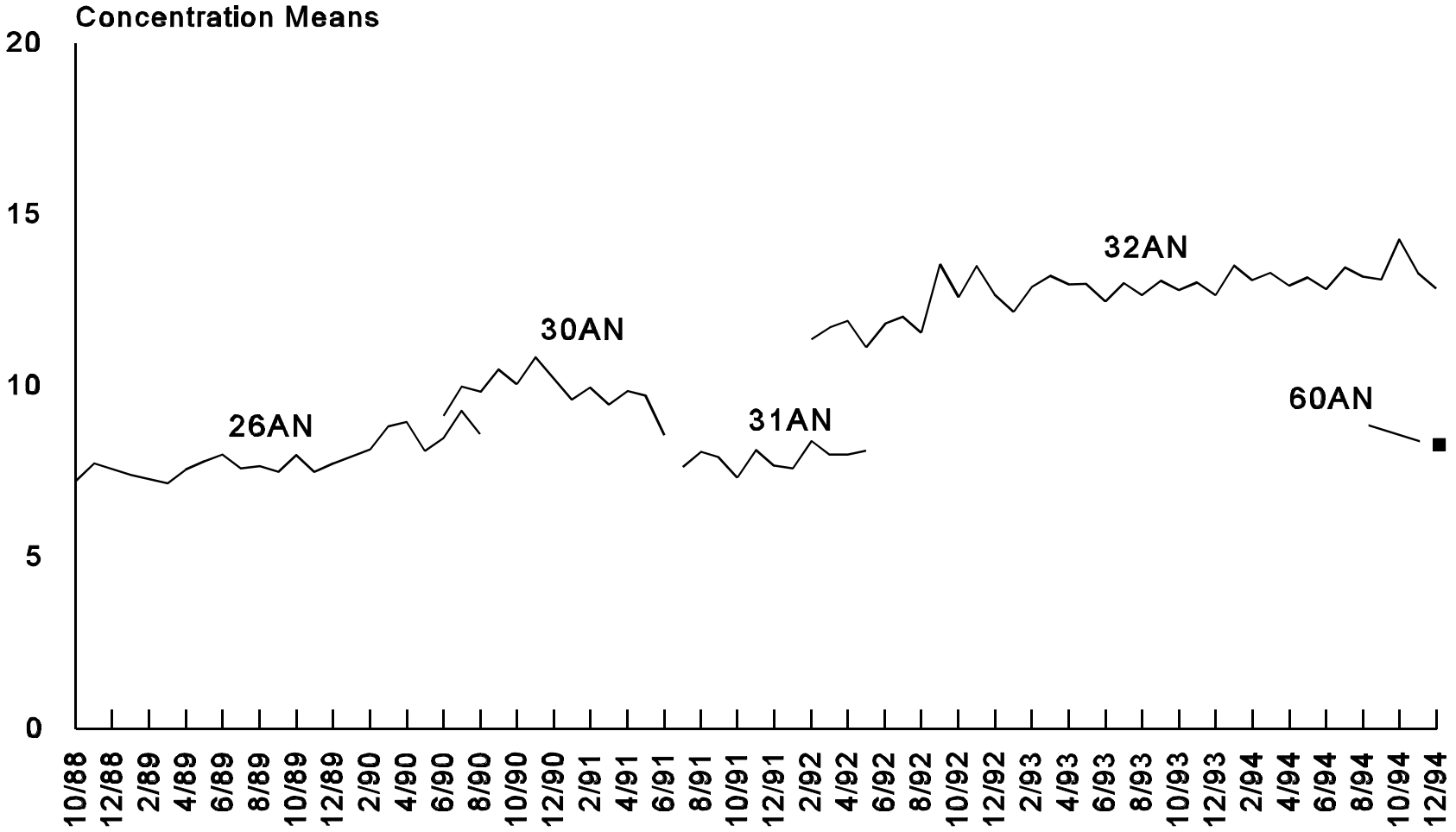
SUMMARY STATISTICS FOR
FERRITIN (LOW POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 26AN | 10/88 - 08/90 | 7.959 | 0.8936 | 11.2283 | 192 |
| 30AN | 06/90 - 06/91 | 9.987 | 1.2057 | 12.0736 | 229 |
| 31AN | 07/91 - 05/92 | 7.899 | 0.8903 | 11.2713 | 122 |
| 32AN | 02/92 - 12/94 | 12.800 | 1.0924 | 8.5343 | 371 |
| 60AN | 12/94 | 8.299 | 0.7995 | 9.6336 | 10 |

# Ferritin Monthly Means - Low Pools

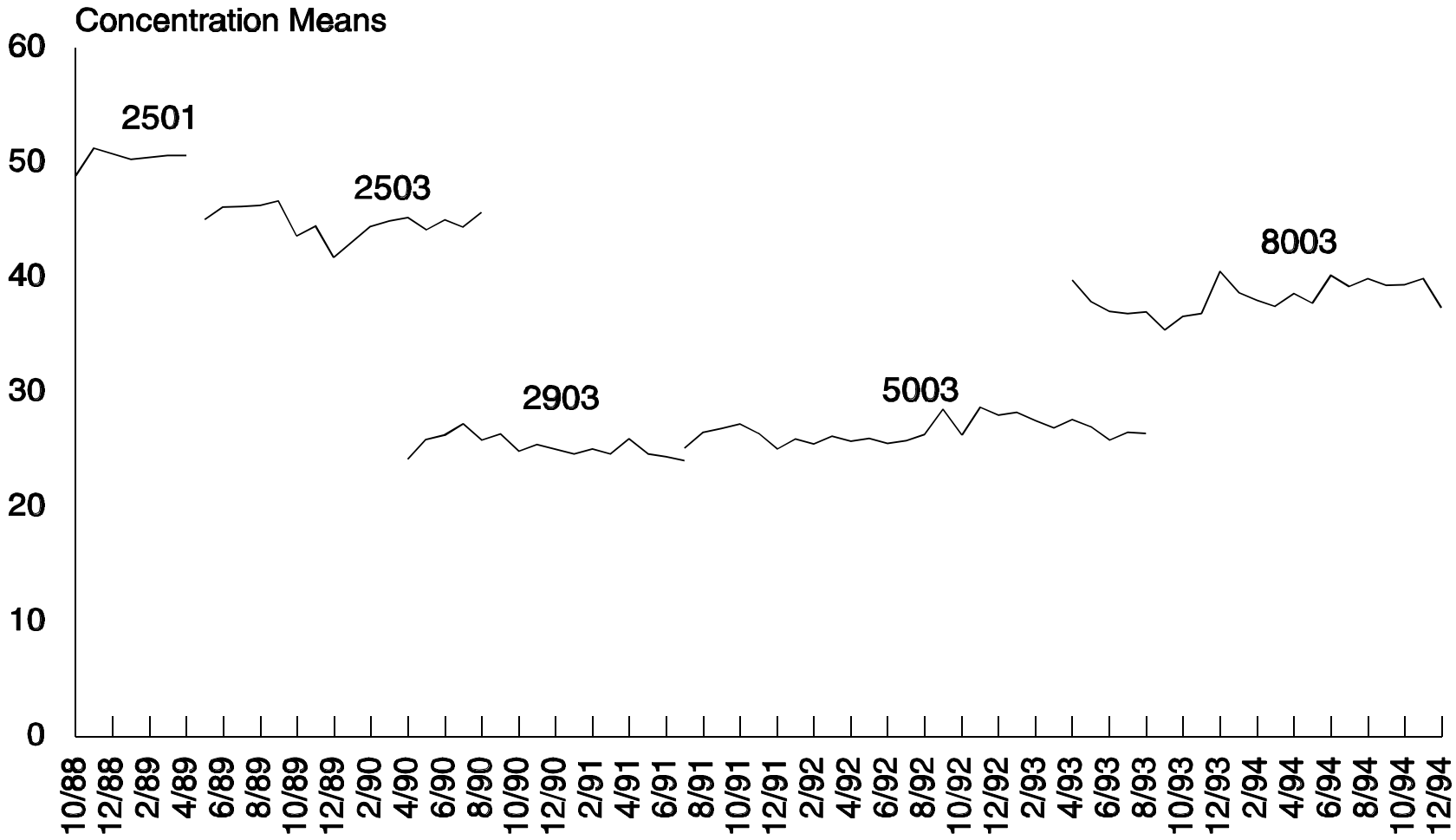



**NOTE: No specimens assayed during the months 12/88, 2/89, 1/90, and 12/90.**

SUMMARY STATISTICS FOR
FERRITIN (MEDIUM POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 2501 | 10/88 - 04/89 | 50.533 | 2.4743 | 4.8964 | 50 |
| 2503 | 05/89 - 08/90 | 45.024 | 2.2229 | 4.9371 | 142 |
| 2903 | 04/90 - 07/91 | 25.556 | 1.6748 | 6.5537 | 180 |
| 5003 | 07/91 - 08/93 | 26.734 | 1.6167 | 6.0475 | 307 |
| 8003 | 04/93 - 12/94 | 38.282 | 2.2403 | 2.2403 | 180 |

# Ferritin Monthly Means - Medium Pools

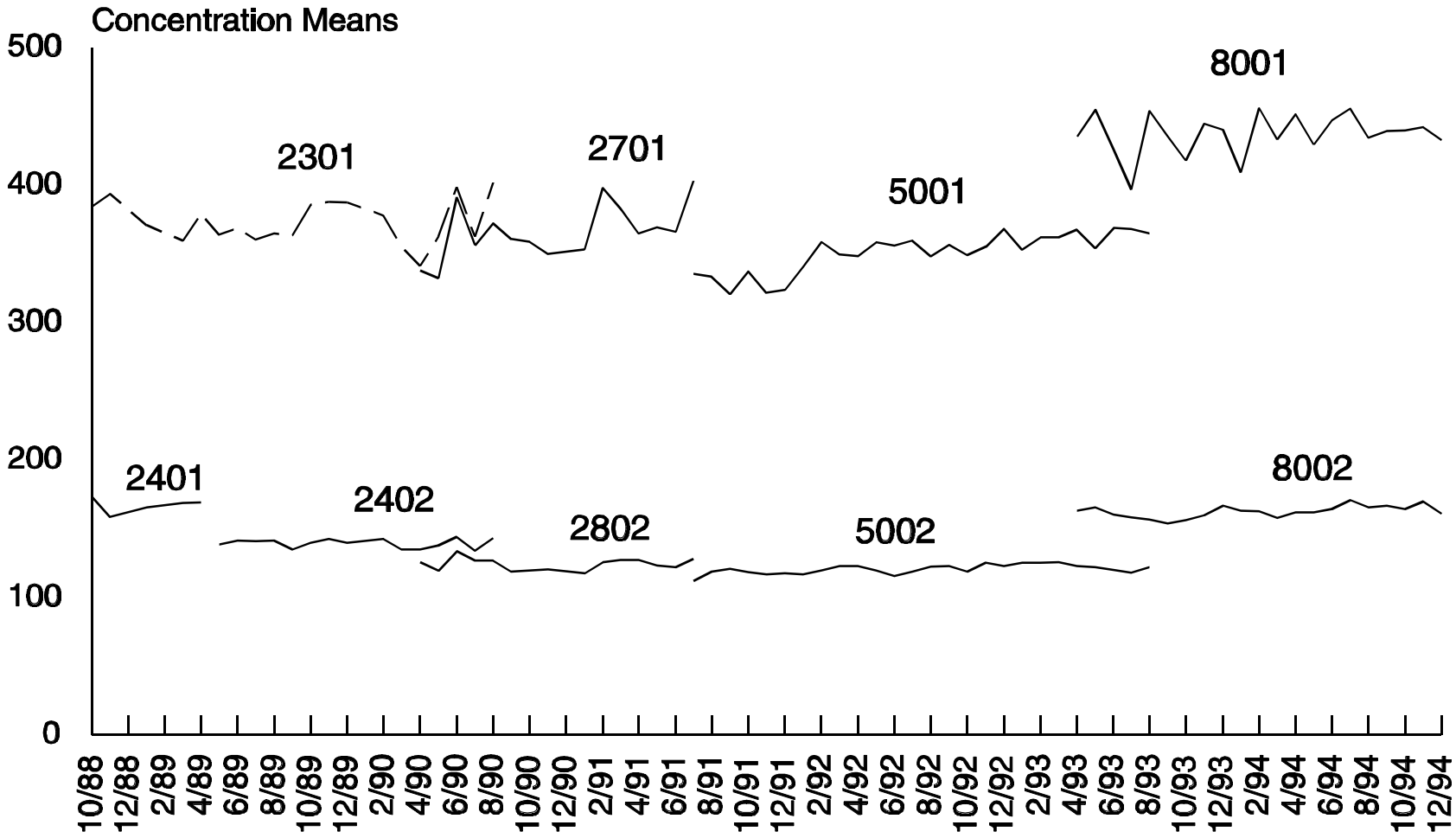



NOTE: No specimens assayed during the months 12/88, 2/89, 1/90, and 12/90.

SUMMARY STATISTICS FOR
FERRITIN (HIGH POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 2301 | 10/88 - 08/90 | 368.493 | 24.4975 | 6.6480 | 192 |
| 2401 | 10/88 - 04/89 | 167.589 | 7.4384 | 4.4385 | 50 |
| 2402 | 05/89 - 08/90 | 138.842 | 6.3854 | 4.5990 | 142 |
| 2701 | 04/90 - 07/91 | 361.321 | 28.6072 | 7.9174 | 180 |
| 2802 | 04/90 - 07/91 | 123.061 | 6.7837 | 5.5124 | 179 |
| 5001 | 07/91 - 08/93 | 351.594 | 22.2020 | 6.3147 | 308 |
| 5002 | 07/91 - 08/93 | 121.026 | 5.1514 | 4.2565 | 308 |
| 8001 | 04/93 - 12/94 | 437.653 | 27.1884 | 6.2123 | 182 |
| 8002 | 04/93 - 12/94 | 162.515 | 7.4396 | 4.5778 | 179 |

# Ferritin Monthly Means - High Pools




NOTE: No specimens assayed during the months 12/88, 2/89, 1/90, and 12/90.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Vitamin C (ascorbic acid) in serum is measured by isocratic high performance liquid chromatography (HPLC) with electrochemical detection at 650 mV. One part serum is mixed with four parts 6% MPA to acidify the serum and stabilize the ascorbate. The specimen is frozen at -70 °C until analysis. After the specimen is thawed at room temperature and centrifuged at 2500 rpm, the supernatant is decanted. This supernatant is mixed with a solution containing trisodium phosphate and dithiothreitol to reduce dehydroascorbate to ascorbate and then re-acidified with 40% MPA to stabilize the ascorbate. The sample is filtered to remove insoluble material. An aliquot is injected onto a C-18 reversed-phase column and eluted with a mobile phase containing 0.15 mol/L monochloroacetic acid, 2 mmol/L disodium ethylenediamine tetraacetate, and 0.13 mmol/L octylsulfonic acid, adjusted to pH 3.00 ± 0.05 with 10 N sodium hydroxide. Quantitation is by peak height and is based on a standard curve generated by using three different concentrations of an external standard (0.005, 0.03, and 0.1 mg/dL).

Ascorbic acid deficiency causes scurvy; however, scurvy is uncommon in the United States. Excess ascorbic acid consumption may cause adverse interactions with some drugs and may also interfere with the results of some clinical chemistry tests. Ascorbic acid has very low toxicity. Adverse effects reported from excess vitamin C consumption include diarrhea and kidney stones.

## 2. SAFETY PRECAUTIONS

Consider all serum specimens received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. We recommend the hepatitis B vaccination series for all analysts working with whole blood and/or serum. Observe universal precautions; wear laboratory coats, safety glasses, and protective gloves during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place all plastic and glassware that contacts serum in an autoclave bag for disposal. Handle acids and bases with extreme care; they are caustic and toxic.

Material safety data sheets (MSDSs) for methanol, ethanol, monochloroacetic acid, disodium ethylenediamine tetraacetate, metaphosphoric acid, sodium hydroxide, sodium phosphate tribasic, L-ascorbic acid, dithiothreitol, and sodium chloride are available through the NCEH/EHLS computer network.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Calculate the values for a given run using the Maxima software installed on the PC. After a run is complete and ready to be submitted to the supervisor, copy the summary table and all data files, data interchange format (DIF) files, and method files to floppy disks. Delete the original files from the hard drive when the analyst is certain that all information has been successfully copied to floppies.

   The supervisor loads the DIF files into a temporary RBASE database where the data are checked, corrected if necessary, and put into the correct format for transfer to the NHANES III database. When the data have been approved, they are transferred to the NHANES III RBASE database located on the NCEH\EHLS PC network. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor copies the values to the NCHS mainframe computer with the other NHANES III data.

b. Data files are uploaded to the NCEH\EHLS PC network and then loaded on a WORM drive for storage. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by Division of Environmental Health Laboratory Science (EHLS) Local Area Network (LAN) support staff, and CDC Data Center staff.

c. Documentation for data system maintenance is contained in printed copies of data records, as well as in "system log" files on the local hard drives used for the archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. For best results, a fasting sample should be obtained.

b. Specimens for vitamin C analysis must be prepared from fresh (not frozen) serum harvested from blood collected in a red-top or serum-separator Vacutainer tube collected by standard venipuncture procedures. One volume of serum is immediately mixed with four volumes of 6 g/dL metaphosphoric acid (MPA) in a polypropylene storage vial (e.g., 100 µL of serum is mixed with 400 µL of 6 g/dL MPA). The vial contents are then vortexed and immediately

frozen, preferably at -70 °C.

c. 500 µL of serum prepared as indicated above (with 2000 µL 6 g/dL MPA added) is preferable, but a sample prepared from 100 µL of serum may be analyzed.

d. The appropriate amount of diluted serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labeled with the participant's ID.

e. Specimens collected in the field are diluted according to directions given in Section 4.b. above, frozen, and then shipped on dry ice by overnight mail. Frozen samples are stored at -70 °C. Samples are stable for at least 5 years at -70 °C and can withstand five freeze/thaw cycles if the total time thawed at room temperature is no more than 4 hours.

f. Specimens generally arrive frozen. Refrigerated samples may be used provided they are kept cold and brought promptly (within 2 hours) from the site of collection.

g. Specimens that have been diluted improperly will give inaccurate test results.

h. Specimen handling conditions are outlined in the EHLS protocol for whole blood collection and handling. (Copies are available in the Special Activities Handling Office.) The protocol describes collection and transport procedures and the special equipment required.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Waters HPLC system (Waters Chromatography Division of Millipore Corporation, Marlboro, MA).

(a) Model 590 or Model 6000 solvent delivery system
(b) WISP 712 auto-sampler with refrigeration unit and 96-vial carriage
(c) TCM temperature control module with an RCM-100/column heater
(d) Model 820 workstation with Maxima software
(e) SIM Box

(2) BAS LC-4B Electrochemical Detector with a thin-layer amperometric detector cell (TL5A) (Bioanalytical Systems, Inc., West Lafayette, IN).

(3) Vortex mixer (American Scientific Products, McGaw Park, IL).

(4) Sorvall GLC-1 centrifuge (DuPont-Sorvall Instruments, Newton, CT).

(5) Magnetic stirrer (Baxter Scientific Products, Stone Mountain, GA).

(6) Digiflex diluter (Micromedic Division, ICN Biomedical, Inc., Costa Mesa, CA).

(7) Ultrasonic cleaner (Mettler Electronics, Anaheim, CA).

(8) Beckman Φ34 pH meter (Beckman Instrument, Inc., Fullerton, CA).

(9) S/P Multi-tube vortexer (Baxter-Scientific Products).

(10) Eppendorf micropipet (Brinkmann Instruments, Inc., Westbury, NY).

(11) Gilson Pipetman micropipet (Rainin Instrument Co., Inc., Woburn, MA).

(12) Mettler PM400 balance (Mettler Instrument Corp., Hightstown, NJ).

(13) Sartorius analytical balance, model 1712 MP8 (Brinkmann Instrument Co.).

b. **Materials**

(1) Rainin "Microsorb" 5-µ C-18 column, 4.6-mm internal diameter X 25-cm length (Rainin Instrument Co., Inc.).

(2) Clear autosampler vials springs and inserts (Sun Microsystems, Wilmington, NC).

(3) 0.45-µm syringe-tip filter (4-mm diameter) (Millipore Corp., Bedford, MA).

(4) Plastic tuberculin syringes (Becton-Dickinson Co., Rutherford, NJ).

(5) Millipore teflon filtration system and 0.22-µm filters, cat. no. H8E85066A (Millipore).

(6) Millex-HA, 0.45-µm filter unit (cat. no. LHA0250S) (Millipore).

(7) 10-mL syringe with regular Luer tip (Becton-Dickinson).

(8) PIC B-8 Reagent, 1-octane sulfonic acid (Waters Chromatography Division).

(9) Methanol, HPLC grade (Fisher Scientific, Inc., Fairlawn, NJ).

(10) Acetone, HPLC grade (Fisher Scientific, Inc.).

(11) Monochloroacetic acid, AR crystal (Mallinckrodt, Inc., Paris, KY).

(12) Disodium ethylenediamine tetraacetate (EDTA), AR grade (Mallinckrodt, Inc.).

(13) Phosphoric acid meta (MPA), AR grade (Mallinckrodt, Inc.).

(14) 10 N Sodium hydroxide (NaOH) (Mallinckrodt, Inc.).

(15) Sodium phosphate, tribasic (TSP), ACS grade (Fisher Scientific, Inc.).

(16) L-ascorbic acid, ACS grade (Fisher Scientific, Inc.).

(17) Dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO).

(18) Sodium chloride (NaCl), ACS grade (Fisher Scientific, Inc.).

(19) Nalgene cryovials (Nalge Company, Rochester, NY).

(20) 10- x 75-mm disposable glass culture tubes (Corning Glassworks, Corning, NY).

(21) 12- x 75-mm disposable glass culture tubes (Corning Glassworks).

(22) 16- x 125-mm disposable glass screw top culture tubes (Corning Glassworks).

(23) 5¾-in pasteur pipets (Kimble, Toledo, OH).

(24) Various glass beakers, volumetric flasks, graduated cylinders, and bottles.

(25) 4-mL clear injection vial with screw top and septum.

c. **Reagent Preparation**

> *Prepare all reagents using deionized water with resistance of at least 15 ohms. Store all reagents at 4 °C, bringing them to room temperature before use.*

(1) Mobile Phase
In a 2-L volumetric flask, combine 28.35 g monochloroacetic acid, 1.489 g EDTA, 52 mL 0.005 mol/L octylsulfonic acid, and enough 10 N NaOH (18-20 mL) to bring the solution to pH 3.00 ± 0.05. Add a stirring bar and deionized water and mix on a magnetic stirrer for about 2 min. Place the flask in a sonicator for about 30 min (shaking after 15 min) until the solution is dissolved. Adjust the solution to pH 3.00 ± 0.05 with 10 N NaOH if needed, remove the stirrer, and dilute to volume. Filter the solution daily. The solution is stable indefinitely.

(2) 40 g/dL Metaphosphoric Acid
Dissolve 40 g MPA in a 100-mL volumetric flask and dilute to volume with deionized water. The solution is stable for 3-4 weeks.

(3) 15 g/dL Metaphosphoric Acid + 0.25 g/dL Dithiothreitol
Dissolve 75 g MPA and 1.25 g DTT in deionized water in a 500-mL volumetric flask and dilute to volume. The solution is stable for 3-4 weeks.

(4) 0.1 mol/L Trisodium Phosphate + 0.25 g/dL Dithiothreitol
Dissolve 19 g TSP and 1.25 g DTT with deionized water in a 500-mL volumetric flask and dilute to volume. The solution is stable for 3-4 weeks.

(5) 0.005 mol/L Octylsulfonic Acid
Dissolve 1 bottle of PIC B-8 reagent in a 1000-mL volumetric flask and dilute to volume. The solution is stable for 6 months.

(6) 3 mol/L Sodium Chloride
Dissolve 17.532 g NaCl in deionized water in a 100-mL volumetric flask and dilute to volume. The solution is used only for storing the reference electrode and is stable for about 3 months.

d. **Standards Preparation**

(1) Stock Standard
Prepare the stock ascorbic acid standard (0.1 g/dL) by dissolving 50 mg of ascorbic acid in 15g/dL MPA + 0.25g/dL DTT in a 50-mL volumetric flask and diluting to volume. Prepare the solution monthly. Store at 4 °C.

(2) Intermediate Standard -- 0.25 mg/dL
Prepare by diluting 125 µL of the stock standard in a 50-mL volumetric flask with 15 g/dL MPA + DTT. Prepare a fresh solution when the bench QC pools are out of control (1-2 times a week). Store at 4 °C.

(3) Working Standard
Prepare these standards (0.005 mg/dL, 0.03 mg/dL, and 0.1 mg/dL) daily from the intermediate standard by using a Digiflex diluter with a 10-mL syringe and an appropriate Gilson pipette in a 16- X 125-mm test tube to dilute, respectively, 100 µL, 600 µL, and 2000 µL of the intermediate standard to 5 mL with 15 g/dL MPA + DTT. Vortex the standard solution and filter it into a 16- X 125-mm test tube, using a 10-mL syringe and a 0.45-µm Millex-HA filter.

e. **Preparation of Quality Control (QC) Materials**

All serum pools are sterile filtered before being dispensed. Serum (500 µL) and 6 g/dL MPA (2 mL) are aliquoted into sterile 5-mL glass Wheaton vials, sealed, and vortexed. The QC pools are stored at -70 °C and are stable for at least 3 years.

The low QC pool is prepared by pooling serum from smokers, who have very low vitamin C levels.

The medium QC pool is prepared by pooling serum from people whose vitamin C level is close to the mean levels observed in normal subjects.

The high QC pool is prepared by pooling serum from volunteers who take doses of >1 g of vitamin C/day for at least 5 days prior to collection.

Limits for all pools are established by analyzing duplicates for at least 20 consecutive days.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

NIST reference materials are not available for vitamin C assays. Each time an analysis is run, the system is calibrated through the use of three different concentrations of working standards (0.005 mg/dL, 0.03 mg/dL, and 0.1 mg/dL) prepared as described in Section 6.d. of this document. A three-point linear standard curve, which is forced through zero, is generated by the Maxima software on the LC system PC. The system is recalibrated after every 20 to 40 specimens, depending on the run length. The standards are reanalyzed as unknowns at the end of each run and must be within 15 g/dL of the established concentration.

The standard curves are saved electronically as part of the method (.MTH) file for the run on a write-once-read-many optical disk.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Procedure**

(1) Instrument Preparation

(a) Turn on the WISP and the refrigeration unit. Set the refrigeration unit to maintain a temperature of 4 °C.

(b) Turn on the BAS electrochemical detector to a positive current of 650 mV with 100 nA full scale.

(c) Set the column heater temperature to 29 ± 2 °C.

(d) De-gas the mobile phase using the Millipore filtration unit.

(e) Place freshly filtered, degassed mobile phase in the reservoir for the pump, turn the pump on, and set it to "manual control" with a flow rate of 0.7-1.0 mL/min.

(f) Allow the column to equilibrate with the mobile phase under flow for 1 to 1.5 hours before starting the analyses.

(g) Turn on the SIM box.

(h) Turn on the computer and access the "Maxima" program.

(i) Press the F6 key, program the flow rate to change to the desired rate (0.7-1.0 mL/min) over 0.25 min, and select "GO." When flow is achieved, exit to the main screen.

(j) Set the pump to "external control."

(k) Load a method that contains the parameters shown in Table 1.

The component table should have all components calibrated on the basis of height, with no weighing, and a linear curve forced through the origin. The retention times will vary with the age and cleanliness of the column, flow rate, and other system variables. Retention times need to be determined and adjusted on a daily basis, and the component table should be adjusted when necessary. The elution order of DTT and uric acid will reverse with column aging. The three components present are ascorbic acid, uric acid, and DTT. The retention times will generally be 4.00 ± 1.0 min for ascorbic acid and 6.25 ± 1.00

min for uric acid. The exact retention times will depend on the length of tubing, the age of the column, the cleanliness of the column, and the flow rate. The DTT peak retention time will vary significantly, because its initial peak on a new column appears at about 15 minutes, which means it is seen not in the first, but in the second chromatogram before the uric acid peak of the second sample. Then, depending on the previously mentioned variables, that peak could be delayed even longer to the third chromatogram. Since the DTT is not quantitated, it is important that the column is regularly cleaned and the flow rate periodically readjusted to prevent this peak from co-eluting with the ascorbic acid or uric acid peaks.

Table 1
Acquisition Parameter

| Parameter | Setting |
|---|---|
| Run time | will vary, typically 5.00-12.50 min |
| Injection volume | 30 µL |
| Sampling frequency | 2.0 points/sec |
| Flow rate | will vary, should be 0.7-1.0 mL/min |
| Trigger | SIM |

The integration parameters will vary with the column type, column age, electrode age and other factors. The parameters shown in Table 2 may be used as a starting point.

Table 2
Integration Parameters

| Parameter | Setting |
|---|---|
| Baseline points | 10 |
| Filter window (in points) | 13 |
| Integration sensitivity (coarse) | 500 µV/sec |
| Integration sensitivity (fine) | 500 $\mu V/sec^2$ |
| Detect negative points | yes |
| Skim ratio | 8.000 |
| Minimum area | 100.0 µV/sec |
| Minimum height | 100.0 µV |
| Minimum width | 1.000 sec |

(l) Edit the Sample Queue making sure all sample numbers, file names, and vial numbers are correct. Enter the three standards (0.005, 0.03, 0.1) and a reagent blank. Insert a set of standards and low, medium, and high controls before every 40 samples. Reanalyze the standards and the reagent blank at the end of the run as unknowns. Enter a dilution factor of 22.25 for all patient samples and bench control specimens on the initial run. Use a dilution factor of 222.5 for repeat analysis of specimens that have had a 10-fold dilution with 15 g/dL MPA because the concentration was at least 3.00 mg/dL on the initial analysis. Save the Sample Queue and method for every run.

(m) Press the F3 key to access the "Execute Current Method" screen. Place an "F" in the left column by the first sample and an "L" by the last sample. (This puts an "X" by all samples to be analyzed.)

(n) Select "EDIT" and edit the Queue-Wide Parameters. Enter the path (drive, directory, subdirectory) of the directory where the data files are stored. Put an "X" by "Save Peak," "Integration," "Append to Database (for a summary), and "Printer" (so the chromatograms will be printed).

(o) Start the method by using the mouse to select the box labeled START.

(2) Sample Preparation

(a) While preparing the specimens, analyze a specimen or a bench QC pool from a previous run in duplicate to establish the run time and the retention times for the analytes, because these will change daily.

(b) Thaw prepared samples at room temperature.

(c) Vortex-mix the samples and centrifuge them for 20 min at 2500 rpm.

(d) Decant supernatants into 12- x 75-mm culture tubes.

(e) Add 100 µL of supernatant to a 10- x 75-mm culture tube using a Gilson pipette with a yellow tip .

(f) Add 300 µL of TSP/DTT with the Digiflex diluter, vortex the solutions on the multitube vortexer, and let the solutions stand 30 min.

(g) Add 45 µL of 40 g/dL MPA with the Digiflex diluter and vortex the solution on the multitube vortexer.

(h) Draw the sample into a 1-mL tuberculin syringe and filter it through a 0.45-µm filter into a 10- x 75-mm culture tube.

(i) Using a pasteur pipet, transfer the sample to a micro-volume insert in an injection vial and seal the vial.

(j) Place the vial in a 96-vial tray, and insert the tray in the WISP.

b. **System Maintenance**

Flush the WISP, column, and BAS detector weekly with deionized water. Take the BAS detector off line. Rinse the reference electrode with deionized water and store it in a 4-mL vial with a septum and screw cap containing 3 mol/L sodium chloride. Polish the working electrode and store it. Re-equilibrate the column by recirculating mobile phase over the weekend at 0.2 mL/min. Every 3-4 weeks (on A Friday afternoon preferably), wash the column with water. Next, pump 50 mL of 80% methanol in deionized water through the column only, followed by flushing with deionized water for 2-3 hours. Pump mobile phase through the column over the weekend to re-equilibrate it.

Purge the WISP before every run. Defrost the refrigeration unit after each run. Monitor the fluid pack for leaks and change it when necessary because the mobile phase is corrosive and damages the valves of the fluid pack. Check the pump seals quarterly and change them if necessary. Monitor the inlet and outlet check valves of the pump and change or rebuild them if visible leaks or variable pump pressure indicates a problem.

c. **Special Method Notes**

(1) Always keep the column at 29±2 °C in a column oven during an analytical run.

(2) Allow the refrigeration unit at least 45 min to stabilize.

(3) Allow the BAS detector at least 1 hour to stabilize.

(4) Allow at least 60 to 90 min for the column to equilibrate to a new mobile phase at a flow rate of 0.8 mL/min.

(5) Turn the system completely off only if it will be idle for more than a week.

(6) If the system will be idle more than 3 to 4 days, take the detector off line, wash the column, and purge the system with deionized water followed by a solution of 80% methanol and 20% water. Before using the system

again, flush it with deionized water and re-equilibrate the column with mobile phase.

(7) Turn the BAS detector to standby whenever the flow is stopped. Flush the detector with deionized water weekly, take it off line, and turn it off. Remove the reference electrode and place it in 3 mol/L sodium chloride in a 4-mL clear screw-top injection vial with the top of the electrode protruding through the septum. It is preferable to have at least two electrodes, one in use and one equilibrating in 3 mol/L sodium chloride. Polish the working electrode with alumina, rinse it with deionized water, sonicate for 5 min, rinse it with deionized water and then with acetone, and air dry it. Then store the electrode until you are ready to reassemble it for analysis.

(8) Plug the BAS detector, SIM, and printer into line voltage conditioners to minimize the effects of electrical current fluctuations.

d. **Calculations**

All calculations are performed by the computer. Calibration curves are linear, forced through zero, and calculated on the basis of a single analysis of three different standard concentrations according to the following formula:

*Concentration = Response Factor x Peak Height x Dilution Factor*

e. **CDC Modifications**

The analysis is the analytical method described in Herman et al. (1) except that only ascorbic acid is measured, and a 25-cm reverse-phase C-18 column is used.

## 9. REPORTABLE RANGE OF RESULTS

The reportable range of results for vitamin C is 0 to 15 mg/dL. Samples with results <0.05 mg/dL or >1.5 mg/dL are reanalyzed before results are released. Linearity for this method has been established for the range 0-3 mg/dL. Samples with vitamin C levels ≥3.0 mg/dL are diluted 10-fold with 15 g/dL MPA + 0.25 g/dL DTT and reanalyzed (see Section 6.c.(3) for preparation instructions). This method has a total coefficient of variation in the range of 5-7%.

## 10. QUALITY CONTROL (QC) PROCEDURES

a. Blind Quality Controls
Blind QC specimens are inserted prior to the arrival of the samples in the Nutritional Biochemistry Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

b. Bench Quality Controls
Bench QC specimens are prepared from three serum pools, which represent high, medium, and low levels of vitamin C in serum. These pools are prepared in the same manner as patient samples and analyzed 2-4 times as part of each run. The results from the pools are checked after each run, and the run is repeated if the mean values of two bench QC specimens are outside the 95% limits or the mean value of one control specimen is outside the 99% limits. The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated annually.

While a study is in progress, the supervisor stores hard copies of the QC results from each analysis in a notebook, a copy of which is also kept by the analyst. Electronic copies of the QC analyses are stored on a write-once read-many optical disc.

Reanalyze samples with vitamin C values outside of the normal range on a subsequent run. Dilute samples that have a vitamin C concentration greater than 3 mg/dL 10-fold prior to reanalysis. Refreeze residual supernatant from the first analysis at -70 °C for repeat analyses, if necessary.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Check to make sure that the hardware is functioning properly. Make sure the pump is operating at the appropriate

pressure with steady delivery. Check the WISP to make sure the injections are being made as programmed.

If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions. Do not report analytical results for runs not in statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Samples that are improperly diluted or have not been promptly diluted after collection will give inaccurate results. Little or no supernatant, no precipitate, or sample volumes inconsistent with the other samples are evidence that a sample has not been diluted correctly. When you encounter any of these situations, write "UNSAX" next to the sample number on the runsheet along with a description of the problem. This indicates that the sample is unsatisfactory and cannot be run.

## 13. REFERENCE RANGES (NORMAL VALUES)

Reference ranges have not been established for vitamin C, but will be established by using the data from NHANES III. The current proposed normal range for this method is 0.05-1.5 mg/dL.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Because there is no evidence that excessively high or low serum concentrations of this analyte constitute an immediate threat to the life of a subject or that they are markers for significant pathological conditions, this analyte is not considered critical, and thus there are no procedures for reporting abnormal results on an emergency basis.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, place the prepared samples in the WISP at 4 °C. Return the unused portion of the patient sample to the freezer.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Because the analysis of serum for vitamin C is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, then we recommend storing the serum at $\leq$ -20 °C until the analytical system is restored to functionality.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

### a. Quality Control Data

Use the "HANES Laboratory Quality Control Summary Sheet" for the vitamin C method to record retention times, standard concentrations, and QC pool concentrations. The QC data are reviewed and approved by the supervisor. Hard copies of the QC results from each analysis are stored in a notebook kept by the supervisor while the relevant study is in progress and for at least 2 years after its completion. A copy is also kept by the analyst. Electronic copies of the QC analyses are stored on a write-once-read-many optical disc.

### b. Analytical Results

Print a hard copy of the summary table for each run from the Maxima software on the PC that runs the LC system. The summary table contains all of the results for standards, QC pools, and participant samples. Give this printout to the supervisor along with the Quality Control Summary Sheet. The hard copy of the summary table is kept in a notebook by the supervisor while the relevant study is in progress and for at least 2 years after its completion. A copy is also kept by the analyst.

The supervisor loads the DIF files for each run from a floppy diskette into a temporary RBASE database where the data are checked, corrected if necessary, and put into the correct format. When the data have been approved by the supervisor, they are transferred to the NHANES III RBASE database located on the NCEH\EHLS PC network. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor copies the values to the NCHS mainframe computer with the other NHANES III data and reports them out through the primary CDC investigator of the study.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

RBASE and the CDC mainframe are used to keep records and track specimens. Records, including related QA/QC data, will be maintained for 10 years after the NHANES study is completed. Only numerical identifiers (e.g., case ID numbers) should be used.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -70 °C. The specimen ID is read off of the vial by a barcode reader attached to the computer used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the DIF file containing the electronic copy of the results is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for keeping a notebook containing the ID numbers of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies.

### REFERENCES

1. Herman HH, Wimalasena K, Fowler LC, Beard CA, May SW, Demonstration of the ascorbate dependence of membrane-bound dopamine B-monooxygenase in adrenal chromaffin granule ghosts. J Biol Chem 1988;263:666-72.

### ACKNOWLEDGMENTS

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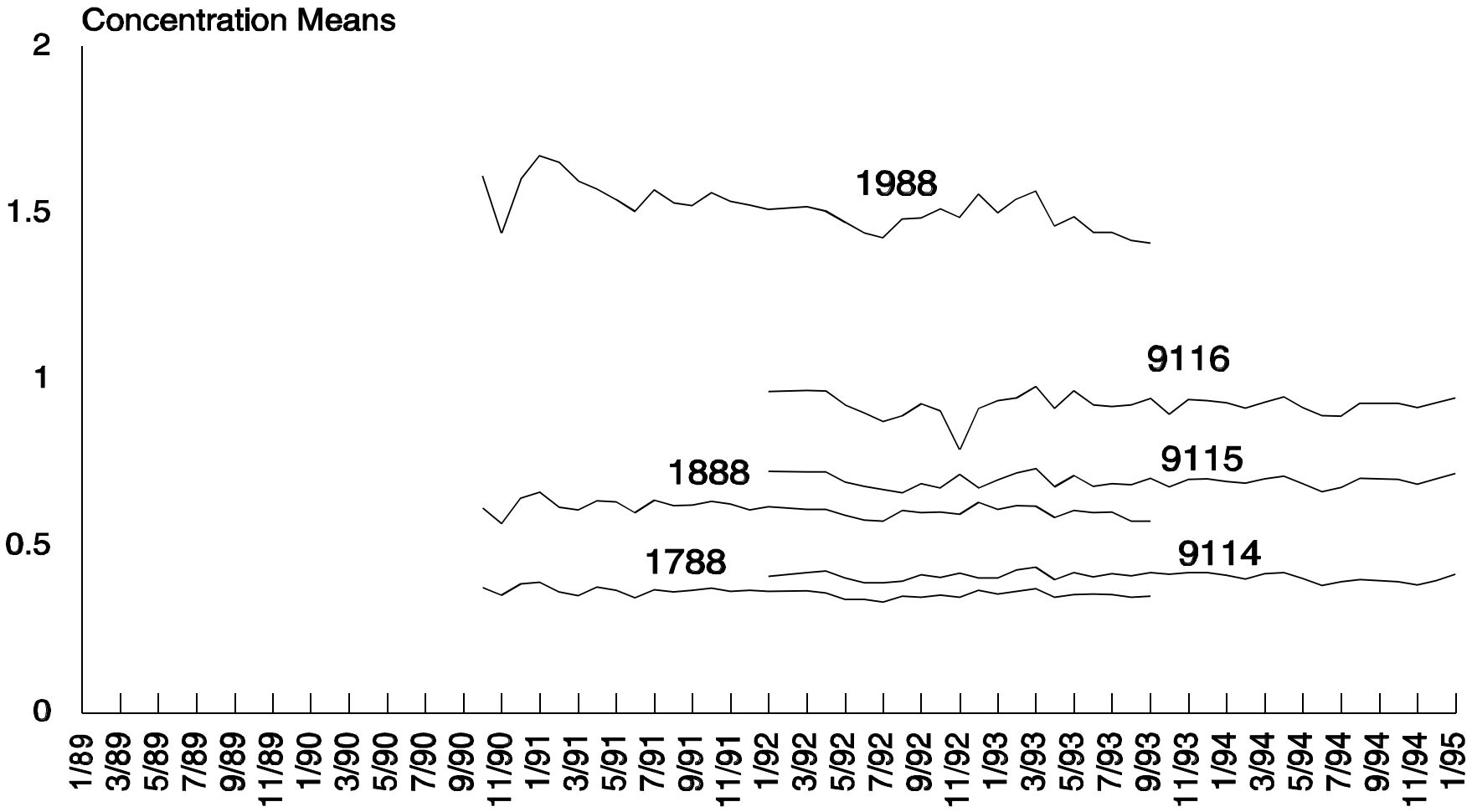
SUMMARY STATISTICS FOR
VITAMIN C
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 1788 | 10/90 - 09/93 | 0.36798 | 0.02887 | 7.8462 | 1531 |
| 1888 | 10/90 - 09/93 | 0.61900 | 0.04729 | 7.6395 | 1530 |
| 1988 | 10/90 - 09/93 | 1.55699 | 0.12566 | 8.0704 | 1531 |
| 9114 | 01/92 - 01/95 | 0.40981 | 0.02363 | 5.7665 | 425 |
| 9115 | 01/92 - 01/95 | 0.69762 | 0.03614 | 5.1810 | 425 |
| 9116 | 01/92 - 01/95 | 0.92986 | 0.05069 | 5.4509 | 421 |

# Vitamin C Monthly Means




NOTE: No specimens assayed for Vitamin C during 2/92 and 9/94.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Vitamin D is functionally a hormone, rather than a vitamin, and is one of the most important biological regulators of calcium metabolism, in conjunction with parathyroid hormone and calcitonin. As calciferol enters the circulation, it is metabolized to several forms, the primary one being 25-hydroxycalciferol (25-OH-D) (1). The first step in the metabolism of vitamin D, 25 hydroxylation, occurs mainly in the liver (2). In humans, only a small amount of 25-OH-D is metabolized in the kidney to other di-hydroxy metabolites (3,4). Because 25-OH-D is the predominant circulating form of vitamin D in the normal population, it is considered to be the most reliable index of people's vitamin D status (5). Vitamin $D_3$ (cholecalciferol) is the naturally occurring form of vitamin D produced in the skin after 7-dehydrocholesterol is exposed to solar UV radiation. Vitamin $D_2$ (ergocalciferol) is produced synthetically by UV irradiation of ergosterol. The two forms differ in the structures of their side chains, but they are metabolized identically and have equivalent biological activities. Both forms are used for fortification of dairy products. Because these two parent compounds provide various contributions to people's overall vitamin D levels, it is important that both forms are measured equally (5,6).

The measurement of 25-OH-D (referred to as the vitamin D assay) is becoming increasingly important in the management of patients with various disorders of calcium metabolism associated with rickets, neonatal hypocalcemia, pregnancy, nutritional and renal osteodystrophy, hypoparathyroidism, and postmenopausal osteoporosis (7-11).

The INCSTAR 25-OH-D assay consists of a two-step procedure. The first procedure involves a rapid extraction of 25-OH-D and other hydroxylated metabolites from serum or plasma with acetonitrile. Following extraction, the treated sample is assayed by using an equilibrium RIA procedure. The RIA method is based on an antibody with specificity to 25-OH-D. The sample, antibody, and tracer are incubated for 90 min at 20-25 °C. Phase separation is accomplished after a 20-minute incubation at 20-25 °C with a second antibody-precipitating complex.

## 2. SAFETY PRECAUTIONS

The vitamin D assay employs $^{125}I$ as a tracer (at a level that does not exceed 4 µCi). Therefore, observe all necessary radiation safety considerations for isotope management and disposal according to the guidelines of the CDC *Radiation Safety Manual*. In addition, all personnel must successfully complete the CDC training course *Radiation Safety in the Laboratory*, or demonstrate knowledge equivalent to those who did. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus, observe Universal Precautions; wear protective gloves during all steps of this method because of both infectious and radioactive contamination hazards. (We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials.) Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines.

Two reagents of special concern in this kit are sodium azide and acetonitrile. Sodium azide may react with lead or copper plumbing to form highly explosive metal azides. When disposing of this reagent, flush it with a large volume of water to prevent azide build-up (12).

Acetonitrile is a flammable substance, and exposure to its liquid or vapor is harmful. If exposure occurs, remove contaminated clothing, flush affected areas with copious amounts of water, and call a physician. If someone inhales acetonitrile, move him or her to fresh air, and give artificial respiration and oxygen if respiration is impaired. In case of a fire, extinguish it with dry chemicals or carbon dioxide. In case of a spill, carefully remove and dispose of the acetonitrile according to environmental regulations.

Material safety data sheets (MSDSs) for all chemicals contained in the kit are available in the MSDS section of the "Working Safely With Hazardous Chemicals" notebook, which is located in the laboratory as well as on the EHLS LAN.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation and calculation of the run are accomplished with the ISODATA software on the Micromedic Apex gamma counter.

   After the data are calculated and the final values are approved by the reviewing supervisor for release, the results are transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network; data entry is verified by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the

values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Although a fasting specimen is recommended, it is not required. No special instructions such as special diets, are required. Diurnal variation is not a major consideration.

b. Specimens for vitamin D analysis should be fresh or frozen serum. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers. Serum specimens should be stored at $\leq$-20 °C

c. A sample volume of 50 µL is required for the assay; 150 µL will permit repeat analysis and adequate pipetting volume as well.

d. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

e. Because vitamin D is very stable, serum samples may be frozen at -20 °C to -70 °C for years before analysis. Several freeze-thaw cycles do not seem to adversely effect the assay, although repeated freeze-thaw cycles should be avoided.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site of collection.

g. Moderately hemolyzed specimens may be used because red blood cells do not contain vitamin D.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and Special Activities Branch specimen handling office). The protocol discusses collection and transport of specimens and the special equipment required. In general, whole blood specimens should be transported and stored at no more than 4 °C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at <-20 °C. Samples thawed and refrozen several times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, transfer the appropriate amount of serum into a sterile Nalge cryovial labeled with the participant's ID.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) Micromedic Apex Automatic Gamma Counter (Micromedic Division, ICN Biomedical, Costa Mesa, CA).
Or: Micromedic Model 10/600 Plus Gamma Counter (ICN Biomedical).

(2) Beckman J6-B centrifuge, 222-tube capacity, temperature-controlled (Beckman Instruments, Inc., Palo Alto, CA).
Or: Beckman TJ-6 centrifuge (Beckman Instruments).

**NOTE**: Centrifuge should be capable of 1800 x g.
$(g = 1118 \times 10^{-8})(\text{radius in cm})(\text{rpm})^2$.

(3) Gilson Pipetman pipettes, 25- and 50-µL sizes (Rainin Instrument Co., Woburn, MA).

(4) Eppendorf Repeater 4780, 1.0- to 5.0-mL size (Brinkmann Instruments, Westbury, NY), to deliver 50-, 500-, and 1000-µL volumes.

(5) Quatro 240 Sample Processor with Quatro Concerto software, IBM compatible computer, and 96 tube sample racks (Matrix Technologies Corporation, Lowell, MA).

(6) Vortex mixer (Fisher Scientific, Fairlawn, NJ).

b. **Materials**

(1) INCSTAR Corporation 25-Hydroxyvitamin D $^{125}$I RIA kit, 100-test size (cat. no. 68100, INCSTAR Corporation, Stillwater, MN).

(2) Disposable 12- x 75-mm borosilicate glass tubes (American Scientific Products, McGaw Park, IL).

(3) Two levels of normal human serum quality control pools (low-normal and high-normal) prepared in-house at CDC to complement two levels of QC pools provided with the kit. Materials are dispensed as 200-µL aliquots in tightly capped Nalge cryovials and stored at $\leq$-70 °C until used.

(4) Corks, size 2 (any vendor).

(5) Parafilm, 4 inch roll (any vendor).

c. **Reagent Preparation**

(1) Donkey-anti-goat (DAG) Precipitating Complex
Donkey anti-goat serum, normal goat serum, and polyethylene glycol are diluted in a bovine serum albumin-borate buffer containing antimicrobial reagents. Although no reconstitution is necessary, mix the reagent for 5-10 min before and during use to ensure that a homogeneous suspension is achieved. If the reagent is not entirely used in one analytical run, store it at 2-8 °C until the expiration date on the label.

(2) Standards, quality control materials, and reagents are supplied ready to use, with no reconstitution necessary. If not used in one run, the kit contents should be stored at 2-8 °C until the expiration date on the label. The 25-OH-$D_3$ tracer contains $^{125}$I and should be properly handled with gloves and disposed of according to CDC radiation safety guidelines.

d. **Standards Preparation**

Vitamin D Standards (0.0, "A"/5.0, "B"/12.0, "C"/20.0, "D"/40.0, and "E"/100.0 ng/mL) are supplied as prediluted 25-OH-$D_3$ in processed human serum in a liquid form, ready to be used. If the entire kit is not used in one run, store the standards at 2-8 °C for up to 10 days. These standards are prepared by INCSTAR and are calibrated independently (13) against HPLC-purified 25-OH D.

e. **Preparation of Quality Control Material**

Five levels of bench quality control materials are used. Normal and elevated levels of 25-OH-D in serum controls (the later produced by spiking with purified 25-OH-D standard solution) are provided with the kit. Three additional levels of serum pools have been prepared by CDC to complement the kit QC materials, in an effort to use in-vivo 25-OH-D concentrations. At present, few other sources of commercial QC materials are available with defined INCSTAR target levels, although the Bio-Rad Laboratories ECS Division (Anaheim, CA) is attempting to develop such materials.

Two levels of blind QC pools may be prepared from pooled, filter-sterilized fasting human serum obtained from donors with elevated or decreased vitamin D levels. Target levels are about 15 ng/mLfor those with decreased levels and 50 ng/mL for those with elevated levels. Pool the serum in acid-cleaned 20-L glass carboys and mix well on a magnetic stirrer. Clean-filter the serum through stacked Millipore filters in a sequential manner using the filters of the following pore sizes, each preceded by a prefilter: 3.00-µm, 1.20-µm, 0.80-µm, 0.65-µm, 0.45-µm, 0.30-µm, and 0.22-µm.

Using sterile technique under a laminar-flow hood and a Micromedic Digiflex, dispense the serum in 1-mL aliquots

into 2.0-mL Nalge cryovials. Cap and label the vials with NHANES barcoded labels that have been specially prepared for the QC pools. Store the pools at ≤-70 °C at the CDC Serum Bank in Lawrenceville where they will be inserted randomly into the NHANES runs. Select twenty vials of each level at random for characterization of the quality control limits and for testing of homogeneity.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

INCSTAR prepares Vitamin D calibration standards from HPLC-purified 25-OH-$D_3$ and reverifies them in its reference laboratory using a reference HPLC assay (13). These standards -- 5, 12, 20, 40, and 100 ng/mL -- are run with each assay. At present, the only available National Institutes of Standards and Technology Standard Reference Material (SRM) with target values assigned for vitamin D is a coconut-milk matrix material, which is not suitable for this assay.

Performance checks for the assay include:

- Nonspecific binding: The zero standard is used as the nonspecific binding. The CPM for the NSB tubes should be <6% of the CPM of the total counts.
- Maximum binding: The CPM of zero ng/mL standard should be >30% of the CPM of the total counts.
- Slope of the standard curve: The 80% and 50% points of the standard curve should be monitored for run-to-run reproducibility.

In addition, the kit standards are run every 6 months as unknowns to verify system calibration.
In accordance with the NCCLS method comparison protocol, a 40-sample comparison between the reference HPLC method (either the fully-validated in-house CDC HPLC method or the HPLC method of Dr. Bruce Hollis, Medical University of South Carolina - developer of the kit) will be performed in order to establish comparability.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Quick Reference Summary Table

Table 1
INCSTAR 25-Hydroxyvitamin D Flow Table

| | Total Cts | NSB | Standards* (ng/mL) | | | | | | Control and Unknown Samples* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | A/5 | B/12 | C/20 | D/40 | E/100 | 1 | 2 | 3 | 4 |
| Tube number | 1-2 | 3-4 | 5-6 | 7-8 | 9-10 | 11-12 | 13-14 | 15-16 | 17-18 | 19-20 | 21-22 | 23-24 |
| Extracted 0 Standard* | | 25 µL | 25 µL | | | | | | | | | |
| Extracted Standards* | | | | 25 µL | 25 µL | 25 µL | 25 µL | 25 µL | | | | |
| Extracted control & Unknown Samples | | | | | | | | | 25 µL | 25 µL | 25 µL | 25 µL |
| $^{125}$I 25-OH-D | 50µL | | | | | | | | | | | |
| NSB buffer | 1 mL | 1 mL | | | | | | | | | | |
| 25-OH-D antiserum | | | 1.0 mL | | | | | | | | | |
| | | Mix well, incubate for 90 min (± 10 min) at 20-25 °C. | | | | | | | | | | |
| DAG Precipitating Complex | | 500 µL | | | | | | | | | | |
| | | Mix well; incubate for 20-25 min at 20-25 °C. | | | | | | | | | | |
| | | Centrifuge using 1800 x g for 20 min. | | | | | | | | | | |
| | | Decant the supernatants. | | | | | | | | | | |
| | Count each tube in gamma counter for 60 sec or longer. | | | | | | | | | | | |

* Standards and control and unknown samples must be extracted with acetonitrile prior to the assay.

b. **Extraction Procedure**

1) Load standards, controls, and samples into a sample rack and place the rack on the Quatro 240.

2) Label a 12- x 75-mm disposable glass tube for each standard and control and patient sample and place the tubes into the recipient rack.

3) Add 500 µL of acetonitrile to each tube, using the Eppendorf Repeater.

4) Using the Quatro 240, transfer 50 µL of the standard, control, or unknown serum specimen to a recipient tube. Make sure that the samples are dispensed slowly and that the pipet tips are below the surface of the acetonitrile while dispensing.

5) After each sample is delivered, place a cork in the top of the tube and vortex the contents for 10 sec.

6) Centrifuge the samples using 1200 x g for 10 min at 20-25 °C.

7) Using the Quatro 240, transfer 25-µL from the supernatant into a second set of appropriately labeled 12- x 75-mm glass reaction tubes in duplicate. (Do not remove the corks until immediately prior to sample transfer.)

**CAUTION:** Take care not to disturb the pellet.

8) Proceed with the assay procedure.

c. **Assay Procedure**

Follow steps 1-2 to prepare the reaction tubes for analysis:

1) Allow all reagents to equilibrate to ambient room temperature. Reconstitute the lyophilized controls. Do not allow any reagents to warm above 25 °C.

2) Using the Eppendorf Repeater pipet, add reagents as follows:

a) Total Counts Tubes

- 50 µL of $^{125}$I 25-OH-D
- 1.0 mL of NSB buffer

b) Nonspecific Binding Tubes

- 25 µL of 0.0 standard (extracted)
- 50 µL of $^{125}$I 25-OH-D
- 1.0 mL of NSB buffer

c) Standards, Controls and Unknown Samples

- 25 µL of standard, control, or unknown sample (extracted)
- 50 µL of $^{125}$I 25-OH-D
- 1.0 mL of 25-OH-D antiserum

3) Cover the tops of the tubes with a sheet of parafilm and vortex them gently without allowing the contents to foam. Incubate tubes for 90 min at 20-25 °C.

4) Using the repeating dispenser, add 500 µL of DAG precipitating complex to all tubes except the total counts tubes.

5) Mix the contents of the tubes well and incubate the tubes for 20-25 min at ambient temperature.

6) Centrifuge using 1800 x g for 20 min at ambient temperature.

7) Decant the supernatants (except the total counts tubes).

8) Using the gamma scintillation counter, count each tube for 1 min to achieve statistical accuracy.

d. **Calculations**

Both the Micromedic Apex and 10/600 Plus counters have full data reduction capabilities. Linear $B/B_0$ vs $\log_{10}$ concentration with a cubic spline curve fit is used in both counters where:

$$B/B_0 = \frac{\text{CPM of Standard or Unknown Sample - CPM of NSB}}{\text{CPM of 0 Standard - CPM of NSB}} \times 100$$

and B = corrected counts/min (blank subtracted) for each tube, and $B_o$ = corrected counts/min of 0 standard (blank subtracted).

This method results in a linearized 6-point standard curve with an inverse relationship of levels of radioactivity (measured in counts per min, or CPM) to concentration of vitamin D in the serum sample. Serum results are expressed as nanograms of vitamin D per milliliter of serum (ng/mL).

e. **CDC Modifications**

This method was brought on line in January 1994 as a service method in the NHANES Laboratory, and no modifications of the manufacturer's instructions were made.

## 9. REPORTABLE RANGE OF RESULTS

The method described here is designed to detect serum 25-OH-D values from 0 to 100 ng/mL, which is beyond the normal range of values expected to be observed for human serum 25-OH-D concentration. Values <5.0 and >70 ng/mL are verified by reassay, including re-extraction. For re-extraction, dilute elevated specimens (>100 ng/mL) with 0 standard prior to extraction. Any samples with CVs >10% are also reassayed.

The limit of detection, when defined as the apparent concentration at 3 standard deviations from the counts at maximum binding, is 2.78 ng/mL. Any values less than the lowest standard, 5.0 ng/mL, are not calculated by the ISODATA software but are reported as "3.0." These levels could occur physiologically and would indicate severe 25-OH-D deficiency. Values greater than 70 ng/mL indicate prolonged exposure to ultraviolet radiation or excessive supplementation.

## 10. QUALITY CONTROL (QC) PROCEDURES

The two levels of 25-OH-D QC materials included with each kit are prepared by the manufacturer from human serum spiked with appropriate amounts of 25-OH-$D_3$ to produce concentrations within specified ranges. Sodium azide is added as an antimicrobial, and the materials are lyophilized for stability.

Like blind QC pools, bench QC pools may also be made from filter-sterilized fasting human serum, as described in Section 6.e.. An effort is made to obtain serum from individuals who are outdoors every day, such as joggers, and from persons who greatly restrict their outdoor activities. In this manner, one may obtain both high-normal and low-normal in-vivo concentrations of QC pools in a matrix identical to that of unknown specimens.

In every batch of 20 specimens analyzed, either one low- or one high-concentration blind quality control pool is randomly inserted. The blind pools are aliquoted and labelled in exactly the same fashion as the NHANES III specimens and are inserted in the specimen batches by the Serum Bank personnel when the specimens are received from the field and racked for analysis.

Long-term estimates of method precision have not yet been established for this method (which was first used in January 1994). Although initial evaluation indicated CVs of 20-30%, it is our experience that once an RIA method is in daily use, CVs may be improved to $\leq$10% fairly quickly.

Quality control data is entered into the mainframe ROSCOE program "QCDATA"; the plotting option of "QCPRTA" or "QCGRA" is used to produced long-term plots by using the Division quality control programs. Analysts retain hardcopy

updates of these plots; the supervisor maintains annual files of data and plots.

Limits are established for new pools after 20 runs.

The system is declared "out of control" if any of the following events occur:

For the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two or more pools fall either both above or both below the 95% limit.
- Two successive run means for a single pool fall either both above or both below the 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

For the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

Table 2
NHANES III Serum Vitamin D Quality Control Pools
1994 Summary

| Pool | Mean (ng/mL) | N | Total Std. Dev. | Total CV (%) | 95% Limits | 99% Limits |
|---|---|---|---|---|---|---|
| 9400 | 21.6 | 29 | 3.23 | 14.96 | 15.51-27.62 | 13.59-29.53 |
| 9401 | 24.9 | 77 | 4.53 | 18.17 | 16.85-32.95 | 14.30-35.50 |
| 9402 | 16.1 | 18 | 3.10 | 19.29 | 10.41-21.72 | 8.62-23.51 |
| 9403 | 57.0 | 16 | 8.17 | 14.33 | 41.51-72.59 | 36.59-77.51 |
| 9404 | 17.2 | 18 | 2.97 | 17.29 | 11.71-22.65 | 9.98-24.38 |
| 9405 | 58.9 | 18 | 10.72 | 18.21 | 39.29-78.47 | 33.09-84.66 |
| 9406 | 12.0 | 26 | 1.63 | 13.66 | 9.21-14.70 | 8.34-15.57 |
| 9407 | 49.3 | 26 | 9.11 | 18.48 | 32.25-66.28 | 26.87-71.66 |
| 9408 | 17.7 | 18 | 3.10 | 17.48 | 11.99-23.51 | 10.17-25.33 |
| 9409 | 54.7 | 19 | 8.60 | 15.74 | 39.37-69.95 | 34.53-74.79 |
| 9410 | 21.6 | 19 | 3.48 | 16.09 | 15.16-28.07 | 13.12-30.11 |
| 9411 | 56.8 | 18 | 8.04 | 14.15 | 41.18-72.45 | 36.23-77.40 |
| 9412 | 19.5 | 8 | 2.94 | 15.06 | 13.67-25.37 | 11.82-27.22 |
| 9413 | 54.3 | 8 | 8.09 | 14.89 | 38.38-69.97 | 33.73-74.92 |

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the system should be declared "out of control," repeat the entire run. If the "out of control" condition still exists, use a new kit and evaluate the autodiluter for pipetting precision and accuracy. Reassay specimens for that analytical run after the system has been reverified to be "in control."

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The greatest source of imprecision in this method is likely to be the extraction step. Other sources of procedural

imprecision may be intermittently imprecise micropipettors or outdated reagents. If the initial concentration of a specimen is greater than that of the highest standard, the specimen should be diluted with 0 standard prior to re-extraction. Counting times should be sufficient to prevent statistical error (e.g., the accumulation of 2,000 CPM will yield 5% error, while the accumulation of 10,000 CPM will yield 1% error). Specimens should not be repeatedly frozen and thawed.

The kit antibody will demonstrate 2.5-100% cross-reactivity with all forms of di-hydroxy-vitamin $D_2$ and $D_3$ steroids; however, in humans these compounds are naturally present only in picomolar concentrations.

**13. REFERENCE RANGES (NORMAL VALUES)**

Season, race, and dietary intake are all known to affect the normal levels of 25-OH-D. 25-OH-D levels correlate well with ultraviolet radiation exposure. The reported difference in 25-OH-D values attributable to seasonal variation in ultraviolet radiation illustrates the importance of personal exposure to sunlight (14,15). The highest levels of 25-OH-D are found during the summer months, and the lowest levels during the winter. Race has also been shown to significantly influence the normal levels of 25-OH-D. It has also been reported that the mean plasma level of 25-OH-D in whites is greater than that in blacks (16).

Published normal ranges from smaller studies in the United States indicate an expected range of approximately 10-40 ng/mL. The NHANES III data will be used to define the normal U.S. levels when statistical analyses of these data are complete.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Because we are collecting data for national prevalence purposes only, we do not routinely notify survey participants with abnormal 25-OH-D values.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should remain at room temperature during preparation and testing, and then be promptly refrozen for storage.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods for performing this test for NHANES III. In case of system failure, store all specimens at $\leq$-20 °C until the system is functioning.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Use the "NHANES Lab Standards and Controls Summary Sheet" for vitamin D to record the quality control data. This reporting sheet has self-explanatory blanks for the standard concentrations and the quality control pool results. Prepare this form in duplicate.

Use the "NHANES III Analytical Worksheet" to record the specimen results. These have been prepared with sample IDs for each preracked run. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is not satisfactory (i.e., cannot be analyzed), write "UNSAX" in the blank. Prepare these forms in duplicate.

Give both types of forms to the supervisor along with the hard copy of the data printout from the gamma counter computer. After the supervisor checks the data, the carbon copies and the data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets. The results are transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network.

Use the "QCDATA6" program in ROSCOE on the CDC mainframe to record quality control data. This program should be updated regularly.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

RBASE and the CDC mainframe are used to keep records and track specimens. Records, including related QA/QC data, are maintained for 10 years beyond the duration of the survey. Only numerical identifiers (e.g., case ID numbers) should be used.

For the NHANES study, serum remaining after ionized calcium analysis is retained at -70 °C for vitamin D analysis.

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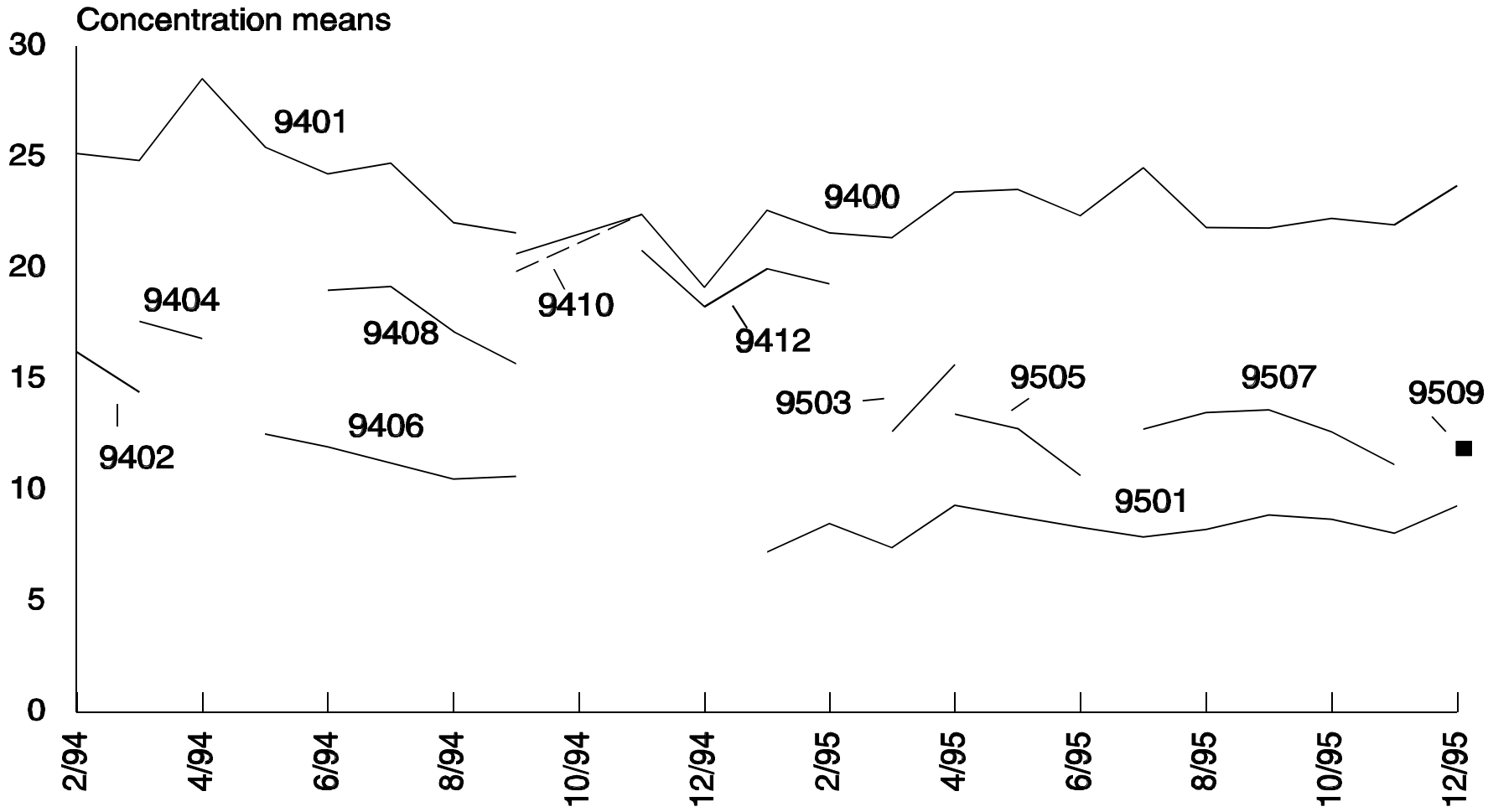
SUMMARY STATISTICS FOR
VITAMIN D (LOW POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 9400 | 09/94 - 12/95 | 22.3037 | 3.3010 | 14.8002 | 598 |
| 9401 | 02/94 - 09/94 | 24.9006 | 4.5253 | 18.1736 | 156 |
| 9402 | 02/94 - 03/94 | 16.0671 | 3.0999 | 19.2936 | 35 |
| 9404 | 03/94 - 04/94 | 17.1789 | 2.9709 | 17.2938 | 36 |
| 9406 | 05/94 - 09/94 | 11.9538 | 1.6327 | 13.6581 | 52 |
| 9408 | 06/94 - 09/94 | 17.7497 | 3.1019 | 17.4757 | 36 |
| 9410 | 09/94 - 11/94 | 21.6174 | 3.4788 | 16.0925 | 38 |
| 9412 | 11/94 - 02/95 | 19.5878 | 2.6775 | 13.6691 | 68 |
| 9501 | 01/95 - 12/95 | 8.4013 | 2.0960 | 24.9487 | 530 |
| 9503 | 03/95 - 04/95 | 13.1766 | 2.7472 | 20.8492 | 56 |
| 9505 | 04/95 - 06/95 | 12.5835 | 2.8743 | 22.8415 | 112 |
| 9507 | 07/95 - 11/95 | 12.8733 | 2.5712 | 19.9735 | 162 |
| 9509 | 12/95 | 11.8700 | 1.1672 | 9.8335 | 12 |

# Vitamin D Monthly Means - Low Pools

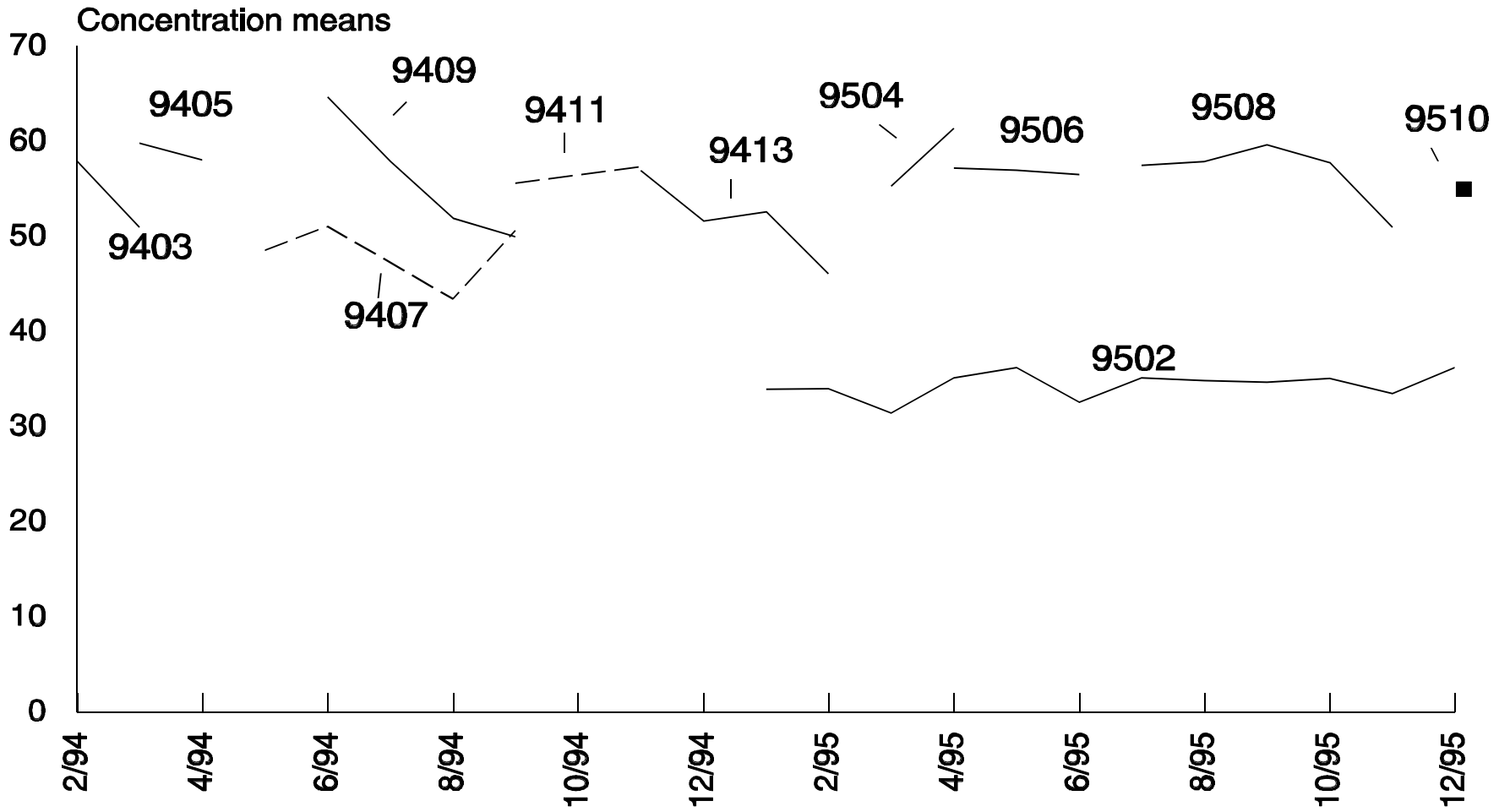



NOTE: Analysis for Vitamin D did not begin until February 1994.

SUMMARY STATISTICS FOR
VITAMIN D (HIGH POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 9403 | 02/94 - 03/94 | 57.0500 | 8.1744 | 14.3285 | 32 |
| 9405 | 03/94 - 04/94 | 58.8761 | 10.1799 | 18.2076 | 36 |
| 9407 | 05/94 - 09/94 | 49.2642 | 9.1063 | 18.4846 | 52 |
| 9409 | 06/94 - 09/94 | 54.6605 | 8.6012 | 15.7356 | 38 |
| 9411 | 09/94 - 11/94 | 56.8167 | 8.0411 | 14.1527 | 36 |
| 9413 | 11/94 - 02/95 | 50.6067 | 7.0498 | 13.9307 | 64 |
| 9502 | 01/95 - 12/95 | 34.3352 | 6.0105 | 17.5055 | 520 |
| 9504 | 03/95 - 04/95 | 56.1861 | 7.8232 | 13.9237 | 54 |
| 9506 | 04/95 - 06/95 | 57.0107 | 9.1190 | 15.9953 | 112 |
| 9508 | 07/95 - 11/95 | 57.1478 | 8.2613 | 14.4560 | 165 |
| 9510 | 12/95 | 54.9283 | 6.5495 | 11.9237 | 12 |

# Vitamin D Monthly Means - High Pools




NOTE: Analysis of Vitamin D did not begin until February 1994.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Lead is measured in blood by atomic absorption spectrometry in a procedure based on the method described by Miller, et al. (1). Quantification is based on the measurement of light absorbed at 283.3 nm by ground-state atoms of lead either from an electrodeless discharge lamp (EDL) or from a hollow-cathode lamp (HCL) source. Blood samples, quality control pools of human and bovine blood, and aqueous standards are diluted with a matrix modifier (nitric acid, Triton X-100, and ammonium phosphate). The lead content is determined by using either a Perkin-Elmer model 5000 graphite furnace atomic absorption spectrophotometer with deuterium background correction or a Perkin-Elmer model 5100 graphite furnace atomic absorption spectrophotometer with Zeeman effect background correction. Lead contamination must be carefully avoided throughout all procedures. All materials used for collecting and processing specimens are screened for possible lead contamination. All processing work is performed under clean conditions, including laminar flow hoods.

Lead analysis is performed to identify cases of lead toxicity.

## 2. SAFETY PRECAUTIONS

Use universal precautions when handling blood products. These include wearing gloves, a lab coat, and safety glasses. We recommend that all analysts working with whole blood or serum samples undergo the hepatitis B vaccination series. Place disposable plastic, glass, and paper (pipet tips, autosampler cups, gloves, etc.) that contact blood in a biohazard autoclave bag. These bags should be kept in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% sodium hypochlorite solution when work is finished. We recommend that analysts use the foot pedal on the Micromedic Digiflex automatic pipettor because it reduces their contact with work surfaces that have been in contact with blood and keeps their hands free to hold the specimen vials and autosampler cups and to wipe off the tip of the Micromedic Digiflex.

*Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis.*

*Take special care when handling and dispensing concentrated nitric acid. Always remember to add acid to water. This material is a caustic chemical capable of causing severe eye and skin damage. Wear metal-free gloves, a lab coat, and safety glasses. If the nitric acid comes in contact with any part of the body, quickly wash with copious quantities of water for at least 15 minutes.*

Material safety data sheets (MSDSs) for nitric acid, Triton X-100, ammonium phosphate, and argon are available through the EHLS computer network.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation and calculation of the run are accomplished with the SAS program "LEAD," which is run in ROSCOE on the CDC mainframe.
After the data are calculated and the final values are approved for release by the reviewing supervisor, the results are transcribed by the data entry clerk into the NHANES III database, which is located in RBASE on the NCEH/EHLS PC network; data entry is proofed by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transport the lead values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC data center staff, respectively.

c. Documentation for system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Specimen donors require no special dietary instructions.

b. Specimen type: whole blood with anticoagulant, preferably $K_3$EDTA at a concentration of 1.5 mg/mL whole blood.

c. The optimal amount of specimen is 2-3 mL (in an unopened collection tube); the minimum is about 500 µL (0.5 mL). Although originally collected in a pre-screened 2-mL EDTA vacutainer, specimens were carefully transferred to a lead-free 2.0 mL cryovial.

d. Acceptable containers include 2- to 7-mL vacuum tubes (e.g., lavender-top Vacutainers) and plastic (metal-free) syringes with plastic caps. The recommended anticoagulants are either $K_3$EDTA or $Na_2$EDTA (1.5 mg/mL). EDTA is preferred to heparin because heparin tends to allow the formation of micro clots. Heparinized blood may be used if necessary. Sterile collection systems should be used for specimen acquisition.

e. Specimen stability has been demonstrated for 1 year at both 4 °C and -20 °C. Stability at -70 °C has been demonstrated for at least 10 years.

f. Specimens are unacceptable if their volume is low (<0.5 mL), if they are suspected of being contaminated because of improper collection procedures or collection devices, or if they are clotted. In all cases, a second whole blood specimen should be requested.

g. The specimen characteristics defined above as "unacceptable" may compromise test results.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in NHANES laboratory and in the Special Activities Specimen Handling Office). Specimen collection and transport, and special equipment required are also discussed in the protocol. In general, whole blood specimens should be transported and stored at 4 °C. Once received, they can be frozen at ≤-20 °C until time for analysis. Portions of the sample that remain after analytical aliquots are withdrawn should be refrozen at ≤-20 °C. Samples thawed and refrozen several times are not compromised unless inadvertent contamination occurs because of improper handling. If the specimen needs to be divided because it contains more than one analyte of interest, transfer the appropriate amount of blood into a sterile Nalgene cryovial labelled with the participant's ID.

i. It is important that each lot of collection tubes and shipping and storage containers be screened for lead contamination.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a. **Instrumentation**

(1) Perkin-Elmer (Norwalk, CT) model 5100 atomic absorption spectrophotometer, including HGA 600 furnace, lead source lamp, EDL power supply (if EDL sources are to be used), AS-60 autosampler, PRS-200 or PR-300 printer, and a UV starter source.

(2) Perkin-Elmer (Norwalk, CT) model 5000 atomic absorption spectrophotometer, including HGA 500 furnace, lead source lamp, EDL power supply (if required), AS-40 autosampler, PRS-10 printer-sequencer, and a UV starter source.

(3) Micromedic Digiflex automatic pipettor equipped with a 2000-µL dispensing syringe, 2000-µL and 200-µL sampling syringes, 0.75-mm tip, and a foot pedal (Micromedic Division, ICN Biomedical, Horsham, PA).

(4) Mettler PL 200 top-loading balance (Mettler Instrument Corp., Hightstown, NJ).

(5) Milli-Q water purification system (Millipore Corporation, Bedford, MA).

(6) Vortex-Genie vortex mixer (Fisher Scientific, Atlanta, GA).

(7) Eppendorf fixed-volume micropipets: 1000-, 500-, 250-, 200-, 50-, and 40-µL volumes (Brinkmann Instruments, Inc., Westbury, NY).

(8) Magnetic stirrer (Corning Glass Works, Corning, NY) and stirring bars (Fisher Scientific).

(9) Oxford automatic dispenser (Monoject Scientific, St. Louis, MO).

b. **Materials**

(1) Stock solution of lead: NIST Standard Reference Material (SRM) 2121-2 or 3128, 10,000 mg/L (National Institute of Standards and Technology, Gaithersburg, MD).

(2) Redistilled concentrated nitric acid (G. Frederick Smith Chemical Co., Columbus, OH).

(3) Triton X-100 (Fisher Scientific, Fairlawn, NJ).

(4) Ammonium phosphate, dibasic ("Baker Analyzed," J.T. Baker Chemical Co. -- or any source found to be low in lead contamination).

(5) Ultrapure water (from the Milli-Q water purification system).

(6) Argon, 99.996% purity (Holox, Atlanta, GA), dispensed from an approved gas regulator (Matheson Gas Products, Secaucus, NJ).

(7) NIST SRM 955a (four levels) trace elements in bovine blood (National Institute of Standards and Technology). These materials are to be tested periodically to verify accuracy.

(8) In-house bovine- and human-blood quality control pools with low and high levels of lead that have reference values established by isotope-dilution mass spectrometry (IDMS).

(9) Pyrolytic graphite tubes, solid pyrolytic graphite L'vov platforms, insertion and alignment tools, and graphite contact rings (Perkin-Elmer).

(10) Small plastic weighing boats (Scientific Products, McGaw Park, IL).

(11) Pipet tips: 1- to 100-µL and 1- to 1000-µL sizes (Rainin Instrument Co., Inc., Woburn, MA).

(12) Acid-cleaned volumetric flasks (1000-, 100-, and 10-mL volumes). The glassware is soaked in a soapy solution (2% solution of Isoclean detergent, Akron, OH) for at least 24 hours, rinsed, soaked in 25% nitric acid for 48 hours, rinsed with ultrapure water, and dried under clean conditions.

(13) Conical-bottom 2-mL polystyrene autosampler cups (Lancer, St. Louis, MO).

(14) Kay-Dry paper towels and Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).

(15) Cotton swabs (Hardwood Products Co., Guilford, ME).

(16) Dehydrated alcohol, USP (Midwest Grain Products of Illinois, Pekin, IL).

(17) Lead-free vinyl examination gloves (Travenol Laboratories, Inc., Deerfield, IL).

(18) Biohazard autoclave bags (Curtin-Matheson Scientific, Inc., Atlanta, GA)

(19) Bleach (10% sodium hypochlorite solution) -- any vendor.

(20) Dental mirror (Perkin-Elmer).

(21) Printer-sequencer tape (Perkin-Elmer).

(22) Bio-Rad "Lyphochek" tri-level lyophilized human whole blood quality control materials (Bio-Rad ECS Division, Anaheim, CA).

c. **Reagent Preparation**

Matrix modifier
0.2% (v/v) nitric acid, 0.5% (v/v) Triton X-100, and 0.2% (w/v) ammonium phosphate. Using Eppendorf pipets, dilute 2 mL redistilled concentrated nitric acid and 5000 µL (5.0 mL) Triton X-100 in approximately 750 mL ultrapure water in an acid-cleaned 1000-mL volumetric flask. Weigh 2.0 g of dibasic ammonium phosphate and add it to the flask by washing down the weighing boat with ultrapure water delivered from a wash bottle. Add a magnetic stirring bar and stir the solution on a stirring plate until the Triton X-100 has dissolved. Remove the stirring bar and bring the solution to volume with ultrapure water. After preparation, this solution should be checked for contamination at the beginning of each analytical run and discarded if an absorbance greater than 0.005 Abs-sec is observed. Store at room temperature and prepare as needed in a flask dedicated to this solution.

d. **Standards Preparation**

(1) 1000 mg/L stock lead standard
If using NIST SRM 2121-2 or 3128, dilute 1.00 mL (delivered with either a Class A volumetric pipet or the Micromedic Digiflex) to 10 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature, and prepare a new standard every 6 months in a flask dedicated to this solution.

(2) 10 mg/L intermediate lead standard
Using either a Class A volumetric pipet or the Micromedic Digiflex, dilute 1.00 mL of the 1000 mg/L stock lead standard to 100 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature, and prepare a new standard monthly in a flask dedicated to this solution.

(3) Working lead standards
Using the Micromedic Digiflex, transfer the intermediate stock standard to 10-mL volumetric flasks as indicated in Table 1 and dilute to volume with ultrapure water:

Table 1
Working Standard Preparation

| Intermediate Stock Standard (µL) | Working Standard Concentration, (µg/dL) |
|---|---|
| 0(*) | 0 |
| 50(*) | 5 |
| 100(*) | 10 |
| 150(*) | 15 |
| 200(*) | 20 |
| 250 | 25 |
| 300(*) | 30 |
| 400(*) | 40 |
| 500 | 50 |
| 600(*) | 60 |
| 750 | 75 |
| 800(**) | 80 |

Store at room temperature, and prepare new standards weekly in flasks dedicated to these solutions.
(*) Use these standard concentrations for NHANES III analysis.
(**) Use this standard when the SRM 955a control materials are to be run.

(4) Calibration standards

(a) Using the Micromedic Digiflex, dispense 100 µL of water followed by 900 µL of matrix modifier into an autosampler cup for use as a process blank. Place this cup in position 1 on the autosampler tray of the model 5000 and position 9 of the model 5100.

(b) Using the Micromedic Digiflex, dispense 100 µL of each of the standards and 400 µL of the matrix modifier into the autosampler cups. Into the same cup, dispense 100 µL of base blood followed by 400 µL of matrix modifier.

(c) Pipet 100 µL of whole blood QC samples into autosampler cups, followed by 900 µL of matrix modifier.

(d) Place the QC samples after position 9 and the standards in positions 1-8 on the 5100 and 2-9 on the 5000.

(e) For NHANES III analyses, the following working standards are used: 0, 5, 10, 15, 20, 30, 40 and 60 µg/dL. When the NIST SRMs are analyzed, an additional 80 µg/dL calibration standard is used. The calibration standard is placed after the 60 µg/dL standard.

e. **Preparation of Quality Control Materials**

Levels of lead in three units of bovine blood were evaluated. One animal (Cow #34) had never been dosed and, therefore, had a very low blood lead level. The blood from this animal is suitable for use as the base blood from which the calibration standards are prepared. The other two animals had been previously dosed; therefore their blood lead levels were somewhat elevated. One of these (Cow #23) had a blood lead level in the normal range for the adult U.S. population. The other animal (Cow #26) was dosed again to bring its blood lead level up to a high range (near the cutoff limit). One liter of blood was drawn from each animal into evacuated bottles containing EDTA as coagulant.

One unit of blood each was drawn from a nonsmoking man and a nonsmoking woman. Both units were in the "low-normal" range, with the lead concentration in the woman's blood being lower than that in the man's. The blood from the man was used to prepare the "low-normal" blood lead pool.

A pool containing 75% blood from Cow #23 and 25% blood from the woman was prepared. The blood was mixed in an acid-cleaned flask by stirring on a magnetic stirrer for at least 1 hour. This pool was used to prepare the medium (or "normal") blood lead pool.

Using an automatic dispenser and sterile technique under a laminar-flow hood, the laboratory workers dispensed the blood into 2-mL cryovials. The base pool was dispensed in 1-mL aliquots. The quality control pools were dispensed in 0.5-mL aliquots. All vials were labelled after capping. The pools were frozen at $\leq$ -20 °C. Twenty vials of each level were randomly selected for characterization of the quality control limits and for testing of homogeneity.

Two levels of blind quality control pools were prepared similarly except that vials and labels identical to NHANES specimen vials were used.

**7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES**

a. Construct a calibration curve using the measured mean values of integrated absorbance (absorbance-seconds, or Abs-sec) of standards at 0-, 5-, 10-, 15-, 20-, 30-, 40-, and 60-µg/dL plotted versus concentration. Choose the NON-LINEAR calibration curve option in the calibration window of the GEM software.

b. Once calibration has been performed, calculate the characteristic mass using the EDIT CALIBRATION function. Acceptable characteristic masses are between 12.0 and 16.0 pg/0.0044 Abs-sec.

c. Display calibration curves in the PLOT CALIBRATON window, both to verify the mathematical fit and to evaluate the slope and intercept. Slopes should be 0.035 Abs-sec/µg/dL and intercepts no more than 0.003 Abs-sec.

d. Verify the calibration using NIST Standard Reference Materials once per week. In addition, analyze Bio-Rad tri-level controls, which have been characterized by IDMS, weekly as a secondary set of reference standards.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) For information regarding the range of linearity and how to handle results outside this range, refer to the Calculations section of this document (Sect. 8.h.).

(2) Allow frozen blood specimens, quality control specimens, and blood calibration material to reach ambient temperature and mix on a vortex mixer for 10 seconds.

(3) While the specimens are thawing, rinse enough autosampler cups for an analytical run with ultrapure water delivered from a wash bottle. Drain the cups upside down on Kay-Dry paper towels.

(4) Prime the Micromedic Digiflex pumps with the matrix modifier solution.

(5) Wipe the tip of the Micromedic Digiflex with a lead-free laboratory tissue after inserting it into any blood specimen.

b. **Sample Preparation**

(1) Using the Micromedic Digiflex, dilute the specimens and controls 10-fold (1:10) with the matrix modifier solution into clean autosampler cups. Use 100 µL of specimen and 900 µL of matrix modifier in the following procedure: pull up 100 µL of blood into the tip; wipe the tip with a Kim Wipe tissue; dispense the blood and 900 µL of matrix modifier into a cup. Observe the tip and release any air bubbles that may become trapped before dispensing the solution into the autosampler cups. To do this, remove the tip from its clamp and hold the tip end up to release air. If air becomes trapped in a blood sample in the tip, dispense it into the waste beaker and take another sample.

(2) Place the autosampler cups containing the specimens in positions 7-34, with an additional blank (100 µL water and 900 µL matrix modifier) in position 6.

(3) Place a cup containing the water used in the preparation of the run on the tray as a sample in position 37.

c. **Instrument Setup for the Model 5100 Spectrophotometer and AS-60 Autosampler.**

(1) Set the instrumant parameters as shown in Tables 2 and 3.

(2) Turn on the argon gas. (The setting may vary from 40 to 50 psi, depending on the instrument used; the furnace will shut off at pressures below 40 psi.)

(3) Turn on the cooling water supply.

(4) Turn on the spectrophotometer, magnet, and HGA 600 furnace.

(5) If necessary, program the temperature program into the memory of the model 7000 computer.

(6) Install a source lamp in position on the turret.

(7) If using an EDL, turn on the EDL power supply. Turn up the power to three-quarters of maximum and wait for the EDL to light spontaneously. (If the lamp does not light, remove it from the turret and expose it to the UV starter source until it lights and return it to its position in the turret.) After the lamp lights, run the power at a high level momentarily, and then turn it down to the recommended wattage.

(8) Check the alignment of the lamp.

(9) Program the model 7000 computer controller.

(10) Check all settings in the PROGRAM ELEMENT mode.

(11) Install a new graphite tube and platform after 2 days of analytical runs or sooner if deterioration in the peak

shape or elevation in the matrix blank indicate the need to do so. Open the furnace by pressing the FURNACE OPEN key on the HGA 600. Install a L'vov platform in the furnace using the insertion tool. Make sure that the platform is properly seated in the tube by holding the tube on end and gently tapping it on a hard surface. If the platform falls out, reinsert it using more pressure. Install the tube in the furnace with the platform at the bottom of the tube. Use the alignment tool inserted in the sample port while pressing the FURNACE OPEN key again to close the furnace. After the furnace closes, press the furnace together to ensure that it has properly closed.

Table 2
Instrument Settings for Model 5100

| Parameter | Setting |
|---|---|
| Wavelength | 283.3 nm |
| HCL Current/EDL Power Supply* | 10 mA/10 W |
| EDL Mode | continuous |
| Slit | 0.7 nm (low) |
| Signal Mode | Peak Area |

* Only one source is used.

Table 3
Representative Furnace Temperature Program*

| Step | Temp °C | Ramp (sec) | Hold (sec) | Gas Flow, mL/min |
|---|---|---|---|---|
| Dry | 100 | 1 | 14 | 300 |
| Dry | 200 | 5 | 20 | 300 |
| Char | 750 | 5 | 30 | 300 |
| Atomize | 2000 | 1 | 4 | 10 |
| Burnout | 2400 | 1 | 4 | 300 |
| Cool | 20 | 1 | 4 | 300 |
| Baseline: -1 sec (Background offset correction (BOC) set automatically) | | | | |
| Read: 0 sec | | | | |

*Dry temperature and hold time may vary with different graphite tube and platform combinations.

(12) Check the quartz windows of the furnace to make sure that they are clean. If there is evidence of sample spattering on the windows, remove the windows and clean them using a cotton swab soaked in denatured alcohol. Wipe the windows dry with a soft tissue (Kim-Wipe) and carefully reinstall them.

(13) Press the STANDBY key; the arm of the autosampler will lift out of the wash cup. Check the alignment of the sampling tip by manually moving the arm to the sample port. If the alignment needs adjustment, unlock the autosampler base by turning the knob on the center front of the base. The knobs on the back left side and front left then can be used to move the base to the necessary position. Lock the base in place with the center front knob. Use the dental mirror to observe the position of the tip in the graphite tube. Check the depth of the autosampler tip in the graphite tube. Use the front adjustment knob to the right of the sampling arm to raise or lower the tip as necessary.

(14) Press the STANDBY key again to return the sampling arm to its position in the wash cup.

(15) Condition the graphite tube. Sequentially type in the following temperatures on the model 7000 keyboard and hold the MANUAL TEMP key for about 5 seconds. Wait about 10 seconds before typing in the next temperature.

1000 °C
1500 °C
2000 °C
2400 °C
2650 °C

(16) Check the drying step of the furnace program. Use the mirror to observe the sample tip as it deposits the sample on the platform. Continue to observe as the sample dries. The drying should be complete 5-10 seconds before the char step begins. If the sample is dry sooner, decrease the hold time of the second dry step appropriately. If it is not dry in time, two options are available: the temperature of the drying step may be increased or the hold time may be increased. Increase the hold time only if increasing the temperature will cause the sample to boil and splatter during the drying step.

(17) If necessary, repeat step 15 until the sample is dry.

(18) When the appropriate drying temperature is determined (even if it is the one already in the program), make sure that the blank is low (less than 0.003 Abs-sec for the mean of two determinations).

(19) Check to make sure that there is enough paper in the PRS-200.

d. **Operation of the Model 5100 and the AS-60**

(1) Press the ANALYZE SAMPLES soft key on the model 5100 to begin a run.

(2) The model 5100 will first run the calibration curve and then the quality control materials. Ensure that the quality control materials are within the specified limits.

(3) If the values observed for the control materials for this analytical run are in control, proceed with the analysis of the diluted blood specimens. (Refer to section 10 of this document for criteria.)

(4) Turn off the system in reverse order.
**Note:** When turning off the EDL power supply, first turn off the power switch. When the power indicator decreases to zero, turn off the variable power knob.

e. **Instrument Setup for the Model 5000 Spectrometer and the AS-40 Autosampler**

(1) Set instrument parameters as shown in Tables 4 and 5.

Table 4
Instrument Settings for Model 5000

| Parameter | Setting |
|---|---|
| Wavelength | 283.3 nm |
| HCL Current/EDL Power Supply* | 10 mA/10 W |
| EDL Mode | continuous |
| Slit | 0.7 nm (low) |
| Signal Mode | Peak Area |

* Only one source is used.

Table 5
Representative Furnace Temperature Program

| Step | Temp °C | Ramp (sec) | Hold (sec) | Gas Flow mL/min |
|---|---|---|---|---|
| Dry | 100 | 5 | 10 | 300 |
| Dry* | 180 | 5 | 20 | 300 |
| Char | 700 | 5 | 30 | 300 |
| Atomize | 2000 | 1 | 5 | 10 |
| Burnout | 2400 | 1 | 4 | 300 |
| Cool | 20 | 1 | 4 | 300 |

Baseline: -8 sec

Read: 0 sec

Recorder: -1 sec

*Dry temperature and hold time may vary with different graphite tube and platform combinations.

(2) Turn on the argon gas. (The setting may vary from 40 to 50 psi, depending on the instrument used; the furnace will shutoff at pressures below 40 psi.)

(3) Turn on the cooling water supply.

(4) Turn on the HGA 500 furnace. Switch the furnace to the run mode by pressing the STANDBY key.

(5) If necessary, program the temperature program into the memory of the HGA 500 furnace. Use the TEMP, RAMP TIME, and HOLD TIME keys to program in each step. Press the STEP key to move to the next step for programming. The default gas flow at all steps is 300 mL/min. At the atomization step (step 4 in the program), type in an alternate gas flow of 20 mL/min and press the INT FLOW key. Also program the BASELINE, REC, and READ functions into the atomization step. Store the program by typing in a number and pressing STORE.

(6) Install a source lamp in position on the turret.

(7) If using an EDL, turn on the EDL power supply. Turn up the power to three-quarters of maximum and wait for the EDL to light spontaneously. (If the lamp does not light, remove it from the turret and expose it to the UV starter source until it lights and return it to its position in the turret.) After the lamp lights, run the power at a high level momentarily, then turn it down to the recommended wattage.

(8) Turn on the model 5000 spectrophotometer. Press the PRINT key on the keyboard. The red light beside the key will illuminate.

(9) Program the model 5000.

- If using an HCL, type in the lamp current on the keyboard and press the LAMP MA key.
- Type in 0.7 and press the SLIT LOW key.
- Type in 283.3 and peak up onto the wavelength by pressing the PEAK key. Press the SET UP key. The display will read about 50; but as the lamp warms up the value will increase. Press the GAIN key to bring the value to approximately 50. (Pressing the GAIN key will either increase or decrease the energy automatically.)
- After the lamp has warmed up for about 20 minutes and while the model 5000 is still in the SET UP mode, twist the lamp in the holder and adjust it to an optimal position by using the knobs on the lamp holder (without clamping on the black end piece of the lamp). This procedure may also require use of the GAIN key. As a check, the ENERGY on the 5000 display should read around 60.
- Press the PEAK AREA key.

- Type in 2 and press the AVG key.
- Type in 6 and press the T key.
- Store the program by typing in a number and pressing the STO key.

(10) Turn on the AS-40 autosampler controller.

(11) Program the AS-40 autosampler controller.

- Type in 20 and press the SAMPLE VOLUME key on the controller.
- Type in the HGA program number and press the HGA PROG key.
- Type in the model 5000 program number and press the INST PROG key.
- Type in the number of the position of the last sample and press the LAST SAMPLE key.

(12) Install a new graphite tube and platform after 2 days of analytical runs or sooner if deterioration in the peak shape or elevation of the matrix blanks indicate the need to do so. Open the furnace by pressing the FURNACE OPEN key on the HGA 500. Install a L'vov platform in the furnace using the insertion tool. Make sure that the platform is properly seated in the tube by holding the tube on end and gently tapping it on a hard surface. If it falls out, reinsert it using more pressure. Install the tube in the furnace with the platform at the bottom of the tube. To close the furnace, use the alignment tool inserted in the sample port while pressing the FURNACE OPEN key again. After the furnace closes, press the furnace together to ensure that it has properly closed.

(13) Check the quartz windows of the furnace to make sure that they are clean. If there is evidence of sample spattering on the windows, remove the windows and clean them using a cotton swab soaked in denatured alcohol. Wipe the windows dry with a soft tissue (Kim-Wipe) and carefully reinstall them.

(14) Press the STANDBY key; the arm of the autosampler will lift out of the wash cup. Check the alignment of the sampling tip by manually moving the arm to the sample port. If the alignment needs adjustment, unlock the autosampler base by turning the knob on the center front of the base. The knobs on the back left and front left then can be used to move the base to the necessary position. Lock the base in place with the center front knob. Use the dental mirror to observe the position of the tip in the graphite tube. Check the depth of the autosampler tip in the graphite tube. Use the front adjustment knob to the right of the sampling arm to raise or lower the tip as necessary.

(15) Press the STANDBY key again to return the sampling arm to its position in the wash cup.

(16) Condition the graphite tube. Sequentially type in the following temperatures on the HGA keyboard and hold the MANUAL TEMP key for about 5 seconds. Wait about 10 seconds before typing in the next temperature.

1000 °C
1500 °C
2000 °C
2400 °C
2650 °C

(17) Check the drying step of the furnace program. Type 1; then press the MANUAL key. Use the mirror to observe the sample tip as it deposits the sample on the platform. Continue to observe as the sample dries. Press the MANUAL key again to keep the autosampler from sampling from this cup indefinitely. The drying should be complete 5-10 seconds before the char step begins. If the sample is dry sooner, decrease the hold time of the second drying step appropriately. If it is not dry in time, two options are available. The temperature of the drying step may be increased or the hold time may be increased. Increase the hold time only if increasing the temperature will cause the sample to boil and splatter during the drying step.

(18) Repeat step 16 until the sample is dry.

(19) When the appropriate dry temperature is determined (even if it is the one already in the program), make sure that the blank is low (less than 0.003 Abs-sec for the mean of two determinations).

(20) Check to make sure that there is enough paper in the PRS-10. Press the paper feed button on the front of the PRS-10 to advance the paper.

f. **Operation of the Model 5000 and the AS-40**

(1) Press the START/STOP key on the AS-40 to begin a run.

(2) The model 5000 will first run the calibration curve and then the quality control materials. Ensure that the quality control materials are within the specified limits.

(3) If the values observed for the control materials for this analytical run are in control, proceed with the analysis of the diluted blood specimens. (Refer to section 10 of this document for criteria.)

(4) Turn off the system in reverse order.
Note: When turning off the EDL power supply, first turn off the power switch. When the power indicator decreases to zero, turn off the variable power knob.

g. **Replacement and Periodic Maintenance of Key Components**

(1) Source lamp. A spare source lamp should be available. Order another if the spare is used for replacement.

(2) Printer tape. A supply of printer tape should be on hand. Order more when the last is installed.

(3) Graphite contact rings. Approximately every 6 months, the graphite contact rings of the furnace housing will need to be replaced. The also need to be repalced if an apparent loss of temperature control occurs or the instrument sounds an alarm indicating a problem with the tube. It is useful to maintain a log book to help keep track of when these replacements are made. At least one spare set of graphite contact rings should be kept on hand.

(4) The sampling tip of the autosampler will need to be repositioned and trimmed every few weeks, depending on how the dispensing is proceeding.

h. **Calculations**

(1) The method described here is linear up to 4 µmol/L, or 80 µg/dL (1). Use the linear regression program in ROSCOE (PESLEAD) to calculate the calibration curve and the specimen concentrations. The program calls for the integrated absorbance values of the standards and samples. It will then subtract the blank and calculate the concentrations of the controls and specimens. The linear regression program generates slopes, intercepts, correlation coefficients, standardized residuals, and plotted and fitted curves. The correlation coefficient, $r^2$, for each curve should be 0.990 or higher. For optimum sensitivity, slopes should be more than 0.035; intercepts should be less than 0.003.

(2) Repeat a specimen analysis when duplicate integrated absorbencies below 0.03 Abs-sec (mean) differ by more than 0.005 Abs-sec or when duplicate integrated absorbencies above 0.03 Abs-sec (mean) differ by more than 0.01 Abs-sec. These correspond to concentration differences of 0.01 µg/dL and 0.025 µg/dL, respectively. Reanalyze specimens containing more than 30 µg/dL lead for confirmation. When reanalyzing any specimen with a concentration greater than 60 µg/dL, prepare a new specimen by diluting it 20-fold (1+19) rather than 10-fold (1+9). The results output from PESLEAD must then be multiplied by 2 to account for this higher dilution.

(3) The detection limit, which is three times the standard deviation of 10 repeat measurements of a sample with low lead concentration, is 0.05 µmol/L (or 1.0 µg/dL) (1,2). Results below the detection limit are reported as nondetectable (ND).

i. **Special Procedure Notes -- CDC Modifications**

A Micromedic Digiflex automatic pipettor is used for many of the dispensing needs in this method because it is less tedious to use and provides better precision and accuracy than Eppendorf pipets, and it also helps to prevent common sources of contamination in the sample preparation steps.

## 9. REPORTABLE RANGE OF RESULTS

Blood lead values are reportable when they exceed the limit of detection (LOD) (as calculated in 8.h.(3)) but are less

than 60 µg/dL. If a blood lead value is less than 1.0 µg/dL (the approximate LOD of this method), it should be reported as "0.5" µg/dL; if it is greater than 60 µg/dL, the specimen should be diluted (1+19) and re-analyzed, with the verified result reported.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the Nutritional Biochemistry Branch for environmental and occupational health studies. The method has proven to be accurate, precise, and reliable. The primary standard used is a NIST SRM, and NIST SRM whole blood materials are used for external calibration. In addition, Bio-Rad tri-level controls that have IDMS target values are measured weekly as a secondary set of standards. Estimates of imprecision can be generated from long-term results from quality control pools.

Two types of quality control systems are used in this analytical method: 1) a system in which "bench" quality control specimens are inserted by the analyst two times in each analytical run ( (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis and 2) a system in which "blind" quality control samples are placed in vials, labelled, and processed so that they are indistinguishable from the subject samples. The results of the measurement of blind specimens are decoded and reviewed by the supervisor. With both systems, all levels of lead concentration are assessed by taking these samples through the complete analytical process. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Two levels of blind quality control pools are used. These pools are prepared in sufficient quantity to last throughout the survey. The levels chosen are in the "low-normal" (approximately 5 µg/dL) and "high-normal" (approximately 20 µg/dL) range so as not to be obvious to the analyst. The pools are prepared in the same way as the bench pools, but they are dispensed in vials identical to those used in the field for NHANES subject samples, labelled with pseudo-participant numbers corresponding to each geographical location of the survey, and stored at $\leq$-20 °C. At least one blind sample is randomly incorporated with every 20 NHANES samples and analyzed according to the method protocol.

The bench quality control pools comprise three levels of concentration spanning the "low-normal," "normal," and "high". Reference materials (blood products with certified values assigned by independent reference methods) are used periodically as a check of accuracy. NIST SRM 955a (levels A, B, C, and D) should be analyzed once a week for this purpose. If the stock of these materials becomes low, another set should be ordered in time to analyze it concurrently with the quality control materials still in use so that a bridge may be formed between the materials. If the material ordered from NIST is from the same lot, a full characterization is not necessary. However, there should be some overlap between the old and new stocks.

Quality control limits are established for each pool. An analysis of variance (ANOVA) is performed for each pool after 20 characterization runs have been performed in which previously characterized NIST SRM and bench quality control pools are used for evaluation. In addition to providing quality control limits, the characterization runs also serve to establish the homogeneity of the pools. Once the homogeneity of the bench and blind materials has been established, it is useful to have them analyzed by another independent reference method (e.g., IDMS). Examples of the long-term precision and accuracy attainable by this method are presented in the quality control results in Table 6.

Table 6
Precision and Accuracy

| Pool | Mean | N | Total SD | Total CV (%) | 95% Limits | 99% Limits |
|---|---|---|---|---|---|---|
| 0588 | 6.0 | 420 | 0.45 | 7.46 | 5.18-6.84 | 4.92-7.10 |
| 0688 | 15.1 | 423 | 0.54 | 3.59 | 14.12-16.15 | 13.80-16.48 |
| 0788 | 35.8 | 422 | 1.18 | 3.29 | 33.52-38.09 | 32.80-38.82 |
| 9551 | 4.86 | 37 | 0.39 | 8.11 | 4.22-5.50 | 4.02-5.71 |
| 9552 | 13.80 | 37 | 0.65 | 4.75 | 12.72-14.80 | 12.39-15.13 |
| 9553 | 31.00 | 37 | 1.12 | 3.62 | 29.14-32.80 | 28.56-33.38 |
| 9554 | 53.50 | 37 | 1.49 | 2.78 | 50.93-56.09 | 50.11-56.90 |

After the standards and bench quality control materials are analyzed (at the beginning of an analytical run), consult the long-term quality control charts for each control material to determine if the system is "in control." Two types of charts are used. The first chart plots the means of the duplicate determinations and compares them with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs). The system is out of control if any of the following events occur:

- The mean from a single pool falls outside the 99% confidence limits.
- The means from two pools fall either above or below the 95% confidence limits.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- The means from eight successive runs for one pool fall either all above or all below the center line.

The second type of quality control chart plots the range of the duplicate determinations and compares them with the 95% and 99% limits as well as with the center line value. The system is out of control if any of the following events occur:

- The range from a single pool falls above the 99% limit.
- The ranges from two pools fall above the 95% limit.
- Two successive within-run ranges for a single pool fall above the 95% limit.
- The ranges from eight successive runs fall above the center line.

If the run is declared "out of control," investigate the system (instrument, calibration standards, etc.) to determine the root of the problem before any analysis of specimens occurs.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more quality control samples fall outside 95% limits for mean or range of duplicate values, take the following steps in succession:

a. Prepare fresh calibration standards, and run the entire calibration curve using freshly prepared standards.

b. Install a new (unused) graphite furnace or pyrolytic graphite L'vov platform -- conditioned as described in Section 8.C.(14) -- and reanalyze the entire sequence of calibrators, blanks, and quality control samples.

c. Prepare and reanalyze fresh dilutions of all whole-blood quality control samples.

If the steps outlined above (a-c) do not result in correction of the "out of control" values for QC materials, consult your supervisor for other appropriate corrective actions. No analytical results should be reported for runs not in statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

This method has been validated with whole blood specimens with target values obtained by isotope dilution mass spectrometry, regarded as the definitive method for measuring lead in whole blood. The range of concentrations reportable has been previously mentioned; no known chemical or physicochemical interferences have been documented for this analytical method. External contamination may limit the accuracy of blood lead values below about 5 µg/dL.

## 13. REFERENCE RANGES (NORMAL VALUES)

The data from NHANES III will be used to establish national norms for the United States.

a. CDC has established a blood lead level $\geq$ 10 µg/dL as the level at which intervention is required for children because some adverse health effects have been documented at this level (3).

b. The overall geometric mean blood lead level for the U.S. population (1988-1991) was 2.8 µg/dL. Blood lead levels were consistently higher for younger children than for older children, for older adults than for younger adults, for males than for females, for blacks than for whites, and for central city residents than for others (4).

c. In the distribution of blood lead levels for all ages, races, and sexes in the US population from 1988 to 1991, as measured during Phase I of NHANES III, the 5th percentile was <1.0 µg/dL, and the 95th percentile was 9.4 µg/dL.

d. For nonoccupationally exposed persons, the median blood lead level should be <20 µg/dL, and 95 % of this population should have levels <35 µg/dL (6).

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

a. Pediatric (age <6 yr): PbB >25 µg/dL -- medical intervention is indicated.

b. Adult: PbB >40 µg/dL -- OSHA regulations require removal from workplace.

The supervising physician of any person who exceeds these "panic values" should be notified of laboratory results.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should reach and maintain ambient temperature during analysis. Take stringent precautions to avoid contamination of samples from external sources of lead.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods of analysis. If the analytical system fails, store whole blood specimens at 4 °C (refrigerated) until the analytical system is restored. If a long-term interruption (greater than 4 weeks) is anticipated, store whole blood specimens at -20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

*As stated in Section 14, for studies other than NHANES, notify the supervising physician as soon as possible of blood lead (PbB) results >25 µg/dL (pediatric) or >40 µg/dL (adults). Use the most expeditious means (telephone, FAX, etc). For NHANES, FAX all values >10 µg/dL to NCHS, which will transmit the results to the donor's physician for follow-up.*

a. Use the "NHANES Laboratory Blood Lead Standard and Quality Control Reporting Sheet" to record QC data. This reporting sheet has self-explanatory blanks for the standard absorbance data, the linear regression information, and results from the quality control pools. Prepare this form in duplicate, using carbon paper.

b. Use the "NHANES III Analytical Worksheet" to record the specimen results. These have been prepared with a list of the sample IDs for each pre-racked run. Record the results for blood lead in µg/dL. If a result is below the detection limit of the method, write ND (for nondetectable) and enter a value of "0.1" in the blank. If a sample is missing from the rack, write NOSAX in the blank. If a sample is not satisfactory (i.e., cannot be analyzed), write UNSAX in the blank. Prepare these forms in duplicate, using carbon paper.

c. Give both types of forms to the supervisor along with the hard copy of the data printout and the hard copy of the printout obtained from running PESLEAD in ROSCOE on the CDC mainframe. After the data is calculated and the final values are approved for release by the reviewing supervisor, the data entry clerk will transcribe the results into the NHANES III data base, which is located in RBASE on the NCEH/EHLS PC network. The carbon copies and the data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets.

d. Use the QCDATA6 program in the NHANES library of ROSCOE to enter the quality control data. This program should be updated regularly.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (e.g., electronic -- RBase; mainframe data files; optical disc or floppy disc) should be used to track specimens. Records are to be maintained for 10 years beyond the completion of the survey for NHANES. This includes related QA/QC data. Duplicate records should be kept (off-site, if sensitive or critical) in electronic or hardcopy format.

## REFERENCES

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## ACKNOWLEDGMENTS

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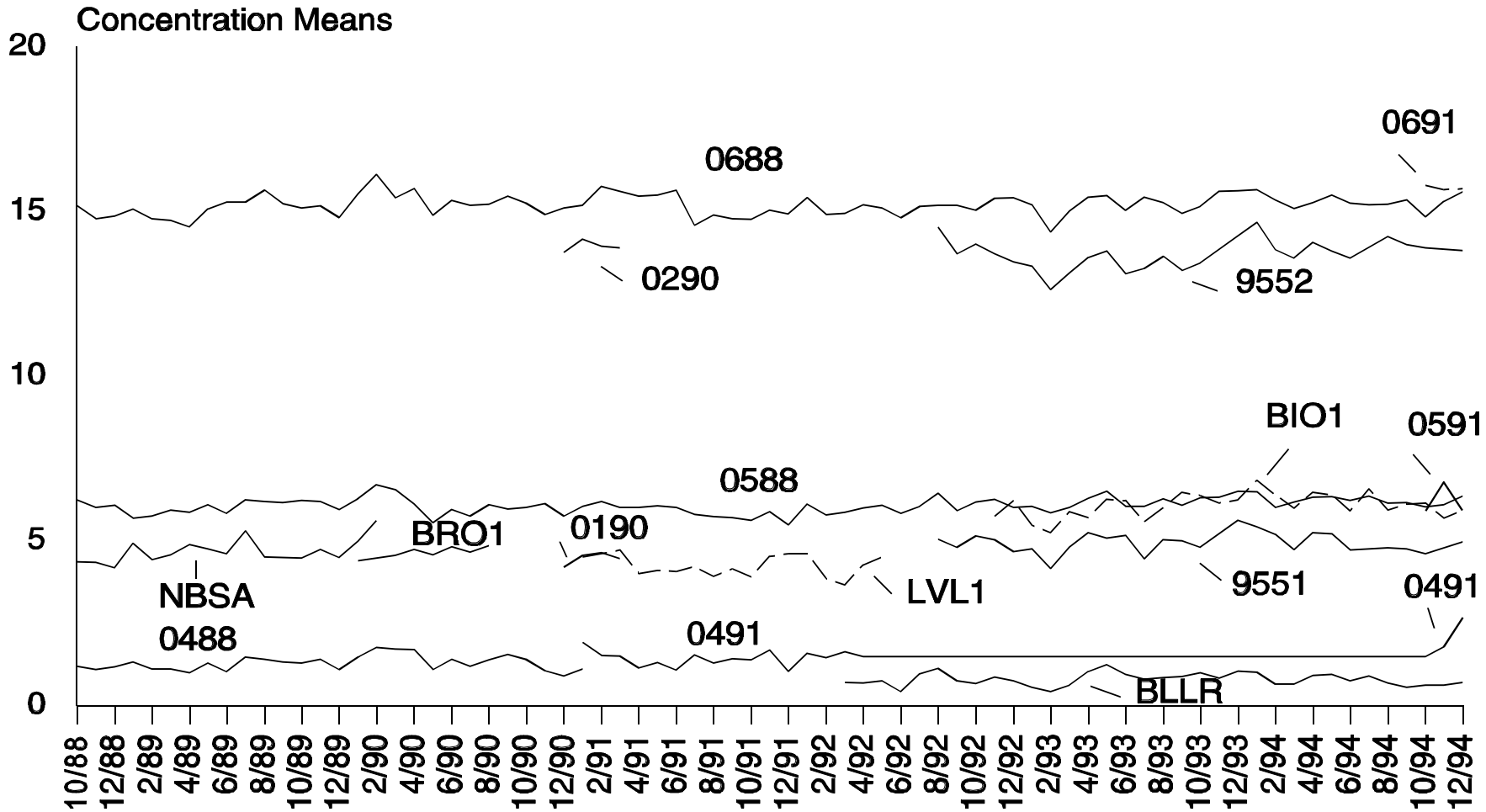
SUMMARY STATISTICS FOR
LEAD (LOW POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0190 | 12/90 - 03/91 | 4.5050 | 0.45616 | 10.126 | 60 |
| 0290 | 12/90 - 03/91 | 13.9805 | 0.47095 | 3.369 | 60 |
| 0488 | 10/88 - 01/91 | 1.2472 | 0.40867 | 32.767 | 478 |
| 0491 | 01/91 - 12/94 | 1.4632 | 0.43462 | 29.703 | 270 |
| 0588 | 10/88 - 12/94 | 6.1027 | 0.46262 | 7.581 | 1860 |
| 0591 | 10/94 - 12/94 | 6.3906 | 0.98434 | 15.403 | 50 |
| 0688 | 10/88 - 12/94 | 15.1917 | 0.60120 | 3.957 | 1864 |
| 0691 | 10/94 - 12/94 | 15.6954 | 0.69884 | 4.453 | 50 |
| 9551 | 08/92 - 12/94 | 4.9154 | 0.43111 | 8.771 | 244 |
| 9552 | 08/92 - 12/94 | 13.6915 | 0.62406 | 4.558 | 243 |
| BIO1 | 11/92 - 12/94 | 6.0920 | 0.55640 | 9.133 | 215 |
| BLLR | 03/92 - 12/94 | 0.8144 | 0.46077 | 56.578 | 1162 |
| BRO1 | 01/90 - 08/90 | 4.5972 | 0.58756 | 12.781 | 68 |
| LVL1 | 01/91 - 05/92 | 4.1226 | 0.43433 | 10.535 | 194 |
| NBSA | 10/88 - 02/90 | 4.5770 | 0.47233 | 10.320 | 90 |

# Lead Monthly Means - Low Pools

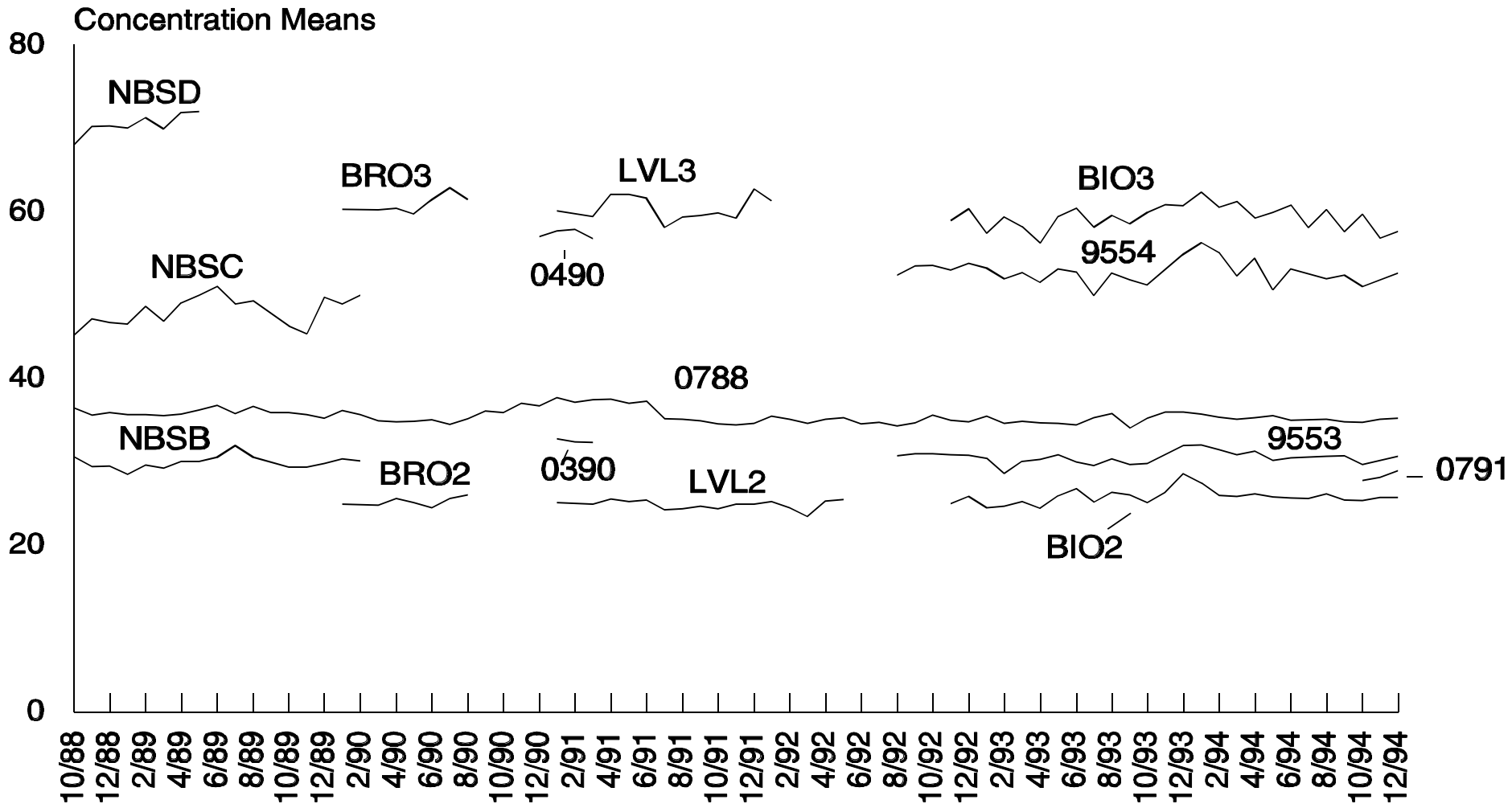



NOTE: No specimens assayed for pool 0491 between 5/92 and 9/94.

SUMMARY STATISTICS FOR
LEAD (HIGH POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0390 | 12/90 - 03/91 | 32.5950 | 0.86929 | 2.667 | 48 |
| 0490 | 12/90 - 03/91 | 57.5425 | 1.26655 | 2.201 | 60 |
| 0788 | 10/88 - 12/94 | 35.4406 | 1.34428 | 3.793 | 1851 |
| 0791 | 10/94 - 12/94 | 28.1304 | 1.30152 | 4.627 | 50 |
| 9553 | 08/92 - 12/94 | 30.7208 | 1.06808 | 3.477 | 244 |
| 9554 | 08/92 - 12/94 | 53.0998 | 1.76694 | 3.328 | 244 |
| BIO2 | 11/92 - 12/94 | 25.8043 | 1.20095 | 4.654 | 219 |
| BIO3 | 11/92 - 12/94 | 59.4179 | 2.35554 | 3.964 | 217 |
| BRO2 | 01/90 - 08/90 | 25.2257 | 0.91006 | 3.608 | 68 |
| BRO3 | 01/90 - 08/90 | 60.8750 | 1.64807 | 2.707 | 68 |
| LVL2 | 01/91 - 05/92 | 24.8772 | 0.94251 | 3.789 | 194 |
| LVL3 | 01/91 - 01/92 | 60.4280 | 2.35668 | 3.900 | 162 |
| NBSB | 10/88 - 02/90 | 29.9301 | 0.99067 | 3.310 | 90 |
| NBSC | 10/88 - 02/90 | 47.8751 | 2.31773 | 4.841 | 90 |
| NBSD | 10/88 - 05/89 | 70.2368 | 1.66563 | 2.371 | 44 |

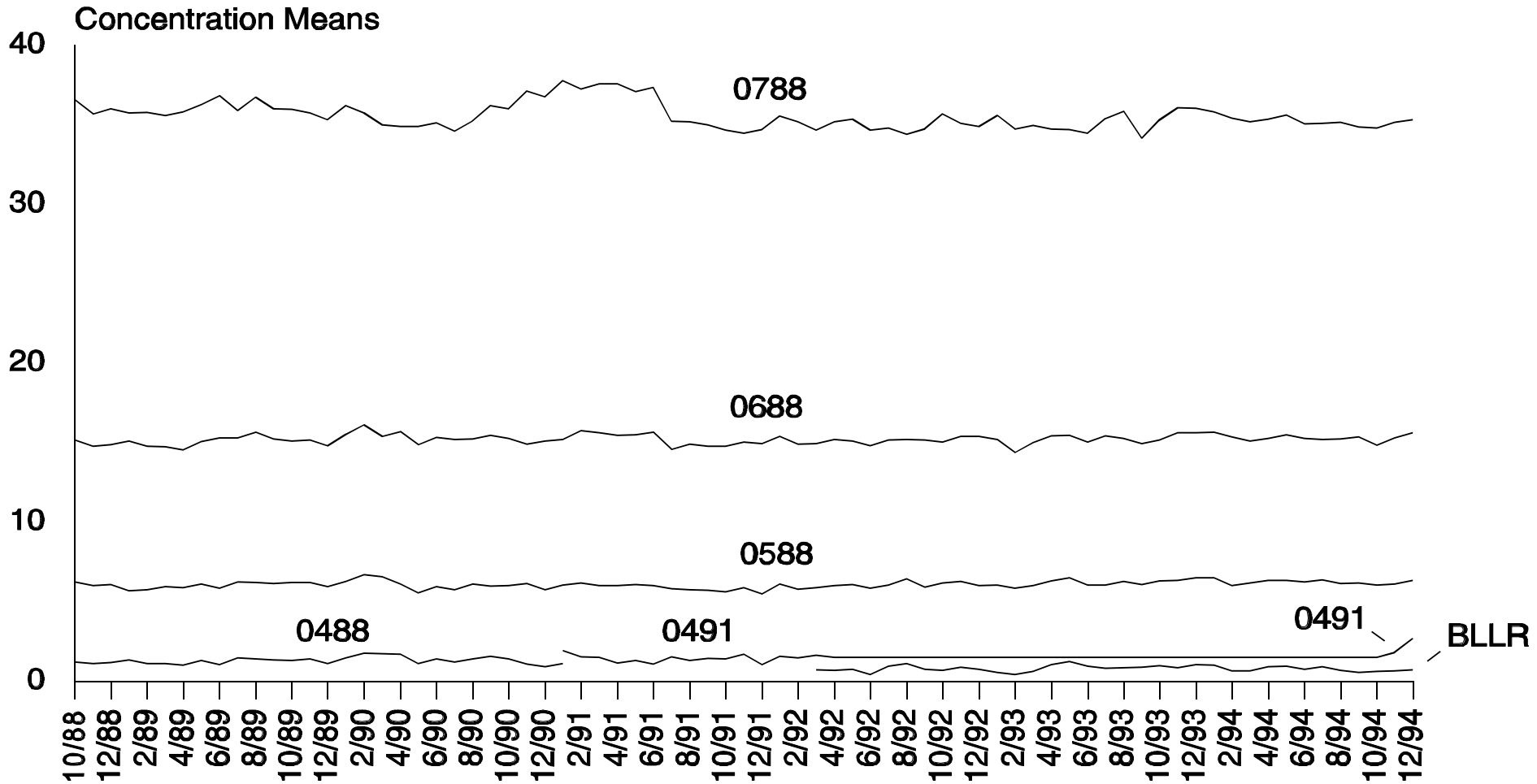
# Lead Monthly Means - High Pools



SUMMARY STATISTICS FOR
LEAD (SELECT POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0488 | 10/88 - 01/91 | 1.2472 | 0.40867 | 32.767 | 478 |
| 0491 | 01/91 - 12/94 | 1.4632 | 0.43462 | 29.703 | 270 |
| 0588 | 10/88 - 12/94 | 6.1027 | 0.46262 | 7.581 | 1860 |
| 0688 | 10/88 - 12/94 | 15.1917 | 0.60120 | 3.957 | 1864 |
| BLLR | 03/92 - 12/94 | 0.8144 | 0.46077 | 56.578 | 1162 |
| 0788 | 10/88 - 12/94 | 35.4406 | 1.34428 | 3.793 | 1851 |

# Lead Monthly Means - Select Pools




NOTE: No specimens assayed for Pool 0491 between 5/92 and 9.94.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Selenium is measured in serum by atomic absorption spectrometry in a procedure based on the methods described by Lewis et al. (1) and by Paschal and Kimberly (2). Quantification is based on the measurement of light absorbed at 196.0 nm by ground state atoms of selenium from a selenium electrodeless discharge lamp (EDL) source. Serum samples, human serum quality control pools, and serum calibration standards are diluted with a matrix modifier (Triton X-100, nickel nitrate, and magnesium nitrate). The selenium content is determined by using a Perkin-Elmer model 5100 graphite furnace atomic absorption spectrophotometer with Zeeman background correction. The Zeeman system offers improved background correction over deuterium arc-corrected systems; use of the latter often results in overcorrection caused by spectral interference from iron or phosphate in the serum (3,4).

Low selenium levels have been linked to Keshan's disease. This is a cardiomyopathy that has been observed in young children who live in areas where the soil is low in selenium, particularly in some regions of China. Selenium has been shown to affect drug metabolism and toxicity. In addition, selenium may have a role in cancer prevention.

## 2. SAFETY PRECAUTIONS

All serum samples received for analysis must be considered potentially positive for infectious agents including HIV and hepatitis B viruses. Observe Universal Precautions. Wear gloves, lab coat, and safety glasses while handling all human blood products. We recommend the hepatitis B vaccine series for all analysts working with intact blood and serum sample materials. Place disposable plastic, glass, and paper (pipet tips, autosampler cups, gloves, etc.) that contact serum in a biohazard autoclave bag. Keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished.

Dispose of all biological samples and diluted specimens in a biohazard bag at the end of the analysis.

> *Special care should be taken when handling and dispensing concentrated nitric acid. Always remember to add acid to water. Nitric acid is a caustic chemical capable of causing severe eye and skin damage. Wear gloves, a lab coat, and safety glasses. If the nitric acid comes in contact with any part of the body, quickly wash with copious quantities of water for at least 15 minutes.*

Material safety data sheets (MSDSs) for nitric acid, Triton X-100, magnesium nitrate hexahydrate, nickelous nitrate hexahydrate, and argon are available through the NCEH computer network.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation and calculation of the run are accomplished with a SAS program, "SELENIUM," which is run in ROSCOE on the CDC mainframe.

   After the data are calculated and the final values are approved for release by the reviewing supervisor, the results are transcribed by the data entry clerk into the NHANES III database, which is located in RBASE on the NCEH/EHLS PC network. Data entry is proofed by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the selenium values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. For best results, a fasting sample should be obtained. Collection of nonfasting specimens can result in samples

that are lipemic and difficult to analyze.

b. Specimens for selenium analysis should be fresh or frozen serum. Since selenium is not an ubiquitous contaminant, blood may be collected in a red-top Vacutainer brand tube by standard venipuncture procedures.

c. A 1-mL sample of serum is preferable. The minimum sample volume required for analysis is 250 µL.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID. The tubes and vials should be screened for the presence of selenium, although contamination from this element is not a common problem.

e. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, they should be stored at ≤-20 °C until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at ≤-20 °C. Samples thawed and refrozen several times are not compromised.

f. Specimens should generally arrive frozen. Refrigerated samples may be used provided they are brought promptly from the site where the blood was collected.

g. Samples collected or processed with equipment contaminated with selenium will not give accurate results.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and Special Activities Branch Specimen Handling Office). The protocol discusses collection and transport of specimens and the special equipment required. In general, serum specimens should be transported and stored at no more than 4-8 °C. Samples thawed and refrozen several times are not compromised. If there is more than one analyte of interest in the specimen and it needs to be divided, the appropriate amount of blood should be transferred into a sterile cryovial labelled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Perkin-Elmer (Norwalk, CT) model 5100 graphite furnace atomic absorption spectrophotometer (GFAAS) with Zeeman background correction, including HGA 600 furnace, selenium EDL source lamp, EDL power supply, AS-60 autosampler, and Epson EX-800 printer.

(2) Micromedic Digiflex Automatic pipettors equipped with a 2000-µL dispensing syringe, and 2000-µL or 200-µL sampling syringes (Micromedic Systems Inc., Horsham, PA).

(3) Mettler PL 200 top-loading balance (Mettler Instrument Corp., Hightstown, NJ).

(4) Milli-Q water purification system (Millipore Corporation, Bedford, MA).

(5) Vortex-Genie vortex mixer (Fisher Scientific, Atlanta, GA).

(6) Eppendorf fixed-volume pipets, 20-, 100-, 400-, 500-, and 1000-µL (Brinkmann Instruments, Inc., Westbury, NY).

(7) Magnetic stirrer (Corning Glass Works, Corning, NY) and stirring bars (Fisher Scientific, Pittsburgh, PA).

(8) UV starter source (Perkin-Elmer).

### b. Materials

(1) Stock solution of selenium: NIST SRM 3149, 10,000 mg/L, (National Institute of Standards and Technology,

Gaithersburg, MD).

(2) Redistilled concentrated nitric acid (G. Frederick Smith Chemical Co., Columbus, OH).

(3) Triton X-100 (Fisher Scientific, Fairlawn, NJ).

(4) Nickelous nitrate, hexahydrate (J.T. Baker Chemical Co.).

(5) Magnesium nitrate, hexahydrate (J.T. Baker).

(6) Ultrapure water (from the Milli-Q water purification system).

(7) Argon, 99.996% purity (supplied as a compressed gas by Holox or other contract agency), equipped with approved gas regulator (Matheson Gas Products, Secaucus, NJ).

(8) Serum Standard Reference Material: NIST SRM 909 (National Institute of Standards and Technology). This material is to be analyzed periodically as a check of accuracy.

(9) Bench and blind human-serum quality-control pools spiked with low and high levels of selenium.

(10) Low-level human serum used as a base for the preparation of standards (NH3 Selenium Base Pool, 9113, prepared by CDC 10/91).

(11) Pyrolytic graphite tubes, solid pyrolytic graphite L'vov platforms, insertion and alignment tools, and graphite contact rings (Perkin-Elmer).

(12) Small plastic weighing boats (Scientific Products, McGaw Park, IL).

(13) Pipet tips: 1- to 100-µL and 1- to 1000-µL sizes (Rainin Instrument Co., Inc., Woburn, MA).

(14) Acid-cleaned volumetric flasks, 1000-, 100-, and 10-mL volumes (Corning Glassworks, Corning, NY). The glassware is soaked in a soapy solution (2% solution of Isoclean detergent, Akron, OH) for at least 24 hours, rinsed, soaked in 25% nitric acid for 48 hours, rinsed with ultrapure water, and dried under clean conditions.

(15) Acid-cleaned volumetric pipets (1- and 5-mL volumes).

(16) Conical-bottom 2-mL polystyrene autosampler cups (Lancer, St. Louis, MO).

(17) Snap-Cap Bio-Vials (Beckman Instruments, Inc., Somerset, NJ).

(18) Floppy disks for storage of analytical software and methods (Maxell Corporation of America, Moonachie, NJ).

(19) Cotton swabs (Hardwood Products Co., Guilford, Maine).

(20) Vinyl examination gloves (Travenol Laboratories, Inc., Deerfield, IL).

(21) Biohazard autoclave bags (Curtin-Matheson Scientific, Inc., Atlanta, GA).

(22) Bleach (10% sodium hypochlorite solution) (any vendor).

(23) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(24) Kay-Dry paper towels and Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).

(25) 50-mL graduated cylinder (Corning).

(26) Dehydrated alcohol, USP (Midwest Grain Products of Illinois, Pekin, IL).

(27) Epson EX-800 printer ribbons (Perkin-Elmer).

c. **Reagent Preparation**

(1) Matrix modifier (1% (w/v) nickelous nitrate, 2% (w/v) magnesium nitrate, and 0.5% (v/v) Triton X-100)
Dissolve 10.0 g nickelous nitrate hexahydrate -- $Ni(NO_3)_2 \bullet 6H_2O$ -- and 20.0 g magnesium nitrate hexahydrate -- $Mg(NO_3)_2 \bullet 6H_2O$ -- in about 500 mL of ultrapure water in a 1000-mL volumetric flask and mix until the salts are dissolved. Using a volumetric pipet, add 5.0 mL of Triton X-100. Add an acid-rinsed magnetic stirring bar; stir the solution gently on a magnetic stirring plate until the Triton X-100 is completely dissolved. Remove the stirring bar and bring the solution to 1000 mL with ultrapure water. Store at room temperature and prepare as needed in a flask dedicated to this solution.

(2) 0.1% (v/v) Triton X-100
Using a volumetric pipet, dilute 1 mL of Triton X-100 to 1000 mL with ultrapure water in a volumetric flask and mix well. Use the magnetic stirrer for adequate mixing. Store at room temperature and prepare as needed.

(3) Flushing liquid for AS-60 Autosampler
Fill the reservoir with deionized water from the appropriate tap at the laboratory sink. Using an Eppendorf pipet, add 1.0 mL of 0.1% Triton X-100. Cap the reservoir and mix the liquid. Prepare as needed.

(4) 10% (vol/vol) bleach solution
In a 500-mL wash bottle, dilute 50 mL of bleach, added with a graduated cylinder, to 500 mL with deionized water. Store at room temperature and prepare as needed.

d. **Standards Preparations**

(1) 1000 mg/L stock selenium standard
Dilute 1 mL NIST SRM 3149 (delivered with either a 1.0-mL Eppendorf pipet or the Micromedic Digiflex) to 10 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature and prepare every 6 months in a flask dedicated to this solution.

(2) 10 mg/L stock selenium standard
Using either a 1.0 mL Eppendorf pipet or the Micromedic Digiflex, dilute 1.00 mL of the 1000 mg/L stock selenium standard to 100 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature and prepare monthly in a flask dedicated to this solution.

(3) Working selenium standards
Using the Eppendorf pipet, transfer the volumes of intermediate standard as outlined in Table 3 to 10-mL volumetric flasks and dilute to volume with ultrapure water. Store at room temperature and prepare daily in flasks dedicated to these solutions.

Table 1
Preparation of Working Standards

| Intermediate Stock (µL) | Working Standard Concentration (µg/L) | Sample Concentration (ng/mL) |
|---|---|---|
| 250 | 250 | 50 |
| 500 | 500 | 100 |
| 750 | 750 | 150 |
| 1000 | 1000 | 200 |

(4) Calibration standards

(a) In a precleaned (rinsed with ultrapure water) Bio-Vial, and using the Eppendorf pipet, prepare a fivefold dilution (1+4) of the serum calibration material: dilute 600 µL of the serum calibration material with 2.40 mL of matrix modifier. Cap the vial and mix the serum well.

(b) Using an Eppendorf pipet, dispense 20 µL of each of the selenium working standards into separate

autosampler cups.

(c) Using an Eppendorf pipet, add 500 µL of the diluted serum and matrix modifier to each of these autosampler cups as well as to an empty cup (to be used as a blank).

(d) Place the blank in position 1 and the standards, in ascending order, in positions 2-5 on the autosampler tray.

e. **Preparation of Quality Control Materials**

Commercially collected serum that has been screened for hepatitis and HIV is obtained from the CDC Serum Bank and stored frozen until time for use. The serum is thawed, pooled in acid-cleaned 20-L glass carboys, and mixed well on a magnetic stirrer. The level of selenium in this pool is assessed by using the protocol described herein.

Prepare the materials as follows: Clean-filter the serum through Millipore filters in a sequential manner by using the following filters (each preceded by a prefilter): 3.00-, 1.20-, 0.80-, 0.65-, 0.45-, 0.30-, and 0.22-µm. Divide the pooled serum into three parts. Retain one part for the normal pool (or "medium" or "base"). To prepare the low pool, dilute the serum by 25-30% with 0.9% NaCl and mix it well. To prepare the high pool, spike the serum with a small volume of 10 mg/L selenium solution and mix it well. Filter each pool through a sterile prefilter and 0.22-µm filter combination.

Using sterile technique under a laminar-flow hood, dispense the serum in 1-mL aliquots into 2.5- or 5-mL wide-mouth tubing vials by using a Micromedic Digiflex dispenser. Cap the vials with rubber stoppers and seal them with tear-away aluminum crimped caps. Store the vials at $\leq$-70 °C. Select 20 vials of each level randomly for characterization of the quality control limits and for testing of homogeneity.

Two levels of blind quality control pools are prepared and similarly dispensed, except that vials and labels identical to NHANES specimen vials are used.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Reference materials (serum products with certified values assigned by independent reference methods) are used periodically as a check of accuracy. NIST SRM 909 should be analyzed once a week for this purpose. NIST SRM 909 comes in lyophilyzed form with a vial of ultrapure water for reconstitutiuon. Once the reference material is reconstituted, it is stable indefinitely as long as it is kept refrigerated. This material should be prepared in the same manner as all other controls and participant samples and inserted in the run following the daily controls. If the stock of this material becomes low, more should be ordered in time for the new pool to be analyzed concurrently with the old one.

This method produces linear results from 0 to 400 ng/mL (2), which is beyond the normal range of values expected to be observed for human serum selenium concentration. The linear regression program ("SELENIUM") in ROSCOE is used to calculate the calibration curve and the specimen concentrations. This program generates slopes, intercepts, correlation coefficients, standardized residuals, and plotted and fitted curves. The correlation coefficient, $r^2$, for each curve should be 0.99 or higher. For optimum sensitivity, slopes should be more than 0.00035 and intercepts should be less than 0.003. Process blanks should be less than 0.005 Abs-sec.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

Allow frozen serum specimens, quality control serum specimens, and serum calibration material to reach ambient temperature.

b. **Sample Preparation**

(1) Prepare a process blank as follows: using the Micromedic Digiflex, dispense 100 µL of ultrapure water and 400 µL of the matrix modifier into an autosampler cup.

(2) Dilute the specimens and controls fivefold (1+4) with the matrix modifier solution in the clean autosampler cups as follows: using the Micromedic Digiflex, dispense 100 µL of the serum control material or specimen and 400 µL of the matrix modifier into the autosampler cups. Prepare duplicate samples of the control

materials.

(3) Place a process blank in positions 6 and 7 on the autosampler tray. Place the autosampler cups containing the controls and specimens (controls first) in positions 8 and following on the autosampler tray.

c. **Instrument Setup for the Model 5100 and AS-60**

Table 2
Instrument Settings

| Parameter | Setting |
|---|---|
| Wavelength | 196.0 nm |
| EDL Power Supply | 5 W |
| EDL Mode | Continuous |
| Slit | 0.7 nm (low) |
| Signal | Peak Area |

Table 3
Furnace Temperature Program

| Step | Temperature, °C | Ramp (sec) | Hold (sec) | Gas Flow, mL/min |
|---|---|---|---|---|
| Dry* | 180 | 5 | 35 | 300 |
| Char | 1180 | 5 | 25 | 300 |
| Atomize | 2280 | 1 | 5 | 0 |
| Burnout | 2600 | 1 | 2 | 300 |
| Cool | 20 | 1 | 2 | 300 |

*Dry temperature and hold time may vary with different graphite tube and platform combinations.

*The program for the atomize step includes instructions for the instrument to turn on the Zeeman magnet at the beginning of the step (Read) and to activate the record function (Recorder) during the step.*

(1) Turn on the argon gas (The setting may vary from 40 to 50 psi, depending on instrument used. The furnace has a low pressure shutoff below 40 psi).

(2) Turn on the cooling water supply.

(3) Turn on the HGA 600 furnace.

(4) Turn on the Zeeman 5100.

(5) Turn on the spectrophotometer.

(6) Turn on the Epson EX-800 printer.

(7) Turn on the Epson CRT.

(8) Turn on the Epson Equity III and computer. The Desktop page will appear.

(9) Click twice on the file 5100 P.C.EXE. The Benchtop page will appear and the autosampler will flush.

(10) Two boxes will appear at the bottom of the page (AS-60 and HGA). Six boxes will appear at the right of the page. When the two bottom boxes turn blue, click twice on the AS-60 box in the top right corner of the page.

(11) Click once on ELEMENT FILE. The "Item Selector Page" will appear. Click once on SE, then once on OK.

(12) The lamp carousel will rotate to SELENIUM EDL.

(13) Plug in the EDL and turn on the power supply. Turn up the power to three quarters of maximum and wait for the EDL to spontaneously light. (If the lamp does not light, expose it to a portable ignitor until it lights.) After the lamp lights, turn it down to the recommended voltage.

(14) Four windows will appear on the screen:

(a) AS-60 CONTROL (green)
(b) DISPLAY CALIBRATION
(c) DISPLAY DATA
(d) DISPLAY PEAKS

A maximum of five windows can appear at one time.

(15) Click once on the DISPLAY CALIBRATION window. Click once on the bow tie in the left corner of the window to remove the window from the screen.

(16) Click once on the DISPLAY PEAKS window. Click and hold on the top portion of the box and move the entire box up into the right top of screen. Shrink the box to fit the space.

(17) Choose the WINDOWS option at the top of the screen. Click once on HGA CONTROL. Move the HGA CONTROL window (yellow) into the bottom right corner of screen.

(18) Choose the WINDOWS option again. Click on ELEMENT PARAMETER.

(19) When the element parameter page appears, click on the HGA box. The furnace program will appear. Check the parameters and update them if necessary. Click on the bow tie (left corner of window) to remove the page from the screen.

(20) Choose the WINDOWS option. Click on ID/WEIGHT parameter.

(21) Type your initials for analyst, then click on the first box under "Position" to bring the cursor down to this location.

(22) Choose the EDIT option. Click on GLOBAL ENTRY. Type in the starting and ending positions (e.g., starting position 1, ending position 30).

(23) Type "blank" under "Sample ID" for positions 6 and 7. Positions 8 through 13 are controls, and position 14 is another blank. After the above information is entered, click on the bow tie in the left corner to remove the screen.

(24) Install a new graphite tube and platform after 2 full days of runs (or sooner if deteriorated peak shape or increased matrix blank absorbance indicate the need to do so). Open the furnace by pressing the FURNACE key on the HGA 600. Install a L'vov platform in the furnace using the insertion tool. Make sure that the platform is properly seated in the tube by holding the tube on end and gently tapping it on a hard surface. If it falls out, reinsert it using more pressure. Install the tube in the furnace with the platform at the bottom of the tube. Use the alignment tool inserted in the sample port while pressing the FURNACE key again to close the furnace. After the furnace closes, press the furnace together to ensure that it has properly closed.

(25) Check the quartz windows of the furnace to make sure that they are clean. If there is evidence of sample spattering on the windows, remove the windows and clean them using a cotton swab soaked in dehydrated alcohol. Wipe the windows dry with a soft tissue (Kim-Wipe) and carefully reinstall them.

(26) Click on SAMPLE STANDBY; the arm of the autosampler will lift out of the wash cup. Check the alignment of the sampling tip by manually moving the arm to the sample port. If the alignment needs adjustment, unlock the autosampler base by turning the knob on the center front of the base. The knobs on the back left and front left sides then can be used to move the base to the necessary position. Lock the base in place with the center front knob. Move the mirror down so that the tip can be observed. Check the depth of the autosampler tip in the graphite tube. Use the front adjustment knob to the right of the sampling arm to raise or lower the tip as necessary. Move the mirror back to the upright position.

(27) Click on SAMPLE STANDBY again. The sampling arm will return to its position in the wash cup and flush for several seconds.

(28) Click on the HGA CONTROL window (yellow). Condition the graphite tube. Type in the temperatures listed below, click on the MANUAL TEMP after each entry, and wait for about 5 seconds. Click the MANUAL TEMP again to turn off the furnace. Wait about 10 seconds before typing in the next temperature.

1000 °C
1500 °C
2000 °C
2400 °C
2650 °C

(29) Click on the AS-60 CONTROL window (green). Go to the bottom of the AS-60 CONTROL page and type 20 after LOCATION and again after VOLUME (µL).

(30) Check the drying step of the furnace program: click on RUN SPECIAL. Lower the mirror and observe the sample tip as it deposits the sample on the platform. Continue to observe as the sample dries. (Return the mirror to upright, out of the light path, after the sample dries.) The drying should be complete 5-10 seconds before the char step begins. If it is dry sooner, decrease the hold time of the dry step appropriately. If it is not dry in time, two options are available: the temperature of the drying step may be increased or the hold time may be increased. Increase the hold time only if increasing the temperature will cause the sample to boil and splatter during the dry step.

(31) If the parameters in the dry step need to be changed, choose WINDOWS. Then when the ELEMENT PARAMETER page appears, click on the HGA box. Change the parameters as necessary.

(32) Continue to check the dry step as in (30) until a successful step is observed.

(33) When the appropriate dry temperature is determined (even if it is the one already in the program), make sure that the blank is low (less than 0.005 Abs-sec).

d. **Operation**

(1) On the AS-60 Control page, click on PRINTER ON/OFF, and then click on RUN STANDARDS.

(2) The Model 5100 will run the calibration curve.

(3) When the curve is finished, run the quality control materials. Type in 6-14 under SAMPLE LOCATIONS. Next click on RUN SAMPLES. Verify that the quality control materials are within the specified limits.

(4) If the values observed for this analytical run are in control, proceed with the analysis of the diluted serum specimens. (See the Quality Control System section of this document for criteria.)

(5) After 20 samples (this includes calibration standards and controls) have been run through the graphite tube, halt the model 5100 from proceeding with analyses. Open the graphite furnace and remove the tube and platform. Clean the charred carbonaceous material from the platform with a cotton swab. First use the cotton end to remove the excess, then use the wooden end to gently scrape off the remaining material. Perform this procedure before each new tray of samples is placed on the autosampler. This procedure should also be done before the system is turned off for the day. Before proceeding with another rack of specimens, run a furnace blank and a process blank to recondition the graphite and ensure that the tube and platform have not been contaminated.

(6) Turn off the system in reverse order.

(7) Turn off the argon gas.

(8) To bleed gas from the line, click on the HGA CONTROL page. Click on the HGA ON/OFF box. The model 5100 will start to run through one cycle. An ERROR window will appear when the gas pressure is low. Click on OK.

(9) Choose the FILE option.

(10) Click on EXIT TO DOS.

(11) A window will appear asking whether you want to save the ID weight parameters. If you do not want to save them, click on OK.

(12) A window will appear asking "Is it OK to exit software?" Before you answer, turn off the EDL power supply and unplug the EDL.

> *When turning off the EDL power supply, first turn off the power switch. When the power indicator decreases to zero, turn off the variable power knob.*

(13) Now click on OK. The lamp carrousel will rotate and the autosampler will flush.

(14) The computer screen will be blank except for the C:\ in the left corner. Type in HDSIT. Wait for three paragraphs to appear on the screen explaining the procedure.

(15) Turn off the printer.

(16) Turn off the CRT and computer.

(17) Turn off the spectrophotometer.

(18) Turn off the Zeeman 5100.

(19) Turn off the HGA-600.

(20) Turn off the cooling water.

e. **Replacement and Periodic Maintenance of Key Components** (in addition to preventive maintenance performed by the service engineer).

(1) Source lamp: Ensure that a spare source lamp is available. Order another if the spare is used for replacement.

(2) Printer ribbon: Keep a supply of printer ribbons on hand. Order more when the last is installed.

(3) Graphite tubes and pyrolytic platforms: Keep at least 3 months' supply on hand. Order more when the inventory falls below this quantity.

(4) Graphite contact rings: Approximately every 6 months, replace the graphite contact rings of the furnace housing. Indications that this procedure needs to be performed are an apparent loss of temperature control or a sounding of the alarm, indicating a problem with the tube. It is useful to maintain a log book to keep track of when these replacements should be scheduled.

(5) Reposition and trim the sampling tip of the autosampler every few weeks, depending on how the dispensing is proceeding.

f. **Calculations**

The program "SELENIUM" run in ROSCOE on the CDC mainframe will correct for the dilution discrepancy between the standard blank and the spiked standards using the dilution factor 1.04. The program will also adjust the integrated absorbance values--measured as peak areas--of the serum standards by subtracting the integrated absorbance of the unspiked serum blank from the integrated absorbances of the selenium-spiked serum standards. It will also adjust the integrated absorbances of the control and specimen samples by subtracting the integrated absorbance of the process blank.

g. **Modifications in the Selenium Method Used for NHANES III**

(1) A Perkin-Elmer Zeeman model 5100 instrument is used in addition to the Zeeman model 3030 originally used for the survey. The 5100 is more useful for routine work and the method is not compromised by using a different instrument. A comparison study (N > 300 specimens over 20 days) performed in 1990 indicated that the method could be performed comparably on the Zeeman model 5100.

(2) A Micromedic Digiflex automatic dispenser is used for many of the dispensing needs in this method because it is easier to use and provides better precision and accuracy than micropipets.

(3) The matrix modifier is added as part of the standard and specimen preparation procedure rather than by separate autosampler addition to save time during the analytical run.

(4) Magnesium nitrate is used in the matrix modifier to aid in charring of the sample during the furnace program.

(5) Since the original publication of the serum selenium method, NIST has made available the SRM 3149, which is used in this version for the preparation of the standards.

(6) The standards and samples are diluted fivefold with matrix modifier (rather than twofold) to reduce matrix effects.

## 9. REPORTABLE RANGE OF RESULTS

a. The method described here is linear between 0 and 400 ng/mL (2), which is beyond the normal range of values expected to be observed for human serum selenium concentration.

b. Duplicate measurements of integrated absorbance or concentration should not differ by more than 10% (± 7 at 70 ng/mL or ± 15 at 150 ng/mL). If they do the analysis is repeated.

c. The detection limit, based on 3 times the standard deviation of 10 repeat measurements of the blank, is 8 µg/L. This value is well below that expected in the normal human adult population. Infants and children may have serum selenium concentrations as low as 20 µg/L, which is detectable by the method described here. Samples with a selenium level >170 ng/mL or <70 ng/mL are reanalyzed for confirmation before results are released. (Samples with concentration levels >200 ng/mL are diluted 1:1 with DI water and reanalyzed.)

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the Nutritional Biochemistry Branch for epidemiological health studies. The method has proven to be accurate, precise, and reliable. The instrumentation used is "state-of-the-art." The primary standard used is a NIST SRM. Estimates of imprecision can be generated from long-term quality control pool results.

Two types of quality control systems are used in this analytical method. One system involves "bench" quality control specimens that are inserted by the analyst two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. The other system involves "blind" quality control samples that are placed in vials, labelled, and processed so that they are indistinguishable from the subject samples. The results of the blind specimens are decoded and reviewed by the supervisor. With both systems, all levels of selenium concentration are assessed by taking these samples through the complete analytical process. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Two levels of blind quality control pools are used. These pools are prepared in sufficient quantity to last throughout the survey. The levels chosen are in the "low-normal" (approximately 100 ng/mL) and "high-normal" (approximately 200 ng/mL) ranges so as not to be obvious to the analyst. The pools are prepared in the same way as the bench pools, but they are dispensed in vials identical to those used in the field for NHANES subject samples, labelled with pseudoparticipant numbers corresponding to each geographical location of the survey, and stored at ≤-70 °C. At least one blind sample is randomly incorporated with every 20 NHANES samples and analyzed according to the method protocol.

The bench quality control pools comprise three levels of concentration spanning the "low-normal" (approximately 100 ng/mL), "normal" (approximately 140 ng/mL), and high (approximately 200 ng/mL) ranges for selenium.

Quality control limits are established for each pool. An analysis of variance (ANOVA) is performed for each pool after 20 characterization runs have been performed in which previously characterized reference materials (a NIST SRM and Nyegaard RM) and bench quality control pools are used for evaluation. In addition to providing quality control limits, the characterization runs also serve to establish the homogeneity of the pools. Once the homogeneity of these bench and blind materials has been established, it is useful to have them analyzed by another independent reference method (e.g., IDMS).

The precision and accuracy of the method are reflected in these long-term quality control pool results:

Table 4
Representative Quality Control Pool Results (ng/mL)

| Pool | Mean | 95% limits | 99% limits | N | Total SD | Total CV (%) |
|---|---|---|---|---|---|---|
| SRM 909 | 103.2 | 95.85-110.45 | 93.54-112.76 | 29 | 4.90 | 4.75 |
| 9110 | 69.6 | 64.26-74.86 | 62.59-76.54 | 55 | 4.36 | 6.26 |
| 9111 | 99.2 | 92.35-106.06 | 90.18-108.23 | 55 | 4.86 | 4.90 |
| 9112 | 153.5 | 141.37-165.73 | 137.51-169.58 | 55 | 7.53 | 4.90 |
| 1188 | 87.3 | 81.18-93.46 | 79.24-95.40 | 96 | 4.56 | 5.23 |
| 1288 | 107.1 | 99.75-114.48 | 97.42-116.81 | 96 | 5.02 | 4.69 |
| 1388 | 148.5 | 139.15-157.85 | 136.20-160.81 | 96 | 5.75 | 3.87 |

After the standards and bench quality control materials are analyzed (at the beginning of an analytical run), the long-term quality control charts for each control material are consulted to determine if the system is "in control." Two types of charts are used. The first chart plots the means of the duplicate determinations and compares them with the 95% and 99% confidence limits and with the center line (the overall mean of the characterization runs). The system is out of control if any of the following events occur for any one of the quality control materials:

- The mean from a single pool falls outside the 99% confidence limits.
- The means from two pools fall either above or below the 95% confidence limits.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- The means from eight successive runs for one pool fall either all above or all below the center line.

The second type of quality control chart plots the range of the duplicate determinations and compares them with the 95% and 99% limits and with the center line. The system is out of control if any of the following events occur for any one of the quality control materials:

- The range from a single pool falls above the upper 99% limit.
- The ranges from two pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- The ranges from eight successive runs for a single pool fall above the center line.

If the run is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the

root of the problem before any analysis of specimens occurs.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

If one or more of the quality control samples fail to meet acceptable criteria for mean or range of duplicate values, take the following steps in succession.

a. Prepare and reanalyze fresh dilutions of all quality control samples.

b. Prepare fresh calibration standards and run the entire calibration curve using the freshly prepared standards.

c. Using a new (unused) graphite furnace and/or pyrolytic graphite L'vov platform, reanalyze the entire sequence of calibrators, blanks, and quality control samples.

If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions. Do not report analytical results for runs not in statistical control.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

a. The range of linearity and limits of detection were previously mentioned in Section 9 of this method.

b. No known chemical or physiochemical interferences have been documented for this analytical method; however, it is very important to avoid external contamination.

**13. REFERENCE RANGES (NORMAL VALUES)**

a. The average serum selenium value for 20 (non-U.S.) adults was listed as 75.3 ± 19.9 ug/L (5).

b. The preliminary unweighted serum selenium concentrations for 14,000 NHANES III participants of all ages, races, and sexes has a 95% confidence interval of 95-150 ng/mL.

The NHANES III data will help to establish the normal ranges of the U.S. population.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Although toxicity is not well defined in humans, the supervisor or designated analyst should routinely report serum selenium values >200 ng/mL by FAX for follow up.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens are allowed to remain at ambient temperature during analysis.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Because the analysis of serum for selenium is inherently complex and challenging, there are no acceptable alternative methods of analysis in the NHANES laboratory. If the analytical system fails, then store specimens and sample materials at ≤-20 °C until the analytical system is restored to functionality.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

a. Use the "NHANES LABORATORY SERUM SELENIUM STANDARD AND QUALITY CONTROL REPORTING SHEET" to record QC data. This reporting sheet has self-explanatory blanks for the standard absorbance data, run information, and quality control pool results. Prepare this form in duplicate, using carbon paper.

b. Use the "NHANES III ANALYTICAL WORKSHEET" to record the specimen results. These have been prepared with a list of the sample IDs for each preracked run. Record the results for serum selenium in ng/mL. If a result is below the detection limit of the method, write "ND" (for nondetectable) in the blank. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is not satisfactory, (i.e., cannot be analyzed), write "UNSAX" in the blank. (NOTE: Attempt to analyze all samples. If a sample proves to be unsatisfactory for analysis, consult with the supervisor before declaring the sample unsatisfactory for analysis or "UNSAX".) Prepare the analytical

worksheets in duplicate, using carbon paper.

c. Give both types of forms to the supervisor along with the hard copy of the data printout from the Zeeman 5100 and the hard copy of the printout obtained from running "SELENIUM." After the supervisor checks the data, the carbon copies and data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets.

d. Use the "QC" program in the HANES library of ROSCOE to enter the quality control data. This program should be updated regularly.

e. Any NHANES specimen with a serum selenium value $\geq$200 ng/mL should be FAXed to NCHS immediately by the supervisor or designee. This is the only patient population for which we are required to notify NCHS.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

RBASE and the CDC mainframe are used to keep records and track specimens. Records for the NHANES survey, including related QA/QC data, should be maintained for 10 years beyond the end of the study. Only numerical identifiers should be used (e.g., case ID numbers).

For other studies, the samples are stored at $\leq$-20 °C for 1 year after analysis. At this time the principal investigator is contacted to make a decision concerning storage or disposal of specimens.

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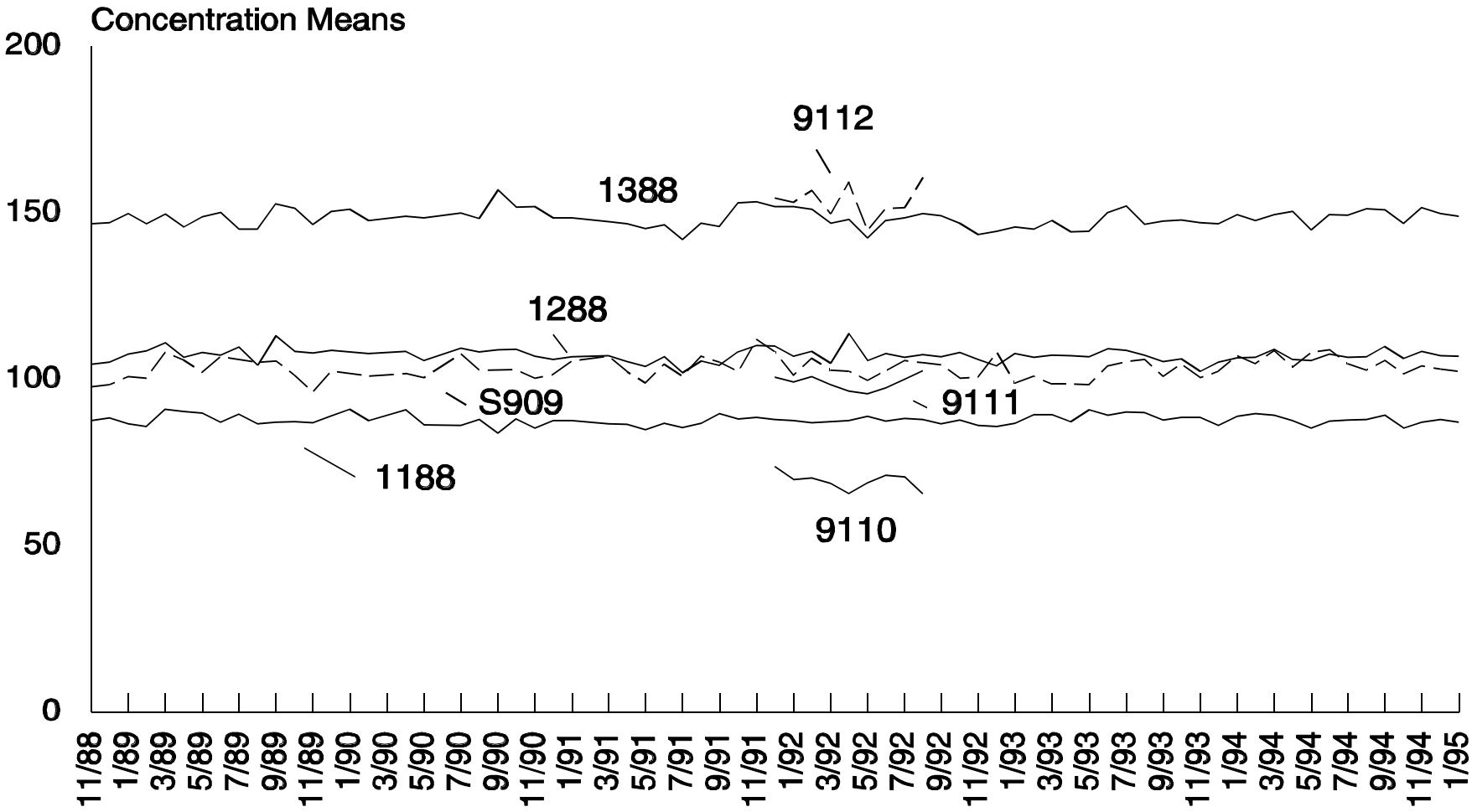
SUMMARY STATISTICS FOR
SELENIUM
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 1188 | 11/88 - 01/95 | 87.814 | 4.7470 | 5.40573 | 1976 |
| 1288 | 11/88 - 01/95 | 107.148 | 5.1305 | 4.78823 | 1976 |
| 1388 | 11/88 - 01/95 | 148.381 | 5.8828 | 3.96466 | 1976 |
| 9110 | 12/91 - 08/92 | 70.196 | 4.5082 | 6.42239 | 360 |
| 9111 | 12/91 - 08/92 | 99.569 | 4.9617 | 4.98315 | 360 |
| 9112 | 12/91 - 08/92 | 153.766 | 7.4324 | 4.83356 | 360 |
| S909 | 11/88 - 01/95 | 102.962 | 5.4104 | 5.25478 | 676 |

# Selenium Monthly Means




NOTE: No specimens assayed for Selenium during the months 3/90, 6/90, and 2/91.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Cadmium analysis is used to identify toxicity. Occupational exposure is the most common cause of elevated cadmium levels.

Cadmium is measured in urine by atomic absorption spectrometry by using a modification of the method described by Pruszkowska et al. (1). Quantification is based on the measurement of light absorbed at 228.8 nm by ground state atoms of cadmium from either a cadmium electrodeless discharge lamp (EDL) or a hollow-cathode lamp (HCL) source. Urine samples, human urine quality control pools, and aqueous standards are diluted with a matrix modifier (nitric acid, Triton X-100, and ammonium phosphate). The cadmium content is determined by using a Perkin-Elmer Model 3030 atomic absorption spectrophotometer with Zeeman background correction. Cadmium contamination must be carefully avoided throughout all procedures. All materials used for collecting and processing specimens are screened for possible cadmium contamination. All processing work is performed under clean conditions, including laminar flow hoods.

## 2. SAFETY PRECAUTIONS

Use universal precautions when handling urine products. Wear gloves, lab coat, and safety glasses. We recommend that all analysts working with whole blood, serum, or other body fluids undergo the hepatitis B vaccination series. Place disposable plastic, glass, and paper (pipet tips, autosampler cups, gloves, etc.) that contacts urine in a biohazard autoclave bag. Keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% sodium hypochlorite solution when work is finished.

*Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analytical run.*

*Special care should be taken when handling and dispensing concentrated nitric acid. Always remember to add acid to water. This material is a caustic chemical capable of causing severe eye and skin damage. Wear metal-free gloves, a lab coat, and safety glasses. If the nitric acid comes in contact with any part of the body, quickly wash with copious quantities of water for at least 15 minutes.*

Material safety data sheets (MSDSs) for nitric acid, Triton X-100, ammonium phosphate, and argon are available through the EHLS computer network.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Statistical evaluation of the run and calculation of the patient values are accomplished with a SAS program, URINCAD run in ROSCOE. The program contains an area in which to enter the standard curve data as well as the QC and patient values.

   After the data are calculated and the final values are approved for release by the supervisor, the results are transcribed by the data entry clerk into the NHANES III database, which is located in RBASE on the NCEH\EHLS PC network. The entered data are proofed by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the cadmium values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe automatically are backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Specimen donors require no special dietary instructions.

b. A first-void urine specimen is preferred, but random ("spot") urine samples are acceptable.

c. A 10-mL sample of urine is collected. It is convenient to collect a larger quantity in a sterile, plastic collection cup and

transfer the required sample to a 15-mL conical-bottom plastic tube.

d. Each lot of collection cups and shipping and storage containers should be screened for cadmium contamination.

e. Since cadmium is a ubiquitous element, the risk of contamination is very high. The following protocol should be strictly followed in order to obtain samples that are free from external contamination.

(1) Urine collection:

(a) If possible, wear metal-free disposable gloves when handling the urine collection materials and specimens. Remove the cup and cap from their plastic wrapping, being careful not to dislodge the cap or touch the inside of the cup or cap.

(b) Ensure that the cap is sealed on the container and affix the participant's preprinted label to the outside of the cup.

(c) Explain the collection instructions to the participant prior to the urine collection. Stress that the inside of the cap not be touched or come in contact with any parts of the body or clothing or external surfaces and that exposure to air should be minimized.

(d) Ensure that participants wash their hands with soap and water before specimens are collected.

(e) Ensure that participants not remove the cap from the collection cup until immediately prior to voiding. Caps should not be left off the cup longer than is necessary to collect the sample. The inside of the cap should not be touched. The cap should be turned up while the participant is voiding; the filled cup should be recapped immediately.

f. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, they should be acidified with $HNO_3$ to 1% (if not previously done) and frozen at $\leq$-20 °C until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at $\leq$-20 °C. Samples thawed and refrozen several times are not compromised. However, care should be taken if other analytes are to be analyzed in the same sample. The cadmium analysis should be performed first because of the high risk of contamination from repeated opening of, dispensing from, and closing of a sample tube or vial.

g. Processing procedure:

(1) Process specimens promptly to prevent microbiological deterioration.

(2) Do not leave the caps off storage containers longer than is necessary to process the sample. Avoid touching the inside of the caps.

(3) Gently swirl the specimen in the capped collection container to resuspend any solids.

(4) Immediately after mixing, pour 10 mL of urine into the plastic tube that has been labelled with the participant's ID.

(5) Cap and tightly seal the tube.

h. Samples that are contaminated with external sources of cadmium because of improper collection or processing will give inaccurate test results. To prevent contamination, strictly follow the above protocol.

i. Specimen handling conditions are outlined in the Division protocol for specimen collection and handling (copies available in the Branch laboratory and the Special Activities Specimen Handling Office.) The protocol discusses collection and transport procedures and any special equipment required. In general, specimens should be transported and stored at no more than 4 °C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at <-20 °C. Samples thawed and refrozen several times are not compromised.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Perkin-Elmer (Norwalk, CT) model 3030 atomic absorption spectrophotometer with Zeeman background correction, including HGA 600 furnace, cadmium source lamp, EDL power supply (if EDL sources are to be used), AS-60 Autosampler, Model 100 printer, and UV starter source.

Table 1
3030 Instrument Parameters

| Parameter | Setting |
|---|---|
| Wavelength | 228.8 nm |
| HCL Current * | 8 mA |
| EDL Power Supply * | 6 W |
| EDL Mode | continuous |
| Slit | 0.7 nm (low) |
| Signal Mode | Peak Area |

* Only one source is used.

Table 2
Furnace Temperature Program

| Step | Temperature (°C) | Ramp (sec) | Hold (sec) | Gas Flow (mL/min) |
|---|---|---|---|---|
| Dry * | 150 | 5 | 35 | 300 |
| Char * | 400 | 5 | 20 | 300 |
| Atomize | 2100 | 1 | 5 | 0 |
| Burnout | 2600 | 1 | 2 | 300 |
| Cool | 20 | 1 | 4 | 300 |

*Dry and char temperatures and hold time may vary with different graphite tube and platform combinations.

*The program for the atomize step includes instructions for the instrument to turn on the Zeeman magnet at the beginning of the step (Read) and to activate the record function (Recorder) during the step.*

(2) Micromedic Digiflex Automatic Dispensers equipped with a 2000-µL dispensing syringe and 2000- and 200-µL sampling syringes (Micromedic Systems, Inc., Horsham, PA).

(3) Mettler model PL 200 top-loading balance (Mettler Instrument Corp., Hightstown, NJ).

(4) Milli-Q water purification system (Millipore Corporation, Bedford, MA).

(5) Vortex-Genie vortex mixer (Fisher Scientific, Atlanta, GA).

(6) Eppendorf fixed-volume micropipets: 1000-, 500-, 250-, 200-, 50-, and 40-µL volumes (Brinkmann Instruments, Inc., Westbury, NY).

(7) Magnetic stirrer (Corning Glass Works, Corning, NY) and stirring bars (Fisher Scientific).

b. **Materials**

(1) Stock solution of cadmium: either NIST SRM 3108 or SRM 2121-1, both 10,000 mg/L (National Institute of Standards and Technology, Gaithersburg, MD).

(2) Redistilled concentrated nitric acid (G. Frederick Smith Chemical Co., Columbus, OH).

(3) Triton X-100 (Fisher Scientific, Fairlawn, NJ).

(4) Ammonium phosphate, dibasic ("Baker Analyzed," J.T. Baker Chemical Co. -- or any source found to be low in cadmium contamination).

(5) Milli-Q ultrapure water (Millipore).

(6) Argon, 99.996% purity (supplied as a compressed gas by Holox or other contract agency), equipped with approved gas regulator (Matheson Gas Products, Secaucus, NJ).

(7) NIST SRM 2670, trace elements in urine (elevated) (National Institute of Standards and Technology). This material is run periodically to verify accuracy and is diluted 1:20 to analyze.

(8) Bench and blind human urine quality control pools (produced in-house), including a base pool and pools spiked with moderately elevated and elevated levels of cadmium.

(9) Pyrolytic graphite tubes, solid pyrolytic graphite L'vov platforms, insertion and alignment tools, and graphite contact rings (Perkin-Elmer).

(10) Small plastic weighing boats (Scientific Products, McGaw Park, IL).

(11) Metal-free pipet tips: 1-100 µL and 1-1000 µL sizes (Bio-Rad Laboratories, Richmond, CA).

(12) Acid-cleaned volumetric flasks (1000-, 100-, and 10-mL volumes). The glassware is soaked in a soapy solution for at least 24 hours, rinsed, soaked in 25% nitric acid for 48 hours, rinsed with ultrapure water, and dried under clean conditions.

(13) Conical-bottom 2-mL polystyrene autosampler cups (Lancer, St. Louis, MO).

(14) Polystyrene 15-mL conical centrifuge tubes with polyethylene seal (Falcon 2099 Tubes, Becton-Dickinson, Oxnard, CA).

(15) Floppy disks for storage of analytical software and methods (Maxell Corporation of America, Moonachie, NJ).

(16) Kay-Dry paper towels and Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).

(17) Cotton swabs (Hardwood Products Co., Guilford, Maine).

(18) Dehydrated alcohol, USP (Midwest Grain Products of Illinois, Pekin, IL).

(19) Metal-free disposable gloves (Oak Technical Inc., Ravenna, OH).

(20) Biohazard autoclave bags (Curtin Matheson Scientific, Inc.)

(21) Bleach (10% sodium hypochlorite solution) -- any vendor.

(22) Polypropylene screw-cap 6-mL vials (Packard Co., Chicago, IL).

(23) Model 100 printer ribbons (Perkin-Elmer).

c. **Reagent Preparation**

> *Before using any disposable pipet tip, first rinse it with 1% nitric acid by drawing up the volume of acid into the tip and then dispensing it into the sink or a waste beaker.*

(1) 0.10% (v/v) Triton X-100
Using a calibrated micropipet such as Eppendorf, dilute 1 mL of Triton X-100 in approximately 900 mL of ultrapure water in an acid-cleaned volumetric flask and mix well, using a stirring bar and stirring plate. Remove the stirring bar before bringing the flask to volume. Store at room temperature and prepare as needed.

(2) 1.0% (v/v) Nitric Acid
Using a calibrated micropipet, add 2 mL of redistilled concentrated nitric acid to 200 mL of ultrapure water in a clean plastic wash bottle and mix well. Using an Eppendorf pipet, add 1 mL of redistilled concentrated nitric acid to 100 mL of ultrapure water in another clean plastic bottle and mix well. Store at room temperature and prepare as needed.

(3) Matrix Modifier (2% (v/v) nitric acid, 0.001% (v/v) Triton X-100, and 0.25% (w/v) ammonium phosphate)
Using calibrated micropipets, dilute 2 mL redistilled concentrated nitric acid and 1 mL 0.10% Triton X-100 in approximately 75 mL ultrapure water in an acid-cleaned 100-mL volumetric flask. Add 0.25 g of dibasic ammonium phosphate to the flask by washing down the weighing boat with ultrapure water delivered from a wash bottle. Bring the solution to volume with ultrapure water. After preparation, check this solution for contamination and discard if an absorbance value greater than 0.01 Abs-sec is observed for the water-matrix modifier blank. Store at room temperature and prepare daily in a flask dedicated to this solution.

d. **Standards Preparation**

(1) 1000 mg/L Stock Cadmium Standard
Dilute 1.00 mL NIST SRM 2121-1 (delivered with either an Eppendorf pipet or the Micromedic Digiflex) to 10 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature and prepare every 6 months in a flask dedicated to this solution.

(2) 10 mg/L Stock Cadmium Standard
Using either an Eppendorf pipet or the Micromedic Digiflex, dilute 1.00 mL of the 1000 mg/L stock cadmium standard to 100 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature and prepare monthly in a flask dedicated to this solution.

(3) 1 mg/L Intermediate Cadmium Standard
Using either an Eppendorf pipet or the Micromedic Digiflex, dilute 1 mL of the 10 mg/L stock cadmium standard to 10 mL with ultrapure water in an acid-cleaned volumetric flask. Store at room temperature and prepare daily in a flask dedicated to this solution.

(4) Working Cadmium Standards
Using the Micromedic Digiflex, transfer the volumes of intermediate standard to 10-mL volumetric flasks and dilute to volume with ultrapure water as shown in Table 3.

Table 3
Preparation of Working Standards

| Intermediate Stock (µL) | Working Standard Concentration (µL) | Sample Concentration |
|---|---|---|
| 125 | 12.5 | 1 |
| 250 | 25.0 | 2 |
| 500 | 50.0 | 4 |
| 750 | 75.0 | 6 |

Store at room temperature and prepare daily in flasks dedicated to these solutions.

(5) Calibration Standards

(a) Prepare 8 mL twofold diluted (1+1) matrix modifier by mixing 4 mL of ultrapure water (delivered with an Eppendorf pipet) and 4 mL of matrix modifier (delivered with an Eppendorf pipet) in a 15-mL polystyrene centrifuge tube and mix well.

(b) Using an Eppendorf pipet, dispense 20 µL of each of the cadmium working standards into separate autosampler cups.

(c) Using an Eppendorf pipet, add 500 µL of the diluted matrix modifier to each of these autosampler cups as well as to an empty cup (to be used as a standard blank).

(d) Place the standard blank in position 1 and the standards, in increasing order, in positions 2-5 on the autosampler tray.

e. **Quality Control Materials Preparation**

Collect urine from normal (i.e., disease free) laboratory workers in sterile collection containers. Pool the urine in acid-cleaned glassware and store at 4 °C. Evaluate the level of cadmium in this base pool using the analytical method described above. Clean-filter the urine in a stack system using a prefilter and the following filter sizes: 0.22-, 0.30-, 0.45-, 0.65-, 0.80-, 1.20-, and 3.00-µm. After filtering, add nitric acid at a concentration of 1% (v/v) to the urine as a preservative. Mix the acidified urine on a magnetic stirrer for several hours. The acidification process causes the urine to become cloudy from precipitation of proteins. Previous experience in this laboratory has shown further filtering to be ineffective. Divide the base pool into three portions. Keep the first as is. Spike the other two to higher levels by adding NIST SRM 2126-1 and mixing on a magnetic stirrer for several hours. Add enough NIST SRM 2126-1 to bring the cadmium concentration for one pool to a mid-range "normal" value (assuming that the concentration of the base pool is in the "low-normal" range), and the other pool to 5.0 ng/mL (the WHO health-based biological limit value (2)). Evaluate the levels of cadmium using the analytical method described below before dispensing the pools.

Dispense 2.5 mL of the bench quality control pools into 6-mL polypropylene screw-capped vials that have been previously screened for cadmium contamination using a Micromedic Digiflex dispensor, with teflon fittings and tubing. (NOTE: Clean the glassware and tubing of the dispenser before use using 1% nitric acid followed by ultrapure water.) Cap the vials immediately after dispensing. Select 20 vials at random from each pool for characterization of the quality control limits. Store the pools at ≤-20°C.

Prepare two levels of blind quality control pools and dispense them into vials identical to those used for the NHANES specimens. The labels must also be identical in appearance to those used in the study.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Use reference materials (urine products with certified values assigned by independent reference methods) to periodically check accuracy. Analyze NIST SRM 2670 once a week for this purpose. Reconstitute the lyophilized material according to the manufacturer's directions and run it in the same way as all other controls and participant samples. If the stock of this material becomes low, order more in time to analyze it concurrently with the quality control materials currently in use so that a bridge may be formed between the materials. If the material ordered from NBS is of the same lot, a full characterization is not necessary. However, there should be some overlap between the old and new stocks.

The method described here is linear up to 6 ng/mL. (The nominal concentration of NIST SRM 2670 is 80 ng/mL above this level, thus we routinely dilute this sample 1:20, so that it's concentration (approximately 4 ng/mL) is within the linear range range of the method.)

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Allow frozen urine specimens and quality control urine specimens to reach ambient temperature and mix gently by inverting or swirling.

(2) While the specimens are thawing, rinse enough autosampler cups for an analytical run with 1% nitric acid delivered from a wash bottle. Drain the cups upside down on Kay-Dry paper towels.

(3) Before using any disposable pipet tip, first rinse it with 1% nitric acid by drawing up the volume of acid into the tip and then dispensing it into the sink or a waste beaker. Before using any plastic container, rinse it with 1% nitric acid delivered from a wash bottle.

b. **Sample Preparation**

(1) When the SRM 2670 is to be used for a quality control specimen, it must first be diluted 20-fold (1+19). Using Eppendorf pipets, pipet 100 µL of the SRM into an autosampler cup. Add 1900 µL of 1% $HNO_3$. Use this diluted urine as a quality control specimen.

(2) Using either the Micromedic Digiflex or an Eppendorf pipet, dilute the specimens and controls twofold (1+1) with the matrix modifier solution in clean autosampler cups. Use 250 µL of specimen and 250 µL of matrix modifier.

> *NOTE: It is important to have the same concentration of matrix modifier in both the standards and in the specimens (controls and unknowns).*

(3) On the autosampler tray, place the autosampler cups containing the specimens in positions 7 and following, with the controls first. Fill an empty autosampler cup with ultrapure water and place it in position 6 (to be used as a blank).

c. **Instrument Setup for the Model 3030 and AS-60**

(1) Turn on the argon gas (The setting may vary from 40 to 50 psi depending on the instrument used; the furnace has a low-pressure shutoff below 40 psi).

(2) Turn on the cooling water supply.

(3) Turn on the HGA 600 furnace.

(4) Install a source lamp in position on the turret.

(5) Turn on the spectrophotometer (Zeeman/3030). After the software has automatically loaded (the element selection page will come up on the screen), press the PRINT key on the keyboard. The red light beside the key will illuminate.

(6) Turn on the PR 100 printer.

(7) Turn on the source lamp.
If using an EDL, turn on the EDL power supply: Turn up the power to three-quarters of maximum and wait for the EDL to spontaneously light. (If the lamp does not light, remove it from the turret and expose it to the UV starter source until it lights and return it to its position in the turret.) After the lamp lights, run the power at a high level momentarily, then turn it down to the recommended wattage.
If you are using an HCL, the 3030 software will light the lamp as part of the setup.

(8) In the SETUP mode, dial in the wavelength until the bar graph on the screen indicates a maximum. It may be necessary to press the GAIN key to bring the bar graph to approximately 50%. (Pressing the GAIN key will either increase or decrease the energy automatically.)
After the lamp has warmed up for about 20 minutes and while still in the setup mode, use the knobs on the lamp holder to optimize the lamp position: twist the lamp in the holder and move it back and forth (without clamping on the black end piece of the lamp). This procedure may also require use of the GAIN key.

(9) Press the USER INDEX key and look up the number of the method for cadmium. Type in the method number and press the RECALL key.

(10) Enter the programming mode by pressing the PROG key. Check the instrument parameters indicated on this page of the screen. Update the parameters if necessary.

(11) Press the PROG key again to get to the HGA 600 page. Check the HGA 600 parameters and update them if necessary.

(12) Press the PROG key again to get to the autosampler page. Check the parameters and update them if necessary.

(13) Install a new graphite tube and platform after 200 firings or about 2 full days' analyses, or if deteriorated peak shape or elevated matrix blank absorbances indicate the need to do so sooner. Open the furnace by pressing the FURNACE key on the HGA 600. Install a L'vov platform in the furnace using the insertion tool. Make sure that the platform is properly seated in the tube by holding the tube on end and gently tapping it on a hard surface. If it falls out, reinsert it using more pressure. Install the tube in the furnace with the platform at the bottom of the tube. Use the alignment tool inserted in the sample port while pressing the FURNACE key again to close the furnace. After the furnace closes, press the furnace together to ensure that it has properly closed.

(14) Check the quartz windows of the furnace to make sure that they are clean. If there is evidence of sample spattering on the windows, remove the windows and clean them using a cotton swab soaked in denatured alcohol. Wipe the windows dry with a soft tissue (Kim-Wipe) and carefully reinstall them.

(15) Press the CONT key to enter the continuous mode. Press the AS STANDBY soft key; the arm of the autosampler will lift out of the wash cup. Check the alignment of the sampling tip by manually moving the arm to the sample port. If the alignment needs adjustment, unlock the autosampler base by turning the knob on the center front of the base. The knobs on the back left side and front left then can be used to move the base to the necessary position. Lock the base in place with the center front knob. Move the mirror down so that the tip can be observed. Check the depth of the autosampler tip in the graphite tube. Use the front adjustment knob to the right of the sampling arm to raise or lower the tip as necessary. Move the mirror back to the upright position.

(16) Press the AS HOME soft key. The sampling arm will return to its position in the wash cup and flush for several seconds.

(17) Condition the graphite tube by heating it in increasing temperature steps. Press the RUN key. Type in the first temperature, press the MANUAL TEMP soft key, and wait for about 5 sec. Press the MANUAL TEMP again to turn off the furnace. Wait about 10 seconds before typing in the next temperature, and repeat the cycle until all five of the following temperature levels have been used to heat the graphite tube.

1000 °C
1500 °C
2000 °C
2400 °C
2650 °C

(18) Press the RUN key.

(19) Press the CHECK soft key.

(20) Check the drying step of the furnace program: type "1" then press the MANUAL POSITION soft key. Lower the mirror and observe the sample tip as it deposits the sample on the platform. Continue to observe as the sample dries. (Return the mirror to upright, out of the light path, after the sample dries.) Press the MANUAL POSITION soft key again to keep the autosampler from sampling from this cup indefinitely. (The system will run through the cycle twice.) The drying should be complete 5-10 seconds before the char step begins. If the sample is dry sooner, decrease the hold time of the drying step appropriately. If it is not dry in time, two options are available. The temperature of the drying step may be increased or the hold time may be increased. Increase the hold time only if increasing the temperature will cause the sample to boil and splatter during the drying step.

(21) If the parameters in the drying step need to be changed, press the PROG key twice to get to the HGA 600 page. Change the parameters as necessary.

(22) Continue to check the drying step as in step 20 until a successful step is observed.

(23) When the appropriate drying temperature is determined (even if it is the one already in the program), make sure that the blank is low (less than 0.01 Abs-sec).

d. **Operation**

(1) In the RUN mode, press the SAMPLER ON/OFF soft key to begin a run.

(2) The Model 3030 will first run the calibration curve and then the quality control materials. Check that the quality control materials are within the specified limits.

(3) If the values observed for this analytical run are in control, proceed with the analysis of the diluted urine specimens. (Refer to the Quality Control System section of this document for criteria.)

(4) Turn off the system in reverse order.

> *When turning off the EDL power supply, first turn off the power switch. When the power indicator decreases to zero, turn off the variable power knob.*

e. **Replacement and Periodic Maintenance of Key Components**

(1) Source lamp: a spare source lamp should be available. Order another if the spare is used for replacement.

(2) Printer ribbon: a supply of printer ribbons should be on hand. Order more when the last is installed.

(3) Graphite tubes and pyrolytic platforms: at least 3 months' supply should be kept on hand. Order more when the inventory falls below this quantity.

(4) Graphite contact rings: approximately every 6 months, the graphite contact rings of the furnace housing will need to be replaced. Indications that this procedure needs to be performed are an apparent loss of temperature control (frequent adjustments to the drying temperature that are not relieved by reseating the platform in the graphite tube) or an alarm sounded by the instrument, indicating a problem with the tube. It is useful to maintain a log book to help keep track of when these rings are replaced.

(5) The sampling tip of the autosampler will need to be repositioned and trimmed every few weeks, depending on how the dispensing is proceeding.

f. **Calculations**

> *The program used next will correct for the dilution discrepancy between the standard blank and the spiked standards by using the dilution factor 1.04.*

(1) The method described here is linear up to 6 ng/mL. (The concentration of NIST SRM 2670 will routinely be above this level, but further dilution of the urine matrix to bring the value into range is not recommended.) Above this level, the calibration curve begins to take on some curvature, and linear regression should not be used for calibration. The cadmium concentration of most of the specimens encountered in NHANES III will be well below 6 ng/mL. If they are not, they should be handled as described below. Use the linear regression program in ROSCOE ("URINCAD") to calculate the calibration curve and the specimen concentrations. Enter the water blank (from position 6 on the autosampler tray) as a specimen. The linear regression program generates slopes, intercepts, correlation coefficients, standardized residuals, and plotted and fitted curves. The correlation coefficient, $r^2$, for each curve should be 0.995 or higher. For optimum sensitivity, slopes should be more than 0.05, and intercepts should be less than 0.02.

(2) Repeat a specimen analysis when duplicate integrated absorbance values differ by more than about 0.005 Abs-sec or concentration values differ more than 0.3 ng/mL. To confirm results, reanalyze specimens containing more than 2.5 ng/mL cadmium (3,4).

(3) If a survey specimen contains more than 6 ng/mL cadmium, reanalyze it at a higher dilution because this value is outside the linear range of the method. Also, some urine specimens may have a particularly difficult matrix because of dissolved solids such as phosphates. This will be manifested by very poor precision in the integrated

absorbance of the analyte or by a background absorbance greater than 1.0 abs-sec. Dilute samples to be reanalyzed for these reasons twofold with 1% $HNO_3$. Dilute the urine 1+1 with 1% $HNO_3$ (250 µL of urine and 250 µL of 1% $HNO_3$). Use this diluted specimen as the sample for dilution with matrix modifier. Since the program URINCAD uses a dilution factor of 2, the results for samples that have been diluted in this way must be multiplied by 2 (for an effective dilution factor of 4). On occasion, the diluted specimen will need further dilution for the same reasons described above. If the 1+1 dilution is not high enough, use a 1+4 dilution (100 µL of urine and 400 µL of 1% $HNO_3$) and proceed as above. In this case, an additional dilution factor of 5 must be applied after the URINCAD program has been run (for an effective dilution factor of 10).

(4) The detection limit, which is 3 times the standard deviation of 10 repeat determinations (5) of a urine sample containing a low concentration of cadmium, is 0.03 µg/L. Report results below the detection limit as nondetectable. (Use 0.01 µg/L for reporting.) If one of the two replicates is lower than the detection limit, report the result as nondetectable, even if the average of the two replicates is $\geq$ 0.03 µg/L. Repeat the analysis of specimens with nondetectable cadmium, preferably in a separate run. If the repeat analysis determines that the sample contains detectable cadmium (it may be just above 0.03 µg/L) and the specimen required dilution, the additional dilution factor may result in a value much higher than that found in the first analysis of this specimen. So that this sample is not mistaken as contaminated, indicate on the repeat run sheet that the sample was diluted because of a matrix interference by placing "(M)" beside the repeat result.

g. **CDC Modifications**

(1) Matrix modifier is added as part of the standard and specimen preparation procedure rather than by separate autosampler addition to save time during the analytical run.

(2) The plasticware is not dried in an oven. We obtain a low analytical blank by rinsing the pipet tips, autosampler cups, and other plasticware with 1% nitric acid immediately prior to use. There is no dilution of the reagents or specimens because the rinsing solution successfully drains from the surfaces.

(3) Aqueous calibration standards are used with no attempt to matrix-match the standards to the specimens because this technique was found to be accurate. The slopes of urine and aqueous calibration curves are not significantly different, which is why we can analyze "difficult" matrices at a higher dilution using the same calibration curve used for less diluted samples. The same concentration of matrix modifier is present in all samples, no matter what the dilution used.

(4) A twofold (1+1) dilution of urine specimens and controls is usually used rather than a fivefold (1+4) dilution because it provides improved sensitivity. A higher dilution is used only for samples with problem matrices; the infrequency of these types of samples does not justify using a higher dilution for all of the specimens.

(5) Triton X-100 is added to the matrix modifier to improve the precision of the autosampler dispensing as well as the "wetting" of the platform by the sample (6).

(6) Maximum power mode is not used for atomization. A 1-sec ramp works well for elements such as cadmium because it allows the analyte to be atomized before the majority of the background is burned off the platform.

## 9. REPORTABLE RANGE OF RESULTS

Urinary cadmium results are reportable in a range from 0.03 µg/L to 6 ng/mL. Samples with a cadmium level >6 ng/mL are outside the linear range of the method. They should be diluted, reanalyzed, and calculated as described in the Calculations section of this document. Samples with a cadmium level below 0.03 µg/L are below the detection limit and are reported as 0.01 µg/L (ND).

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the Nutritional Biochemistry Branch for environmental and occupational health studies. The method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-the-art. The primary standard used is a NIST SRM. Estimates of imprecision can be generated from long-term quality control pool results. Two types of quality control systems are used in this analytical method: 1) "bench" quality control specimens that are inserted by the analyst two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis and 2) "blind" quality control samples that are placed in vials, labelled, and processed so that they are indistinguishable from the subject samples. The

results of the blind specimens are decoded and reviewed by the supervisor. With both systems, all levels of cadmium concentration are assessed by taking these samples through the complete analytical process. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Two levels of blind quality control pools are used. These pools are prepared in sufficient quantity to last throughout the survey. The levels chosen are in the "low-normal" (approximately 1 ng/mL) and "high-normal" (approximately 5 ng/mL) range so as not to be obvious to the analyst. The pools are prepared in the same way as the bench pools, but they are dispensed in vials identical to those used in the field for NHANES subject samples, labelled with pseudo-participant numbers corresponding to each geographical location of the survey, and stored at $\leq$-20 °C. At least one blind sample is randomly incorporated with every 20 NHANES samples and analyzed according to the method protocol.

The bench quality control pools comprise three levels of concentration, spanning the "low-normal," "normal," and "high" (at the World Health Organization health-based biological limit) ranges for cadmium.

Quality control limits are established for each pool. An analysis of variance (ANOVA) is performed for each pool after 20 characterization runs have been performed in which previously characterized NIST SRM and bench quality control pools are used for evaluation. In addition to providing quality control limits, the characterization runs also serve to establish the homogeneity of the pools. Once the homogeneity of the bench and blind materials has been established, it is useful to have them analyzed by another independent reference method (e.g., IDMS).

Table 4
Precision and Accuracy (1992 data)

| Pool | Mean (ng/mL) | 95% limits | 99% limits | N | Total SD | CV (%) |
|---|---|---|---|---|---|---|
| 0188 | 0.285 | 0.22-0.35 | 0.20-0.37 | 141 | 0.04 | 13.57 |
| 0288 | 1.42 | 1.32-1.51 | 1.29-1.55 | 141 | 0.06 | 4.00 |
| 0388 | 5.53 | 5.25-5.81 | 5.16-5.90 | 141 | 0.16 | 2.83 |
| SRM 2670 | 81.78 | 76.84-86.42 | 75.27-88.29 | 48 | 2.84 | 3.47 |

After the standards and bench quality control materials are analyzed (at the beginning of an analytical run), the long-term quality control charts for each control material are consulted to determine if the system is "in control." Two types of charts are used. The first type plots the means of the duplicate determinations and compares them with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs). The system is out of control if any of the following events occur for the quality control materials:

- The mean from a single pool falls outside the 99% confidence limits.
- The means from two of two or more pools fall either both above or both below the lower 95% limit.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- The means from eight successive runs for one pool fall either all above or all below the center line.

The second type of quality control chart plots the range of the duplicate determinations and compares them with the 95% and 99% limits as well as with the center line. The system is out of control if any of the following events occurs for any one of the quality control materials:

- The range from a single pool falls above the 99% limit.
- The within-run ranges from two pools fall above the 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- The ranges from eight successive runs fall above the center line.

If the run is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any further analysis of specimens occurs.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more of the quality control samples falls outside the 99% limits for mean or range of duplicate values, take the following steps in succession.

1) Prepare fresh dilutions of all urine quality control samples and reanalyze them. This is especially important if the controls are high because it will rule out the possibility of contamination from an outside source.

2) Prepare fresh calibration standards and run the entire calibration curve using the freshly prepared standards.

3) Install a new (unused) graphite furnace and/or pyrolytic graphite L'vov platform and then reanalyze the entire sequence of calibrators, blanks, and quality control samples.

If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions. Do not report analytical results for runs not in statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. The range of linearity and limits of detection were previously mentioned. (See Section 7 and Section 9.)

b. No known chemical or physiochemical interferences have been documented for this analytical method; however, it is very important to avoid external contamination.

## 13. REFERENCE RANGES (NORMAL VALUES)

a. WHO Recommendations:
The World Health Organization has recommended 10 µg Cd/g creatinine as the critical level for cadmium in urine. A value of 5 µg Cd/g creatinine has been considered the health-based biological limit (6).

b. Other References:
Ewers et al. proposed 2 µg Cd/g creatinine as the upper normal limit of cadmium in urine (7).

c. CDC Raw Data (uncorrected for creatinine):
The results of the first 4 years from NHANES III ranged from 0.01-15.57 ng/mL with a 95% range of 0.01-1.96 ng/mL. A total of 16,606 specimens were analyzed.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

According to OSHA Biological Workplace Laboratory standards, urinary cadmium levels > 5 µg cadmium/g creatinine in urine may indicate toxicity. However, because we are collecting data for national prevalence purposes only, participants in the NHANES survey are not routinely notified of elevated cadmium levels.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Keep specimens at room temperature during testing. Take care to avoid external contamination as previously described.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods for performing this test. In case of system failure, store all urine specimens at -20 °C until the system is functioning.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

a. **Quality Control Data**
Use the "HANES Laboratory Urine Cadmium Standard and Quality Control Reporting Sheet" to record these data. This reporting sheet has self-explanatory blanks for the standard absorbance data, the linear regression information, and the quality control pool results. Prepare this form in duplicate, using carbon paper.

b. **Analytical Results**
Use the "NHANES III Analytical Worksheet" to record the specimen results. These have been prepared with a list of the sample IDs for each preracked run. Record the results for urine cadmium in ng/mL. If a result is below the detection limit of the method, write "ND" (for nondetectable) in the blank. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is not satisfactory, (i.e., cannot be analyzed), write "UNSAX" in the blank. Prepare these forms in duplicate using carbon paper.

For samples that are repeated, use an additional report sheet (the "NHANES III Repeat Worksheet"). Prepare in duplicate using carbon paper.

c. Give all forms to the supervisor along with the hard copy of the data printout from the Zeeman 3030 and the hard copy of the printout obtained from running "URINCAD." After the supervisor checks the data, the carbon copies and data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets. When the data is calculated and the final values are approved for release by the supervisor, the results are transcribed by the data entry clerk into the NHANES III database, which is located in RBASE on the NCEH\EHLS PC network.

d. Use the "QC" program in the HANES library of ROSCOE to enter the quality control data. These data should be updated regularly.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

RBASE and the CDC mainframe are used to keep records and track specimens. Records including related QC\QA data, are maintained for 10 years after the completion of the survey for NHANES III. Only numerical identifiers should be used (e.g., case ID numbers).

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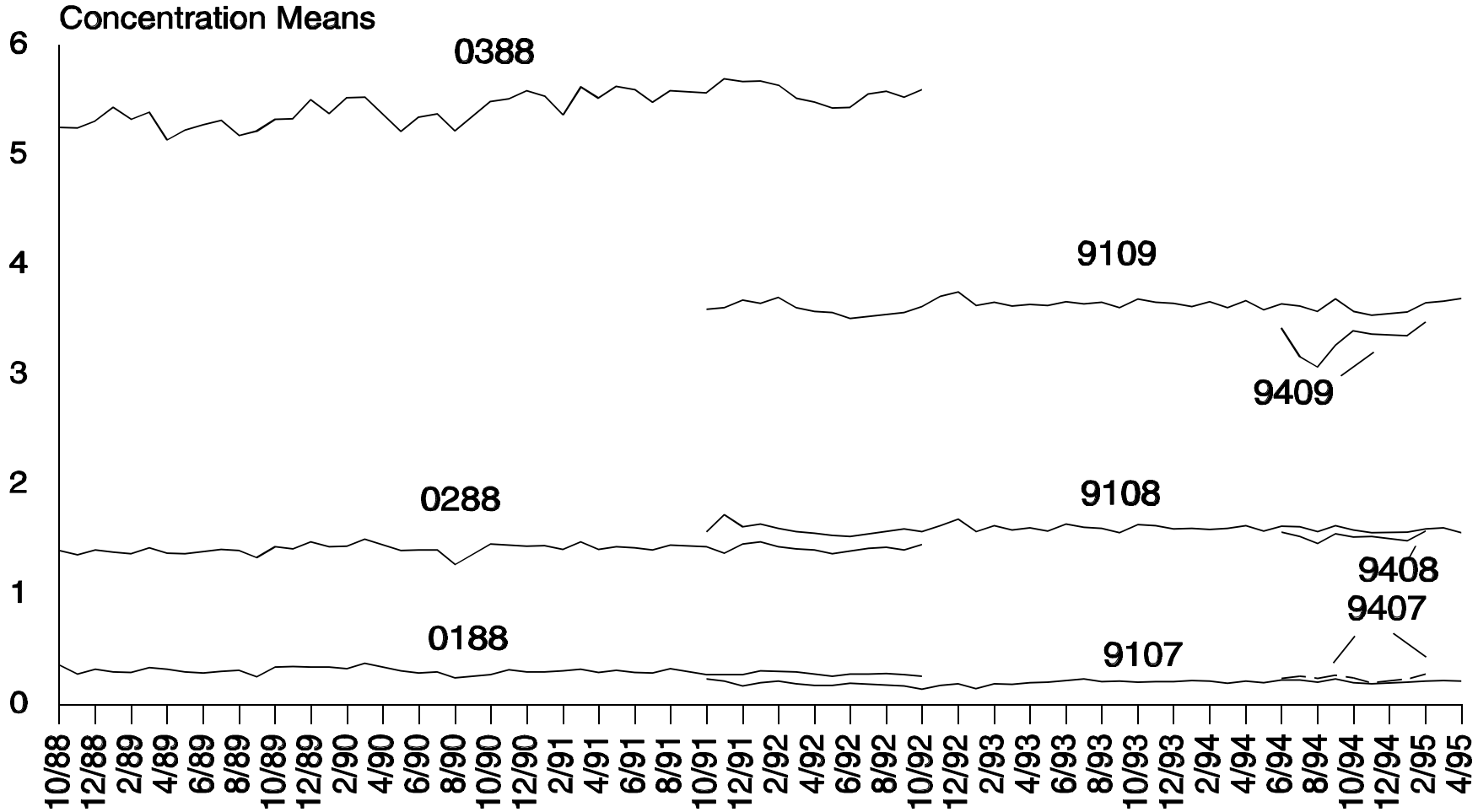
SUMMARY STATISTICS FOR
URINARY CADMIUM (ALL POOLS)
BY POOL

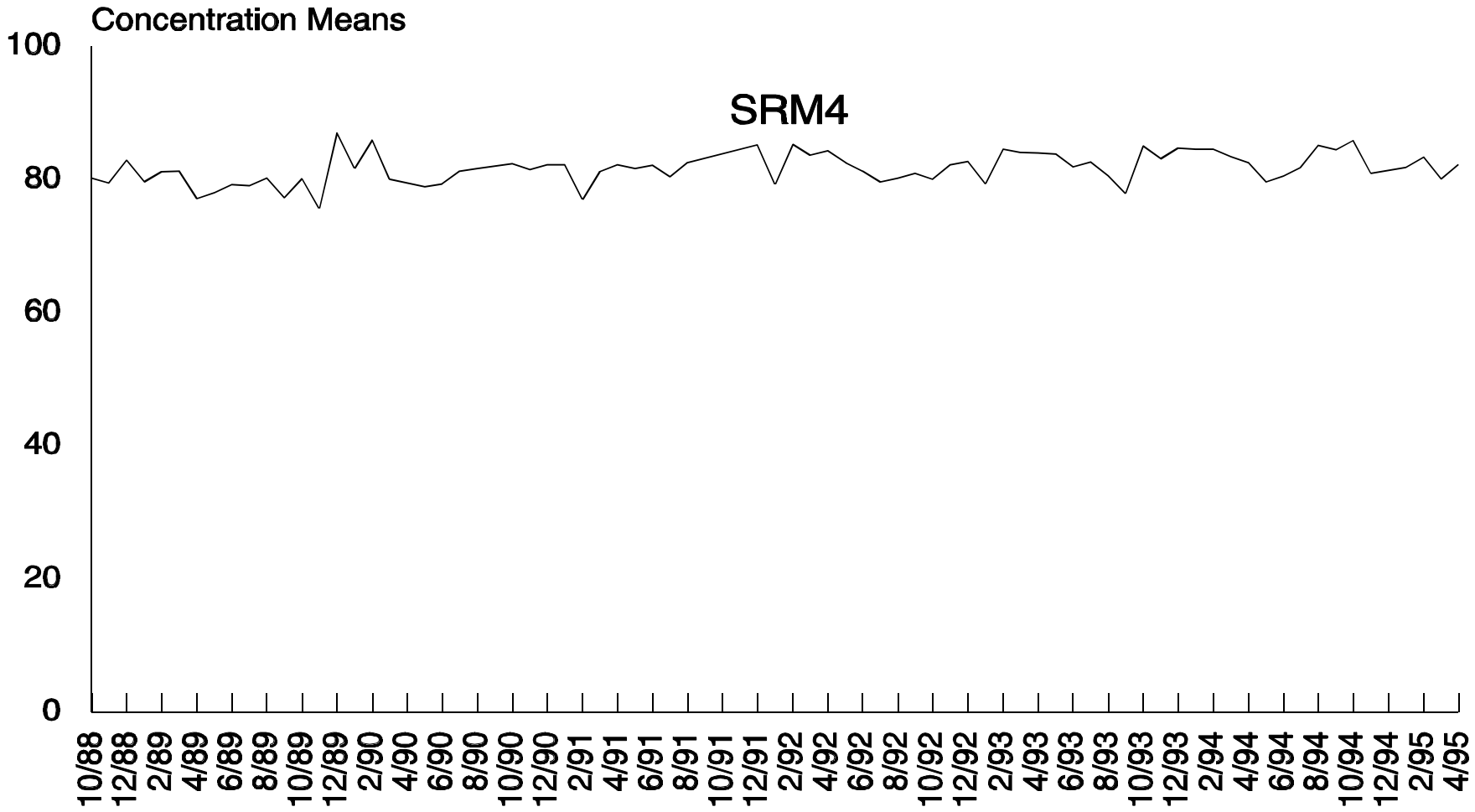
| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0188 | 10/88 - 10/92 | 0.3029 | 0.05345 | 17.6742 | 1824 |
| 0288 | 10/88 - 10/92 | 1.4230 | 0.07154 | 5.0276 | 1824 |
| 0388 | 10/88 - 10/92 | 5.4478 | 0.21702 | 3.9836 | 1818 |
| 9107 | 10/91 - 04/95 | 0.2061 | 0.04597 | 22.3025 | 1344 |
| 9108 | 10/91 - 04/95 | 1.6017 | 0.06947 | 4.3375 | 1344 |
| 9109 | 10/91 - 04/95 | 3.6374 | 0.14403 | 3.9597 | 1344 |
| 9407 | 06/94 - 02/95 | 0.2541 | 0.04836 | 19.0337 | 224 |
| 9408 | 06/94 - 02/95 | 1.5301 | 0.07714 | 5.0415 | 224 |
| 9409 | 06/94 - 02/95 | 3.2945 | 0.31326 | 9.5086 | 212 |
| SRM4 | 10/88 - 04/95 | 81.6200 | 3.19039 | 3.9088 | 940 |

# Urinary Cadmium Monthly Means - All Pools Except High Pool SRM4




NOTE: No specimens assayed for Urinary Cadmium during 4/90, 9/90, 9/91, and 12/94.

# Urinary Cadmium Monthly Means - High Pool SRM4




NOTE: No specimens assayed for Urinary Cadmium during 4/90, 9/90, 9/91, and 12/94.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

More than 99% of the calcium in the body is present in bone. Essentially all of the remaining calcium is present in the blood serum in three distinct forms: approximately 40% is present as protein-bound calcium, Ca-P; 45% as ionized calcium, $Ca^{++}$; and 15% as diffusible complexed calcium, Ca-R. To measure the total calcium concentration, one must release the protein-bound and complexed calcium. This is accomplished with hydrogen and other cations that compete for the binding sites. Then the released calcium, which is completely ionized, is measured against a standard solution with a known calcium concentration. Both total and ionized calcium are measured by ion-selective electrodes.

The pH is defined as the negative logarithm of the hydrogen ion activity. The pH of the serum specimen is measured with an electrode, and the value is used to calculate the normalized calcium level, or what the ionized calcium value would be if the pH were 7.4. Ionized calcium is very pH-dependent; a sample exposed to room air loses carbon dioxide to the atmosphere and exhibits a rise in pH. Within the pH range of 6.9-8.0, the NOVA 7 analyzer can predict the normalized calcium level within 2% of the theoretical value (1).

Decreased ionized calcium levels are seen in hypothyroidism and in vitamin D deficient rickets. Elevated levels are indicative of hypercalcemia. Patients undergoing hemodialysis and individuals with hyperparathyroidism have elevated ionized calcium levels (2).

## 2. SAFETY PRECAUTIONS

All serum specimens received for analysis must be considered potentially positive for infectious agents, including HIV and the hepatitis B virus. Observe universal precautions and wear laboratory coats, safety glasses, and protective gloves during all steps of this method. We recommend the hepatitis B vaccination series for all analysts working with whole blood or serum samples. After the analysis is completed, discard any residual sample material by autoclaving. Place all plastic and glassware that comes in contact with serum in an autoclave bag for disposal. The serum for this analysis is submitted frozen, in an unopened serum separator evacuated tube. Take care when opening the tube so as not to create potentially hazardous aerosols. Also, be careful when working with the fluids pack reagent: it contains sodium hydroxide, which can be caustic on contact with the skin. The material safety data sheet (MSDS) for the fluids pack is kept in the laboratory along with the method procedure. Additional copies are available through the manufacturer, and are accessible through the Division LAN.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The NOVA 7 analyzer directly measures both total (TCa) and ionized calcium ($Ca^{++}$) and pH and records values for total calcium, sample pH, ionized calcium, and normalized calcium on the instrument printout.

   After the data is recorded and the final values approved for release by the reviewing supervisor, the results are transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network. Data entry is verified by the supervisor and clerk. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the values to the NCHS mainframe computer along with the other NHANES III data.

b. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

c. Documentation for data system maintenance is contained in hard copies of data records.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special participant instructions such as fasting or special diets are required prior to blood collection for calcium testing.

b. Specimens for calcium analysis should be fresh or frozen serum from an unopened Vacutainer. Samples should be collected in a serum separator tube such as a Becton-Dickenson SST tube (2).

c. Sample size is 400 µL. Although automated analysis can be conducted with the Nova 7, we do not recommend it because of the rise in pH and alteration of ionized calcium values that occur as samples are exposed to ambient air. Samples may be manually sampled fairly rapidly and with minimal error.

d. Because serum pH is measured as a part of the analysis, the blood must be allowed to clot for 1/2 hour, chilled in wet ice for 5 min, then centrifuged for 15 min at 2500 x g. The tube must not be opened, but it should be refrigerated (placed on its side) for 1 hour, and then frozen at -20 °C until shipped to CDC on dry ice. Upon receipt, the tube is stored frozen until immediately prior to analysis, when it is allowed to thaw carefully at room temperature, opened, and the serum contents sampled immediately to prevent loss of $CO_2$ and an aberrant increase of serum pH (3-4).

e. Serum may be stored for at least 1 year prior to analysis provided it is kept frozen at -20 °C to -70 °C.

f. Specimens for calcium analysis in NHANES III must be frozen until they are ready to be analyzed. In a hospital setting, the Nova 7 would be used for STAT analysis on fresh serum or whole blood.

g. Hemolysis and lipemia do not adversely affect test results for this assay. The pH of the sample must be between 6.9 and 8.0 to obtain an accurate correction of ionized calcium to normalized calcium.

h. Specimen handling conditions are outlined in the Division protocol for whole blood collection and handling (copies available in the NHANES laboratory and in the Special Activities Branch Handling Office). The protocol discusses the collection and transport of specimens as well as any special equipment required. In general, whole blood specimens should be transported and stored at no more than 4 °C. Portions of the sample that remain after the analytical aliquots are withdrawn should be frozen at <-20 °C. If there is more than one analyte of interest, the specimen needs to be divided. Transfer the appropriate amount of serum into a sterile Nalge cryovial labeled with the participant's ID.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

NOVA 7+7 electrolyte analyzer (NOVA Biomedical, Waltham, MA).

### b. Materials

The following materials used on the NOVA 7+7 analyzer are all required for analysis and are available only from NOVA Biomedical unless otherwise noted:

(1) Ion selective electrodes for pH, total calcium, and $Ca^{++}$, and a reference electrode.

(2) Fluids packs (for 750 analytical samples) (installed in the instrument and replaced as emptied). Each Pack contains the following:

(a) Internal Standards A (1.04 mmol/L TCa, 1.00 mmol/L $Ca^{++}$, pH 7.45) and B, (3.12 mmol/L TCa, 3.06 mmol/L $Ca^{++}$, pH 6.94).
(b) Reference solution.
(c) Waste bottle.

(3) External standards, three levels.

(4) Total/ionized calcium electrode internal-filling solution.

(5) $Na^+$/pH electrode-conditioning solution (to condition the surfaces of the pH electrode).

(6) Pump tubing conditioner solution (to prevent the buildup of static electricity).

(7) Cleaning solution (to remove protein buildup from mixing chamber).

(8) NOVA-Stat controls -- three levels of bovine albumin-based liquid control materials.

(9) Conical bottom 2.0-mL polystyrene sample cups (Baxter Scientific Products, Atlanta, GA).

(10) Wood applicator sticks, cotton-tipped ends, disposable (any vendor).

c. **Reagent Preparation**

Bring all liquid reagents to room temperature prior to analysis, and check the level of the fluids pack. Ensure that the pH and total calcium electrodes are properly conditioned. Do not open external standards and control bottles until ready to begin the analysis. Promptly recap these materials after sampling and store them at refrigerated to ambient temperatures to minimize $Ca^{++}$ and pH changes.

d. **Standards Preparation**

Use fresh aliquots of external standards during each day of the analysis. There are three levels of external standards:

(1) STD1: TCa 1.08 mmol/L, $Ca^{++}$ 1.00 mmol/L, pH 7.45 @ 37 °C

(2) STD2: TCa 1.59 mmol/L, $Ca^{++}$ 1.48 mmol/L, pH 7.27 @ 37 °C

(3) STD3: TCa 3.24 mmol/L, $Ca^{++}$ 3.06 mmol/L, pH 6.94 @ 37 °C

These are aqueous solutions prepared by the manufacturer for calibration as well as trouble-shooting and maintenance purposes.

e. **Preparation of Quality Control Material**

Control materials are provided by the manufacturer. Lot numbers and expiration dates are recorded on the standard/controls result sheet.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

The NOVA 7 uses Standards A and B for a two-point calibration to measure the electrode slope and verify the electrode performance. Calibration is performed daily and can be initiated manually by pressing CALIBRATE. It is also initiated automatically by the NOVA 7 microcomputer at defined intervals (approximately every 2 hours). There are no NIST standards available for calibration verification. Standards are obtained from the instrument manufacturer and are traceable to reagent grade chemicals.

In addition, a one-point calibration with Standard A is automatically initiated by the NOVA 7 whenever an analysis is performed to monitor and minimize the effect of electrode drift on analytical results. The results can also be verified by analyzing the three external standard solutions and preparing a linear regression of the actual concentration versus their theoretical values.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Use the maintenance log for record-keeping.

a. **Procedure**

(1) Perform the following steps daily prior to analysis:

(a) Flush the electrode train.

(b) Flush the sampler probe.

(2) Clean the reference electrode, micromixer, and upper air detector.

(3) Check the level of the fluids in the fluids pack and replace if indicated.

(4) Use a clean, water-moistened, cotton-tipped applicator to clean the sample inlet port.

(5) Press mV DISPLAY, then press CALIBRATE, and record the Standard A millivolt (mV) reading.

(6) Press and hold SLOPE, then record TCa, $Ca^{++}$, and pH electrode slope performance numbers, checking that the numbers are within acceptable ranges.

(7) Press and hold SHIFT and OFFSET ADJ., and then record the offset % factor.

(8) Put the sampler into the UP position and follow the single analysis procedure outlined in chapter 9 of the NOVA 7/7+7 instruction manual. Manually analyze three NOVA 7 external standards (one level each) and three NOVA 7 control serum samples (one level each) in duplicate by pouring each into a sample cup and pressing ANALYZE. Record the results and check that the values are within the acceptable ranges.

(9) Press and hold FLOW, then record the two numbers that appear in the pH and $Ca^{++}$ displays. Check that these numbers are within optimal operating limits.

(10) Single sample analysis:

(a) Put the sampler in the UP position.

(b) Press ANALYZE to extend the sampler probe for sample aspiration.

(c) Position the sample over the sampler probe.

(d) Press ANALYZE a second time to initiate sample aspiration and analysis. Results will be printed in 75 seconds.

b. **Preventive Maintenance**

Perform these procedures according to the schedule in Table 1, and record the results in the maintenance log.

Table 1
Maintenance Schedule

| Service | Frequency |
|---|---|
| Change septa | As required* |
| Flush electrode train | Daily |
| Flush sampler probe | Daily |
| Clean reference electrode, micromixer, & upper air detector | Daily |
| Recondition TCa electrode | Every 2 days |
| Replace R tube | Weekly |
| Replace W tube | Weekly |
| Lubricate sampler lead screw | Monthly |
| Replace S tube | Monthly |
| Replace all tubing and interconnect tubes | Every 6 months |
| Service peristaltic pump | Every 6 months |

* Change septa each time the fluids pack is replaced and again when the level of internal Standard A drops to half volume.

c. **Calculations**

Calculate the pH of the test solution by comparing the electrode potential of the unknown solution with a ratio of the potentials of standards A and B (contained in the fluids pack).

The normalized calcium value between pH 7.2 and 7.6 is calculated with this equation:

$$\log [Ca^{++}]_{7.4} = \log [Ca^{++}]_x - .24 ( 7.4 - X)$$

where X is the measured pH of the sample, $[Ca^{++}]_x$ is the ionized calcium concentration in the test solution, and $[Ca^{++}]_{7.4}$ is the normalized calcium concentration at pH = 7.4. Between pH 6.9-7.2 and 7.6-8.0, correction factors are employed by the microprocessor to predict normalized calcium values.

d. **CDC Modifications**

No CDC modifications have been made to the instrumental procedure. Use of the Becton-Dickenson SST Vacutainer tubes was required to preserve specimen integrity during shipment from the NHANES field laboratory sites. The careful cooling/chilling/freezing steps in the field lab are necessary to prevent cracking the tube glass. The use of an autosampler on the NOVA 7 has been eliminated because studies in-house showed a rise in pH and loss of $Ca^{++}$ as replicates of the same specimen sat in the full carousel before analysis.

## 9. REPORTABLE RANGE OF RESULTS

The manufacturer reports the following linearity and reproducibility data for the NOVA 7 (1).

a. The Nova 7 is linear for total calcium between 0.1 and 6.25 mmol/L and for ionized calcium between 0.1 and 4.9 mmol/L. pH determination is linear between 6.00 and 9.00.

b. Reproducibility Data:

Within-run imprecision
- Total calcium: < 1.5 %
- Ionized calcium: < 1.09 %
- pH: ± 0.005

Day-to-day imprecision
- Total calcium: < 2.5 %
- Ionized calcium: < 1.5 %
- pH: ± 0.010

## 10. QUALITY CONTROL (QC) PROCEDURES

Freshly opened aliquots of three levels of NOVA bovine albumin-based, quality control materials are used with each assay. Because of the requirement that specimens be prepared under a vacuum to prevent pH changes, it is not feasible to prepare in-house bench and blind quality control pools for this assay for any component other than total calcium.

Quality control data is entered in the program "QCDATA," and long-term plots are generated at periodic intervals from the program "QC" by using the plot option. The abbreviations for analytes to be used are IOCA, TCAL, NOCA, SEPH. Limits for new lots of NOVA pools are provided by the manufacturer, and results are monitored for acceptable performance.

The system is declared "out of control" if any of the following events occur:

a. On the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two of two or more pools fall either both above or both below the lower 95% limit.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a

trend.

b. On the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two of two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the instrument is "out of control," UC will appear on the display of the Nova 7 and an error code will be recorded on the instrument printout. The error code can be looked up in the NOVA 7 instruction manual (1), which is located in the laboratory area provided for the instrument. A set of troubleshooting instructions are available for each error code. After completing the troubleshooting procedures, verify that the instrument is "in control" by analyzing an external standard or control before reanalyzing a sample.

When the quality control materials fail to meet the accepted criteria, use new materials. After the system has been verified to be "in control," reanalyze all specimens for that analytical run and report the results of reanalysis rather than the original values.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

It is critical that all routine and preventive maintenance procedures be strictly followed for optimum instrument performance. To prevent $CO_2$ loss, do not load more than 20 specimens on the autosampler carousel. If manually sampling, do not open specimens until immediately before sampling. Recap standards and controls immediately after sampling.

The measurement of total and ionized calcium is a well-established process. There are no significant interfering conditions or substances affecting routine analysis. If sample donors consume calcium supplements, total calcium levels may be elevated, but clinical test results for ionized calcium are not affected.

## 13. REFERENCE RANGES (NORMAL VALUES)

The manufacturer's suggested ranges (1) for total and ionized (normalized) calcium and pH at 37 °C are:

Total Calcium: 2.25-2.75 mmol/L (9.0-11.0 mg/dL)
Ionized Calcium: 1.13-1.32 mmol/L (4.5-5.3 mg/dL)
pH @ 37 °C: 7.35-7.45

Total and ionized calcium values are strictly defined for this well-established clinical test. NHANES analyses of data from the representative cross section of the U.S. population surveyed will further contribute to a determination of expected normal values for calcium/ionized calcium levels; results of the final data analysis are not available at this time. Preliminary CDC data corroborate the expected normal ranges indicated.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Since this is not considered a critical test for NCHS, abnormal results are not reported. Patients with severely abnormal calcium levels would already be under a physician's care.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Frozen samples should be allowed to thaw slowly, unopened, at room temperature prior to testing. Once opened, the serum contents should be sampled immediately to prevent loss of $CO_2$ and an aberrant increase of serum pH.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Because consistency of results is important, there are no acceptable alternative methods for performing this test for NHANES III. In case of system failure, store all unopened specimens at -20 °C until the system is functioning.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

a. Record quality control data on the "NHANES Laboratory Standard and Control Summary Sheet." Enter the standards values and the quality control pool results. Prepare this form in duplicate.

b. Record analytical results on the "NHANES III Analytical Worksheet." Enter the values for total calcium, serum pH, and normalized calcium in the blanks provided next to the appropriate sample ID. If a sample is missing from the rack, write "NOSAX" in the blank. If a sample is unsatisfactory, (i.e. cannot be analyzed), write "UNSAX" in the blank. Prepare these forms in duplicate.

c. Give all forms to the supervisor along with the hard copy of the data printout from the NOVA 7. After the supervisor checks the data, the carbon copies and data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting sheets. The results will be transcribed by the data entry clerk into the NHANES III data base that is located in RBASE on the NCEH/EHLS PC network.

d. Ionized calcium cannot be correctly normalized for samples with a pH of less than 6.9 or greater than 8.0. For these samples, only the total calcium, pH, and ionized calcium values are entered into the NHANES data base. The abnormal pH value will indicate the reason for the missing normalized calcium result.

e. Use the QC program, "QCDATA6," in the NHANES library in ROSCOE on the CDC mainframe to enter the quality control data.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

RBASE and the CDC mainframe are used to keep records and track specimens. Records, including related QC/QA data, are maintained for 10 years after completion of the survey. Only numerical identifiers (e.g., case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality.

**REFERENCES**

1. NOVA 7+7 Electrolyte Analyzer instruction manual. Waltham (MA): NOVA Biomedical, 1986.

2. Gibson, RS. Principles of nutrition assessment. New York:Oxford University Press,1990:490.

3. Graham GA, Schoen I, Johnson L. The effect of specimen choice, collection, processing, and storage on ionized calcium determinations. In: Moran R, editor. Ionized calcium: its determination and clinical usefulness. Galveston (TX): UTMB Press, 1986:88-92.

4. Bowers GN, Brassard C, Sena SF. Measurement of ionized calcium in serum with ion-selective electrodes: a mature technology that can meet the daily service needs. Clin Chem 1986;32:1437-47.

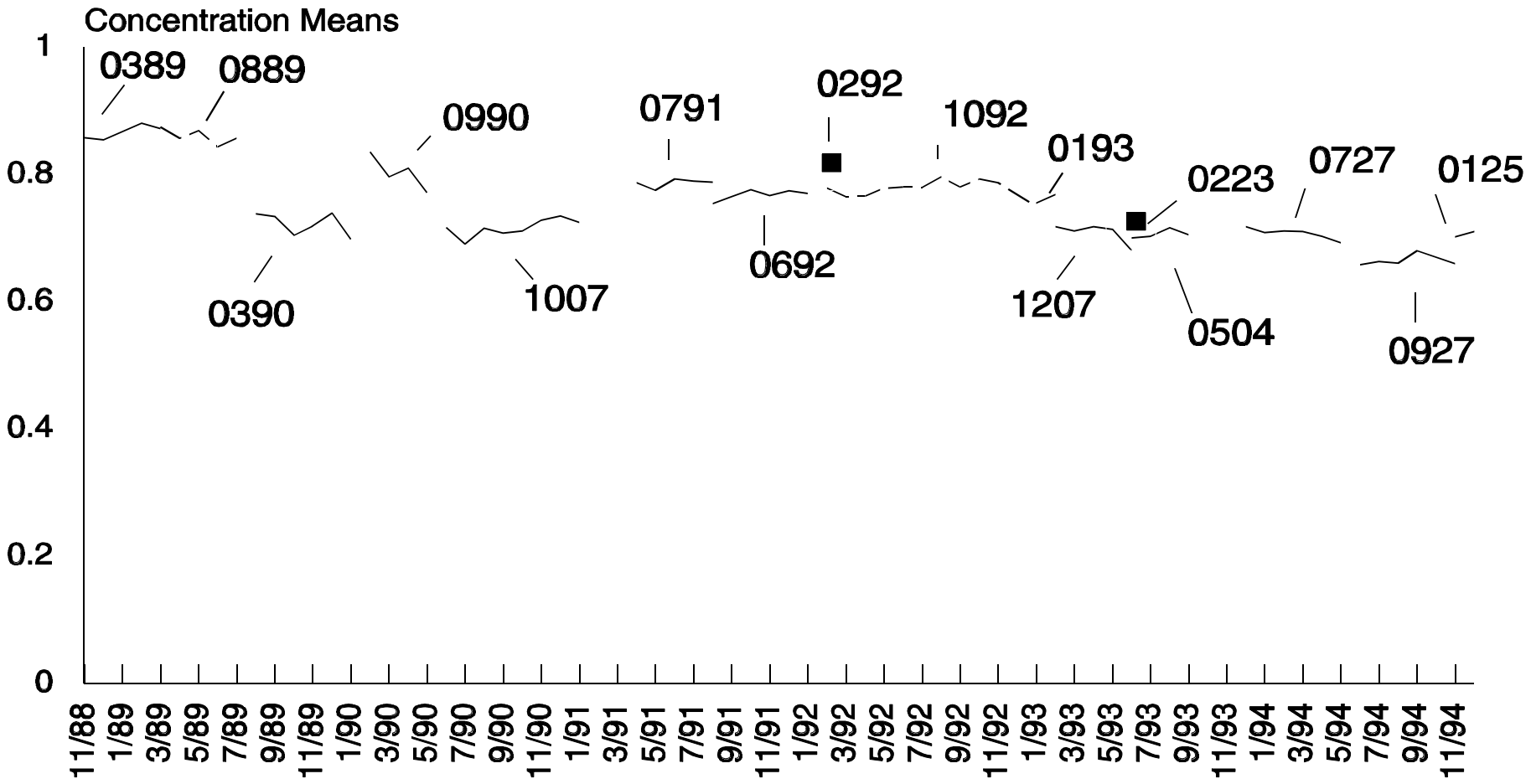
**ACKNOWLEDGMENTS**

We gratefully acknowledge the contributions of Barbara Bowman, Ph.D., Elaine Gunter, Dayton Miller, Ph.D., Eric Sampson, Ph.D., and Emmalen Smith who assisted in developing the methodology, performing the analyses for serum total and ionized calcium in the NHANES III study, and preparing the manuscript for this chapter.

SUMMARY STATISTICS FOR
IONIZED CALCIUM (LOW POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0389 | 11/88 - 03/89 | 0.86906 | 0.022518 | 2.59103 | 64 |
| 0889 | 03/89 - 07/89 | 0.85792 | 0.025871 | 3.01554 | 96 |
| 0390 | 08/89 - 01/90 | 0.72273 | 0.022679 | 3.13791 | 88 |
| 0990 | 02/90 - 05/90 | 0.79656 | 0.024509 | 3.07685 | 65 |
| 1007 | 06/90 - 01/91 | 0.71327 | 0.023388 | 3.27894 | 110 |
| 0791 | 04/91 - 08/91 | 0.78577 | 0.018418 | 2.34390 | 104 |
| 0692 | 08/91 - 01/92 | 0.76776 | 0.014926 | 1.94406 | 152 |
| 0292 | 02/92 | 0.81063 | 0.007719 | 0.95223 | 16 |
| 1092 | 02/92 - 01/93 | 0.77832 | 0.018639 | 2.39481 | 184 |
| 0193 | 01/93 - 02/93 | 0.75667 | 0.010072 | 1.33113 | 24 |
| 1207 | 02/93 - 06/93 | 0.71250 | 0.014797 | 2.07675 | 96 |
| 0223 | 06/93 | 0.71813 | 0.010468 | 1.45771 | 16 |
| 0504 | 06/93 - 09/93 | 0.70734 | 0.015760 | 2.22810 | 64 |
| 0727 | 12/93 - 05/94 | 0.70848 | 0.017096 | 2.41303 | 112 |
| 0927 | 06/94 - 11/94 | 0.66353 | 0.017083 | 2.57459 | 68 |
| 0125 | 11/94 - 12/94 | 0.70368 | 0.013000 | 1.84745 | 19 |

# Ionized Calcium Monthly Means - Low Pools

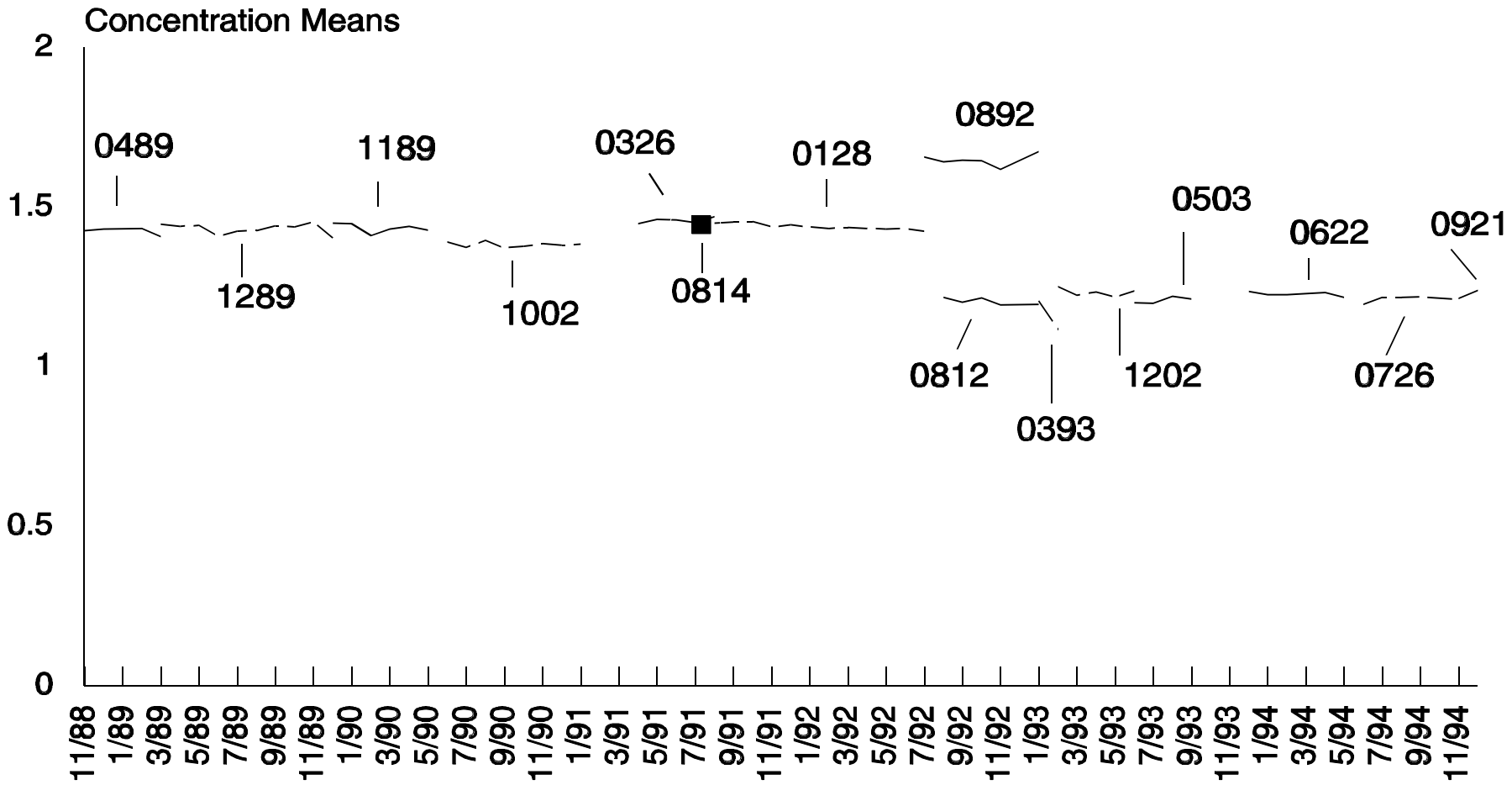



NOTE: No specimens assayed for Ionized Calcium during 1/89, 2/91-3/91, 12/92, and 10/94.

SUMMARY STATISTICS FOR
IONIZED CALCIUM (MEDIUM POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0489 | 11/88 - 03/89 | 1.42469 | 0.022037 | 1.54677 | 64 |
| 1289 | 03/89 - 12/89 | 1.43155 | 0.021969 | 1.53461 | 168 |
| 1189 | 12/89 - 05/90 | 1.43450 | 0.013585 | 0.94703 | 80 |
| 1002 | 06/90 - 01/91 | 1.38109 | 0.014736 | 1.06698 | 110 |
| 0326 | 04/91 - 08/91 | 1.45710 | 0.015393 | 1.05645 | 100 |
| 0814 | 07/91 | 1.43500 | 0.023805 | 1.65887 | 4 |
| 0128 | 08/91 - 07/92 | 1.44081 | 0.014882 | 1.03291 | 272 |
| 0892 | 07/92 - 01/93 | 1.64042 | 0.038359 | 2.33836 | 96 |
| 0812 | 08/92 - 01/93 | 1.20388 | 0.032276 | 2.68103 | 80 |
| 0393 | 01/93 - 02/93 | 1.19042 | 0.040052 | 3.36454 | 24 |
| 1202 | 02/93 - 06/93 | 1.22920 | 0.020363 | 1.65663 | 112 |
| 0503 | 06/93 - 09/93 | 1.21109 | 0.020634 | 1.70376 | 64 |
| 0622 | 12/93 - 05/94 | 1.22741 | 0.019625 | 1.59887 | 112 |
| 0726 | 06/94 - 11/94 | 1.21044 | 0.019580 | 1.61761 | 68 |
| 0921 | 11/94 - 12/94 | 1.21850 | 0.022542 | 1.85001 | 20 |

# Ionized Calcium Monthly Means - Medium Pools

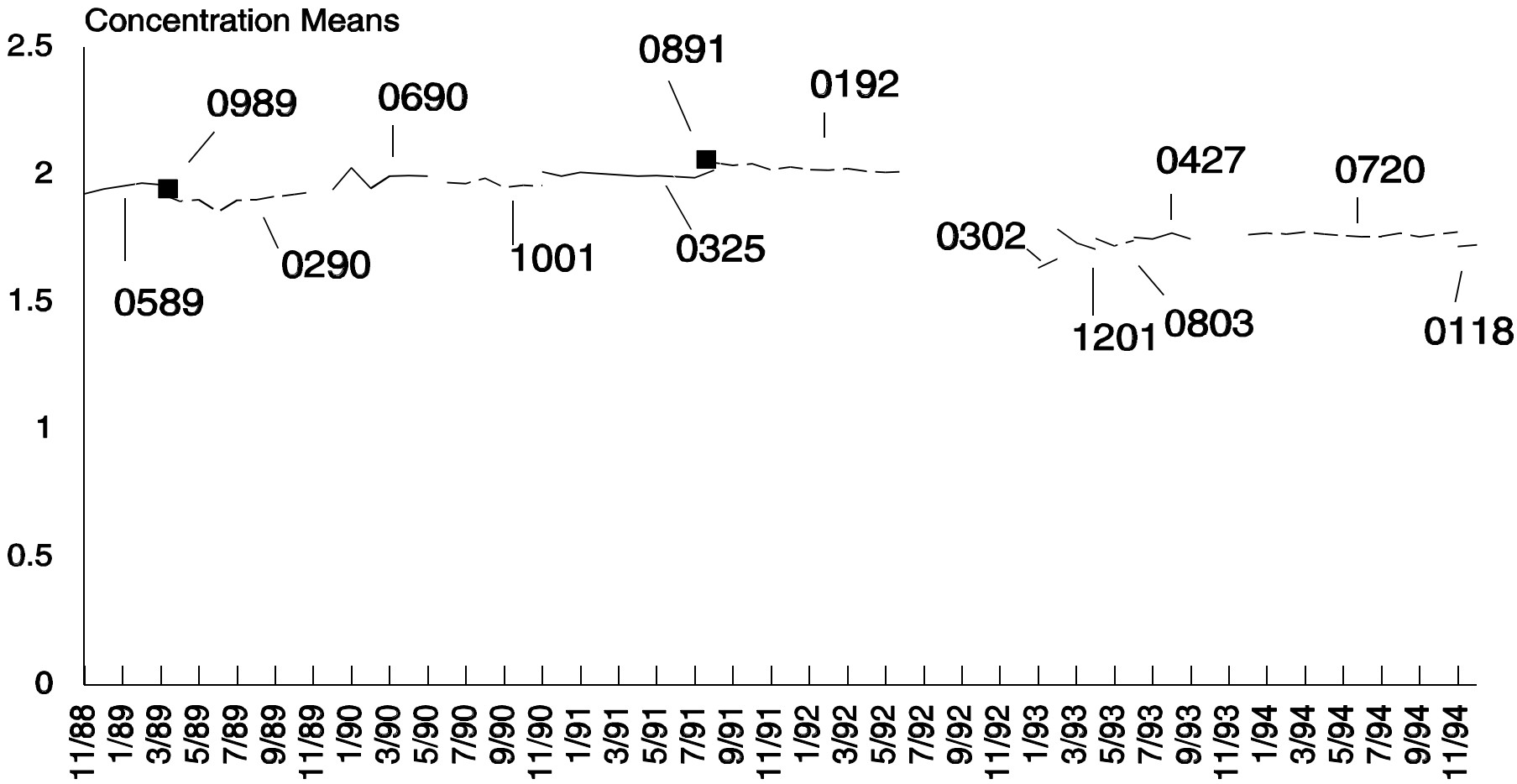



NOTE: No specimens assayed for Ionized Calcium during 1/89, 2/91-3/91, 12/92, and 10/94.

SUMMARY STATISTICS FOR
IONIZED CALCIUM (HIGH POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0589 | 11/88 - 03/89 | 1.95308 | 0.028252 | 1.44655 | 52 |
| 0989 | 03/89 | 1.94167 | 0.030401 | 1.56574 | 12 |
| 0290 | 03/89 - 11/89 | 1.90231 | 0.035756 | 1.87963 | 156 |
| 0690 | 12/89 - 05/90 | 1.98707 | 0.052467 | 2.64045 | 92 |
| 1001 | 06/90 - 11/90 | 1.96329 | 0.024997 | 1.27319 | 82 |
| 0325 | 11/90 - 08/91 | 1.99797 | 0.020634 | 1.03275 | 128 |
| 0891 | 07/91 | 2.04750 | 0.043493 | 2.12421 | 4 |
| 0192 | 08/91 - 06/92 | 2.02777 | 0.023816 | 1.17450 | 260 |
| 0302 | 01/93 - 02/93 | 1.64100 | 0.037262 | 2.27066 | 20 |
| 1201 | 02/93 - 04/93 | 1.73732 | 0.036705 | 2.11272 | 56 |
| 0803 | 04/93 - 06/93 | 1.73857 | 0.030117 | 1.73226 | 56 |
| 0427 | 06/93 - 09/93 | 1.75609 | 0.027523 | 1.56728 | 64 |
| 0720 | 12/93 - 11/94 | 1.76683 | 0.029341 | 1.66063 | 180 |
| 0118 | 11/94 - 12/94 | 1.71950 | 0.025021 | 1.45513 | 20 |

# Ionized Calcium Monthly Means - High Pools

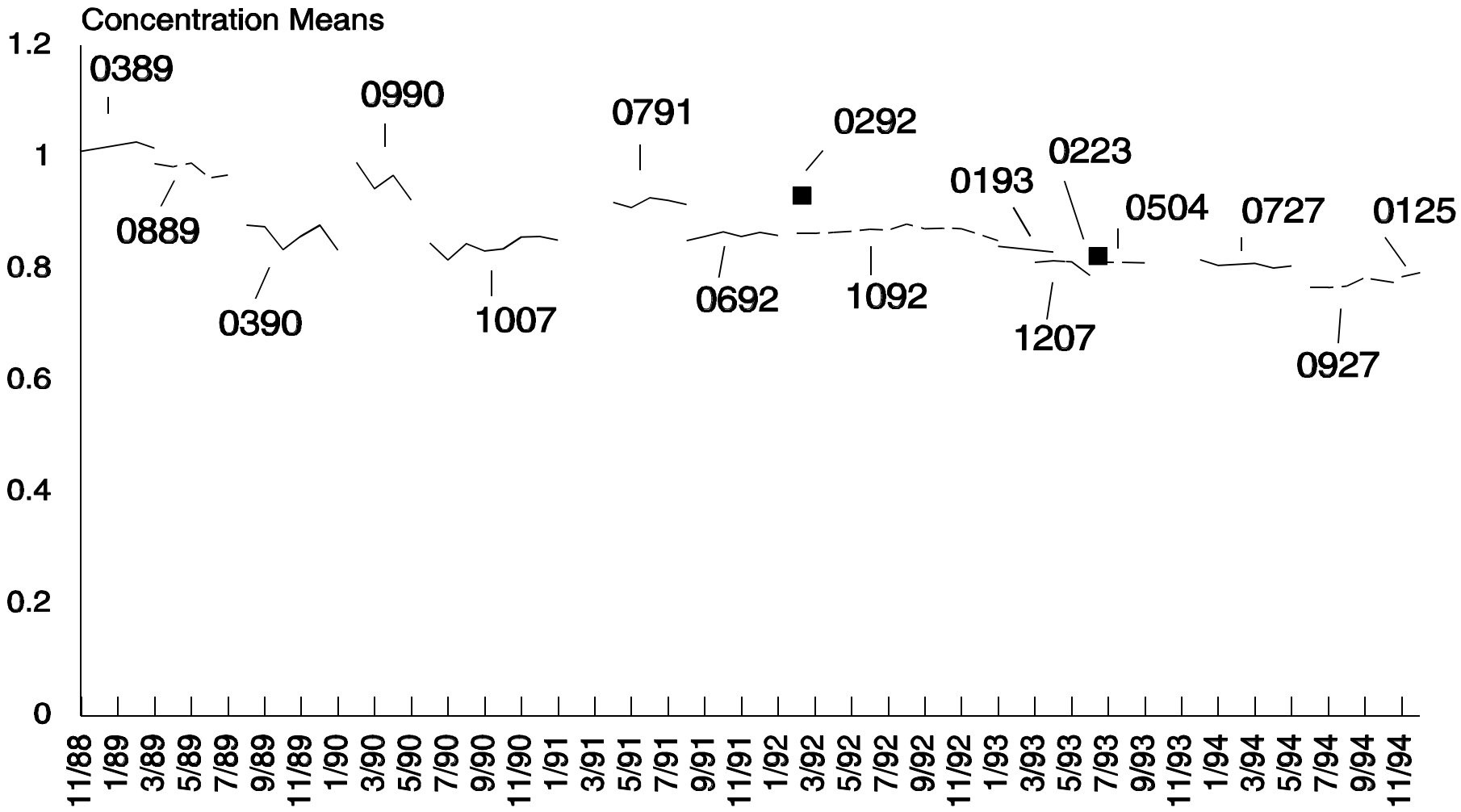



NOTE: No specimens assayed for Iodized Calcium during 1/89, 2/91-3/91, 12/92, and 10/94.

SUMMARY STATISTICS FOR
NORMALIZED CALCIUM (LOW POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0389 | 11/88 - 03/89 | 1.01922 | 0.014288 | 1.40184 | 64 |
| 0889 | 03/89 - 07/89 | 0.97604 | 0.015727 | 1.61126 | 96 |
| 0390 | 08/89 - 01/90 | 0.86023 | 0.028243 | 3.28316 | 88 |
| 0990 | 02/90 - 05/90 | 0.94883 | 0.028409 | 2.99413 | 60 |
| 1007 | 06/90 - 01/91 | 0.83946 | 0.024636 | 2.93469 | 112 |
| 0791 | 04/91 - 08/91 | 0.91880 | 0.015390 | 1.67503 | 108 |
| 0692 | 08/91 - 01/92 | 0.85972 | 0.010508 | 1.22222 | 144 |
| 0292 | 02/92 | 0.93188 | 0.007500 | 0.80483 | 16 |
| 1092 | 02/92 - 01/93 | 0.86978 | 0.010932 | 1.25685 | 180 |
| 0193 | 01/93 - 04/93 | 0.84042 | 0.009991 | 1.18881 | 24 |
| 1207 | 02/93 - 06/93 | 0.81219 | 0.010583 | 1.30306 | 96 |
| 0223 | 06/93 | 0.81813 | 0.008342 | 1.01961 | 16 |
| 0504 | 06/93 - 09/93 | 0.81625 | 0.011886 | 1.45613 | 64 |
| 0727 | 12/93 - 05/94 | 0.80929 | 0.015580 | 1.92512 | 112 |
| 0927 | 06/94 - 11/94 | 0.77044 | 0.016878 | 2.19073 | 68 |
| 0125 | 11/94 - 12/94 | 0.78684 | 0.012933 | 1.64360 | 19 |

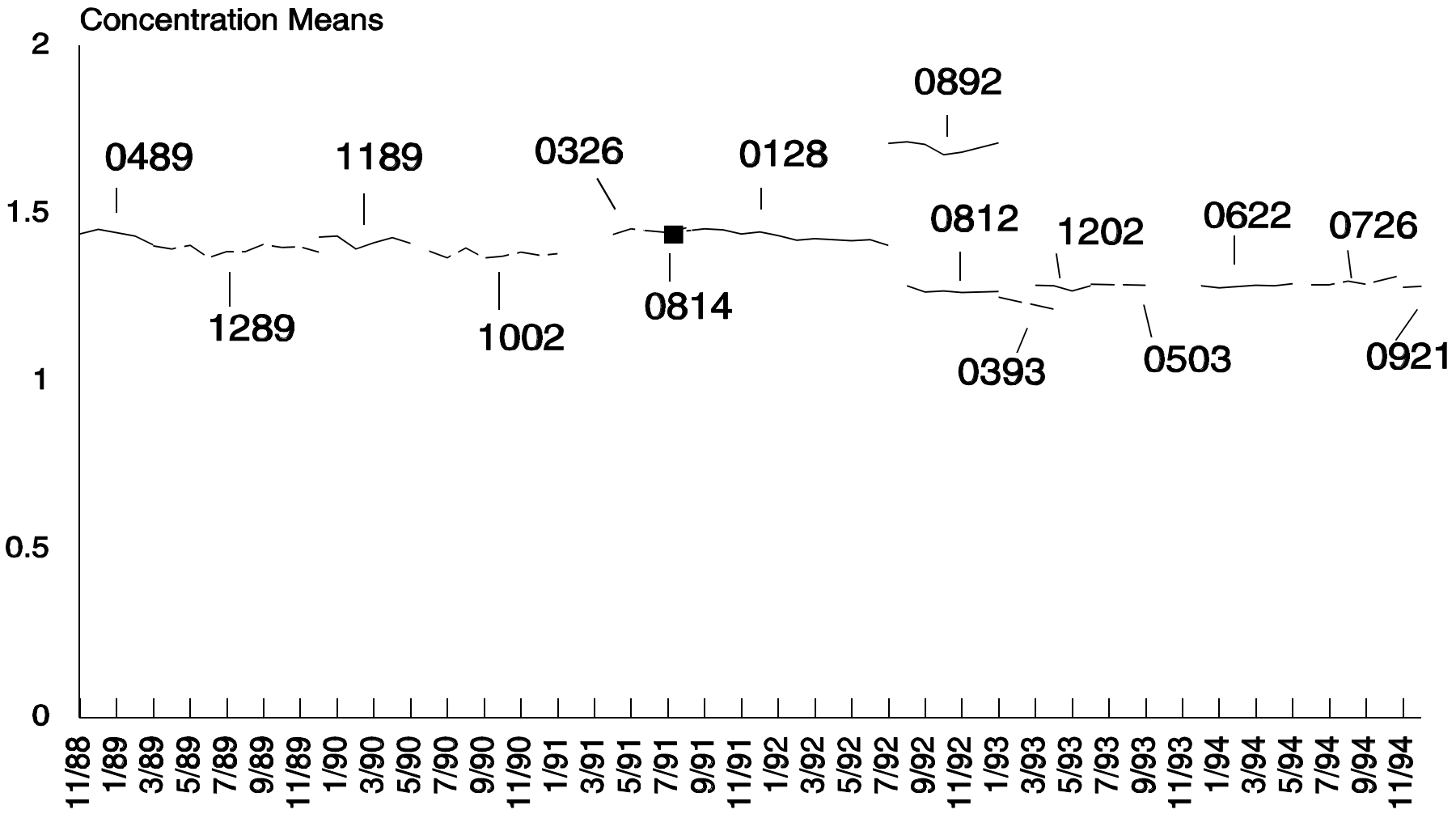
# Normalized Calcium Monthly Means - Low Pools



SUMMARY STATISTICS FOR
NORMALIZED CALCIUM (MEDIUM POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0489 | 11/88 - 03/89 | 1.43172 | 0.028203 | 1.96987 | 64 |
| 1289 | 03/89 - 12/89 | 1.39292 | 0.019495 | 1.39960 | 168 |
| 1189 | 12/89 - 05/90 | 1.41947 | 0.014226 | 1.00222 | 76 |
| 1002 | 06/90 - 01/91 | 1.37857 | 0.016971 | 1.23109 | 112 |
| 0326 | 04/91 - 08/91 | 1.44846 | 0.014666 | 1.01251 | 104 |
| 0814 | 07/91 | 1.43000 | 0.023094 | 1.61497 | 4 |
| 0128 | 08/91 - 07/92 | 1.43477 | 0.019120 | 1.33264 | 260 |
| 0892 | 07/92 - 01/93 | 1.70052 | 0.024511 | 1.44137 | 96 |
| 0812 | 08/92 - 01/93 | 1.27150 | 0.015103 | 1.18781 | 80 |
| 0393 | 01/93 - 02/93 | 1.24375 | 0.017892 | 1.43852 | 24 |
| 1202 | 02/93 - 06/93 | 1.28482 | 0.015006 | 1.16798 | 112 |
| 0503 | 06/93 - 09/93 | 1.28844 | 0.013120 | 1.01825 | 64 |
| 0622 | 12/93 - 05/94 | 1.28491 | 0.014765 | 1.14912 | 112 |
| 0726 | 06/94 - 11/94 | 1.29294 | 0.017625 | 1.36313 | 68 |
| 0921 | 11/94 - 12/94 | 1.28050 | 0.012763 | 0.99672 | 20 |

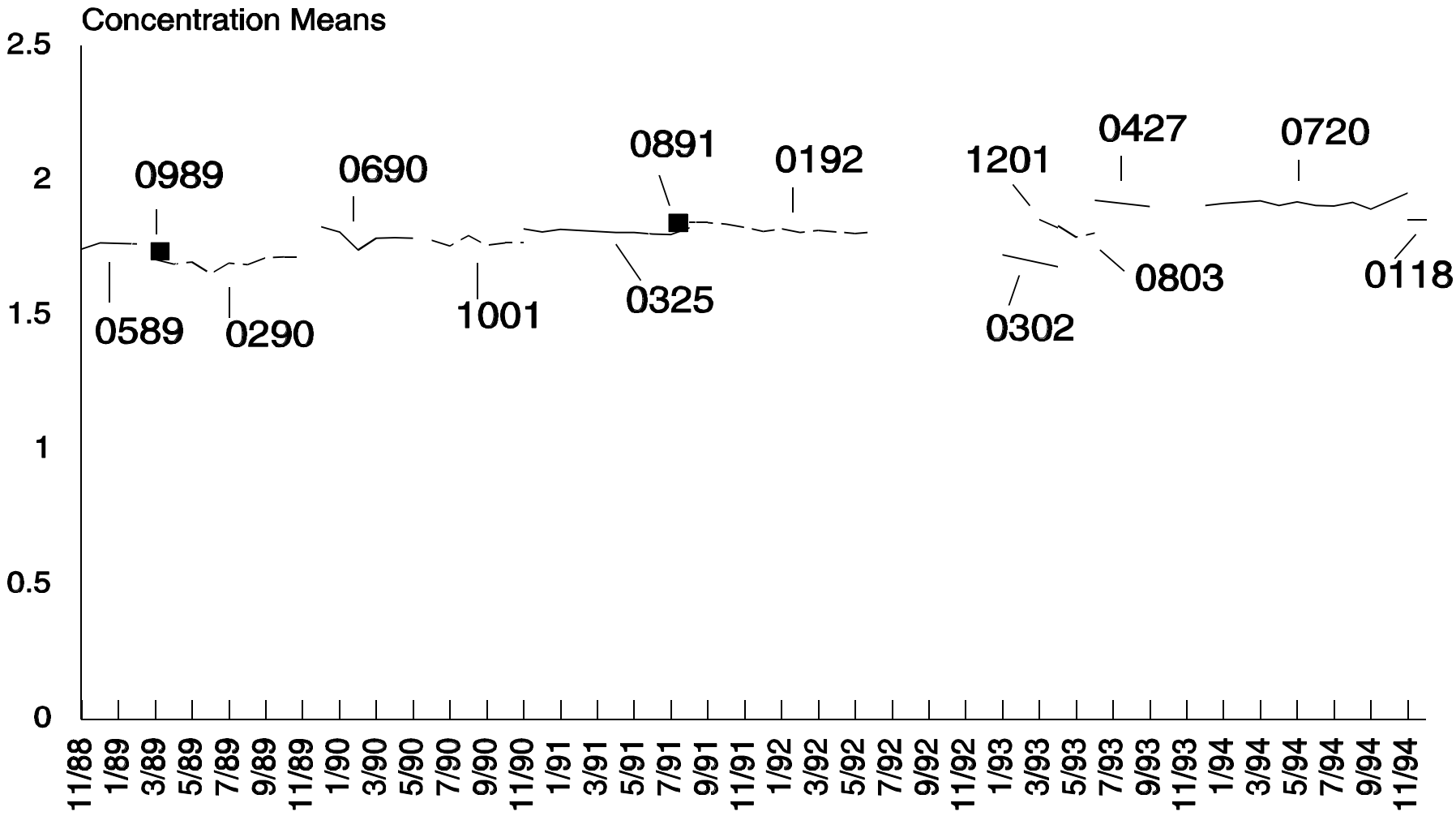
# Normalized Calcium Monthly Means - Medium Pools



SUMMARY STATISTICS FOR
NORMALIZED CALCIUM (HIGH POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0589 | 11/88 - 03/89 | 1.76146 | 0.024407 | 1.38561 | 48 |
| 0989 | 03/89 | 1.73438 | 0.025290 | 1.45816 | 16 |
| 0290 | 03/89 - 11/89 | 1.69462 | 0.030318 | 1.78910 | 156 |
| 0690 | 12/89 - 05/90 | 1.79435 | 0.049731 | 2.77155 | 92 |
| 1001 | 06/90 - 11/90 | 1.76667 | 0.026857 | 1.52019 | 84 |
| 0325 | 11/90 - 08/91 | 1.80727 | 0.018497 | 1.02345 | 132 |
| 0891 | 07/91 | 1.84000 | 0.034641 | 1.88266 | 4 |
| 0192 | 08/91 - 06/92 | 1.82214 | 0.037154 | 2.03905 | 248 |
| 0302 | 01/93 - 02/93 | 1.71650 | 0.032811 | 1.91152 | 20 |
| 1201 | 02/93 - 04/93 | 1.85679 | 0.034118 | 1.83747 | 56 |
| 0803 | 04/93 - 06/93 | 1.80982 | 0.029449 | 1.62717 | 56 |
| 0427 | 06/93 - 09/93 | 1.91297 | 0.031956 | 1.67051 | 64 |
| 0720 | 12/93 - 11/94 | 1.91422 | 0.028617 | 1.49498 | 180 |
| 0118 | 11/94 - 12/94 | 1.85200 | 0.022384 | 1.20865 | 20 |

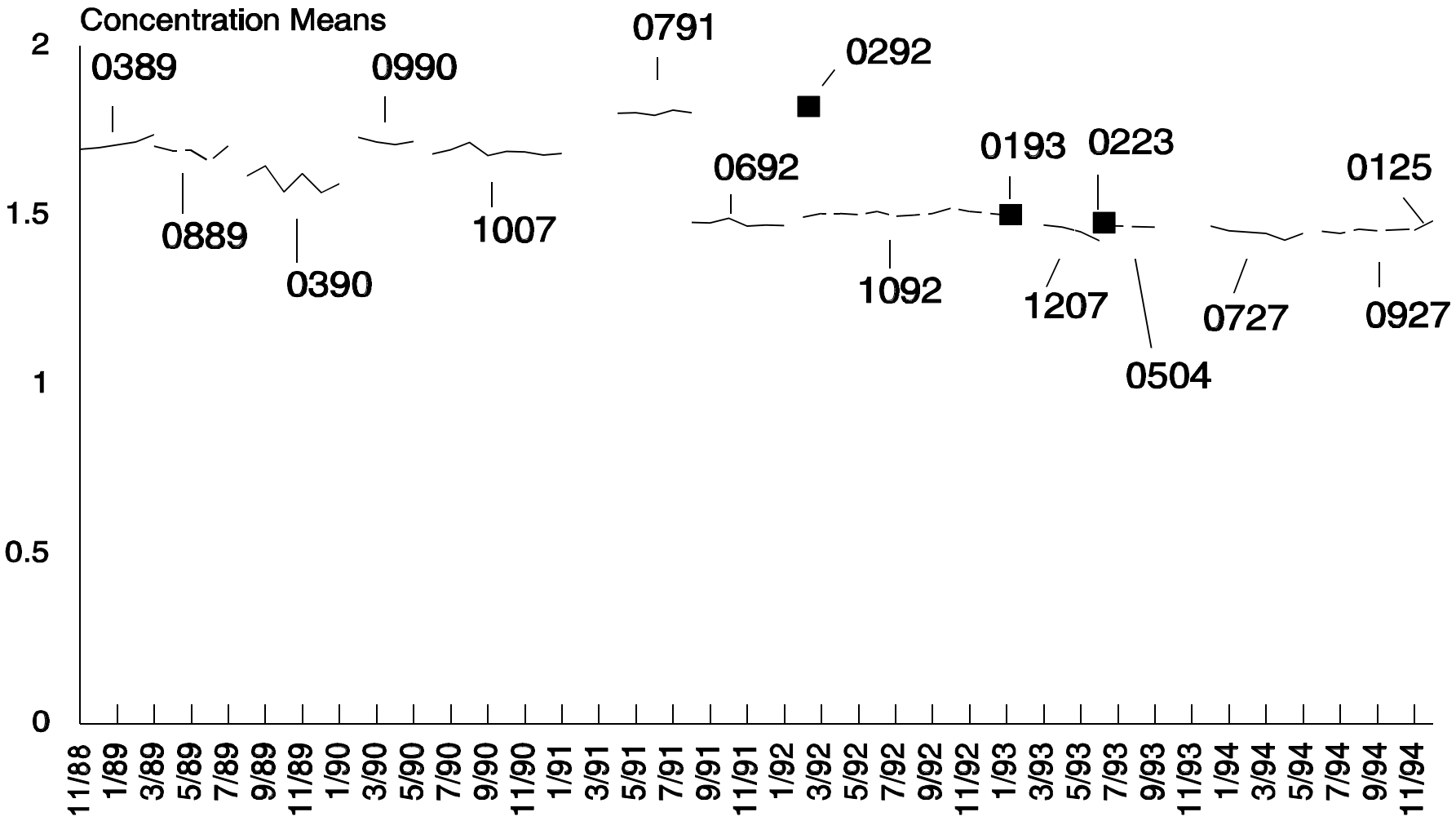
# Normalized Calcium Monthly Means - High Pools



SUMMARY STATISTICS FOR
TOTAL CALCIUM (LOW POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0389 | 11/88 - 03/89 | 1.71500 | 0.03371 | 1.96572 | 64 |
| 0889 | 03/89 - 07/89 | 1.68667 | 0.03772 | 2.23609 | 96 |
| 0390 | 08/89 - 01/90 | 1.60398 | 0.04520 | 2.81771 | 88 |
| 0990 | 02/90 - 05/90 | 1.71500 | 0.02275 | 1.32640 | 64 |
| 1007 | 06/90 - 01/91 | 1.68866 | 0.02422 | 1.43411 | 112 |
| 0791 | 04/91 - 08/91 | 1.80056 | 0.02254 | 1.25190 | 108 |
| 0692 | 08/91 - 01/92 | 1.47939 | 0.01758 | 1.18851 | 148 |
| 0292 | 02/92 | 1.80813 | 0.02287 | 1.26470 | 16 |
| 1092 | 02/92 - 01/93 | 1.50603 | 0.01805 | 1.19833 | 184 |
| 0193 | 01/93 | 1.49208 | 0.03230 | 2.16477 | 24 |
| 1207 | 02/93 - 06/93 | 1.46352 | 0.02349 | 1.60508 | 88 |
| 0223 | 06/93 | 1.46438 | 0.01315 | 0.89798 | 16 |
| 0504 | 06/93 - 09/93 | 1.46719 | 0.01397 | 0.95192 | 32 |
| 0727 | 12/93 - 05/94 | 1.44946 | 0.02265 | 1.56245 | 92 |
| 0927 | 06/94 - 11/94 | 1.45441 | 0.02333 | 1.60429 | 68 |
| 0125 | 11/94 - 12/94 | 1.46100 | 0.01744 | 1.19381 | 20 |

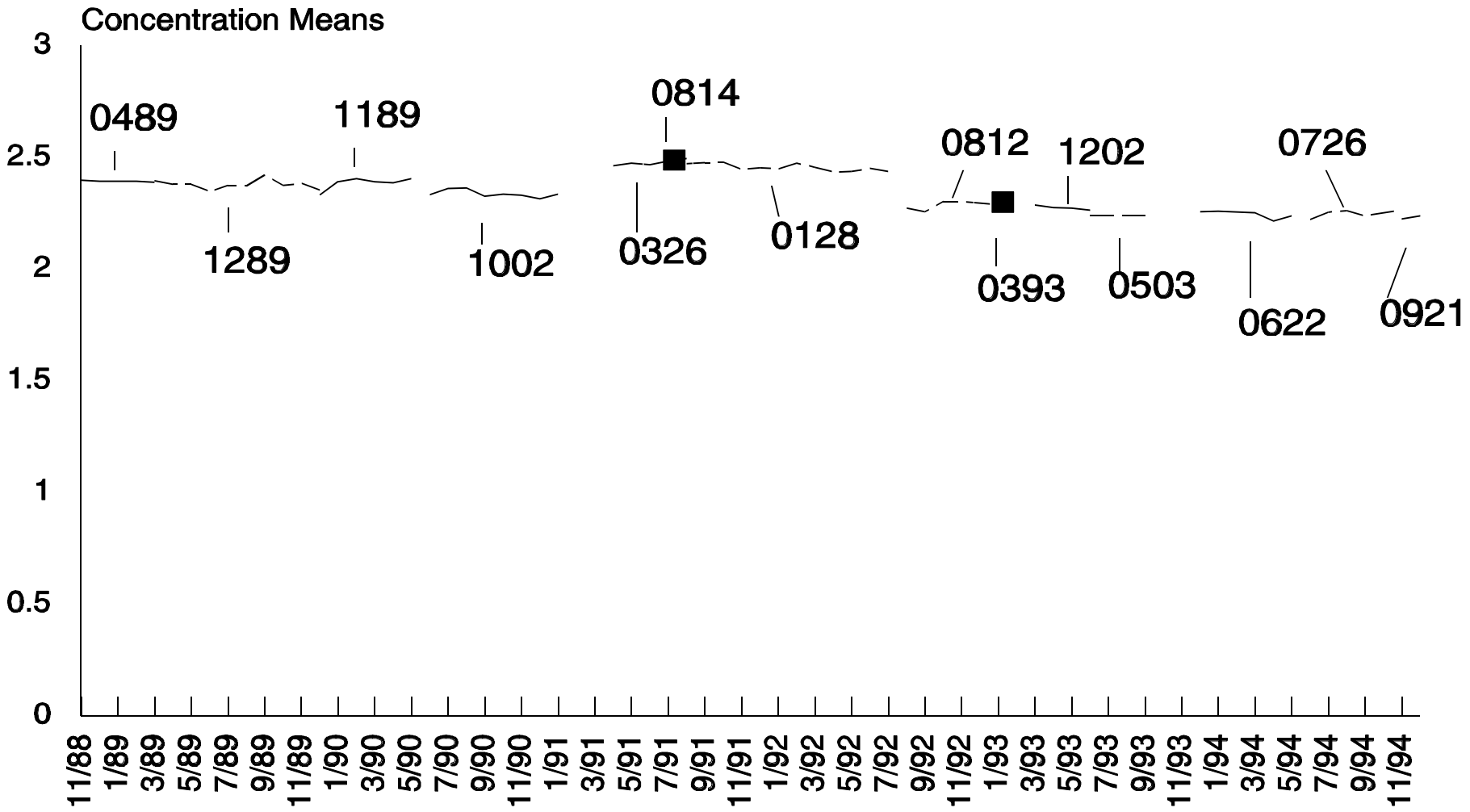
# Total Calcium Monthly Means - Low Pools



SUMMARY STATISTICS FOR
TOTAL CALCIUM (MEDIUM POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0489 | 11/88 - 03/89 | 2.39188 | 0.04346 | 1.81685 | 64 |
| 1289 | 03/89 - 12/89 | 2.37780 | 0.04328 | 1.82037 | 168 |
| 1189 | 12/89 - 05/90 | 2.38987 | 0.02646 | 1.10705 | 80 |
| 1002 | 06/90 - 01/91 | 2.33973 | 0.03425 | 1.46401 | 110 |
| 0326 | 04/91 - 08/91 | 2.46962 | 0.02360 | 0.95576 | 104 |
| 0814 | 07/91 | 2.46750 | 0.06652 | 2.69587 | 4 |
| 0128 | 08/91 - 07/92 | 2.45716 | 0.02774 | 1.12891 | 268 |
| 0812 | 08/92 - 01/93 | 2.28000 | 0.03975 | 1.74325 | 80 |
| 0393 | 01/93 | 2.28167 | 0.04967 | 2.17672 | 24 |
| 1202 | 02/93 - 06/93 | 2.27558 | 0.03571 | 1.56939 | 104 |
| 0503 | 06/93 - 09/93 | 2.24031 | 0.01448 | 0.64619 | 32 |
| 0622 | 12/93 - 05/94 | 2.24685 | 0.04060 | 1.80689 | 92 |
| 0726 | 06/94 - 11/94 | 2.24353 | 0.02981 | 1.32887 | 68 |
| 0921 | 11/94 - 12/94 | 2.22650 | 0.03856 | 1.73200 | 20 |

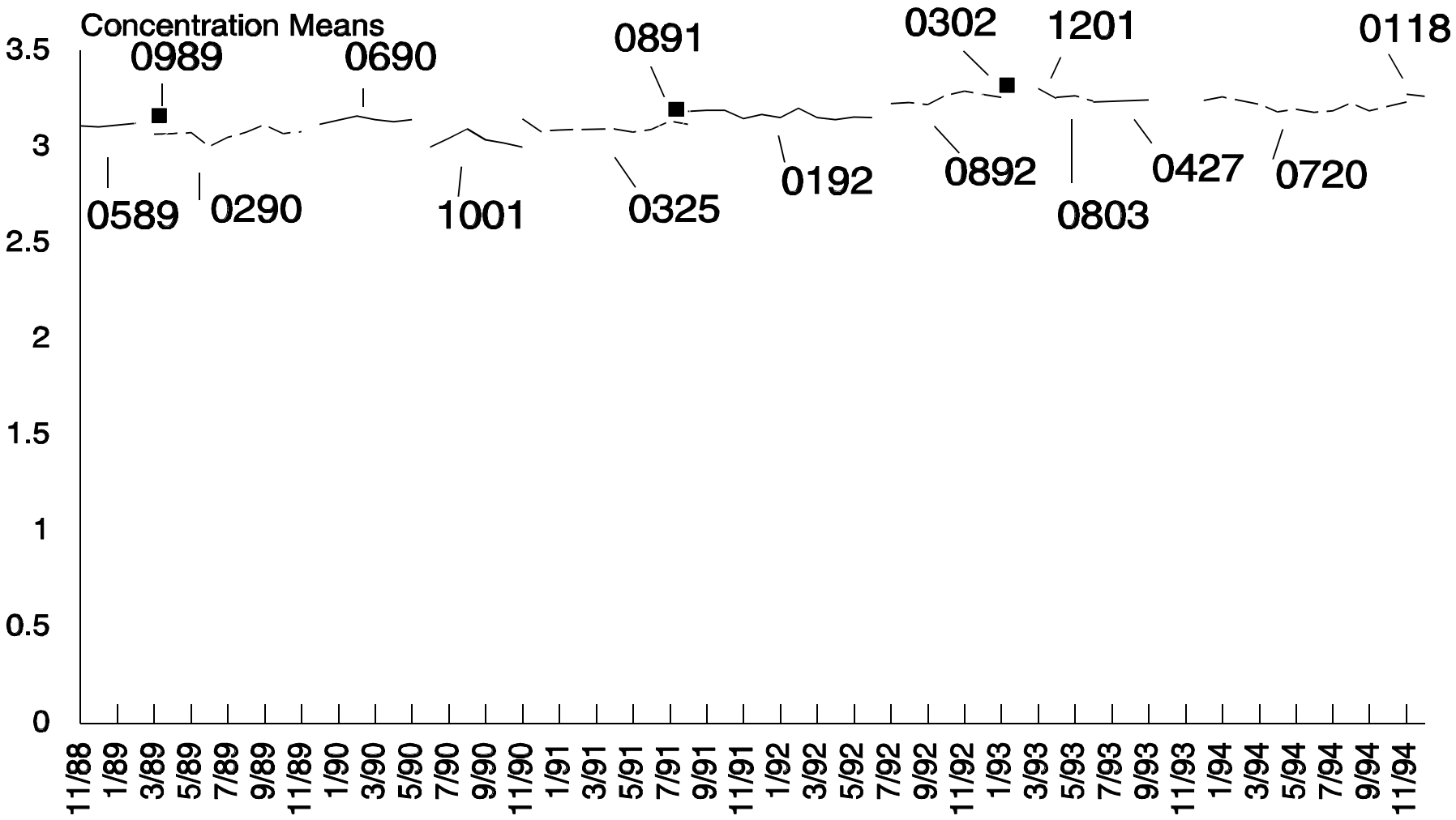
# Total Calcium Monthly Means - Medium Pools



SUMMARY STATISTICS FOR
TOTAL CALCIUM (HIGH POOLS)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 0589 | 11/88 - 03/89 | 3.11625 | 0.07913 | 2.53941 | 48 |
| 0989 | 03/89 | 3.14625 | 0.03948 | 1.25469 | 16 |
| 0290 | 03/89 - 11/89 | 3.06468 | 0.07352 | 2.39906 | 156 |
| 0690 | 12/89 - 05/90 | 3.13685 | 0.04000 | 1.27510 | 92 |
| 1001 | 06/90 - 11/90 | 3.03241 | 0.05377 | 1.77332 | 83 |
| 0325 | 11/90 - 08/91 | 3.09606 | 0.03608 | 1.16542 | 132 |
| 0891 | 07/91 | 3.19250 | 0.11871 | 3.71835 | 4 |
| 0192 | 08/91 - 06/92 | 3.17055 | 0.04197 | 1.32367 | 256 |
| 0302 | 01/93 | 3.31550 | 0.09162 | 2.76343 | 20 |
| 1201 | 02/93 - 04/93 | 3.29563 | 0.08455 | 2.56549 | 48 |
| 0803 | 04/93 - 06/93 | 3.25411 | 0.04615 | 1.41830 | 56 |
| 0427 | 06/93 - 09/93 | 3.24219 | 0.03309 | 1.02066 | 32 |
| 0720 | 12/93 - 11/94 | 3.21625 | 0.06911 | 2.14868 | 160 |
| 0118 | 11/94 - 12/94 | 3.27400 | 0.04465 | 1.36380 | 20 |
| 0892 | 07/92 - 01/93 | 3.25000 | 0.06321 | 1.94499 | 96 |

# Total Calcium Monthly Means - High Pools



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

### a. Clinical Relevance

Cotinine is a major metabolite of nicotine, created in the body as a result of a person inhaling tobacco smoke, either by smoking or by passively inhaling environmental tobacco smoke (ETS). While the half-life of nicotine is only 0.5-3.0 hours (3-5), that of cotinine is approximately 15-20 hours (1-3), thus allowing a longer measurement window to occur. Cotinine may be measured in serum, urine, or saliva -- the half-life of cotinine in all three fluids is essentially the same (1). Cotinine concentrations tend to be three to eight times greater in urine than in serum; however, for studies requiring a quantitative assessment of exposure, plasma or serum is regarded as the fluid of choice (6). Therefore, serum was chosen for the NHANES cotinine analyses. The enzyme immunoassay (EIA) method is used as a screening method for differentiating "low" (<25 ng/mL) and "high" ($\geq$25 ng/mL) cotinine concentrations in serum for confirmatory, definitive, mass spectrometry analysis.

### b. Assay Principle

The cotinine assay is a competitive EIA for the qualitative and semiquantitative determination of cotinine in serum, urine, and saliva. The test relies on the competition between free cotinine in the sample and cotinine bound to enzyme, for antibody fixed on a polystyrene plate. After excess enzyme is washed away and substrate added, the amount of free cotinine present is inversely proportional to the amount of free cotinine in the biological sample (7).

### c. Special Precautions

Because of the nature of these analyses, all analysts in this study must be nonsmokers, and analyses must be conducted in a nonsmoking laboratory environment.

## 2. SAFETY PRECAUTIONS

### a. Biological Hazards

Follow universal precautions while working with serum specimens. Use proper technique and avoid any direct contact with the sample. Wear lab coats, gloves, and protective eye wear while handling the specimens, and use a biological safety cabinet when aliquoting samples. Treat all specimens as potentially positive for HIV and Hepatitis B. Autoclave all leftover blood specimens before disposal, as well as all plastic, glassware, and paper products that have come in contact with the blood. Collect all washings from ELISA plates and disinfect them using bleach. Disinfect laboratory work surfaces with a 10% bleach solution after a spill of blood or serum and after work activities are completed.

### b. Chemical Hazards

Reagents and solvents used in this study include those listed below. Material data safety sheets (MSDSs) for these chemicals are readily accessible through the Division LAN CD-ROM system.

(1) Avoid contact with 3,3',5,5'-tetramethylbenzidine. It is toxic if inhaled, touched or swallowed. Sensitization of the skin may occur after contact. Wash the contacted area well with water. If feeling unwell, contact a doctor or seek medical advice.

(2) Sulfuric acid is a corrosive oxidizing acid used as a stopping reagent. Avoid contact with skin. Wash areas of contact with water. All leftover acid solutions should be disposed of as hazardous wastes.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Unknown sample concentrations are determined with the Mikrofit software and a four-parameter (log/linear) logistics fit standard curve. Data from the absorbance readings on the ANTHOS microtiter plate reader are communicated to the computer, which uses Labsoft Mikrotek microtiter plate software. A standard curve is fitted by using a four-parameter equation.

b. After the data is calculated and the final values are approved by the reviewing supervisor for release, the results are transcribed by the data entry clerk into the NHANES III COTININE data base, which is located in RBASE on

the NCEH/EHLS PC network; data entry is verified by the supervisor and clerk.

c. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff and CDC Data Center staff, respectively.

d. Documentation for data system maintenance is contained in hard copies of data records, as well as in "system log" files on the local hard drives used for archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Specimen donors require no special dietary instructions.

b. Specimens for serum cotinine analysis should be fresh or frozen. For NHANES samples, blood is collected into standard red-top Vacutainers. Specimens are stored in 2-mL Nalge polypropylene vials, with caps tightly sealed to prevent desiccation of the sample.

c. Required specimen volume is 100 µL.

d. Cotinine is stable for at least several years at -20 °C and below. Quality control (QC) pools for the NHANES Lab are normally stored at -70 °C for maximum stability. Several freeze-thaw cycles appear to have minimal effect on the specimen.

e. Specimen handling and transport is handled by the NHANES program according to the standardized protocols outlined in the laboratory technician's manual. In general, blood should be processed as soon as possible after clotting, and the sample should be maintained in the frozen state during shipment and subsequent storage. The NHANES serum cotinine specimens are maintained at $\leq$-70 °C prior to analysis.

f. At this time, there is no evidence that atypical specimen characteristics such as hemolysis or lipemia influence the EIA analysis of serum cotinine. However, unusual characteristics are recorded on the analysis runsheet, and this information is maintained in the records for tracking purposes.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Microtiter plate reader, model 2001 (Anthos Labtec Instruments, Frederick, MD).

(2) Vortex mixer (Fisher Scientific Co., Fairlawn, NJ).

(3) Thelco Model 4 incubator (Precision Scientific, GCA Corp. Chicago, Il).

(4) Micromedic Digiflex automatic pipettor/dilutor with 2.0-mL dispensing and 200-µL sampling syringes (Micromedic Division, ICN Biomedical Co., Costa Mesa, CA).

(5) Mettler P1200 analytical balance (Mettler Instrument Co, Hightstown, NJ).

(6) Wellwash 4 automatic plate washer (Denley Instruments Inc., Durham, NC).

(7) Labsoft Microplate software with Mikrofit program (Mikrotek Laboratory Systems, Denley Instruments Inc., Durham, NC).

(8) Magnetic 9" x 9" magnetic stirrer (Cole-Parmer Instrument Co., Chicago, Il).

(9) Tecan Model RSP 5031 pipettor (Tecan, Triangle Park, NC).

b. **Materials**

(1) STC cotinine EIA kits, 480-test size (STC Diagnostics, Bethlehem, PA). The kit contains the following reagents, in quantities adequate for 5 plates:

(a) Anticotinine coated microtiter plates, antibody immobilized on polystyrene plates, supplied in dry form.

(b) Enzyme conjugate, horseradish peroxidase labeled with cotinine and diluted in a protein matrix of fetal bovine serum with protein stabilizers.

(c) Substrate reagent, one bottle of 3,3',5,5' tetramethylbenzidine

(d) Stopping reagent, one bottle of 2.0 mol/L sulfuric acid

(2) Bovine serum albumin, catalog # A-7906, lot # 45F-0076 (Sigma Chemical Co., St. Louis, MO).

(3) (-)-Cotinine, lot # 07314-4PW (Aldrich Chemical Company, Milwaukee, WI).

(4) Gilson Pipetman, Models P200 and P1000 (Rainin).

(5) Pipet tips, disposable, RT20 and RT200 (Rainin).

(6) Pipet, Titertek, digital multichannel, variable volume 50-200 µL, cat. #77-705-00 (Flow Laboratories).

(7) Reservoirs, reagent, cat # 224-4872 (BioRad).

(8) Film, pressure-sensitive, Falcon 3073 (Becton-Dickenson, Vallejo, CA).

(9) Tubes, disposable, 12- x 75-mm (Corning Glass Works, Corning, NY).

(10) Reagent bottles, graduated cylinders, Erlenmeyer flasks, and gloves and biohazard bags (various vendors).

(11) Serum quality control pools (made "in-house" by pooling serum with known levels of cotinine to obtain desired reference ranges).

c. **Reagent Preparation**

*NOTE: All necessary reagents are prepared in working form by the manufacturer. No modification is made to the assay protocol for reagents.*

8 g/dL Bovine Serum Albumin

(Used as dilution matrix for standards and elevated-concentration specimens.) Dissolve 80 g of purified bovine serum albumin (BSA) with 500 mL deionized water in a 1-L volumetric flask by gently stirring on a magnetic stirrer. Dilute to 1 L volume with deionized water, and mix again to ensure solution homogeneity. Store at 4-8 °C for 1 month.

d. **Standards Preparation**

*NOTE: Although certain standard solutions are supplied by the kit manufacturer, they are in a concentration range intended for qualitative or semiquantitative confirmation of active, rather than passive, smoke exposure status (cotinine concentration greater than 20-25 ng/mL). In order to use the EIA test as a screening tool for mass spectrometry confirmation of cotinine concentration, we had to prepare additional cotinine standards from National Institutes of Standards and Technology (NIST) primary standard cotinine perchlorate ranging from 0.0- to 50.0 ng/mL.*

(1) 50,000 ng/mL Cotinine Stock Standard
Dissolve 5 mg NIST cotinine perchlorate in 100 mL of 8 g/dL BSA in a 100-mL volumetric flask. Mix well; store at 4-8 °C. The standard will be stable for 6 months.

(2) 100 ng/mL Cotinine Spiking Stock Solution. Dilute 1 mL of 50,000 ng/mL cotinine stock standard to 500 mL with 8 g/dL BSA. Mix well; store at 4-8 °C. This solution will be stable for 6 months.

(3) 0-50 ng/mL Working Cotinine Standards. Using the Micromedic Digiflex dilutor and reagent dispenser, prepare the following working standards as needed by diluting the 100 ng/mL standard with 8 g/dL BSA according to the dilution scheme in Table 1.

Table 1
Dilutions for Working Cotinine Standards

| Working Standard Concentration (ng/mL) | Volume 100 ng/mL Standard (mL) | Volume 8 g/dL BSA Diluent (mL) | Final Volume (mL) |
|---|---|---|---|
| 0.0 | 0.0 | 200.0 | 200 |
| 1.0 | 2.0 | 198.0 | 200 |
| 2.0 | 4.0 | 196.0 | 200 |
| 5.0 | 10.0 | 190.0 | 200 |
| 10.0 | 20.0 | 180.0 | 200 |
| 16.0 | 32.0 | 168.0 | 200 |
| 24.0 | 48.0 | 152.0 | 200 |
| 40.0 | 80.0 | 120.0 | 200 |
| 50.0 | 100.0 | 100.0 | 200 |

e. **Preparation of Quality Control Material**

Quality control materials are prepared from serum processed from whole blood collected with no anticoagulant. The blood is collected from normal humans whose previous smoking/nonsmoking status has been verified. The various pools are well mixed and prescreened for final cotinine concentrations. Aliquots of 0.25 mL are usually dispensed in 2.0-mL high-density polypropylene vials. Pools are stable at -70 °C for more than 3 years; we normally do not prepare more than a 3-year supply at one time because of space limitations.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration**

See Section 6.d. of this document. In an EIA, each plate is considered an independent "run" and contains its own standards. The plate reader is factory calibrated and receives preventive maintenance twice yearly. The tolerance

limits for standards are ±15% of the expected values.

b. **Calibration Verification**

This is a newly developed assay. Existing NIST materials are available only in a urine matrix. However, the stock standard described earlier has been analyzed by GC/MS and has been found to agree within 5% of its expected concentration when compared with a highly-purified cotinine perchlorate standard. Finally, the measured absorbance values for three of the calibration standards (50, 25, and 12.5 µg/L) included in each run as "unknowns" are used to calculate the actual concentrations of these three standards. The run is considered acceptable when the calculated concentrations are within 15% of the expected values.

Additionally, every time new lots of standards are prepared (usually two times per year), multiple aliquots are analyzed as unknowns against the previous lot of standards to verify the accuracy of their preparation.

Rigorous review of quality control data ensures the accuracy and reliability of results on a run-by-run basis.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Procedure**

> *To protect hands against acids and solvents during sampling, wear latex gloves. To avoid evaporation or degradation of specimens, process samples as rapidly as possible.*

(1) Thaw specimens and quality control materials of frozen serum at room temperature. Mix well using a vortex mixer.

(2) Dilute all specimens to be analyzed 1:20 with 8 g/dL BSA.

(3) Add 50 µL of standard, control, or sample to the wells of the microtiter plate as shown in Figure 1. Specimens will be analyzed in singlicate as both undiluted serum and 1:20 diluted serum.

Fig 1
Plate Layout

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | p27 | p22 | p22 | p23 | p23 | p24 | p24 | p25 | p25 | p26 | p26 | p31 |
| B | p27 | s1 | s2 | s3 | s4 | s5 | s6 | s7 | s8 | s9 | q1 | p31 |
| C | p28 | s2 | s2 | s3 | s4 | s5 | s6 | s7 | s8 | s9 | q1 | p32 |
| D | p28 | q2 | q3 | q4 | q5 | p1 | p2 | p3 | p4 | p5 | p6 | p32 |
| E | p29 | q2 | q3 | q4 | q5 | p1 | p2 | p3 | p4 | p5 | p6 | p33 |
| F | p29 | p7 | p8 | p9 | p10 | p11 | p12 | p13 | p14 | p15 | p16 | p33 |
| G | p30 | p7 | p8 | p9 | p10 | p11 | p12 | p13 | p14 | p15 | p16 | p34 |
| H | p30 | p17 | p17 | p18 | p18 | p19 | p19 | p20 | p20 | p21 | p21 | p34 |

s1-s9 = standards 0, 1, 2, 5, 10, 16, 24, 40, and 50 ng/mL, respectively
q1-q5 = quality control pools low1, low2, med, high1, and high2, respectively
p1-p34 = unknown patient samples

(4) Add 100 µL enzyme conjugate to all wells, and start the stopwatch when the conjugate is added to the first well.

(5) Incubate the plate for 30 minutes at room temperature.

(6) Wash the plate six times with deionized water using the microtiter plate washer.

(7) Add 100 µL of substrate to all wells and incubate 30 minutes at room temperature in the dark.

(8) Add 100 µL stopping reagent to all wells.

(9) Measure the absorbance of the well solution at 450 nm within 30 minutes after the reaction stops. Consult the microtiter plate reader manual for operating instructions (8).

b. **Calculations**

Unknown sample concentrations are determined by using the Mikrofit software and a four-parameter (log/linear) logistics fit standard curve.

Data from the absorbance readings on the ANTHOS microtiter plate reader are communicated to the computer, which uses Labsoft Mikrotek microtiter plate software. A standard curve is fitted by using a four-parameter equation.

Refer to the Mikrofit software manual for further information regarding software features and operations (9).

c. **CDC Modifications**

The following modifications of the original STC kit instructions are based on CDC optimization experiments:

(1) The sample size has been increased from 10 µL to 50 µL to enhance sensitivity.

(2) Additional standards are added in order to lower the detection range and permit improved quantitation.

(3) All specimens are assayed undiluted, in duplicate, and those with concentrations >50 ng/mL are diluted 1:20 and reassayed in duplicate in order to increase throughput and decrease specimen volume requirements.

(4) A comparison study was performed by using 2,137 serum specimens from Phase I of the NHANES III survey (1988-1991) with cotinine concentrations >3.0 ng/mL. The study compared results from the EIA screening method and the reference liquid chromatography/mass spectrometry (LC/MS) method. The data were compared on a $\log_{10}$ basis. The regression line was:

$$\log_{10}(\text{EIA}) = 0.9175 * \log_{10}(\text{MS}) + 0.247,\ r^2 = 0.9517$$

These data indicated good agreement overall, with an absolute (intercept) bias of < 2 ng/mL. As expected, the EIA values were slightly higher on overall than the MS results, and the percentage bias decreased as the concentration increased.

(5) The accuracy of the EIA method will be validated by comparing the results of 500 specimens assayed by this technique are to be compared with the results of the same specimens assayed by the reference LC/MS method.

## 9. REPORTABLE RANGE OF RESULTS

The reportable range for the EIA screening method for cotinine is 0-1000 ng/mL (based on sequential dilutions), however, the limit of detection for this assay has been statistically determined to be 3 ng/mL. The linear range of the standard curve is 2-40 ng/mL. If a sample value is still outside of this range with the 1:20 dilution, the samples are further diluted to bring the values into the linear range. Accuracy and reliability have been documented for a dilution factor up to 1:100, calculated on the basis of quality control materials of known concentration (unpublished in-house

data).

## 10. QUALITY CONTROL (QC) PROCEDURES

EIA is a "batch" method (i.e., all specimens, standards, and QC pools are treated to the same processes, such as extraction, simultaneously). During an average day, 340 specimens are analyzed in duplicate, with replicate analyses of five levels of bench QC pools (a "low" pool consisting of serum from nonsmokers, a "high" pool consisting of serum from smokers, and several intermediate pools prepared by blending serum from smokers and nonsmokers to desired cotinine concentration levels). In every rack of 20 specimens, one blind QC specimen will be inserted randomly. Blind QC pools are prepared in the same manner as the unknown specimens, with the same types of labels and vials being used. Two levels, low-normal and high-normal, are prepared in order to verify values reported in the near abnormal concentration ranges for a given analyte.

Quality control limits are established with the mainframe programs "QCLIMIT" and "QC." Preliminary limits are established with 20 consecutive runs and updated periodically thereafter. (Quality control data are maintained and stored electronically; hardcopy records of these data are filed and stored in the supervisor's office for a minimum of 2 years.)

Examples of the precision and accuracy of the method are reflected in the long-term quality control pool results shown in Table 2.

Table 2
NHANES III Serum Cotinine EIA Method QC Pools

| Pool | Mean (ng/mL) | 95% limits | 99% limits | N | SD | CV |
|---|---|---|---|---|---|---|
| Low1 | 2.5 | 0.07-4.96 | -0.71-5.73 | 60 | 1.25 | 52.4 |
| Low2 | 5.8 | 3.18-8.46 | 2.35-9.30 | 60 | 1.35 | 26.2 |
| Med | 27.2 | 18.65-35.66 | 15.96-38.35 | 60 | 4.34 | 17.1 |
| High1 (1:20) | 211.4 | 126.65-296.35 | 99.83-323.07 | 60 | 43.26 | 22.2 |
| High2 (1:20) | 345.1 | 219.50-470.61 | 179.78-510.33 | 60 | 64.06 | 20.9 |

Blind QC materials are examined for similar criteria. The supervisor also evaluates the slope, intercept, and $r^2$ values for trends. For 225 runs conducted January through April 1993, the overall coefficient of variation for this method was 10-30% over the entire analytical range.
The system is declared "out-of-control" if any of the following events occur:

On the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two of the two or more pools fall either both above or both below the lower 95% limit.
- Two successive run means for a single pool fall either both above or both below the lower 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

On the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two of the two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single-pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the system should be declared "out of control," take the following remedial action(s):

a. Check the kit expiration date; only use viable kits.

b. Make sure standards, controls, and any sample dilutions are fresh. Using old preparations can lead to variable results.

c. Verify that the plate washer is working properly. Uneven or incomplete washing will lead to false results. Make sure all areas of the plate receive an equal number of washes. Different numbers of washes will give different OD readings.

d. Check tips on the multichannel pipet. Make sure each tip is dispensing the same volume of reagent.

e. Ensure that all reagents are at room temperature; cold reagents will give false results.

f. The most crucial step in EIA is the color development step. If the color development is inadequate, (if the reaction stopped too soon), the curve will be flat, differences between concentrations will be small, and controls will vary greatly especially at the low end. If the color is too dark (i.e., if the reaction was stopped too late), the curve will be steep and accuracy at the high end may be lost. Improper color development is the most frequent cause of out-of-control results. Color development should be stopped so that curves are shaped so that the zero point (after the addition of acid) gives an absorbance between 1.2 and 1.8 OD value. This assures a standard curve with good differentiation along all points of the curve.

After troubleshooting procedures have been completed and the system has been verified to be "in control," reanalyze all specimens for that analytical run, and report the values obtained during the reanalysis rather than the original values.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

a. The linear range of the standard curve is 2-40 ng/mL.

b. Interfering substance: 3-hydroxycotinine, 10% cross reactivity.

**13. REFERENCE RANGES (NORMAL VALUES)**

Because the NHANES population includes both smokers and nonsmokers, a wide range of serum cotinine values may be expected, from less than 0.1 ng/mL to greater than 1000 ng/mL. The following ranges for cotinine are used in evaluating data: cotinine concentrations less than 5 ng/mL are generally accepted to indicate nonsmokers; concentrations greater than 15 ng/mL generally indicate smokers; concentrations from 5 to 15 ng/mL may indicate recent ETS exposure (10).

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Critical (life-threatening) levels of cotinine have not been established for this screening method. Test results, as determined by this method, are intended as screening tools to detect populations actively exposed to smoke by inhalation, as opposed to those passively exposed (nonsmokers).

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens are allowed to remain at room temperature during sampling.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods of cotinine screening analysis for the NHANES survey. If the analytical system fails, specimens must be stored at $\leq$ -20 °C until the analytical system is restored.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

a. Record QC data in the NHANES III serum cotinine database, and print out a report sheet for the supervisor's review. This reporting sheet contains absorbance and concentration results for quality control pools and standards. Prepare this form in duplicate.

b. Use the "NHANES III Analytical Worksheet" to record the analytical results. These have been prepared with a list of the sample IDs for each preracked run. Record the results for cotinine in ng/mL. If a sample is missing from the

rack, write "NOSAX" or "-" in the blank. If a sample in not satisfactory (i.e., cannot be analyzed), write "UNSAX" in the blank. Prepare these forms in duplicate.

c. Give both types of forms to the supervisor along with the hard copy of the data printout from the computer. After the supervisor checks the data, the copies and data printouts will be returned for filing in a notebook. The supervisor will keep the original copies of the reporting and standards/QC sheets.

d. Enter QC data in the SERUM COTININE database on the EHLS LAN. The NHANES cotinine database will be updated regularly.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

RBASE and the CDC mainframe are used to keep records and track specimens. Records for the NHANES survey (including QA/QC data) are maintained for 10 years beyond the end of the study. Only numerical identifiers (e.g., case ID numbers) should be used. No personal identifiers are provided to EHLS at any time.

After EIA screening analysis, the specimens are reracked for LC/MS confirmatory analysis according to their concentration (i.e., those <25 ng/mL in "low" runs, and those ≥25 ng/mL in "high" runs). Runsheets for the new runs are provided to the LC/MS supervisor, and specimens are stored at -70 °C until analysis. After analysis, they are retained at -70 °C until all survey data for cotinine are released, in case repeat analyses are required.

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Clinical Relevance

Cotinine is a major metabolite of nicotine that may be used as a marker for both active smoking, and as an index to environmental tobacco smoke (ETS) exposure, or "passive smoking." Cotinine is generally preferred over nicotine for such assessments because of its substantially longer half-life. The half-life of cotinine in plasma has been estimated to be about 15-20 hours (1-3); by contrast, the half-life of nicotine is only 0.5-3 hours (3-5). Cotinine may be measured in serum, urine, or saliva -- the half-life of cotinine in all three fluids is essentially the same (1). Cotinine concentrations tend to be higher (3-8x) in urine than in serum; however, for studies requiring a quantitative assessment of exposure, plasma or serum is regarded as the fluid of choice (6). Therefore, serum was chosen for NHANES cotinine analyses.

Assay Principle

Serum cotinine is measured by an isotope dilution-high performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometric (ID HPLC-APCI MS/MS) method. Briefly, the serum sample is spiked with methyl-$D_3$ cotinine as an internal standard, and following an equilibration period, proteins are precipitated with trichloroacetic acid, and cotinine is extracted from the alkalinized supernatant with methylene chloride. The organic extract is concentrated, and the residue is injected onto a short C18 HPLC column. The eluate from these injections is monitored by APCI-MS/MS, and the m/z 80 daughter ion from the m/z 177 pseudo-molecular ion is quantitated, along with additional ions for the internal standard, and for confirmation. Cotinine concentrations are derived from the ratio of native to labeled cotinine in the sample as compared with a standard curve.

Special precautions

Because of the nature of these analyses, all analysts in this study must be nonsmokers.

## 2. SAFETY PRECAUTIONS

*Eye protection and suitable protective clothing must be worn during the extraction and processing of samples by this method.*

a. **Biological Hazards**

This assay involves human serum samples. Observe universal precautions. Wear a lab coat, gloves, and protective eyewear while handling the specimens. Perform all sample aliquoting in a biological safety cabinet. We recommend the hepatitis B vaccination series for all analysts working with intact serum samples.

b. **Chemical Hazards**

Reagents and solvents used in this study include those listed below. Material safety data sheets (MSDSs) for these chemicals are readily accessible through the LAN CD-ROM system of the Division of Environmental Health Laboratory Sciences (EHLS), National Center for Environmental Health (NCEH), Centers for Disease Control and Prevention (CDC); hard copies are filed in the supervisor's office.

(1) Methylene chloride

This solvent is chemically stable and relatively unreactive; it poses a relatively low hazard. It is not flammable, but the vapor can be irritating to the eyes, nose and throat, and skin or eye contact with the liquid should be avoided. Flush copiously with water if any contact should occur. Evaporation of significant volumes of this solvent must be performed in the Savant evaporator or in a chemical fume hood.

(2) Trichloroacetic acid

This is a relatively strong and corrosive organic acid that gradually decomposes at room temperature in aqueous solution to form chloroform and $CO_2$. It is nonflammable, but it may be poisonous if inhaled or swallowed or absorbed through the skin. Prolonged contact may cause severe burns to the skin or eyes. Wear safety glasses while working with this reagent.

(3) Methanol

This is a flammable solvent and may form explosive vapors. The vapor may be irritating to the eyes, nose, and throat; liquid methanol is poisonous and may be absorbed through the skin. Any exposed skin areas should be immediately flushed with water.

(4) Toluene
This is a flammable liquid and also may form explosive vapors. Remember that the vapor is heavier than air and may travel some distance to an ignition source. Toluene forms an irritating vapor. As a liquid, it is a skin irritant and may be absorbed through the skin. Wear gloves and work in a chemical fume hood when working with large volumes of toluene.

*NOTICE: POTENTIAL EXPLOSION HAZARD IN THIS ASSAY*

c. **Special Hazard**
Exercise particular care when 80% methanol is used as the mobile phase during analyses involving the API III heated nebulizer. It is possible for explosive mixtures of methanol vapor and air to accumulate in the ionization chamber, and if arcing should occur, this mixture can ignite. This has already happened on several occasions during these analyses. Although recent inlet source modifications that the manufacturer has installed on this instrument should significantly reduce the possibility of an explosion, observe the following precautions when using the API III nebulizer:

(1) Never change between heated nebulizer and IonSpray state files while the electronics are on and the mobile phase is flowing or within 1-2 minutes after the flow has been stopped (because of the danger of arcing).

(2) Be very careful when opening and closing the orifice shutter. If the plate passes close to the corona discharge needle, arcing may occur. Electronics-1 should be OFF when the shutter is opened or closed.

(3) Make certain that the sample pump is on whenever the system is in operation and solvent is flowing.

(4) Be careful at all times when working in the region between the heated nebulizer inlet and the HPLC. If an explosion should occur, the entire heated nebulizer inlet could be ejected with sufficient force to cause a potentially serious injury if it were to strike a person.

**3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT**

a. This procedure has been designed to incorporate computerized data handling to the maximum extent possible, both to address the relatively high throughput demands of the assay and to eliminate manual data entry errors. At no time is it necessary (or desirable) to manually record a sample ID number or an analytical result. Nevertheless, manual checks and proofreading of information are essential parts of the protocol.

The entire database for the NHANES study consists of several files. The main database is an RBase file that contains participants' demographic information, the enzyme immunoassay (EIA) screening results, the link between the NHANES sample ID (a seven-digit number) and the cotinine LC/MS laboratory working ID (which is a seven character alphanumeric label), and the final, validated cotinine result. The sample cleanup file is a simple custom database that records sample preparation information for each run, such as ID, date of analysis, analyst, sample volume, and special notes. Finally, analytical results are maintained in a SAS data file that contains the merged data from both the sample cleanup and LC/MS results files. The latter two databases are maintained by the Cotinine LC/MS Laboratory in the Clinical Biochemistry Branch.

b. The sample cleanup file is maintained on a laboratory PC and copied via the EHLS Network to a second PC for processing. Thus two copies on separate hard drives are maintained. In addition, a second backup copy is maintained on the same drive on the laboratory PC, and both machines are periodically (generally weekly) backed up to the Network tape archive system. A printout of each run of samples is made and manually checked for validity immediately following entry. A simple record locking procedure is used to restrict any further changes in the Cleanup files after the records have been transferred and merged with the analytical results data.

The SAS data file (NHCOT.DAT) is the main results file maintained by this laboratory. This file, and associated files maintained for standards results and for "extra" (non-NHANES data) results, are automatically copied by a DOS batch file to a separate directory on the same hard drive and to an L:\LINK directory on a Network drive each time the processing program is run (generally daily). In addition, separate copies of each file are maintained on 3.5" floppies that are updated weekly, and the PC itself is backed up to the Network tape drive periodically as described above. Raw instrument data files are archived on both magnetic and optical disks on the Macintosh network as described in Section 8 below. The supervisor should be contacted for emergency assistance with these files or with the Macintosh LocalTalk network; the EHLS LAN manager should be contacted for assistance with any

EHLS network problems.

c. Malfunction reports and documentation for system maintenance are in the "Sample Log" files maintained on the Macintosh and in printed form in the associated notebook; in the processing documentation file (COTCALC.DOC); and in the electronic system log files maintained by the archiving software.

Both commercial and custom programs are used in support of this method. All serum cotinine custom programs are written in either QuickBasic 4.5, Turbo Pascal 6.0 (DOS), or Think Pascal 4.0 (Macintosh). Commented source code for these programs is maintained on the appropriate system (PC or Mac) and in printed form in the "HANES Cotinine Programs" folder. All source files contain revision history headers. SAS processing files are also maintained on the PC.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. There are no special requirements such as fasting or adherence to special diets for participants in this assay.

b. The specimen for these analyses is serum.

c. This assay involves both an EIA prescreening analysis that requires 0.2 mL of serum, and an LC/MS analysis that normally requires 1.0 mL of serum for samples with "low" cotinine concentrations in the passive exposure range and 0.05 mL of serum for samples with "high" cotinine concentrations, generally those from active smokers. Therefore, minimum requirements may vary according to the nature of the individual sample, but in general, 1.5-2 mL of serum is needed for this assay. The optimum sample size is 3 mL or greater, the amount needed to include repeat analysis if indicated.

d. No anticoagulants, special preservatives, or unusual sterility procedures are required for sample processing. For NHANES samples, blood is collected with standard Vacutainer equipment, and the serum is stored in plastic, cryogenic, screw-cap vials.

e. The long-term stability of cotinine in serum is still under investigation as part of the NHANES study. However, literature reports suggest that serum cotinine is stable when the samples are stored frozen at low temperatures (6), and both normal and accelerated stability studies of pure cotinine conducted at CDC indicated that the compound is relatively stable under a variety of conditions.

f. Specimen handling and transport is handled by the NHANES program according to standard protocols. In general, blood is processed as soon as possible after clotting, and the sample is maintained in the frozen state during shipment and subsequent storage. The NHANES serum cotinine samples at CDC are maintained in low-temperature freezers at -70 °C.

g. At this time, there is no evidence that atypical specimen characteristics such as hemolysis or lipemia influence the LC/MS analysis of serum cotinine. However, unusual sample characteristics are recorded in the sample cleanup file, and this information is maintained in the database files for tracking purposes.

h. Although an attempt is made to analyze all samples received whenever possible, a small volume (<0.5 mL) of a low cotinine sample may not generate reliable results. The results from such samples in the NHANES study are maintained in the laboratory database, but they are not validated for release until final evaluations have been completed as described below (Section 10).

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Major Instrumentation and Other Equipment**

(1) Hewlett-Packard model 1090L, Series II, HPLC system equipped with the binary DR5 solvent delivery system, variable volume injector, and an autosampler (Hewlett Packard Company, Palo Alto, CA).

(2) Sciex API III Triple Quadrupole mass spectrometer with heated nebulizer interface (Perkin Elmer, Norwalk, CT).

(3) Three Macintosh computers: IIci, IIfx #1, and IIfx #2, interfaced to the mass spectrometer and interconnected via a LocalTalk network (Apple, Cupertino, CA).

(4) Savant SpeedVac SC200 (CDC # 70583), equipped with a VP190 high vacuum pump, RT4104 refrigerated trap, and a VaporNet VN100 auxiliary trap (Savant Instrument Co., Farmingdale, NY).

(5) Eberbach orbital shaker (Eberbach, Ann Arbor, MI).

(6) VWR Multi-Tube Vortexer; cat # 58816-115 (VWR Scientific, Atlanta, GA).

(7) IEC Centra 7R centrifuge (International Instrument Co., Needham Heights, MA).

(8) Gilson Pipetman P-200 and P-100 pipets, Rainin Instrument Company, Woburn, MA)

(9) Gilson Microman M-25 pipet (Rainin).

b. **Materials**

**Note:** Class A glassware such as pipets and volumetric flasks are used unless otherwise stated.

(1) Trichloroacetic acid, cat. no. #25,139-9, 99+% ACS reagent (Aldrich Chemical Company Milwaukee, WI).

(2) Potassium hydroxide cat. no. P-250, 85-90% reagent, FW = 56.11 (Fisher Scientific, St. Louis, MO).

(3) Ammonium acetate cat. no. 37,233-1 (99.999%), FW = 77.08 (Aldrich).

(4) HPLC grade water (Burdick and Jackson Laboratories, Muskegan, MI).

(5) Methanol, HPLC grade (Burdick and Jackson).

(6) Cotinine, Aldrich 28,471-8 [486-56-6]. Labeled as (-)-cotinine, 98%, FW=176.22, BP=250 deg @ 150 mm, MP=40-42$^{o}$, $\alpha$ D20= -16$^{o}$ (C=1,EtOH) (Aldrich).

(7) Cotinine-$D_3$, DLM-1819, lot #F-0549 (Cambridge Isotopes Laboratories, Cambridge, MA).

(8) Toluene, Burdick & Jackson Microsolve VLSI #MS80863-4,
lot #BB355, $H_2O$=0.011%, residue < 1, GC 99.9% (Burdick & Jackson).

(9) Isopropanol, cat. no. 323-4, Lot no. AU658, $H_2O$=0.024% (Burdick and Jackson).

(10) Methylene chloride, "$GC^2$," capillary GC-GC/MS grade (Burdick and Jackson).

(11) "Micro" cleaning solution (International Products Corp., Trenton, NJ).

c. **Reagents**

Cleanup reagents (#1 and #2) are prepared fresh each Friday for the following week's analyses. The diluent is Organo-Pure water, which is also prepared fresh each Friday according to the written procedure attached to the still. The ammonium acetate stock used to prepare the HPLC mobile phase (#3) is prepared fresh each Monday.

(1) 10% trichloroacetic acid
Weigh out 25.0 g of trichloroacetic acid in a beaker, carefully slurry in about 150 mL of Organo-Pure water, transfer the solution to a 250 mL volumetric flask, and bring it to volume with Organo-Pure water. Transfer the solution to a clean glass bottle and label it with the contents, the preparation date, and the preparer's initials. Store the solution at room temperature.

(2) 5 N potassium hydroxide (KOH)
To prepare 100 mL of reagent, you will use 28.06 g of KOH. Using the percentage purity listed on the bottle, calculate the needed weight by dividing 28.06 by the (decimal) purity. For example, if the reagent is 85% pure, then the needed weight of KOH is 28.06 g ÷ 0.85 = 33.01 g. Weigh out the indicated amount of KOH, dissolve it in 50-60 mL of Organo-Pure water, transfer the solution to a 100-mL volumetric flask, and bring it to volume with Organo-Pure water. Transfer the solution to a polypropylene bottle and label it with the contents, the preparation date, and the preparer's initials. Store the solution at room temperature.

(3) 2 mM ammonium acetate
This reagent is used with methanol to form the HPLC mobile phase. Prepare fresh stock of 500 mL each Monday. Weigh out 77.1 mg of the crystalline material and bring to volume in a 500-mL volumetric flask using Burdick & Jackson HPLC water. Filter the stock through a 0.45-µm filter, and store it in a labeled, glass-stoppered (HPLC) bottle at 4 °C.

(4) HPLC mobile phase
Methanol / 2 mM ammonium acetate, 80:20 by volume. Make up the HPLC mobile phase daily or as needed. Using 100-mL and 500-mL graduated cylinders, place 400 mL of Burdick & Jackson methanol in a clean LC solvent flask and add 100 mL of the 2 mM ammonium acetate solution. Store residual mobile phase in the LC flask with an aluminum foil cover in the refrigerator.

d. **Standards**

One complete set of cotinine calibration standards was prepared for use throughout the entire NHANES study. These standards (Series SN) were prepared in June 1992 as described in detail below. The standards were analyzed over a period of 2 weeks to confirm their suitability and then flame-sealed in washed and silanized ampules on June 26-29, 1992, and stored at -20 °C. The cotinine-$D_3$ internal standard was prepared at the same time and was aliquoted, sealed, and stored in the same manner. A total of 14 standards ranging in concentration from 0 to 50 ng/mL, were prepared. However, at present only the first 12 standards, ranging from 0 to 25 ng/mL, are used.

Original stocks were prepared on June 5, 1992. Stock C0: dissolved 255.1 mg of (98%) cotinine in toluene; q.s. to 100 mL. Final concentration = 2500 µg/mL. Stock D0: dissolved 25.97 mg of cotinine-$D_3$ in toluene; q.s. to 10 mL. Nominal final concentration = 2597 µg/mL.

Working Stocks: Stock A: dilute stock C0 1:100 with toluene, 5 mL of C0 q.s. 500 mL with toluene; 25 µg/mL. Stock B: dilute stock A 1:10 with toluene, 10 mL of A, q.s. to 100 mL with toluene; 2.5 µg/mL. Stock C: dilute stock B 1:10 with toluene, 10 mL of B, q.s. to 100 mL; 0.25 µg/mL. Stock D: dilute stock C 1:10 with toluene, 10 mL of C, q.s. to 100 mL; 0.025 µg/mL. Stock DA: dilute stock D0 1:100 with toluene, 2 mL of D0, q.s. to 200 mL; 25 µg/mL. Stock DB: dilute stock DA 1:10 with toluene, 10 mL of DA, q.s. to 100 mL with toluene; 2.5 µg/mL.

ISTD Spiking Solution: Combine 15 mL of stock solution DA and 30 mL of isopropanol (final concentration = 4%) and bring the volume to 750 mL with toluene. (375,000 ng ÷ 750 mL = 500 ng/mL therefore 10 µL contains 5 ng.)

All standards were sealed in ampules on June 26 and June 29, 1992. The ampules are stored in labeled boxes at -10 °C. Calibration standards were prepared with ca. 2.3 mL in 5 mL ampules. Eighty ampules of standards 1-12 were prepared, 40 ampules each of standards 13 and 14 (35 and 50 ppb) and 248 ampules (3 mL) of the internal standard (ISTD). The residues of the following were sealed and stored at -60 °C:

Stock A 14 x 50 mL ampules (ca. 1/2 full)
Stock DA 5 x 50 mL ampules
Stock DB 2 x 50 mL ampules
Stock C0 3 x 50 mL ampules
Stock D0 2 x 5 mL ampules

e. **Controls**

Four QC serum pools were prepared for the NHANES study: pools #800000, 900000, BQ1, and BQ2. The first two are bench QC pools for runs containing low or high cotinine samples, respectively. The latter two are the corresponding blind QC pools that have been inserted in all of the NHANES runs. Pools BQ1 and BQ2 were prepared by the CDC HANES Laboratory in August 1992 from fresh serum; pools #800000 and 900000 were

prepared in the Cotinine LC/MS Laboratory as described below.

Table 1
Preparation of Working Standards

| Concentration (ng/20µL) | Concentration (ng/mL) | Final Volume (mL) | Cotinine (ng) | Stock | mL | ISTD VolDA | ISTD ng/20 µL |
|---|---|---|---|---|---|---|---|
| 50.000 | 2500 | 100 | 250000 | A | 10 | 1 | 5 |
| 35.000 | 1750 | 100 | 175000 | A | 7 | 1 | 5 |
| 25.000 | 1250 | 200 | 250000 | A | 10 | 2 | 5 |
| 17.500 | 875 | 200 | 175000 | A | 7 | 2 | 5 |
| 10.000 | 500 | 200 | 100000 | A | 4 | 2 | 5 |
| 5.000 | 250 | 200 | 50000 | B | 20 | 2 | 5 |
| 2.500 | 125 | 200 | 25000 | B | 10 | 2 | 5 |
| 1.000 | 50 | 200 | 10000 | B | 4 | 2 | 5 |
| 0.500 | 25 | 200 | 5000 | C | 20 | 2 | 5 |
| 0.250 | 12.5 | 200 | 2500 | C | 10 | 2 | 5 |
| 0.100 | 5 | 200 | 1000 | C | 4 | 2 | 5 |
| 0.050 | 2.5 | 200 | 500 | D | 20 | 2 | 5 |
| 0.025 | 1.25 | 200 | 250 | D | 10 | 2 | 5 |
| 0.000 | 0 | 200 | 0 | — | 0 | 2 | 5 |

Pools 800000 and 900000 were prepared from two pools of serum: "NHANES III Cotinine, 9201-Low, 8/92," and "NHANES III Cotinine, 9203-High, 7/92." The target concentration for pool 800000 was about 2 ng/mL. On the basis of preliminary analyses of the two stocks, 23 mL of the high stock pool was added to 2,775 mL of the low stock pool; the resulting pool was stirred overnight at 4 °C in the cold room. The following day (September 10, 1992), the pool was further mixed at room temperature for about 5 hours, then continuously stirred while being dispensed into 2-mL cryovials labeled "800000". A digiflex automatic pipettor was used for dispensing.

After the Digiflex pipet was thoroughly cleaned, the remainder of the high-stock pool was dispensed in the same manner into cryovials labeled "900000." There were twenty-four 50-sample racks of 800000 prepared (ca. 1,200 vials), and eight 50-vial racks of 900000 (ca. 400 vials). Random samples from both pools were removed for homogeneity testing, and the remaining samples were stored in the -70 °C freezer. Residual bulk volumes of both pools were placed in 500-mL Wheaton serum bottles, labeled, and stored in the same freezer.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

The calibration curve for this assay is based on the analysis of the standards set described above in section 6.d. A set of 12 standards contained in silanized 2-mL glass vials and ranging from 0-25 ng/mL are assayed under standard conditions a total of four times with each day's samples. The standards are analyzed in order from 0-25 ng/mL and then repeated in reverse order (from 25-0 ng/mL). After the day's samples have been analyzed, the standards are again assayed in both forward and reverse direction. Therefore, a total of 48 standards are assayed with each day's samples. These data are used to establish calibration curves as described below in section 8.d.

b. Verification

(1) Initial

The accuracy of cotinine measurements based on standards SN was evaluated by analyzing NIST RM 8444 (cotinine in freeze-dried urine) and aqueous standards prepared gravimetrically from a primary standard of cotinine perchlorate (purity > 99 mol% ) obtained from the National Institute of Standards and Technology (NIST). In these analyses, the reference samples were processed as unknowns through the entire cleanup and analysis procedure.

(2) Daily

The results from the first two sets of standards assayed each day are evaluated immediately, and the results are verified before any samples are assayed. The 24 data files are processed with the Sciex quantitation software (MacQuan and Method-3) as described below in section 8.c.3. The "HANES Check" program is then run on these data. This program 1) reformats and organizes the integrated area counts for all three ions for each standard, 2) calculates the quantitation ion ratios, 3) back-calculates a cotinine concentration for each standard using a 5-point moving regression as described in section 8.d, 4) determines the mean internal standard area counts, and (5) checks the quantitation ratios measured for the "zero" standard. The results of this evaluation, including a system-generated interpretation of the operational status of the assay, are displayed on screen, and sent to the printer for archiving. Acceptable results at this point are:

- Standard calculated value = nominal concentration ± 10% (30% for standards less than 0.1 ng/mL).
- Mean area counts for m/z 180→80 ≥ 182,655
- "Zero" standard ratio < 0.0219

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

The NHANES laboratory prescreens samples using an EIA procedure. The samples are classified as being either above or below a nominal cut-off of 25 ng/mL on the basis of the screening results and then are incorporated into either "low" or "high" runs as indicated by a computerized sorting algorithm. Each run consists of a prepared rack of 50 samples of the same type (either "low" or "high"). At the time the run is prepared, each sample or control is assigned an alphanumeric laboratory ID with a four-character prefix, and a two-digit suffix in the format XXXX-nn, where XXXX is the run designation (e.g., B001), and -nn is the position of the sample in the run (e.g., 25). For NHANES samples, these working ID numbers are linked to the NHANES IDs in the NHANES database in the Nutritional Biochemistry Branch. The first run series in the NHANES cotinine analysis has been designated B001, with the number of each subsequent run increased by one. Each run is set up in the following format: samples 01 and 50 are water blanks, samples 02 and 49 are bench QC samples, and samples 03 through 48 are unknowns (including blind QC samples).

a. Sample Preparation

*Note: Rinse all clean glassware and teflon tubes with methanol and air dry them before use.*

(1) Remove the designated rack of samples from the freezer and allow the samples to thaw at room temperature. The samples may be placed in a few inches of cool water in the sink to facilitate thawing. During the week, the next day's samples are generally placed in the refrigerator the night before and allowed to thaw overnight.

(2) Enter the sample information for the run into the lab computer. Start the cleanup sample log program (type CLEANUP at the DOS prompt) and choose the option "New Samples." There are seven fields of information that must be entered for each run:

Run #. Enter the current designation (e.g. B001).
Samples. Enter the number of samples (normally = 50).
ISTD. Enter the ampule number of the current ISTD.
Date. Defaults to the current date; can be changed if necessary.
Analyst. Enter the initials of analyst(s) (3 characters maximum).
Serum Volume. The default is 1.0; set to 0.05 for HIGH runs.
Diluent. The default is '---'; set to 0.95 for HIGH runs.

After filling in the required fields, press F10 to enter the samples into the cleanup database. After the data

have been entered, prepare and check a printout of the data; then choose 'Edit Data' from the main menu to change any values as necessary.

(3) Label a set of 13- x 100-mm silanized culture tubes using the preprinted labels prepared on the BLP 3152 label printer. Check the current settings of the M-25 Microman, P-200 Pipetman and P-1000 Pipetman pipets and confirm that they are set to the proper volumes (normally 10 µL, 50 µL, and 1 mL, respectively). Remove the current ISTD stock solution from the refrigerator, warm it to room temperature, mix well, and add 10 µL of the solution directly to the bottom of each tube using the Gilson Microman pipet reserved for this purpose.

(4) Check the ID number on the sample vial against the number listed on the run sheet and confirm that they match. Note any unusual characteristics of the sample (e.g., lipemic, hemolyzed) and record them in the Notes field of the cleanup file. Mix the samples well by vortexing. For LOW runs, place 1.00 mL of each sample in a sample tube using the P-1000 pipet. For HIGH runs, place 0.05 mL of each sample in a sample tube using the P-200 pipet. Use a fresh tip for each sample. If there is insufficient sample volume in a low run, remove a smaller aliquot (e.g., 0.5 mL) as necessary and record the actual volume used in the sample cleanup file. Cap the tube with a 13-mm polyethylene snap cap (Precision Labs). Repeat for each sample in the run.

(5) If less than 1 mL of sample was taken for analysis, dilute to volume with Organo-Pure water. For example, in HIGH runs, add 0.95 mL of water to each tube (except for samples 01 and 50, the water blanks).

(6) Place the entire rack of samples on the Eberbach orbital shaker and turn on (low speed). Allow the samples to equilibrate with the internal standard for at least 20 minutes but no longer than 45 minutes.

(7) Add 1 mL of 10% trichloroacetic acid (TCA) to each sample using an automated pipetor, recap the vial and return it to the rack. After adding TCA to all of the samples, place the rack in the VWR multi-tube vortexer and vortex at medium speed for 30 seconds. Centrifuge at full speed (3000 rpm = ca. 1900 x g) for 20 minutes in an IEC Centra 7R centrifuge.

(8) Label a set of 16- x 80-mm teflon tubes. Decant the TCA supernatant into the labeled tube. Then, using an Eppendorf "Repeater" pipet, add 0.5 mL of 5 N KOH to each sample and mix.

(9) Add 6 mL of methylene chloride using a Labindustries Repipet. Cap the tubes, place a hard spacer between the tops of the tubes in the rack and the upper foam pad of the shaker, and agitate on the VWR multi-tube vortexer for 30 minutes. Note: gradually increase the motor speed until all of the tubes are mixing well.

(10) Remove the tubes from the vortexer and centrifuge at full speed for 10 minutes in the IEC Centra centrifuge. Remove the upper aqueous layer almost to the interface by using a water aspirator. Be careful not to contact the lower phase with the aspirator tip; if any contact occurs, replace the tip with a fresh pasteur pipet.

(11) Place a set of sodium sulfate columns (1.1 - 1.2 g EM SC-0760-E $Na_2SO_4$ in 10 mL polypropylene columns; packed by Analytichem) in the column rack, and prerinse with about 4 mL of methylene chloride. Place the methylene chloride extract (from step 9) above on the washed column and collect the eluant in a clean, labeled, 13- x 100-mm silanized glass tube. Do not attempt to force out the residual liquid from the column. If any aqueous material is eluted from the column it will generally be visible as white droplets in the methylene chloride. In that case, carefully transfer the methylene chloride layer (avoiding the droplets) to a clean, labeled 13- x 100-mm tube before drying the sample. Reseal any unused columns in their original foil container.

(12) Place the tubes in the Savant and take to dryness as follows:

*Note: Check the oil in the sight glass and add more if necessary.*

(a) Turn on the oil circulator and then the vacuum pump.

(b) The arrow on the bleeder valve for the concentrator will be facing the concentrator chamber. Wait for the vacuum to pull down below 1 torr. Turn on the Vapornet VN100.

(c) Make sure the samples all have the same volume of methylene chloride (for balance). If necessary, add

sufficient "$GC^2$" methylene chloride to ensure balance. Also be sure to place tubes in the Savant rotor at intervals such that rotor balance is maintained.

(d) Close the lid and turn on the rotor switch. Allow sufficient time for the rotor to come up to its maximum speed (ca. 45 sec).

(e) Slowly rotate the bleeder valve so that the arrow is facing to the right and perpendicular to the vacuum line.

(f) When the samples are dry (normally this will require about 60 min), turn the bleeder valve so that the arrow is facing the concentrator. (At this point the rotor will still be spinning, and a hissing sound will be heard as air is introduced into the chamber.)

(g) Turn off the rotor. When the rotor stops, check the samples for dryness. If samples have not dried, resume with step (d).

(h) If the samples are dry, turn off the pump and then turn off the oil circulator and the Vapornet. Rotate the Vapornet valve to bleed the vacuum.

(13) Add 200 µL of methylene chloride to each sample using a Hamilton multipipet. Swirl gently to ensure that the bottom region of the tube is well rinsed.

(14) Carefully decant the contents of the tube into an autosampler vial. Allow the solvent to evaporate by placing the uncapped vials in a bench-top hood at room temperature overnight.

Note-1: Make certain that any anomalies in the cleanup or in the appearance or behavior of the samples are recorded in the samples "Note" field of the cleanup database.

Note-2: All of the sample-specific supplies used in this assay are disposable except for the teflon tubes and caps. Those are soaked after use in cleaning solution (3% Micro) for at least 2 hours but usually overnight, cleaned, and rinsed with tap water by hand. The tubes (and caps) are then sent to glassware for final cleaning. As a precaution, they are shipped in a box with an enclosed label indicating that the tubes are dirty. The returned, clean tubes are prerinsed with methanol as described above before being used. Tubes used for 'LOW' and 'HIGH' runs are kept separate at all times.

Note-3: The sodium sulfate columns are not reused in this assay. However, the used columns are emptied, washed, and stored for possible later use in other analyses.

b. **LC/MS/MS Analysis**

(1) Remove the ammonium acetate buffer solution from the refrigerator and allow it to warm to room temperature. On Mondays, make up 500 mL of fresh stock as described above in section 6.c.3. Also on Mondays, (to prevent interference from the growth of organisms in the system) clean the HPLC reservoir that holds the buffer solution and pour out fresh HPLC-grade water to rinse the column. Perform additional routine maintenance on Mondays as described in the next paragraph.

Remove the heated nebulizer interface, clean the interface and behind the gate valve with a KimWipe soaked with methanol, clean the corona discharge needle with emory paper and rinse with methanol, swab the heated nebulizer quartz tube with methanol and toluene, and clean the orifice. Perform an injector wash on the HPLC with pure methanol.

(2) Record the pressures of all gas cylinders, and record the temperature and pressure of the He pump and the MS ("$N_2$ Off" condition). Note: The temperatures and pressures of the MS are recorded in the 'Vacuum Log' Excel file; all other values are recorded in the 'Sample Log' MacWrite file; both of these files are maintained on the IIfx (#1).

(3) Turn on ELECTRONICS 1, and then ELECTRONICS 2. Start the IIci and open the TUNE or RAD software as appropriate (TUNE on Mondays, RAD on other days). Set M1 and M3 in the State file to 200 and 100, respectively. Note that in order to activate a state file in RAD, a batch has to be loaded and started. Turn off Ar in the state file. Allow to warm up for about 1 hour.

(4) The cotinine analysis state file is termed COT-TEST. (Note that some tune parameters (e.g., RE1, DM1, MU etc) will change with time.

(5) After at least 10 minutes of warm up, record the pressure of the mass spectrometer. (This value has been reading less than the initial pressure.)

(6) Turn off ELECTRONICS 1, then turn on the curtain gas and simultaneously open the gate valve. Turn ELECTRONICS 1 back on and reset M1 and M3 if necessary. (Note: ELECTRONICS 1 is turned off while the gate valve is opened to avoid possible arcing between the corona discharge needle and the gate.) After an additional 10 minutes, record the temperature and pressure of MS ("$N_2$ On").

(7) Turn on the HPLC, load METHOD #1, and turn on the $N_2$ and He gas valves. (Open the second-stage valves only on the gas cylinders and open the He valve at the back of the LC.) Put the premixed mobile phase (section 6.a.4) in reservoir A. Allow the mobile phase to degas for at least 10 minutes.

(8) Switch on the HN TEMP. (It should already be set to about 500°.) Turn on the auxiliary and nebulizer gas flows. (One valve for both flows and it should be set already).

(9) Confirm that the HN TEMP is 500°, turn on the sample pump, turn on the HPLC pump, and pump the mobile phase (100% A) at 1 mL/min for about 10 minutes. Note: If the solvent flow is stopped for more than a minute or two, reduce the HN TEMP until the flow is restarted.

(10) On Mondays, do Q1 & Q3 scans of the mobile phase only from 10 to 220 amu in 0.1 amu steps with a dwell time of 2 msec, collecting 10 scans in MCA mode. Do this twice, and make a hard copy of the second set of scans and retain the results in the "Q1 & Q3 Scans" folder. Check the tune and mass assignments against the anilide standards (6.8 ng/mL acetanilide and 7.4 ng/mL benzanilide) and a (5 ng/mL) cotinine standard using flow-injection analysis, and perform other maintenance analyses as indicated. Then quit TUNE and open RAD.

(11) In RAD, load batch file "SN00X Standards" (located in the RAD Files folder). Change the file name and the sample name to the current run number, close the batch window and activate the START button on the control panel. The Ar will switch on. After about 10 minutes, record the MS temp and pressure ("CG On"). Also record the initial collision gas thickness (CGT) value.

(12) Set up the run on the HPLC: press the START button, assign the proper start and stop vial numbers (remember vial numbers begin with 0, not 1), and then press ENTER. Record the HPLC HP and LP (high pressure / low pressure) values at start up.

(13) Prepare the current set of 12 calibration curve standards, and analyze the standards twice -- first in the forward direction (standards 1-12), and then in the reverse direction (standards 12-1).

(14) On Mondays, the standards analysis may be repeated twice more.

(15) On other days, process the first two standards runs using MacQuan on the IIfx (see below), and screen the results using the 'HANES Check' program on the IIfx. If the results are OK, reconstitute the current set of samples by adding 20 µL of toluene to each vial. Cap the vials, vortex them and place them in the autosampler rack. Prepare and load a new batch file for the current set of samples (normally, a run will consist of 50 samples) and analyze. To avoid loss of data, monitor the samples during the automated analysis, and abort the run immediately if a problem should arise. After the samples have been analyzed, analyze the standards twice more in the same pattern (i.e. from 1-12, and then from 12-1). Record any anomalies in individual sample behavior or handling in a note in the "sample log" file on the IIfx and in the cleanup database file.

Sciex Shutdown

(16) Record the MS temperature and pressure ("End of day CG On"). Turn off ELECTRONICS 2 and then ELECTRONICS 1.

(17) Switch the mobile phase to 100% MeOH. Pump at 1 mL/min for about 10 min (using pump B only). Meanwhile, remove the buffer reservoir (A) from the LC, cover it and store it in the refrigerator. Replace

reservoir A with HPLC water and degas, then pump 100% water at 1 mL/min for about 10 min (using pump A only). Switch to pump B and replace reservoir A with methanol, degas, and pump 100% MeOH using pump A for about 5 minutes prior to storage. (Note: Because pump A is stored with the strong HPLC solvent, be certain to flush thoroughly with buffer the next day before beginning the analyses in order to avoid RT drift). Close the gate valve.

(18) Turn off HN TEMP. When the temperature is < 100 degrees, set the LC pump flow rate to 0 and turn off the sample pump. When the HN TEMP has dropped to about 60 °C, turn off the auxiliary and nebulizer gases.

(19) Turn off the HPLC metering pump. Turn off the LC, close the $N_2$ and He gas cylinder valves (second stage only), and close the He valve behind the HPLC.

(20) On Monday-Thursday, set the recycle switch to 10 hours, on Fridays set it to 60 hours. Then press the RECYCLE button.

c. **Data Processing**

(1) There are 3 generic names for data files: A00X, SN00X and NON00X. The A00X files contain the actual NHANES sample run data. SN00X files are standard curve runs of 12 data files each. NON00X files are all other runs that don't fall into either of the previous categories. These include runs that test one aspect of the workup, runs of standards that are not going to be used in standard curve calculations (e.g., those with more than 12 data points, or standards used to test the MS and/or LC systems), and blank injections.

(2) After each batch of samples has been analyzed, copy the data files from the IIci to the hard drive on the IIfx (either #1 or #2, whichever computer will be used for data processing). Leave a copy of the data files on the IIci in the RAD data files folder.

(3) Process samples using MacQuan and Method 3. The method parameters are shown in Table 2.

Table 2
Method Parameters

| Parameter | Setting |
|---|---|
| Noise threshold | 6.0 |
| Quant threshold | 3.0 |
| Minimum width | 2 |
| Multiplet width | 0 |
| Baseline width | 200 |
| R.T. window | 8.0 |

(4) Process all data files using Method 3 parameters, then visually check and manually redraw all baselines on peaks with area counts less than 10,000. If the data are very noisy, visually check all of the integrations and redraw the baselines as necessary.

(5) Prepare an AQ file in TeachText and, using the clipboard, transfer the information (ion, sample ID, time, RT, area, etc) on each ion to the file.

(6) For A00X files only, process the samples on the IIfx-#2, and then start the print macro to print out a hardcopy of each integrated peak from each sample. On alternate Mondays only, initiate the standards print macro to obtain a hardcopy of a complete set of the day's standards.

(7) At the end of each day, store all of the data files in the HANES Data Folder on the Mac 205 external drive (connected to the IIci), and in the HANES Data Folder on the IIfx-#1. Store an additional copy of the A00X.AQ data files (the actual NHANES samples) in the Completed AQ Files folder on the IIfx-#2. Then delete the day's files from the Iici.

(8) Once a month, copy all data files from the 205 external drive to two separate optical disks. The files may then be deleted from the IIfx and 205 hard drives. Following the collection of the data, there should be at least two copies of each NHANES data file maintained on separate drives (or disks) at all times. In addition, all files (program, system files, etc) are backed up from all three computers to an optical disk on an annual basis.

(9) Back up the current cleanup database (CLEANUP.RND) on the laboratory PC, and transfer a copy through the network to the processing computer. Using the Apple File Exchange program (with the 'Cotinine' parameters loaded), transfer a copy of all of the *.AQ files from IIfx-#1 to a (PC) formatted 1.44 Mb floppy disk into the 'Current' sub-directory. At this time, also print a hardcopy of the Sciex Sample Log.

(10) With the processing computer logged onto the local printer, place the 1.44 Mb transfer disk in drive 'B' and type "AQ". This will initiate a batch file that backs up and archives the previous set of data, and then runs the program "AQCOT.EXE."

(11) Using the appropriate options in AQCOT, transfer the selected files from drive "B" to the hard drive and process them. As part of this processing, the integrity of the data files will be checked, the MS data files will be merged on a sample-by-sample basis with the information in the sample cleanup files, a formatted print out of the results for each sample will be generated, and the resulting data will be appended to the SAS data file for subsequent analysis. Similar processing is performed for standards and "NON" files but without cleanup information merging, and with the results stored in separate SAS data files.

d. **Calculations**

The calibration of this analysis is not completely linear over the more than 3 orders of magnitude covered by the standard curve. Therefore, the results are calculated by comparing the quantitation ratio (m/z 177→80 / m/z 180→80) of the sample with the standard curve by using a 5-point moving regression. The same basic algorithm for this calculation has been incorporated into "HANES Check," "AQCOT," and the SAS program used for final data processing (NHCOT.PGM). In each case, the sample ratio is compared with the individual standard ratios, and that standard concentration whose mean ratio has the minimum difference from the sample ratio is taken as the midpoint of a 5-point curve. The regression parameters derived from that subregion of the standard curve are then used to calculate the result.

## 9. REPORTABLE RANGE OF RESULTS

Because this analysis may be applied to both smokers and nonsmokers, a rather large range of serum cotinine values may be expected, from less than 0.1 ng/mL to greater than 1000 ng/mL. These assays are performed on prescreened samples that have been divided into two groups on the basis of the preliminary EIA results, with a nominal cutoff of 25 ng/mL. Any sample from a "LOW" run with a nominal cotinine concentration greater than 25 ng/mL -- or from a "HIGH" run with a calculated concentration greater than 500 ng/mL -- must be diluted and reanalyzed. In addition, any sample classified as "High" with an LC/MS value less than 25 ng/mL should be reanalyzed without dilution in a subsequent run.

## 10. QUALITY CONTROL (QC) PROCEDURES

Four human serum pools are used in this study. Their preparation was described previously in section 6.e. In addition, two water blanks are included in each analytical run of 50 samples. All cotinine data are reviewed immediately when processed by using the AQCOT program. Obvious problems or invalid QC results are used to identify potential repeat runs at that point. In addition, before a set of data is released, all samples are subjected to a final evaluation according to the following criteria:

a. All QC results (blanks, bench, and blind QC) are confirmed once more for the mean and range values using SASQC. For the run to be accepted, it must meet the following criteria based on the characterization (A-series) data, which may be updated periodically as indicated:

- Blanks. Reject if any blank in the run is >3 SD from the mean or if both are >2 SD above the mean.
- Bench & Blind QC. Reject if any pool is outside of 3-SD limits (mean or range), or if any two pools are outside of 2-SD limits in the same direction.

b. The relative retention time (RRT), defined as the retention time for the quantitation ion divided by the retention time for the ISTD, must be 1.000 $\pm$ 0.050. The results from any sample with an RRT outside of these limits are regarded as unconfirmed, and the sample must be reanalyzed.

c. Confirmation ratios are the ratios of the confirmation ion (m/z 177→98) divided by the quantitation ion (m/z 177→80). Because these values are not fundamental and may be influenced by several factors such as small variations in Q2 fragmentation conditions, they can vary somewhat over time; therefore, the individual values are compared with the mean obtained for samples in that run. Because of low ion counts for the confirmation ion, these evaluations are limited to samples with a calculated cotinine concentration of $\geq$0.5 ng/mL. Samples are selected for further evaluation if they have a cotinine concentration of 0.5 ng/mL but not more than 10 ng/mL and a confirmation ratio greater than 3-SD from the mean, or if they have a cotinine concentration >10 ng/mL and a confirmation ratio outside of 2-SD limits.

d. Concentrations are checked to make certain that the values are within the range of the method; in general, that means the actual measured value (prior to correction for dilution) must be no greater than 25 ng/mL. Samples with (uncorrected) cotinine values greater than 25 ng/mL are selected for repeat analysis at a greater dilution.

e. The mean recovery estimated for serum cotinine samples in this method is about 60%. The recovery of each sample is estimated from the raw ion counts observed for the ISTD relative to the mean observed for all of the standards (generally n=48) assayed that day. Any sample with an estimated recovery of less than 20% is reanalyzed if sufficient residual sample is available. However, low recovery alone is not grounds for rejection of a sample.

f. All LC/MS data are plotted vs. the EIA screening results in both linear and logarithmic form, and any major deviations between results for samples with a nominal cotinine concentration greater than about 2-3 ng/mL (LOD for the EIA) are noted. Samples producing the main outliers are reanalyzed if possible (note: outliers are estimated visually from the plots, and by comparison with the individual 99% confidence limits.)

g. All nonempty note fields in the raw data file are printed out and examined for limiting and excluding factors affecting individual samples. Also, the hardcopies are examined once more for abnormal ion chromatograms and for other indications of possible problems.

On the basis of the above criteria, a set of samples is generated. For each set, the (lab) ID numbers and raw cotinine concentrations (in ng/mL) of all samples are included. This list is edited as necessary to replace either individual values or values for entire runs with repeat analysis values where indicated. A log is generated that lists every sample ID (B-Series) and the actual sample (either B- or X-series) that was used for the report. A reason is entered into the log for every X-series (repeat) result that is included.

These values are then adjusted in a two-step process. First, the data are sorted by concentration and a "check value" is generated for each sample by multiplying the cotinine concentration times the sample volume in mL taken for the assay. Then the samples are corrected by subtracting the mean blank value from each result (both corrected and check value). Samples with a check value less than the LOD (defined as 3 * SD of the blanks) that were based on a volume of 1 mL are changed to -999 (below LOD), while values with a check value less than the LOD that were based on sample volumes less than 1 mL are changed to -997 (QNS). In addition, samples with unconfirmed cotinine values are marked as -998 at this time. The latter group of samples would include, for example, samples with deviant confirmation ratios that could not be confirmed on repeat analysis.

For NHANES samples, the results are then linked to the NHANES database, and the NHANES and lab ID numbers are matched by computer. A printout of the merged data is obtained and the data are spot checked throughout the file to confirm that the correct lab and NHANES ID numbers were matched. A final results file is then generated with two fields: the NHANES ID number and the cotinine concentration in ng/mL. This final file is again spot checked for accuracy.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. **Calibration**

System calibration and general readiness is assessed on a daily basis from a review of the instruments' operating conditions (temperature, pressure, etc) and from the results of the "HANES Check" evaluation of the first two sets of standards. In the latter case, the program notes potential problems (such as low sensitivity as indicated by the mean ISTD ion counts, or high background levels of cotinine in the blank) and suggests possible corrective actions. Following corrective actions, the system is re-evaluated with standards as before, and the "HANES Check" is run again until acceptable results are obtained.

b. **Quality Control**

If two or more quality control sample values from either the bench or blind QC samples are outside of 95% limits in the same direction, or if any values are outside of 99% limits, then the following steps are taken:

(1) The run is flagged in the processing file.

(2) An attempt is made to identify reasons for the apparent problem and these are recorded. If indicated, further NHANES sample processing is halted until the problem can be corrected.

(3) The run is repeated as an "X-series" run at some later date. Note that this is NOT always possible with low sample runs because of limited residual volumes. Repeats of high runs are not a problem in this regard.

Any questionable sample identified by either QC or individual sample evaluation that cannot be confirmed by repeat analysis is flagged in the database by changing the cotinine value to -998.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Other nicotine metabolites such as trans-hydroxycotinine, and physiological substances such as caffeine have been reported to interfere with immunoassay and/or chromatographic assays of cotinine in some studies. However, no known interferents have yet been reported when mass spectrometric methods are used. Two physiological substances with MW = 176 that might be encountered in blood are ascorbic acid and serotonin. No interference in the analysis of cotinine was noted when standards of these substances were analyzed according to our usual procedures. In addition, the presence of an interfering substance in a particular sample would be indicated by a deviation in the expected confirmation ratio of m/z 177→98 / mz 177→80 for that sample.

## 13. REFERENCE RANGES (NORMAL VALUES)

Because the NHANES population includes both smokers and nonsmokers, a rather large range of serum cotinine values may be expected. Several investigators have estimated urinary cotinine cut-off values likely to distinguish between smokers and nonsmokers, but relatively few comparisons of serum or plasma levels have been reported. Jarvis et al. (7) estimated a cut-off value of 13.7 ng/mL for plasma cotinine levels as measured by gas chromatography, a value approximately 3.6-fold lower than the urine estimate in the same population. Benkirane et al. (8) estimated cut-offs of 17-50 ng/mL by an ELISA method, and 28-70 ng/mL by RIA, whereas Van Vunakis (9) reported a serum cotinine cut-off of 8-20 ng/mL by RIA. In general, serum cotinine concentrations greater than 10-20 ng/mL are probably indicative of a currently active smoker.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable for this procedure.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples are stored frozen at -70 °C until they are analyzed. The rack of samples is removed from the freezer and allowed to thaw overnight in a refrigerator. The samples are brought to room temperature on the morning of the analysis, and the vials are vortexed briefly immediately prior to sampling. All handling of intact serum samples is carried out in a biological safety cabinet. The residual samples are replaced in the racks and refrozen.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Generally, if a problem with the method exists, samples are held in the freezer until the problem can be resolved. If necessary, extracted samples ready for analysis can be stored at -70 °C for at least 1 week before they are assayed. In principle, these samples could also be analyzed by capillary gas chromatography/mass spectrometry; however, samples with relatively low cotinine levels (ca. ≤ 2-5 ng/mL) require the use of high-resolution mass spectrometers to attain sufficient sensitivity for the assay, and the daily throughput of GC/MS assays would be significantly lower than that achieved with this method.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Analytical results are reported as ng cotinine/mL serum for each sample. Data are cross-checked for accuracy by the assigned analyst and the supervisor. The supervisor releases final, validated results for the NHANES samples, which are transferred electronically to the NHANES RBase file for release to NCHS. Critical call reporting is not applicable for this method.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Samples that have been prescreened and classified by EIA are periodically transferred from the HANES laboratory to the Analytical Chemistry Laboratory, Clinical Biochemistry Branch (CBB), accompanied by a transfer sheet that is maintained on file.

Following analysis, residual sample from these assays is maintained in the same freezer (CDC # 70666) that is used to hold samples waiting to be processed. Periodically, racks of completed samples are transferred to dense-pack boxes, which are labeled with the contents and transferred for archive storage in a -70 °C freezer. A sample tracking system documents the status and location of completed samples in a series of "HANESx" Lotus-123 files, which are maintained on the laboratory PC. These files contain the laboratory ID, NHANES ID #, current freezer location, estimated residual volume, and date of archiving (if applicable) of each sample that has been transferred to the Analytical Chemistry Laboratory for analysis.

## 19. QUALITY CONTROL SUMMARY CHARTS AND GRAPHS

Because the screening data won't be released to the public the EIA qc information is not included in this section.

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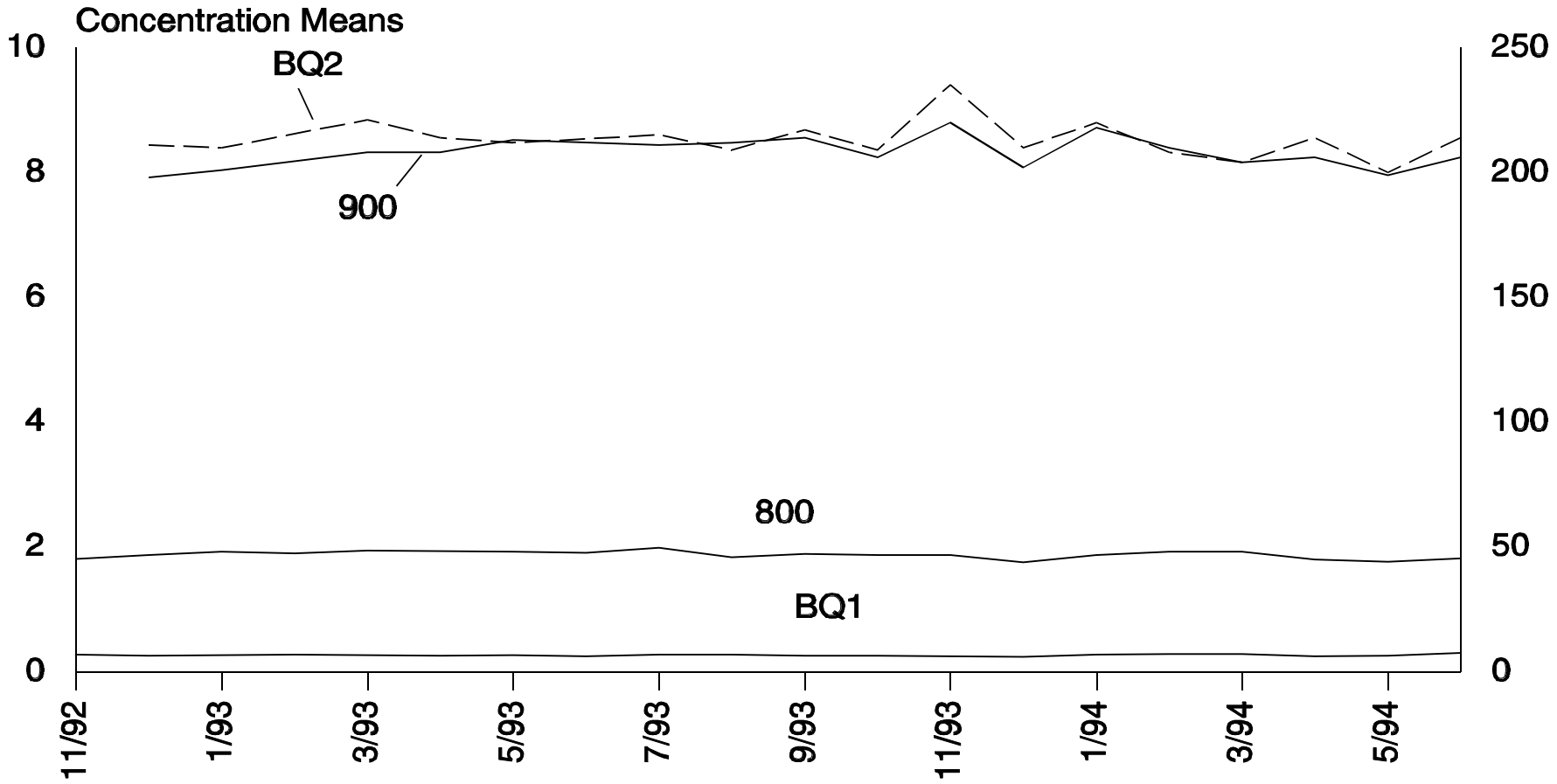
SUMMARY STATISTICS FOR
COTININE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 800 | 11/92 - 06/94 | 1.881 | 0.06287 | 3.34315 | 20 |
| 900 | 12/92 - 06/94 | 208.000 | 6.26498 | 3.01201 | 17 |
| BQ1 | 11/92 - 06/94 | 0.270 | 0.01539 | 5.70340 | 20 |
| BQ2 | 12/92 - 06/94 | 213.118 | 7.70456 | 3.61517 | 17 |

# Cotinine Monthly Means




Pools BQ1 and 800 use the Y-axis values on the left and
Pools BQ2 and 900 use the Y-axis values on the right. This
representation was necessary due to large variation in magnitude between low and high pools.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The enzyme hexokinase (HK) catalyzes the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP). In the presence of nicotinamide adenine dinucleotide (NAD), G-6-P is oxidized by the enzyme glucose-6-phosphate dehydrogenase (G-6-PD) to 6-phosphogluconate and reduced nicotinamide adenine dinucleotide (NADH). The increase in NADH concentration is directly proportional to the glucose concentration and can be measured spectrophotometrically at 340 nm (1-3).

Glucose is the major carbohydrate present in the peripheral blood. Glucose derived from dietary sources is either oxidized to provide energy or converted to glycogen or fatty acids for storage in the liver and tissues. The most frequent cause of hyperglycemia is diabetes mellitus. Some other factors that contribute to elevated blood glucose are pancreatitis, pituitary or thyroid dysfunction, renal failure, and liver disease. Hypoglycemia is less frequently observed, but is found in conditions such as insulinoma, hypopituitarism, neoplasms, or insulin-induced hypoglycemia (4).

## 2. SAFETY PRECAUTIONS

Wear gloves, lab coat, and safety glasses for handling all human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container. Discard all disposable glassware into sharps waste containers. These containers are collected and disposed of twice weekly by University of Missouri waste management personnel.

Protect all work surfaces with absorbent benchtop paper. Discard benchtop paper into the biohazard waste container weekly or whenever blood contamination occurs. Disinfect all work surfaces with a 10% sodium hypochlorite (bleach) solution weekly.

*Dispose of all biological samples and diluted specimens in a biohazard waste container at the end of the analysis.*

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (JOUTPUT.TXT) on a 5¼" floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. A backup copy is made of the JOUTPUT.TXT file onto a 3½" floppy diskette.

c. Each specimen vial is given a unique sequential University of Missouri, Columbia (UMC) lab accession number, which is also recorded next to the NHANES ID number on the transmittal sheet.

d. All data and quality control (QC) files are stored on the University of Missouri mainframe computer system which uses the CMS operating system. Backup copies of all files are also kept on high-density 5¼" diskettes.

e. Records of specimen tracking and system maintenance are kept on flow sheets in the "NHANES FLOW LOG" notebook.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Random, fasting or 2-hour post-oral glucose tolerance bloods are collected for glucose analysis following NHANES sample collection criteria. Each type of specimen is identified by a unique vial identification number (i.e. A = fasting, B = 2 hour, * = random, blank = fasting only).

b. Specimen type: 1.5 mL plasma with NaF as preservative.

c. Optimal amount of specimen required is 1.5 mL; minimum is 0.2 mL.

d. 3- to 5-mL of whole blood is collected in a vacuum tube containing the glycolytic inhibitors potassium oxalate and

sodium fluoride (e.g., gray-top Vacutainers). Specimens are centrifuged immediately at 1500 g for 10 min. Plasma is transferred to a 2-mL polypropylene screw-cap vial and frozen at ≤-70 °C. Frozen plasma specimens are shipped weekly in batches in styrofoam-insulated shipping containers with dry ice to the University of Missouri Diabetes Diagnostic Laboratory via over-night courier.

e. Upon receipt, all specimens are stored in a ≤-70 °C freezer until analysis. Specimen stability has been demonstrated for 1 year at ≤-70 °C. Multiple freeze-thaw cycles should be avoided.

f. The criteria for unacceptable specimens are either a low volume (<0.2 mL) or gross hemolysis. Specimens collected without NaF, or those that are thawed upon arrival, are also unacceptable.

g. Specimens that do not meet the acceptable criteria are not analyzed. The reasons are noted in the transmittal sheet.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Cobas Mira Chemistry System (Roche Diagnostic Systems, Inc., Montclair, New Jersey).

(2) Sorvall Model GLC-2B general purpose centrifuge (DuPont Instrument, Newton, CT).

(3) Milli-Q Plus ultra-pure water system (Millipore, Bedford, MA).

(4) Gilson Pipetman adjustable pipet, 200- to 1000-µL (Rainin Instrument Co., Woburn, MA).

(5) Thermolyne Varimix mixer (Thermolyne Inc., Dubuque, IA).

b. **Other Materials**

(1) Glucose reagent, cat. no. 44558 (Roche).

(2) Glucose standard (Sigma Chemical, St. Louis, MO).

(3) Pipet tips, 200- to 1000-µL sizes (Fisher Scientific, St. Louis, MO).

(4) Pipet-Aid (Drummond Scientific Co., Broomail, PA).

(5) Pyrex 20-mL disposable pipet (Fisher Scientific).

(6) 10-mL class "A" volumetric pipet (Fisher Scientific).

(7) The following items are all supplied by Roche Diagnostic Systems,Inc. (Branchburg, NJ): sample cups, thermal paper, reagent probe, 1000-µL reagent syringe, replacement plunger tip for both reagent and sample syringes, sample needles, reagent containers, cuvette segments, sample racks, calibration rack, reagent rack, and thermal printer paper.

(8) Disposable gloves (Fisher Scientific).

(9) Biohazardous waste storage bags and boxes (Fisher Scientific).

(10) Absorbent benchtop paper (any vendor).

(11) Bleach (10% sodium hypochlorite solution) (any vendor).

(12) 12- x 55-mm polypropylene storage tubes (Clinical Plastics, Leominster, MA).

(13) Colored caps for 12- x 55-mm polypropylene storage tubes (Bio-Rad Laboratories, Richmond, CA).

(14) Lyophilized serum controls BC1 and BC2 (Baxter Scientific Products, St. Louis, MO).

(15) NIST SRM909 lyophilized serum reference material (National Institute of Standards and Technology, Gaithersburg, MD).

c. **Reagent Preparation**

Glucose Reagent
Using a volumetric pipet, reconstitute a vial of glucose reagent with 20.0 mL distilled water. Swirl or invert the vial gently until the reagent is completely dissolved. If more than one vial of reagent is needed, solutions from different vials with identical lot numbers may be pooled and mixed prior to analysis.

Glucose reagent is stored at 4-8 °C prior to reconstitution. After reconstitution, the reagent is stable at 4-8 °C for 14 days.

d. **Standards Preparation**

(1) Glucose standard
100 mg/dL, 150 mg/dL, and 500 mg/dL.

(2) Calibration standard
150 mg/dL of Certified Glucose (D-glucose) Standard Solution is purchased from Sigma Chemical Company and calibrated against the National Institute of Standards and Technology (NIST) standard reference material SRM 909. Store the standards in 2 mL-aliquots in tightly capped polypropylene tubes at ≤-20 °C until the day of assay. A thawed and well-mixed standard is placed at the cup position 1 in the calibration rack. A new calibration standard tube is used for each assay.

e. **Preparation of Quality Control Materials**

The two commercial lyophilized serum controls BC1 (Lot XLS536) and BC2 (Lot XPS636) are purchased from Baxter. Tap the vial gently to dislodge lyophilized cake. Using a volumetric pipet, reconstitute each vial with 10 mL of diluent, which has been brought to 20-25 °C prior to usage. Allow the vials to stand at 20-25 °C for 10 min and then swirl gently to mix contents. Invert the vial several times until the contents are completely dissolved. Store tightly capped vials at 4-8 °C. (They are stable for 5 days.) Reconstitute new vials every week.

Two in-house controls, IHH2, and IHL2, are prepared by collecting 450 mL (one unit) of whole blood from one diabetic and one nondiabetic subject. The blood is collected in blood bags containing EDTA as an anticoagulant. The plasma is separated immediately from the red blood cells by centrifugation in a refrigerated centrifuge (4 °C) for 25 min at 1500 g. The plasma is removed from the red blood cells and aliquoted in 0.5-mL portions and stored at ≤-70 °C in polypropylene storage tubes. One vial of each control is thawed and used in each assay. Reconstitution is not required for the in-house controls.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

(1) The Cobas Mira glucose assay uses a single 150-mg/dL calibration point, which is analyzed at the beginning of each run as a sample. The glucose value for the calibrator should fall within limits of 148 to 152 mg/dL. The instrument requires recalibration if the value of the calibrator is outside the specified limits.

(2) To calibrate the instrument, place a well-mixed glucose standard in Calibrator Cup Position 1 on the Calibration Rack and work under the ROUTINE worklist. Choose "CA" for the sample position. The screen will respond with "CAL." The cursor/highlighter will move to accept the test entry.

(3) Select GLUCOSE for the test key, and press ENTER.

(4) The calibration entry will disappear, but the procedure is programmed and the calibration will be performed automatically.

(5) Recalibration of the instrument is also performed when (a) the means or ranges of two or more controls exceed the 2 SD limits and a repeat of the controls results in the same error, or (b) an above- or below-the-mean trend (8 runs in a row) is observed in one of the four levels of control. (See sections 10 and 11.)

b. **Verification**

(1) In order to verify the calibrator, use Sigma glucose (D-glucose) standards (100 and 500 mg/dL) purchased from Sigma Chemical Company and calibrated against NIST standard reference material SRM 909. These standards are analyzed and the results compared to certified or accepted target values.

(2) Agreement with certified values should be ±5%; suggested frequency of verification is quarterly or whenever it is necessary for troubleshooting the system.

(3) The calibrator value is confirmed on an in-house Beckman Glucose Oxidase Analyzer every 6 months. The expected comparability between the hexokinase and glucose oxidase methods should be ±5%.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) For information regarding the range of linearity and how to handle results outside this range, refer to the Calculations section below.

(2) Allow frozen plasma samples, quality control specimens, and the glucose calibration material to reach 20-25 $^{o}$C and mix on a Varimix mixer 8 to 10 times.

(3) While specimens are thawing, reconstitute the glucose reagent and allow it to reach 20-25 $^{o}$C.

(4) Use a fine-point permanent marker to label the first and the last sample cups in each sample rack with the UMC ID numbers corresponding to the specimens to be analyzed.

(5) Check the reservoir and waste bottle level. Fill or empty the bottles as needed. Only Type II reagent grade water is used in the reservoir bottle.

(6) Fill the analyzer with clean cuvette segments. Press down the segments and be sure that they are seated properly.

b. **Sample preparation**

(1) To prevent fibrinogen from clotting in the sample pipeting system, centrifuge specimens at 1500 g for 10 min prior to analysis.

(2) Using a Gilson Pipetman, transfer 300 µL of controls and samples into the corresponding sample cups. Close the caps tightly and load them into the sample racks following the sample positions in the order set-up in the worklist.

c. **Instrument setup**

(1) Under PROG, set up system parameters and instrument configuration as shown in Table 1.

(2) Press the ON switch on the Cobas Mira analyzer.

(3) Under INFO and SYSTEM CHECKS, prime the tubing and syringe with water.

(4) Streams from both sample and reagent probes should be straight and continuous. Syringes should be free of air bubbles.

(5) Observe the sample needle and reagent probe. They should not appear damaged. Replace them if necessary. Check that the sample tubing is seated properly.

(6) Use the PROG section to check the glucose test profile. The current list for the glucose profile is shown in Table 2.

Table 1
Cobas Instrument Parameters

| | Parameter | Setting |
|---|---|---|
| Analytical Parameters | Temperature | 37°C |
| | Operation Mode | Sample Selective |
| | Control Interval | Each Day |
| | Time | No |
| Output Mode (Printer) | Status | On |
| | Number of Copies | 1 |
| | Sample Auto Mode | SPL/CAL/CS |
| | QC Auto Mode | OFF |
| Instrument Configuration | Software-Version | 8735C |
| | Interface | Installed |
| | DENS/Quality | Installed |

d. **Operation of Assay Procedure**

(1) The two commercial control pools BC1 and BC2 are always placed in cup positions 2 and 3 on the calibration rack. At the beginning of the worklist, before the sample list, request control analysis by typing "CS GLUC."

(2) Program the sample worklist under ROUTINE menu. Enter the first and the last numbers of the specimens to be analyzed, and press the test GLUC. The worklist will appear on the screen.

(3) Load the glucose standard in cup position 1 in the sample rack followed by the two in-house controls IHH2 and IHL2 in cup positions 2 and 3.

(4) Place the first 26 specimens to be analyzed in positions 4 through 29 on sample rack #1. Cup position 30 is reserved for a control. Place the rest of the specimens in sample racks #2 through 5.

(5) A sample rack always starts and ends with one of the four levels of controls, alternating between high- and low-level controls.

(6) A run always ends with the two commercial controls BC1 and BC2.

(7) The first and last cups in each sample rack are identified by their corresponding UMC accession numbers. The rest of the specimen cups are not marked, but a colored sample cup is used to identify every tenth sample in a rack.

(8) Check the sample cup identification numbers against the worklist.

(9) Place the reagents into the appropriate positions on the reagent rack.

(10) Place the calibration rack, the reagent, and the sample racks on the rack platform.

(11) Lift the analyzer cover. Insert the empty cuvette segments into position. Press the segments down firmly. Close the analyzer cover.

(12) Start the analysis by pressing START.

(13) Press the STATUS screen to display TRANSFER and ANALYZER operation status. The status screen will indicate the appropriate times when rack handling and segment handling are allowed during analysis.

(14) Check the glucose standard and the two control values. The calibrator value should be between 148 and 152 mg/dL, while the two control values should be within their established 2-SD limits. If any of the control values are outside their specified limits, abort the run and perform instrument calibration.

(15) After calibration, if the glucose standard and the two controls are within their specified values, proceed with the run.

Table 2
Glucose Test Profile

| Parameter | Setting | Parameter | | Setting |
|---|---|---|---|---|
| Measurement Mode | Absorb | Test Range | Low | 0.0 mg/dL |
| Reaction Mode | R-S | Test | High | 600.0 mg/dL |
| Calibration Mode | Calibrator | Normal Range | Low | 60.0 mg/dL |
| Reagent Blank | Reag/Dil | | High | 120.0 mg/dL |
| Cleaner | No | Number of Steps | | 1 |
| Wavelength | 340 nm | Calculation Step A | | Endpoint |
| Decimal Position | 1 | Reading, First | | T1 |
| Unit | mg/dL | Reading, Last | | 6 |
| Analysis | | Calibration Interval | | On Request |
| Sample Diluent Name | $H_2O$ | Reagent Blank Low | | -0.0270 A |
| Post Dilution Factor | 2.00 | Reagent Blank High | | 0.2700 A |
| Conc. Factor | 2.00 | Blank Low | | -0.0600 A |
| Sample Volume | 3.0 µL | Blank High | | 0.0600 A |
| Cycle | 1 | Calibrator Cup Pos. | | 1 |
| Dilution | 50.0 µL | Calibrator 1 | | 150.0 mg/dL |
| Reagent Volume | 200 µL | Replicate | | Duplicate |
| Calc. Sample Limit | 0.2400 A | Deviation | | 5.0% |
| Point | T1 | | | |
| Reaction Direction | Increase | BC1 Control Pos: 2 (Low) | | 70.0 mg/dL |
| Check | On | BC1 Control Pos: 2 (Assign) | | 74.4 mg/dL |
| Conversion Factor | 1.00000 | BC1 Control Pos: 2 (High) | | 79.0 mg/dL |
| Offset | 0.00000 | BC2 Control Pos: 3 (Low) | | 264.0 mg/dL |
| | | BC2 Control Pos: 3 (Assign) | | 283.0 mg/dL |
| | | BC2 Control Pos: 3 (High) | | 302.0 mg/dL |

(16) If the glucose standard or one of the two controls is still outside the established limits, repeat the calibration process using fresh glucose standard and fresh control specimens. Do not analyze patient samples until the calibrator and the two controls are all within their acceptable ranges.

(17) When the analysis is complete, glucose results are printed automatically on the printer tape.

(18) Discard the used cuvette segments, reagent, and sample cups in the appropriate waste container.

(19) Turn off the instrument.

e. **Recording of Data**

(1) Quality Control Data
All replicate values of QC data plus all pertinent assay information (date of analysis, reagent lot number, technician ID, samples ID etc.) are recorded on the daily worksheet. The calibrator value is also recorded.

(2) Analytical Results
Use the "NHANES III Transmittal Worksheet" to record the glucose results. Record glucose results, matching sequential UMC ID numbers on the print-out tape with corresponding numbers on the transmittal sheet. Record the result for plasma glucose in mg/dL. If a result is below the detection limit of the method, write "BD" (for below detection limit) in the comment field. If a sample is missing or if the sample volume is less than 200 µL, write "NES" (not enough sample) on the comment field. Lipemic or hemolyzed samples are also noted in the comment field. The original transmittal sheet is kept in the NHANES Glucose Data Book.

(3) Enter the data into a file using a custom-written DBASE III+ program. To minimize data entry error, obtain the specimen information directly from the JOUTPUT.TXT file provided by the NHANES mobile unit.

(4) During the data entry process, check the lab accession number to ensure it matches the correct NHANES specimen ID.

(5) The data file is saved on the diskette under both DBASE (DT#####.DBF) and ASCII (DT#####.TXT) formats. The prefix DT identifies the file as a data containing file. ##### identifies the unique shipment ID number.

(6) Use the "LABQC" program provided by NHANES to enter the quality control data. Two LABQC files (one labeled as MOmmddyy.DBF and one labeled as MOmmddyy.TXT) are saved on the same 5¼" diskette as the sample file and sent to NCHS.

(7) Give a copy of the transmittal sheet, the lab report, the daily assay worksheet, and the data-file diskette, as well as the original data tape obtained from the Cobas Mira to the supervisor. After the data is checked and corrected, backup copies of the diskette and the lab report are made. Send the original diskette and report to NCHS using an anti-static floppy disk mailer. The back-up diskette and report are kept at the Diabetes Diagnostic Laboratory at the University of Missouri.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Perform tube cleaning and syringe priming procedure on the day of assay. Use 10% bleach for cleaning solution. Select the TC test file for the procedure. After tube cleaning, prime the syringes for 5 min with Type II reagent grade water.

(2) Perform precision tests monthly and after any maintenance on the sample or reagent pipetting pathway. Two different concentrations of potassium dichromate are used for the precision testing. The P150 and P250 precision tests check the pipetting precision at two different sample volumes and two different reagent volumes. The expected coefficient of variation for the P150 precision test is $\leq$1.5%. The expected coefficient of variation for the P250 precision test is $\leq$2.5%. (See instrumentation manual.)

(3) Replace both the 100-µL sample syringe and the 1000-µL reagent syringe plunger tips as needed to ensure good pipetting precision.

(4) Replace the reagent probe, sample needle, and sample tubing loop as needed.

(5) Performance verification inspections are performed by the Roche Service Engineer every 6 months as part of the routine preventive maintenance.

g. **Calculations**

(1) The Cobas Mira glucose analysis is linear up to plasma glucose concentrations of 600 mg/dL. Reanalyze samples containing more than 600 mg/dL by diluting the specimen two-fold (1+1) with distilled water. The result output must then be multiplied by 2 to account for the dilution.

(2) The detection limit, based on 10 repeat measurements of zero standard (Type II reagent grade water) and serial dilution of a sample with a low glucose concentration, is 2 mg/dL.

(3) Glucose values less than 50 mg/dL or greater than 200 mg/dL are considered abnormal and analysis must be repeated for confirmation.

## 9. REPORTABLE RANGE OF RESULTS

Plasma glucose values taken at fasting, 2-hour post-OGTT, or at random are reportable in the range 2 mg/dL to 600 mg/dL without dilution. If a plasma glucose value is less than 2 mg/dL, it should be reported as below the lower detection limit; if greater than 600 mg/dL, the specimen should be diluted (1+1) with water and reanalyzed.

## 10. QUALITY CONTROL (QC) PROCEDURES

Two types of quality control (QC) systems are used in this analytical method: 1) "sample QC" and 2) "batch QC." For sample QC (i.e., precision estimation), 5% of specimens are randomly selected and analyzed either within-assay or between-assay for quality assurance purposes. If the CV between duplicates is greater than 5%, the specimen is reanalyzed. If more than three paired replicate samples have CVs greater than 5%, the instrument is checked and the calibration process is performed. Batch QC specimens are placed in the calibration rack at the beginning and the end of each sample rack and at the end of the entire run.

The batch QC pools consists of four levels of control pools, which cover the full range of plasma glucose concentrations for normal and diabetic populations. Two are commercial lyophilized serum controls, BC1 and BC2, purchased from Baxter Scientific Company. BC1 and BC2 are placed in the calibration rack and are identified to the instrument as the system controls.

Two other controls, IHH2 and IHL2, are prepared in-house and stored at $\leq$-70 °C. One vial of each is thawed and used in each assay. Reconstitution is not required for the in-house controls. All four levels of controls are assayed at the beginning and the end of each analytical run. One of the in-house controls is assayed at least once in each sample rack.

If the stock of these controls becomes low, another batch is ordered or prepared in time to analyze it concurrently with the current QC materials. The new controls are used only after their means and the ranges have been established by performing 20 characterization runs.

The bias limit is set at 1 SD or the 67% limit; the warning limit (WL) is the 2-SD or the 95% limit and the control limit (CL) is the 3-SD or the 99% limit. An example of the precision and accuracy for the controls used for NHANES specimens is shown in Table 3.

Table 3
Precision and Accuracy

| Pool | Mean | 95% limits | 99% limits | 95% limits (range) | 99% limits (range) | Runs | Total CV, % |
|---|---|---|---|---|---|---|---|
| BC1 | 74.4 | 71-77 | 70-79 | 5.9 | 7.9 | 20 | 1.9 |
| BC2 | 283.0 | 270-295 | 264-302 | 15.6 | 20.7 | 20 | 2.2 |
| IHH2 | 499.3 | 482-517 | 473-526 | 11.5 | 14.9 | 20 | 1.8 |
| IHL2 | 94.5 | 92-98 | 90-99 | 4.8 | 6.5 | 20 | 1.6 |

After each assay run, all control data are recorded on the daily worksheet. The results of the analysis are accepted or rejected according to the guidelines established by NHANES.

Two types of QC charts are used in assessing the quality of an assay. The first chart plots the mean of all the replicate determinations in a run and compares it with the established target mean, which is the overall mean established by the 20 characteristic runs.

The NHANES guideline declares a system as "out-of-control" if any of the following events occur for any one of the QC materials:

- The mean from a single control falls outside the 99% confidence limits.
- The means from two controls fall either both above or both below the 95% confidence limits.
- The daily means of one control from eight successive runs (excluding the runs in which the mean is within 1 SD or bias range) fall either all above or all below the center line.

The second type of QC chart plots the range of the replicates (the difference between the highest and the lowest value of a single control within a run) and compares it with the established target range, which is the overall mean of daily ranges established by the 20 characteristic runs.

The NHANES guideline declares a system as "out-of-control" if any of the following events occur for any one of the QC materials:

- The daily range from a single control falls outside the 99% confidence limits.
- The daily ranges from two controls fall either both above or both below the 95% confidence limits.
- The daily range of one control from eight successive runs fall either all above or all below the mean line.

If a run is declared out of control, investigate the system (instrument, standards, controls etc.) to determine the cause of the problem. Do not perform any analysis until the problem has been resolved.

The Laboratory also participates in an external QC program offered by Baxter Diagnostics Inc. (QAP) for BC1 and BC2. The individual control values obtained in all glucose assays performed each month are submitted to Baxter. These values are then compared with our own cumulative mean as well as the group cumulative mean (divided by method). Up to 12 months of statistictical data is available in each monthly report.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. When the QC results fail to meet the acceptable criteria, check the sample cup containing the QC specimen for bubbles and reanalyze the QC specimen.

b. If the QC results meet the acceptable criteria, accept the run and report the results.

c. If steps 1-2 do not result in correction of the "out-of-control" values for QC materials, perform precision testing and replace the syringe plunger tips as needed.

d. Recalibrate the system using a new vial of glucose standard.

e. Reanalyze the calibrator, controls, and specimens. Specimens are stable at 4-8 °C overnight. If the system requires more than 24 hours before it can be restored to functionality, use new aliquots of standard, controls, and specimens for analysis.

If the above steps do not correct the "out of control" condition, consult the supervisor for further corrective actions. Do not perform glucose analysis until the system is declared "in-control" again.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

An extensive list of drugs and other factors that may interfere with the determination of glucose is reported by Young et al.(5). Examples are improper collection technique, alcohol ingestion, and bilirubin.

**13. REFERENCE RANGES (NORMAL VALUES)**

a. Other references: In a study of 83 males and 64 females, from the New York metropolitan area, researchers using the Roche Reagent for Glucose on the Cobas Mira found the expected values to be 64-112 mg/dL.

b. Reference ranges for glucose were established at the Diabetes Diagnostic Laboratory in May 1991 on 31 nonobese, nondiabetic subjects (mean age=28.1 years). Each person was given a 360-cal standard meal challenge (Sustacal), followed a week later by a 75-gram oral glucose load (OGTT).

Table 4
Mean and Observed Ranges for Glucose (mg/dL)

| | | Fasting | 1 hour | 2 hour |
|---|---|---|---|---|
| Sustacal | Mean | 89.0 | 73.9 | 81.2 |
| Sustacal | Range | 79.0-99.0 | 47.5-133.5 | 55.0-102.5 |
| OGTT | Mean | 89.7 | 109.7 | 85.1 |
| OGTT | Range | 71.5-100.5 | 57.0-201.3 | 67.0-127.5 |

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

a. Fasting specimen $\geq$140 mg/dL.

b. 2-hour post-OGTT specimen $\geq$200 mg/dL.

c. Random plasma glucose concentrations $\geq$200 mg/dL are considered diabetic, in accordance with World Health Organization (WHO) guidelines (6).

d. Medical intervention may be necessary. Subjects with glucose values above these specified limits are reported weekly by phone to NCHS.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach and maintain 20-25 $^{o}$C during analysis. Specimens are returned to storage at $\leq$-70 $^{\circ}$C as soon as the analyses are completed.

**16. ALTERNAIVTE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

If the analytical system fails, specimens are to be stored at 4-8 $^{o}$C (refrigerated) until the Cobas Mira system located in the Special Chemistry Laboratory, Department of Pathology is available for analysis. If long-term interruption (more than 24 hours) is anticipated, we recommend that specimens be stored at $\leq$-70 $^{o}$C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

NCHS is notified weekly by telephone of all subjects with glucose values in the diabetic ranges specified by WHO (6). The supervising physicians are then notified by NCHS.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

All specimens are tracked on both laboratory log books and electronic data files kept on the University of Missouri mainframe system and floppy diskettes. Hard copies of all transmittal sheets containing the specimen information, test results, and daily assay worksheets are kept in bonded laboratory notebooks. The lab reports, as well as all corresponding QC data, are stored in a separate notebook. Only the NHANES ID number, age, and sex of the individual are known to the laboratory. Other personal identifiers are not provided to the laboratory in order to protect the confidentiality of the participants.

Residual samples are stored at ≤-70 °C for 1 year and returned to the NCHS serum repository in Rockville, MD.

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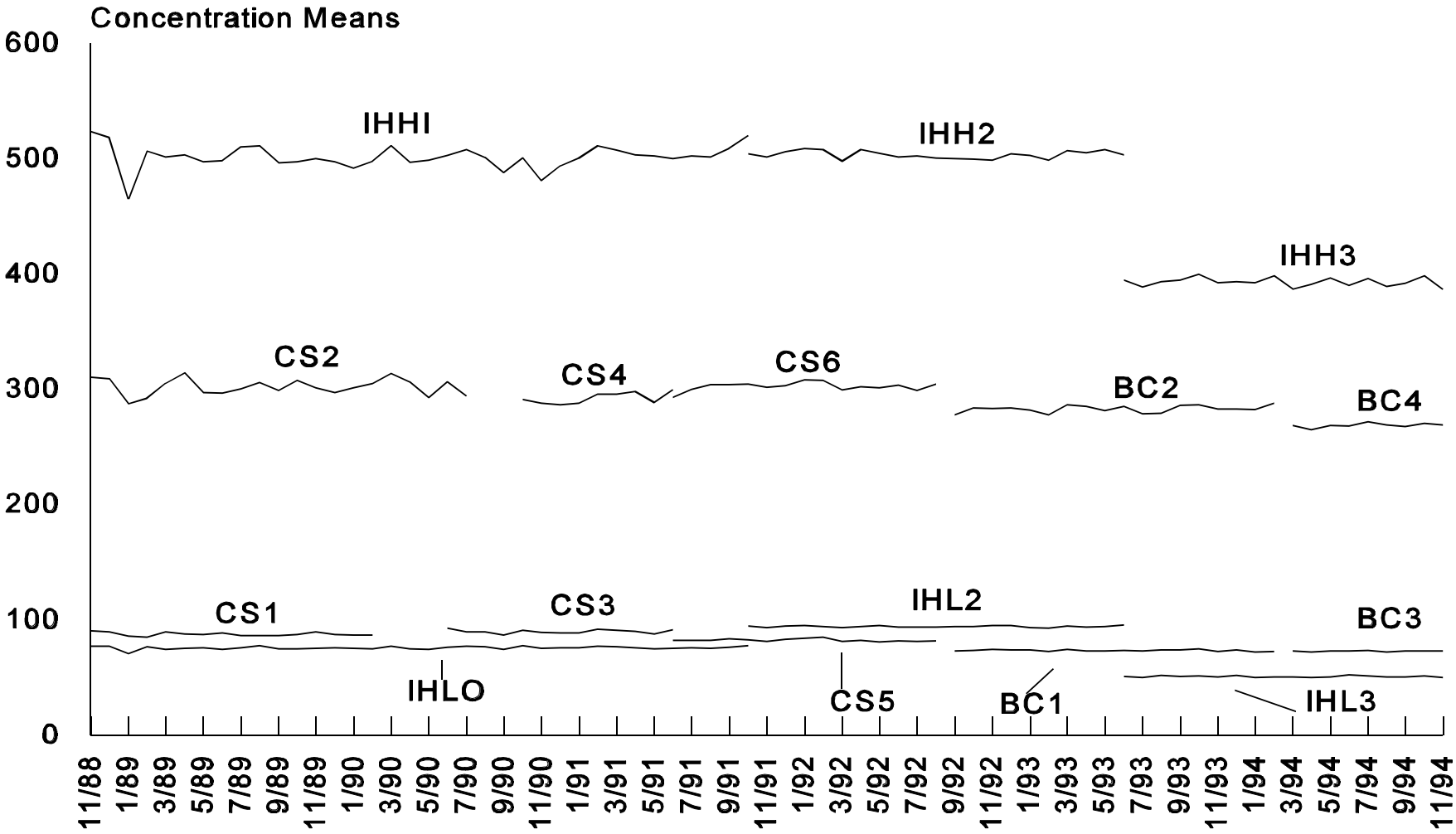
SUMMARY STATISTICS FOR
GLUCOSE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| CS1 | 11/30/88 - 02/21/90 | 87.848 | 2.7630 | 3.14520 | 163 |
| CS2 | 11/30/88 - 07/05/90 | 302.459 | 9.5689 | 3.16369 | 225 |
| CS3 | 06/14/90 - 06/18/91 | 90.028 | 3.2903 | 3.65476 | 170 |
| CS4 | 10/24/90 - 06/25/91 | 292.356 | 10.8106 | 3.69775 | 125 |
| CS5 | 06/26/91 - 08/27/92 | 82.640 | 2.3372 | 2.82811 | 235 |
| CS6 | 06/26/91 - 08/27/92 | 303.093 | 7.8254 | 2.58184 | 233 |
| IHHI | 11/30/88 - 10/02/91 | 500.880 | 14.3223 | 2.85942 | 438 |
| IHLO | 11/30/88 - 10/02/91 | 76.010 | 2.2119 | 2.91007 | 434 |
| IHH2 | 10/03/91 - 06/07/93 | 503.796 | 8.0931 | 1.60642 | 324 |
| IHL2 | 10/03/91 - 06/07/93 | 94.466 | 1.8274 | 1.93445 | 327 |
| IHH3 | 06/10/93 - 11/21/94 | 393.133 | 7.8317 | 1.99213 | 232 |
| IHL3 | 06/10/93 - 11/21/94 | 51.153 | 1.2499 | 2.44336 | 232 |
| BC1 | 09/17/92 - 02/28/94 | 73.789 | 1.8313 | 2.48185 | 293 |
| BC2 | 09/17/92 - 02/28/94 | 283.472 | 6.6550 | 2.34768 | 289 |
| BC3 | 03/15/94 - 11/21/94 | 73.136 | 1.5095 | 2.06391 | 146 |
| BC4 | 03/15/94 - 11/21/94 | 268.857 | 5.9802 | 2.22430 | 148 |

# Glucose Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human insulin is a polypeptide hormone that originates in the ß-cells of the pancreas and serves as a principal regulator for the storage and production of carbohydrates. Its secretion is normally stimulated by increases in the amount of glucose in the circulation.

Insulin radioimmunoassay (RIA) is a double-antibody batch method. Insulin in the specimen competes with a fixed amount of $^{125}$I-labelled insulin for the binding sites of the specific insulin antibodies. Bound and free insulin are separated by adding a second antibody, centrifuging, and decanting. The radioactivity in the pellet is then measured. The radioactivity is inversely proportional to the quantity of insulin in the specimen (1-3).

This test is used to measure insulin levels in the bloodstream and is also useful in determining pancreatic ß-cell activity.

Conditions such as obesity, a high-carbohydrate diet, and inactivity tend to increase expected normal values. Values are found to be elevated shortly after food intake and in cases of acromegaly, Cushing's syndrome, and thyrotoxicosis.

## 2. SAFETY PRECAUTIONS

Wear gloves, a laboratory coat, and safety glasses when handling all human blood specimens. Discard all plastic tips, sample cups, gloves and contaminated assay materials into a biohazard waste container. Discard all disposable glassware into a waste container for sharps. These containers are collected and processed twice a week by the University of Missouri-Columbia (UMC) hazardous waste management personnel.

$^{125}$I-labelled insulin has approximately 2 µCi $^{125}$I radioactivity per kit. A laboratory coat, safety glasses, and gloves are required while handling all radioactive materials. Wear a film badge that monitors radioisotope dosage on the lapel during the RIA procedures. The work area is surveyed for contamination monthly. Discard liquid and solid radioactive waste into their properly labelled containers. The containers are collected and disposed of by the UMC health physics/environmental health personnel.

Protect all work surfaces with absorbent benchtop paper, which is discarded weekly into biohazard waste containers or whenever blood contamination occurs. Disinfect all work surfaces with 10% sodium hypochlorite (bleach) solution weekly.

> *Dispose of all biological sample and diluted specimens in a biohazard waste container at the end of the analysis. Smoking, eating or drinking are not permitted in areas where radioactive materials are being handled. Dispose of all radioactive waste in properly labelled radioactive waste containers.*

Material Safety Data Sheets (MSDSs) for sodium hypochlorite are available through the University of Missouri-Columbia.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

The integrity of specimen and analytical data generated by this method is maintained by the following protocol:

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e. whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. A backup copy is made of the KOUTPUT.TXT file onto a 3½" floppy diskette and given a file name of "SP#####.DBF." The prefix SP identifies the file as an a incoming file containing sample information, and ##### identifies the unique shipment ID number.

c. Each specimen vial is given a unique sequential UMC lab accession number, which is recorded adjacent to the NCHS ID number on the transmittal sheet.

d. The CMS operating system is used to store all data and QC files on the UMC mainframe computer system. Backup copies of all files are also kept on 5¼" diskettes.

e. Specimen tracking and system maintenance records are kept on flow sheets in the "NHANES III Flow Log" notebook.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Random, fasting or 2-hour post-oral glucose tolerance test (OGTT) blood specimens are collected for insulin analysis in accordance with NHANES sample collection criteria. Each type of specimen is identified by a unique vial identification number ("6" and "6A" for fasting, "6B" for 2-hour post-OGTT, and "6*" for random samples) .

b. Specimen type: serum without anticoagulants or preservatives.

c. The optimal volume for this test is 1.5 mL; the minimum volume is 0.5 mL.

d. Whole blood, 3 to 5 mL, is collected in vacuum tubes (red-top tube or serum-separator tubes). Specimens are allowed to clot at room temperature for 15-30 min, and then are centrifuged at 1500 g for 10 min. Serum is transferred to a 2-mL polypropylene screw-top vial and frozen at ≤-20 °C. Frozen serum samples are sent weekly in batch in a styrofoam insulated shipping container with dry ice to the UMC Diabetes Diagnostic Laboratory via an overnight courier.

e. Insulin is stable for at least 1 month at -20 °C and 1 year at ≤-70 °C (4). Specimens should be processed within 1 hour of collection. Upon receipt, the processing laboratory will store the specimens in a ≤-70 °C freezer until analysis. Repeated freeze/thaw cycles, except as needed for analysis, should be avoided.

f. Samples with insufficient volume or samples thawed upon arrival are rejected. No analyses are performed on specimens that do not meet acceptance criteria. These conditions are noted on the transmittal sheet.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) Berthold model LB 2111 and model LB2104 multi-crystal gamma counter with sodium iodide 1-1/8" x 1-1/4" crystals (Berthold, Nasua, NH). Counting efficiency is approximately 75% for $^{125}I$ when plastic tubes are used; the measuring range is up to 250,000 CPM per detector.

(2) Jouan refrigerated centrifuge model GR4-22 (Winchester, VA). Temperature range: -8 °C to 60 °C, temperature accuracy: <±2 °C, maximum RPM: 8,000 maximum, timer range: 0-99 min in 1-min increments.

(3) Eppendorf tip-ejector fixed-volume pipettes, 50- and 100-µL volume (Fisher Scientific, St. Louis, MO).

(4) Eppendorf repeater pipet, 10-µL to 5-mL, precision to 0.1% (Fisher).

(5) Combitips for Eppendorf repeater with adapter (2.5-mL tips, graduated in 50 µL increments (Fisher).

(6) Cornwall repeating dispenser, 2-mL volume (Fisher).

(7) Pipetman adjustable pipet, 200-1000 µL (Rainin Instrument, Woburn, MA).

(8) Milli-Q Plus ultra-pure water system (Millipore, Bedford, MA).

b. **Materials**

(1) Pharmacia Insulin RIA kit (Pharmacia Diagnostics AB, Uppsala, Sweden). Reagents are stable until the expiration date, which is printed on each bottle. The recommended storage temperature for all reagents is 2-8 °C (5). Each kit contains reagents for assaying 100 tubes. Each kit contains the following:
Diluent: 50 mL phosphate buffer containing bovine serum albumin, EDTA, and detergent, pH 7.0.
Insulin antiserum (guinea pig):Guinea pig antiserum in assay buffer. *Color coded yellow*.
$^{125}$I-Insulin : approximately 2.6 ng (1.0 µCi at date of manufacture). *Color coded blue*.
Decanting suspension: Sepharose anti-guinea pig IgG raised in sheep. Store at 4-8 °C and mix well before use.

(2) Veronal buffer (World Health Organization (WHO), Geneva).

(3) 12-x 75-mm Borosilicate glass culture tubes (any vendor).

(4) Racks for radioimmunoassay tubes (Fisher, St. Louis, MO).

(5) Waterproof markers for labelling tubes (any vendor).

(6) Pipette stand (Fisher).

(7) Class A 5.0-mL volumetric pipet, calibrated "to deliver" (Fisher).

(8) Reusable glass beakers, various volumes, accurate to ±5% (Fisher).

(9) Disposable gloves (any vendor).

(10) RIA decanting rack (Pharmacia).

(11) 12-well plastic counting rack for gamma counter (Berthold).

(12) Berthold multi-calibrator matched $^{125}$I sources (Berthold).

(13) Prescored general purpose ampules, 1-mL (Fisher).

(14) Aluminum foil (any vendor).

(15) Absorbent benchtop paper (Whatman Lab Sales, Hillsboro, OR).

(16) Bleach (10% sodium hypochlorite) (any vendor).

(17) Computer printout paper (any vendor).

(18) Calculator TI-60X, (Texas Instruments, Houston, TX).

c. **Reagent Preparation**

For assays of more than 100 tubes, pool kit reagents bearing identical lot numbers and mix well before use. Mix gently to avoid foaming.

d. **Standards Preparation**

Human insulin standard materials, 0, 3, 10, 30, 100, and 240 micro-International Units (µU/mL), from Pharmacia Diagnostics are supplied as part of the kit.

e. **Preparation of QC Materials**

Three levels (low, medium, high) of lyophilized human serum controls are purchased from Bio-Rad Laboratories, Anaheim, CA. Using a Class A volumetric pipet, reconstitute each vial of control serum at room temperature by adding 5.0 mL distilled water. Allow the vials to stand for 10 minutes; invert the vials several times to mix contents.

Do not shake the vials. If more than one vial of each control is reconstituted, pool and mix vials with identical lot numbers together. Transfer 750-µL aliquots of each pool into polypropylene storage tubes. Cap the tubes tightly and freeze the aliquots at ≤-70 °C. Thaw each aliquot one time only.

The in-house lot no. 6 (IH6) pool is prepared by collecting one unit of whole blood from six nondiabetic volunteers at Columbia Blood Donor Center. All blood is screened for HIV and hepatitis B. Serum is separated from red blood cells at the Center. Serum from all six donors are pooled, and 500 KIU trasylol/mL of serum is added as a preservative. Transfer 750-µL aliquots to polypropylene storage tubes. Cap the tubes tightly and freeze the aliquots at -70 °C. Reconstitution of IH6 is not required. Thaw each aliquot one time only.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

(1) A calibration curve is constructed by using the linear $\%B/B_0$ (B=%bound, $B_0$=maximum binding) of standards at 0, 3, 10, 30, 100, and 240 µU/mL plotted against the logit of insulin concentrations.

(2) A cubic spline curve option is chosen in the Berthold "Create Protocol" option in the gamma-counter software.

(3) The calibration curve is displayed immediately following the standard curve summarization. To verify the mathematical fit, the smoothing factor must be less than 16 for assay acceptance.

(4) Percent $B_0$/total counts (TC) is monitored to verify the binding activity of the antibody and labelled $^{125}$I insulin solution. It should be between 45% to 55%. If the $B_0$/TC value is outside of those limits, notify the supervisor prior to accepting a run.

### b. Verification

The World Health Organization's (WHO's) international standard for insulin (first International Reference Preparation, 66/304) is used monthly to verify the insulin calibration curve. Prepare the stock solution by dissolving the ampoule supplied by WHO in 0.75 mL of 0.07 M veronal buffer (pH 8.6). Class A volumetric pipets are used to prepare subsequent working standards of 3, 10, 30, 100, and 240 µU/mL with Pharmacia diluent. Store both stock and working standards in sealed 1-mL ampule vials at -20 °C. Thaw one set of working standard monthly and, using a regular insulin assay, analyze it simultaneously with Pharmacia standard. The validation is monitored in three areas:

(1) The Pharmacia kit standard curve is compared directly with WHO insulin standards.

(2) A group of samples is analyzed by using both sources of standards. The means and linear regression relationships between the paired insulin values are calculated and charted.

(3) Five consecutive WHO standard curves are overlaid to monitor any long-term drift in the assay.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Allow frozen samples and quality control materials (three levels of Bio-Rad controls and one level of in-house control) to reach ambient temperature. Invert each gently to mix.

(2) Allow the standards, antibody, $^{125}$I tracer, and assay buffer to reach ambient temperature. *(Note: keep the precipitating reagent cold until ready to be used)*.

(3) Label a set of 12-x 75-mm borosilicate glass tubes in a RIA rack in the following sequence: TC, nonspific binding (NSB), and 6 levels of standards (all in triplicate); followed by one set of controls, specimens, and another set of controls (all in duplicate).

### b. Sample preparation

(1) All samples are analyzed initially without being diluted.

(2) For information regarding the range of linearity and how to handle results outside this range, refer to the calculations section of this document.

c. **Procedure**

All single-volume dispensing is done with an Eppendorf fixed-volume pipets. A new tip is used for each new specimen. All multiple-volume dispensing is done with an Eppendorf 2.5-mL Repipet with Combitips graduated in 50-µL increments (e.g., a setting of "1" will dispense a volume of 50 µL; a setting of "3" will dispense a volume of 150 µL).

(1) Pipet 150 µL of diluent in triplicate into NSB tubes.

(2) Transfer 100 µL of standards, controls, and unknown specimens into their respective borosilicate test tubes.

(3) Add 50 µL of $^{125}$I-insulin to all tubes.

(4) Add 50 µL of insulin antiserum to all tubes *except* the TC and NSB tubes. *Checkpoint: Contents of all tubes should now be green.*

(5) Shake the rack vigorously to ensure that the samples are well mixed. Cover the rack with aluminum foil and incubate for 2 hours at room temperature.

(6) At the end of the incubation period, remove the bottles containing the decanting solution from the cold room; however, do not allow the solution to warm up to room temperature. Shake the bottles vigorously to make the suspension homogenous before using the solution.

(7) Using a Cornwell repeating syringe, add 2 mL of the COLD (4-8 °C) decanting reagent to each tube *except* the TC tube.

(8) Vortex all tubes. Cover the rack with aluminum foil and incubate the tubes for an additional 30 min at room temperature.

(9) Centrifuge the tubes for 10 min at 2000 rpm at 4 °C. Do not allow the tubes to stand after centrifugation.

(10) Transfer all tubes, *except* the TC tube, to a specially designed decanting rack. Press the tubes down so they are seated securely inside the rack. Invert the rack in one smooth motion and decant the supernatant into a waste container. Invert the tubes for 30 sec on plastic-backed absorbent paper to blot off excess liquid, then turn the tubes upright. Dispose of contaminated waste liquid properly.

(11) Check the background activity of the counting racks by counting the empty racks for 2 min prior to placing any RIA tubes in the racks. Background activity should be less than 100 CPM. If the background exceeds 100 CPM, do not use the rack for the assay. The rack must be set aside or cleaned with decontaminant wash until its radioactivity has decreased below 100 CPM. See Section 8.g.2 for instrument calibration and efficiency measurement procedures.

(12) Count the pellets for 2 min. See section 8.d and 8.e for how to set up and operate the Berthold gamma counter.

d. **Instrument Set-Up**

(1) Berthold Multi-Crystal Gamma Counter (6)

(a) An evaluation method has to be assigned to each protocol number prior to operation. At the main menu, choose CHANGE DIRECTORY.

(b) Using the space bar, select RIA for the protocol number that is used for RIA INSULIN. Enter it into the directory by pressing RETURN.

(c) Create a protocol for insulin RIA by choosing CREATE PROTOCOL from the main menu.

(d) Enter the parameters as indicated in Table 1.

Table 1
Parameters for the Berthold Gamma Counter

| Parameter | Setting | Parameter | Setting |
|---|---|---|---|
| Name of test | Insulin | Number of standards | 5 |
| Measurement time | 2.00 min | Units of Concentration | µU/mL |
| Isotope | $^{125}$I | Standard 1 | 3 |
| Curve fit | Spl-auto | Standard 2 | 10 |
| Type of assay | Bound | Standard 3 | 30 |
| | | Standard 4 | 100 |
| TC | 3 Replicates | Standard 5 | 240 |
| NSB | 3 Replicates | Lower threshold | 1.00 |
| $B_0$ | 3 Replicates | Upper threshold | 100.00 |
| Standards | 3 Replicates | No. of QC Controls* | 3 |
| Pat-NSB | 0 Replicates | NSB/T | Calculated |
| Unknowns | 2 Replicates | $B_0$/T | Calculated |
| Dilution factor | 1 | Bn/REF | Calculated |
| Ether interference | Off | Slope at 50% | Calculated |
| Quality control | Full QC | ED20 | Calculated |
| | | ED50 | Calculated |
| | | ED80 | Calculated |

* Maximum allowed by the program is 3; if additional controls are used, specify the number used in assay set-up mode under OPERATION.

(2) Jouan Refrigerated Centrifuge model GR4-22

(a) All controls and indicators are located on the front panel.

(b) Press PROG to program the parameters for various centrifuge conditions. The initial set up is needed only once for each program.

(c) Press ENTER to move from one parameter to next.

(d) Make changes when it is necessary. Press ENTER.

(e) Press the program number for SAVE PROG NUMBER.

(f) After the initial set up has been completed, choose the required centrifuge parameters for RIA procedures by pressing RCL and PROG NUMBER.

Table 2
Parameters for Jouan Centrifuge

| Parameter | Setting | Parameter | Setting |
|---|---|---|---|
| Program number | (1-9) | Acceleration rate | 9 |
| Radius | 172 mm | Brake | 9 |
| Time | 10 min 00 sec | RPM/RCF | RPM |
| Temperature | 4 °C | Speed | 3000 RPM |
| Temp deviation | 0 | Saved program # | (1 to 9) |

e. **Operation of Gamma Counter**

(1) At the main menu, select MEASUREMENT UNINTERRUPTED.

(2) Choose the protocol number identified as "RIA Insulin".

(3) The parameter inputs for operational set up are shown in Table 3.

Table 3
Operational Set Up for Gamma Counter

| Parameter | Setting |
|---|---|
| Comment | (NHANES batch #) |
| Outlier rejection | On |
| Standard curve | Create new curve |
| Curve plot | Linear-log |
| Patient ID file | Not used |
| Auto incr. numbers start with | (1st sample ID) |
| Blank lines | 1 |
| Control position setup total # | 8 |

Table 4
Position of Controls

| Control | Position |
|---|---|
| QC1 | 1 |
| QC2 | 2 |
| QC3 | 3 |
| QC4 | 4 |
| QC5 | # of samples + 5 |
| QC6 | # of samples + 6 |
| QC7 | # of samples + 7 |
| QC8 | # of samples + 8 |

Press HOME to exit.

(4) After the protocol information is entered, the gamma counter screen displays the tube sequence. Place the first 12 RIA tubes in the plastic counting rack. Match the tube sequence with the screen display.

(5) Press the red button to initiate counting. The counting time is indicated on the gamma counter.

(6) At the end of 2 min, a beep will sound to alert the operator to place the next 12 RIA tubes in the rack.

(7) Proceed until all tubes have been counted.

(8) All data reduction is done automatically.

f. **Recording of Data**

(1) Quality Control Data
All replicate values of QC data plus all pertinent assay information (i.e., UMU batch ID, NHANES shipper and box numbers, date of analysis, reagent lot #, technician ID, sample ID, etc.) are recorded on the insulin daily work sheet. Calculate the daily mean and range for each control.

(2) Analytical Results
Use the "NHANES III Transmittal Worksheet" to record the serum insulin results. The sequential UMC ID number is used in the assay, and the matching test result is transcribed onto the transmittal worksheet. Record the result for insulin in µU/mL. If a result is below the detection limit of the assay (2.5 µU/mL), note "< 2.5" in the comments field. If a sample is missing or there is not enough sample to run the test, write "NES" (not enough sample) in the comments field. Also note if any of the samples are lipemic or hemolyzed. Keep the original transmittal sheet in the NHANES RIA book.

(3) Enter the data in a file using a custom-written DBASE III+ program. To minimize data entry error, obtain the specimen information directly from the KOUTPUT.TXT file provided by the NHANES mobile unit.

(4) During the data entry process, check the lab accession number to ensure it matches the correct NHANES specimen ID.

(5) Save the data file on the diskette under both DBASE (DT#####.DBF) and ASCII (DT#####.TXT) formats. The prefix DT identifies the file as a data-containing file, and ##### identifies the unique shipment ID number.

(6) Use the "LABQC" program provided by NCHS to enter the quality control data.o LABQC files, one labeled as MOmmddyy.DBF and one labeled as MOmmddyy.TXT are saved on the same 5¼" diskette as the sample file and sent to NCHS.

(7) Give copies of the transmittal sheet, lab report, daily assay worksheet, and data file diskette, as well as the original gamma counter printout, to the supervisor. After the data are checked and corrected, backup copies of the diskette and lab report are prepared. The original diskette and report are sent to NCHS in an antistatic floppy-disk mailer. The back-up diskette and report are kept at the Diabetes Diagnostic Laboratory, UMC.

g. **Replacement and Periodic Maintenance of Key Components**

(1) The centrifuge RPM and timer are checked and the centrifuge buckets are greased quarterly. The brushes are checked semiannually and changed as needed. Centrifuge maintenance is recorded in the equipment maintenance log book.

(2) A monthly calibration program is performed on the Berthold gamma counter by using a matching set of multicalibrator reference sources. Choose CALIBRATION OPTIONS under the gamma counter main menu. Place the 12 matching $^{125}$I reference sources in the counting rack. Perform INSTRUMENT QC, STANDARDIZATION, and HV ADJUST following the instructions displayed on the screens. Press START, and the calibration program will be performed automatically. A report will be generated listing the efficiency and the background measurements of all 12 wells. Standardization of all wells is performed via high-voltage standardization. The report is kept in the gamma counter log book. If the calibration program fails, the supervisor is notified immediately.

The air filter for the gamma counter is cleaned monthly. All calibrations and maintenance records are recorded in the equipment maintenance log book.

(3) All pipets are calibrated quarterly by using the gravimetric method. The method measures the performance of a pipet by using an analytical balance with distilled water. The accuracy and precision of the pipet at a specified volume are calculated by using a Lotus 1-2-3 spreadsheet program. Pipets that do not meet the accuracy and precision criteria are returned to the manufacturer for repair. All calibration results are recorded in the pipet calibration log book.

h. **Calculations**

(1) The Berthold gamma counter has full data reduction capabilities. The calibration curve is obtained with a smoothed cubic spline function. The curve plots linear $\%B/B_0$ versus logit of the concentrations, where

$\% B/B^0 = (B - NSB)/(B_0 - NSB)*100.$

$B_0$ = Mean bound counts at maximum binding
B = Mean bound counts of specimen
NSB = Mean bound counts of non-specific binding

The Berthold data-reduction program generates a 6-point sigmoidal splined standard curve ($B_0$ included). Control and unknown sample values are obtained from the curve.

The program also provides a smoothing factor, which indicates the deviation permitted between measuring points and curve points, or the deviation required for a good curve fit. Generally, the smoothing factor should be between 0.125 and 4. *Smoothing factors exceeding 16 require the supervisor's approval before the assay is accepted*.

(2) Freshly iodinated $^{125}$I-Insulin should provide at least 10,000 CPM for total counts (TC). *Do not use any labelled materials in which the TC activity has deteriorated below 3,000 CPM*.

(3) Percent $B_0$/TC can be used as a check on the binding activity between the antiserum and the labelled materials. For Pharmacia insulin antiserum, $\%B_0$/TC usually is in the range of 45% to 55%. If it falls outside those limits, notify the supervisor.

(4) Test results are expressed as micro International Units of Insulin per milliliter of serum (µIU/mL). The insulin RIA has a low detection limit of 2.5 µL/mL and is linear up to 100 µIU/mL.

(5) Dilute and reanalyze any specimen with a concentration greater than 100 µIU/mL. Depending on the concentration, prepare a new specimen using either 1 part serum with 1 part assay buffer with a dilution factor of 2 (DF2) or 1 part of serum with 3 parts of assay buffer (DF4). Multiply the measured value by the appropriate dilution factor before reporting.

(6) The low detection limit, based on 10 repeat measurements of zero standard and serial dilution of a sample containing a low insulin concentration, is determined to be 2.5 µIU/mL. All specimens with insulin values less than 2.5 µIU/mL are reanalyzed for confirmation and then reported as "<2.5."

(7) Duplicate specimen tubes for which the coefficient of variation is greater than 10%, indicating unacceptable imprecision, are flagged by the gamma counter, and the specimen is reanalyzed.

## 9. REPORTABLE RANGE OF RESULTS

Serum insulin values are reportable in the range of 2.5 to 100 µIU/mL. Values below the detection limit (2.5 µIU/mL) are repeated for verification and values above 100 µIU/mL are reanalyzed at an appropriate dilution factor.

## 10. QUALITY CONTROL (QC) PROCEDURES

Two types of quality control systems are used in this analytical method: 1) "sample QC" systems and 2) "batch QC" systems. For sample QC systems, 5% of specimens are randomly selected and analyzed either within-assay or between-assay for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is

reanalyzed. "Batch QC" specimens are placed before and after all specimens to be analyzed.

The batch QC pool consists of four levels of control pools that cover the spectrum of insulin concentrations for both normal and diabetic populations. Three are commercial lyophilized serum controls (L7, L8, and L9) purchased from Bio-Rad Laboratories. The other control, IH6, is prepared in-house and stored at ≤-70 °C. One vial of each control is thawed and used in each assay. Reconstitution is not required for the in-house control.

If the stock of these controls becomes low, another batch is ordered or prepared in time to analyze it concurrently with the current quality control materials. The new controls are used only after their means and ranges are established following 20 characterization runs. Sometimes, more runs are used to update control means and ranges if 20 runs are deemed to be inadequate to represent the overall performance of the RIA over time. All updates of control means and ranges are performed after approval from NCHS.

The bias limit is the 1 SD or 67% limit; the warning limit (WL) is the 2 SD or 95% limit, and the control limit (CL) is the 3 SD, or 99% limit. Examples of precision and accuracy estimates established for controls during NHANES III are shown in Table 4.

Table 4
Daily Means and Ranges

| Pool | Mean | 95% limits | 99% limits | 95% limits (range) | 99% limits (range) | Runs | CV,% |
|---|---|---|---|---|---|---|---|
| L7 | 11.45 | 9.92 - 12.99 | 9.15 - 13.75 | 2.35 | 2.85 | 68 | 6.39 |
| L8 | 40.74 | 36.31 - 45.18 | 34.09 - 47.70 | 7.87 | 9.67 | 68 | 5.46 |
| L9 | 101.52 | 91.60 - 111.43 | 86.65 - 116.38 | 22.92 | 28.39 | 68 | 4.88 |
| IH6 | 15.36 | 13.38 - 17.34 | 12.39 - 18.33 | 3.31 | 4.07 | 20 | 6.47 |

(L7, L8, and L9 were updated on 03/25/93, and IH6 was established on 06/02/92.)

After each assay run, all control data are recorded on the daily worksheet. The analysis is accepted or rejected according to the guidelines established by NHANES.

The quality of an assay is assessed by two types of QC charts (Levey-Jennings). The first chart plots the *mean* of all the replicate determinations in a run. This mean is then compared with the target mean, which is the overall mean established by the 20 characterization runs.

The NHANES guidelines define a system to be "out of control" if any of the following events occur for any of the QC materials:

- The mean from a single control falls outside the 99% confidence limit.
- The means from two controls fall either above or below the 95% confidence limit.
- The daily means of one control from eight successive runs (excluding the runs in which the mean is within 1 SD or bias range) fall either all above or all below the center line.

The second type of QC chart plots the *range* of the replicates (the difference between the highest and lowest value of a single control within a run). This range is then compared with the target range, which is the overall mean of daily ranges established by the 20 characterization runs.

The NHANES guidelines define a system as "out of control" if any of the following events occur for any one of the QC materials:

- The daily range from a single control falls outside the 99% confidence limit.
- The daily ranges from two controls fall either both above or both below the 95% confidence limits.
- The daily ranges of one control from eight successive runs fall either all above or all below the center line.

If the system is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the cause of the problem before any additional specimens are analyzed.

The Diabetes Diagnostic Laboratory also participates in an external QC program conducted by the College of American Pathologists (CAP). Two levels of survey materials are analyzed quarterly for insulin in a routine RIA run, and results are submitted to CAP for interlaboratory comparison.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

When the system is declared "out of control", take the following steps:

a. Recount the run.

b. Troubleshoot the system to locate probable cause of the problem (i.e., spilled tubes, partial loss of a pellet, pipeting error, or problem with one level of standard, etc.). If a cause can be identified and the problem corrected, notify the supervisor. The supervisor will evaluate the situation and determine whether to accept or reject the run.

c. If no obvious cause of a problem can be identified, reject the run and reanalyze all of the specimens.

d. Document the problem and any actions taken on the daily worksheet.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

No significant interference from specimens containing anticoagulants, or from thos that are lipemic or hemolyzed has been observed. However, patients taking insulin injections typically develop anti-insulin antibodies that will interfere with the assay. The cross-reactivity of Pharmacia insulin antibody with proinsulin is approximately 40%.

Obesity, consumption of a high carbohydrate diet, and inactivity tend to increase expected normal values. Values are increased following food intake and are higher among patients with acromegaly, Cushing's syndrome, or thyrotoxicosis.

## 13. REFERENCE RANGES (NORMAL VALUES)

*During the course of analyses for insulin during NHANES III, several changes in assay kit were required. From 10/88 to 1/4/90, the original Cambridge Laboratories (Cambridge, MA) RIA kit was used. The Cambridge Corporation was bought by Ventrex, Inc. (Also of Cambridge , MA), and the kit as sold by Ventrex was used from 1/25/90 to 9/6/90. During this time period a trend characterized by elevation of values was noted, and the University of Missouri decided to switch to an RIA kit marketed by Pharmacia (Fairfield, NJ), and this assay was used from 11/90 through the end of the survey.*

*To establish an overall correction for the data, the relationships between the 3 assays were established using 109 specimens assayed simultaneously. Three linear regression equations were calculated:*

*Ventrex = 1.315 (Cambridge) - 1.48, r2 = 0.96*

*Pharmacia = 0787 (Cambridge) + 0832, r2 = 0.93*

*Pharmacia = 0.597 (Ventrex) + 1.746, r2 = 0.96*

*Since the Pharmacia kit was used for the majority of the survey, both the Cambridge data and the Ventrex data were successively converted to Pharmacia equivalence*

a. The manufacturer indicates that mean fasting levels for healthy individuals lie below 20 µIU/mL (5).

b. The Diabetes Diagnostic Laboratory established internal reference ranges in May 1991, using 31 non-obese, nondiabetic adults (mean age 28.1 years)(6). Volunteers drank Sustacal Challenge (Mead-Johnson) (360 kcal), and 1 week later they drank a 75-gm glucose load solution as part of the Oral Glucose Tolerance Test. Observed reference ranges for both studies are shown in Table 5.

Table 5
Mean and Observed Ranges for Insulin (uIU/mL)

| Glucose Challenge | Fasting | 1-hour | 2-hour |
|---|---|---|---|
| Sustacal mean (range) | 7.41 (3.08 - 11.92) | 29.15 (11.57 - 12.37) | 16.08 (5.46 - 57.36) |
| OGTT mean (range) | 6.83 (3.48 - 11.09) | 42.96 (11.84 - 99.45) | 24.91 (9.59 - 55.99) |

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable for this procedure.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach and maintain ambient temperature during analysis. Return specimens to ≤-70 °C storage as soon as the analysis is completed. Avoid repeated freeze/thawing, except as necessary for specimen analysis.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Because the radioimmunoassay is complex and the characteristics of antibodies are very different from one another, there is no acceptable alternative method of analysis. If the analytical system fails, return all specimens to storage at ≤-70 °C. Reanalyze the specimens when the system is back in control.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (eg. electronic - DBASE, floppy disk) are used to track the specimens. We recommend that records (including specimen data, QC data, and shipping information), be maintained for 3 years after specimen analysis.

The only means of identifying a specimen is its unique NCHS and/or UMC identification numbers. No personal identifiers are available as this information is kept confidential by NCHS.

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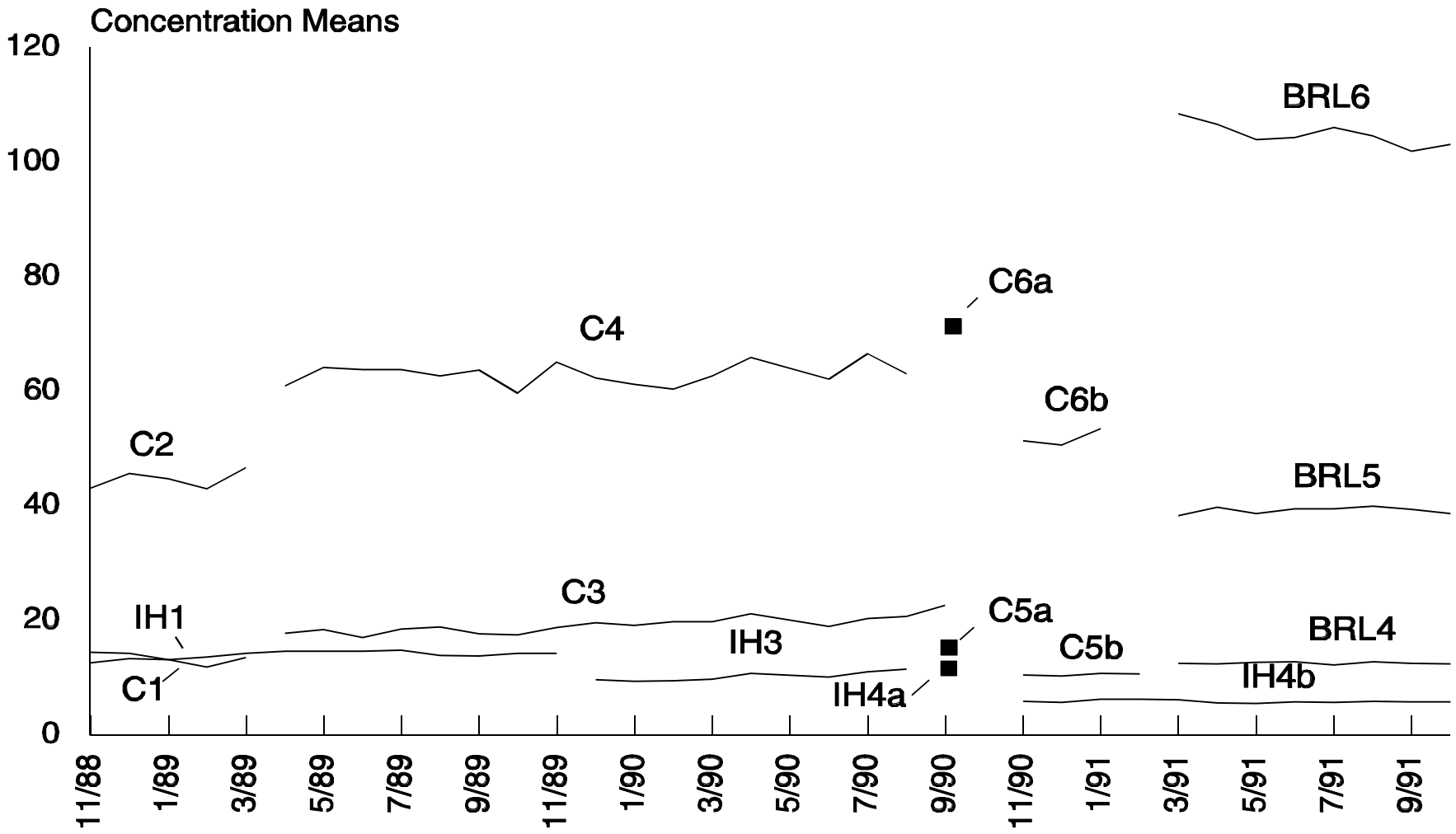
SUMMARY STATISTICS FOR
INSULIN - PHASE 1 POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| C1 | 11/18/88 - 03/24/89 | 13.004 | 1.72828 | 13.2907 | 91 |
| C2 | 11/18/88 - 03/24/89 | 44.966 | 4.77559 | 10.6203 | 91 |
| C3 | 04/07/89 - 08/21/90 | 19.168 | 2.24229 | 11.6981 | 417 |
| C4 | 04/07/89 - 08/21/90 | 62.912 | 7.31712 | 11.6307 | 410 |
| C5a | 09/06/90 | 14.123 | 1.82923 | 12.9526 | 4 |
| C5b | 11/09/90 - 02/18/91 | 10.511 | 0.79185 | 7.5337 | 119 |
| C6a | 09/06/90 | 71.885 | 5.96166 | 8.2933 | 4 |
| C6b | 11/09/90 - 01/24/91 | 51.441 | 3.93192 | 7.6435 | 103 |
| IH1 | 11/18/88 - 07/29/89 | 14.153 | 1.73658 | 12.2701 | 229 |
| IH3 | 12/14/89 - 08/31/90 | 10.251 | 1.41947 | 13.8470 | 217 |
| IH4a | 09/06/90 | 11.215 | 0.76370 | 6.8096 | 4 |
| IH4b | 11/09/90 - 10/31/91 | 5.865 | 0.62993 | 10.7413 | 370 |
| BRL4 | 03/14/91 - 10/31/91 | 12.541 | 0.82116 | 6.5478 | 220 |
| BRL5 | 03/14/91 - 10/31/91 | 39.240 | 2.44039 | 6.2192 | 220 |
| BRL6 | 03/22/91 - 10/31/91 | 104.491 | 6.12832 | 5.8649 | 208 |

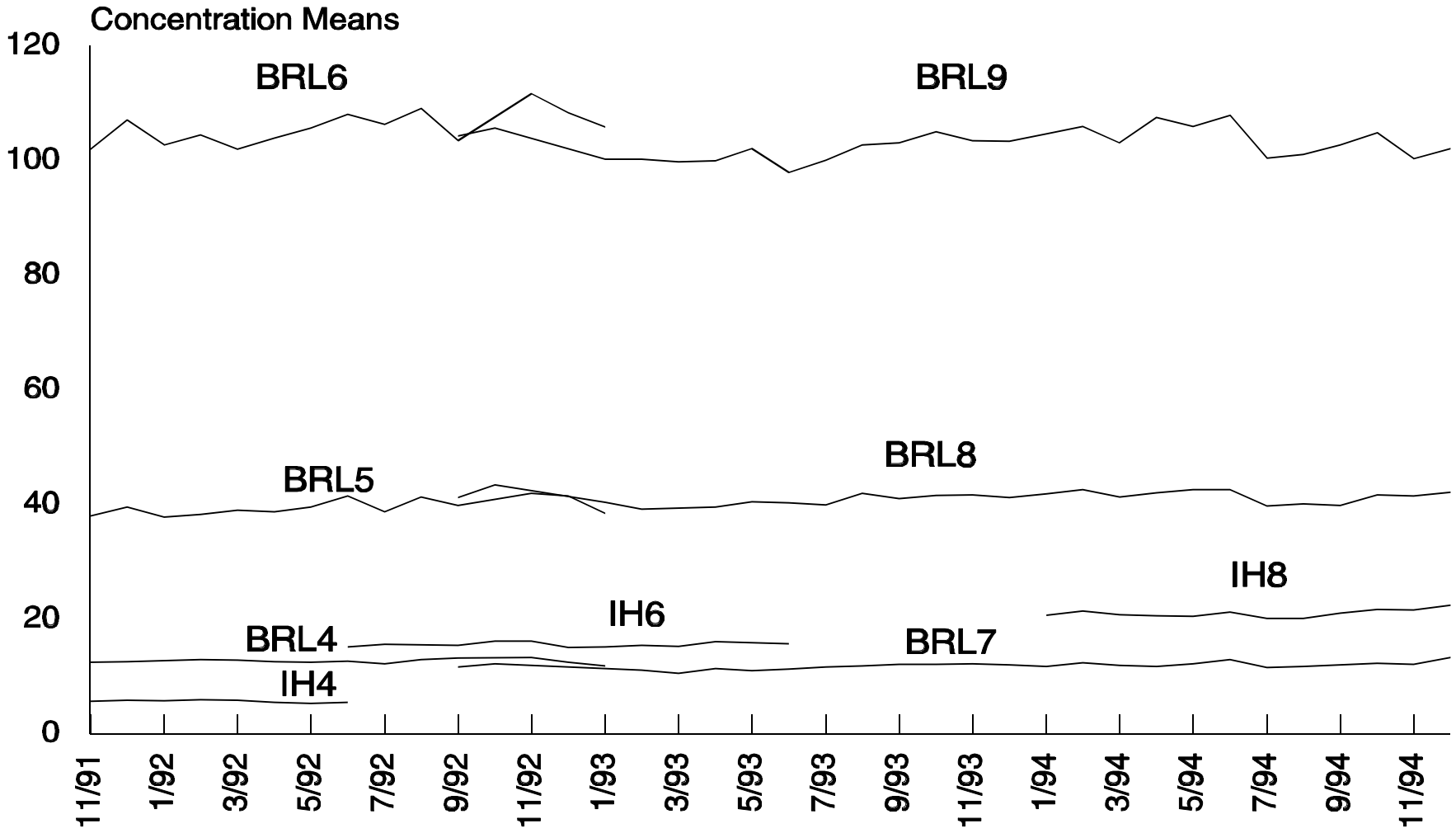
# Insulin Monthly Means - Phase 1



SUMMARY STATISTICS FOR
INSULIN - PHASE 2 POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| IH4 | 11/05/91 - 06/18/92 | 5.693 | 0.62604 | 10.9959 | 284 |
| IH6 | 06/19/92 - 12/01/94 | 15.589 | 1.22518 | 7.8592 | 372 |
| IH8 | 01/04/94 - 12/01/94 | 20.871 | 1.36955 | 6.5619 | 296 |
| BRL4 | 03/14/91 - 09/05/92 | 12.729 | 0.88468 | 6.9502 | 427 |
| BRL5 | 03/14/91 - 09/05/92 | 39.595 | 2.96695 | 7.4933 | 428 |
| BRL6 | 03/14/91 - 09/05/92 | 105.499 | 7.19922 | 6.8240 | 428 |
| BRL7 | 09/17/92 - 12/01/94 | 11.818 | 0.94861 | 8.0265 | 719 |
| BRL8 | 09/17/92 - 12/01/94 | 41.083 | 2.73366 | 6.6540 | 719 |
| BRL9 | 09/17/92 - 12/01/94 | 102.833 | 6.85733 | 6.6684 | 720 |

# Insulin Monthly Means - Phase 2



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Hemoglobin A1c is a glycohemoglobin formed by the nonenzymatic glycosylation of hemoglobin A. The reaction occurs in two steps. First, a Schiff base is formed by the reversible condensation of the carbonyl group of glucose and the amino group at the N-terminal valine of the β chain of hemoglobin A (1). The quantity of Schiff base (labile) formed depends on the ambient blood glucose concentration. Second, some of the Schiff base then undergoes an Amadori rearrangement to form a stable ketoamine (hemoglobin A1c). Because the Schiff base concentration changes acutely with fluctuations in blood glucose concentration and not all of the Schiff base is converted to stable HbA1c, it is essential to distinguish stable from labile HbA1c (2-5).

The Bio-Rad DIAMAT glycosylated hemoglobin analyzer system uses the principles of ion exchange high-performance liquid chromatography (HPLC). It enables the automated measurement of hemoglobin A1c without interference from lipemia, labile HbA1c (Schiff base), or temperature fluctuations. The analyzer makes use of a single-piston pump with a step-gradient valve system allowing three phosphate buffers of increasing ionic strength to pass through the analytical column in a timed sequence. The analytical column contains spherical cation exchange resin. The HbA1c fraction is separated from the HbA1a+b, HbF, and HbAo fractions based on the differences in net ionic charge. The %HbA1c is calculated as a percentage of all subfractions. Hemolyzed samples are maintained at a constant 6-10 °C in the autosampler chamber until they are injected into the analysis stream. Detection is performed at two wavelengths, 415 nm and 690 nm, to ensure a stable baseline.

All operations are controlled by a microprocessor. A built-in integrator performs data reduction of test data and sends the information to the printer, which produces a chromatogram and report summarizing %HbA1c and HbA1(a+b+c) values, as well as relative percentages (by area) of all resolved hemoglobin subfractions. Each sample is completely processed in 8 min.

The Schiff base is eliminated by incubating the patient sample in a borate-containing hemolysis reagent for 30 min at 37 °C (5). Borate binds to the vicinal diols of glucose promoting dissociation of the labile component.

Glycosylated hemoglobin is an index of average blood glucose level for the previous 2 to 3 months. It is widely used as an indicator of glycemic control in the care and treatment of patients with diabetes mellitus.

## 2. SPECIAL SAFETY PRECAUTIONS

Wear gloves, lab coat, and safety glasses when handling human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container. Discard all disposable glassware into a sharps waste container. Place all liquid hazardous waste materials in closed containers labelled as hazardous waste and stating the composition of waste being contained. These containers are collected and disposed of weekly by University of Missouri hazardous waste management personnel.

Protect all work surfaces with absorbent benchtop paper. Discard benchtop paper into a biohazard waste container weekly or whenever blood contamination occurs. Disinfect all work surfaces with 10% sodium hypochlorite weekly.

Material Data Safety Sheets (MSDSs) for human whole blood hemolysates, octyl-phenoxy polyethoxyethanol, and sodium hypochlorite are available through the University of Missouri-Columbia.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit arrives with a corresponding transmittal sheet and an ASCII data file on a 5¼" floppy diskette (ioutput.txt). The ASCII file containing the specimen ID, collection date, and type of sample (i.e. whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. A backup copy is made of the ioutput.txt file onto a 3½" floppy diskette.

c. Each specimen vial is labelled with a unique sequential University of Missouri-Columbia (UMC) lab accession number. This number is recorded adjacent to the NHANES ID number on the transmittal sheet.

d. All data and quality control (QC) files are stored on the UMC mainframe computer system which uses the CMS operating system. Backup copies of all files are also kept on 5¼" high-density (HD) diskettes.

e. Specimen tracking and system maintenance records are kept on flow sheets in the "NHANES Flow Log" notebook.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Samples are collected in accordance with NHANES specimen collection criteria for the glycosylated hemoglobin test. No special instructions such as fasting or special diets are required.

b. Specimen type: whole blood with anticoagulant, preferably $K_3$EDTA at a concentration of 1.5 mg/mL whole blood.

c. The optimal amount of specimen is 0.5 mL; minimum amount is 100 µL (0.1 mL).

d. An acceptable container is a 2-mL or larger vacuum tube (i.e., a lavender-top Vacutainer). $K_3$EDTA is the recommended anticoagulant.

e. Clotted specimens are unacceptable for this test.

f. Any samples that exhibit undesirable chromatograms (i.e., deterioration peaks, hemoglobin variants) are to be analyzed by the alternative standardized affinity method (6,7).

g. Samples are refrigerated at 4-8 °C immediately after collection and transported at 4-8 °C on refrigerant packing. Once received and analyzed, samples may be frozen and stored at ≤-70 °C. Do not freeze samples at -20 °C. Samples are stable at 4-8 °C for 7 days, and at ≤-70 °C for one year. Do not freeze/thaw samples more than one time (8).

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Diamat automated HPLC system, model 723 (Bio-Rad Laboratories, Hercules, CA).

(2) Diamat autodilutor, model AD-7 (Bio-Rad Laboratories).
Sample volume: 5-100 µL, reproducibility <±0.5%
Reagent volume: 125-250 µL, reproducibility ≤±0.2%
Overall accuracy: <±1.0%

(3) Water bath, model MW1120A-1 (Blue M Electric Company, Blue Island, IL).
Temperature Range: 0-100 °C
Accuracy: <±1.0 °C

(4) Jouan model 4.12 refrigerated centrifuge with swing-out rotor (Jouan Inc., Winchester, VA).
Temperature range: 0 to 40 °C
Temperature accuracy: <± 2 °C
Maximum RPM: 4,200
Timer: 0-30 min in 1-min increments

(5) Rainin Pipetman P-1000 and P-5000 adjustable pipets. (Rainin Instrument Company, Woburn, MA).

(6) Corning Mega-Pure water distillation system, model MP-4S (Corning Inc., Corning, NY).

(7) Thermolyne Vari-mix mixer (Thermolyne Inc., Dubuque, IA).

(8) Fluke electronic thermometer, model 51, with type K thermocouple (John Fluke Manufacturing Co., Everett, WA).

b. **Other Materials**

(1) Diamat reagents, (Bio-Rad Laboratories).

(a) Hemolysis reagent -- polyoxyethylene ether $R(OCH_2CH_2)_xOH$, (0.1% V/V) in a borate buffer. Store at room temperature; stable until expiration date printed on bottle.

(b) Elution buffers 1, 2, and 3 -- phosphate buffers containing 0.015% sodium azide ($NaN_3$) as a preservative. Store at room temperature; stable until expiration date printed on bottle.

(c) Wash solution -- phosphate buffer (pH 6.4) containing 0.015% sodium azide as a preservative. Store at room temperature; stable until expiration date.

(d) Bio-Rad reference standard diluent -- potassium cyanide (KCN)/borate solution. Store at 2-8 °C. Refrigerate until used; stable until expiration date printed on vial.

(2) Bio-Rad HbA1c Reference Standard (Bio Rad Laboratories).

(3) Analytical column, 4-mm x 15-cm, containing spherical cation-exchange resin. 4500- analyses capacity (Bio-Rad Laboratories).

(4) Column prefilters (Bio-Rad Laboratories).

(5) Sample vials, 1.5-mL capless microcentrifuge tubes (Outpatient Services, Petaluma, CA).

(6) Fisherbrand Redi-Tip pipet tips for P-1000 and P-5000 Pipetman (Fisher Scientific, St. Louis, MO).

(7) 100% nitrile rubber gloves (Best Manufacturing, Menlo Park, GA).

(8) Biohazardous waste storage bags and boxes (Jefferson Smurfit Corporation, Highland, IL).

(9) Bleach (10% sodium hypochlorite solution) (any vendor).

(10) Diamat thermal printer paper (Bio-Rad Laboratories).

(11) Absorbent benchtop paper (Whatman LabSales, Hillsboro, OR).

(12) Gauze sponges, 4"x 3", 16-ply, nonsterile (Johnson and Johnson, New Brunswick, NJ).

(13) 12- x 55-mm polypropylene tubes (Clinical Plastics, Leominster, MA).

(14) Caps for 12- x 55-mm polypropylene tubes (Bio-Rad Laboratories).

(15) Singlefold paper towels, cat. no. 227-04 (Fort Howard Corp., Green Bay, WI).

(16) Kim-Wipe lintless tissues (Kimberly Clark Corp., Roswell, GA).

(17) Stainless steel incubation racks for incubating up to 48 1.5-mL microcentrifuge vials (UMC Science Instrument Shop).

(18) 30- x 6-mL circular centrifuge racks with 30 x 1.5-mL inserts for Jouan model 4.12 centrifuge (Jouan, Inc.).

(19) Tygon 3603 tubing (Cole-Parmer Instrument Co.).

(20) 500-mL pyrex filtering flask (Fisher Scientific).

(21) Hazardous waste containers (empty 20-L plastic bottles surrounded by corrugated cardboard supplied by the UMC Dept. of Pathology).

(22) 20-mL graduated cylinder (Fisher Scientific).

(23) Timer capable of measuring up to 30 min in 1-sec intervals (any vendor).

(24) 10-mL volumetric pipet (Fisher Scientific).

(25) Sharps container (Devon Industries, Chatsworth, CA).

c. **Reagent Preparation**

Reagents are supplied ready to use.

d. **Standards Preparation**

(1) Bio-Rad HbA1c Reference Standard
Lypholized hemolysate derived from human blood. Prior to reconstituting the standard, store it at 2-8 °C. It is stable until the expiration date printed on the vial. Reconstitute a vial of the standard with 10-mL Bio-Rad reference standard diluent using a 10-mL volumetric pipet. After reconstitution, transfer 2.5-mL aliquots into 12- x 55-mm polypropylene storage tubes, cap the tubes tightly, and store them at ≤-20 °C until the day of assay. Reconstituted standards are stable for 14 days at 4-8 °C and for 90 days when stored at ≤-20 °C (5).

(2) Calibration Standard

(a) Pipet 750 µL of newly reconstituted or thawed Bio-Rad HbA1c reference standard into each of three 1.5-mL capless microcentrifuge vials using Rainin Pipetman P-1000. Place vials in positions 1, 2, and 3 in the autosampler.

(b) Place QC specimens in positions 4 (WB5) and 5 (WB6) of the autosampler tray. Place additional QC specimens after every twelfth sample, alternating between WB5 and WB6. Place one QC sample at the end of each run.

e. **Preparation of Quality Control Materials**

Six 7-mL EDTA Vacutainer tubes of venous whole blood were drawn from a known nondiabetic individual (WB5) and six were drawn from a known diabetic individual possessing an elevated HbA1c level (WB6). For each HbA1c level, the six tubes were pooled, dispensed in 25-µL aliquots at 4 °C, and stored at ≤-70 °C. This quantity of QC materials lasts for 2 years at ≤-70 °C.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

The Diamat HPLC system uses a single-level calibration. Three consecutive samples of HbA1c Reference Standard are analyzed at the beginning of each run. The first injection of reference standard is used as a system primer and discarded. The second injection is used to adjust the A1c calibration parameter. The HbA1c value for the Reference Standard should fall within ± 0.2% of the assigned value established for the particular lot of standard. The third value is used to confirm proper calibration and must also be within ± 0.2% of the assigned value for the standard.

b. **Verification**

The %HbA1c values for the original lot number of Reference Standard used in the Diabetes Diagnostic Laboratory was assigned by Bio-Rad Laboratories in 1987 (Lot# 60747). %HbA1c values for subsequent lots of Reference Standard were established by 20 interassay determinations, in duplicate, with the previous lot of standard used for calibration. The mean of the 40 determinations is calculated and used as the assigned %HbA1c value for that lot of standard.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Ensure that all reagents are at room temperature (20-25 °C) before beginning the assay.

(2) Allow frozen reference standard, QC specimens, and any frozen blood specimens to thaw. Mix each vial thoroughly prior to preparation.

(3) Using a fine-point permanent marker, label 1.5-mL sample vials with the corresponding UMC ID numbers on the blood specimens to be analyzed. Place the vials in a stainless steel incubation rack in the sample order as they are marked.

(4) Place blood specimens on the Thermolyne Vari-Mix mixer. Allow specimens to mix a minimum of 5 min prior to preparation.

### b. Sample Preparation

(1) Using the Diamat autodiluter, prepare control and patient hemolysates by diluting 5 µL of well-mixed whole blood with 1.0 mL hemolysis reagent in the sample vial. Wipe the outside of the dilutor tip with gauze dampened with distilled water. After inserting the tip of autodilutor into the blood specimen, press the button on top of the handle to draw 5 µL of specimen into the tip. Insert the tip into the corresponding 1.5-mL sample cup, and press the button again to dispense the sample and reagent into the sample vial. Wipe the outside of the tip with another wet gauze. Repeat the procedure for all patient specimens and QC specimens to be assayed.

(2) To remove labile HbA1c, place the hemolysates in 37 °C water bath for a minimum of 30 but no more than 45 min.

(3) After incubating the hemolysates, transfer them into racks of 30 6-mL centrifuge tubes and centrifuge them 15 min at 2500 rpm at 4 °C to precipitate cellular debris. After being centrifuged, the hemolysates are ready for analysis.

(4) Place the vials for QC samples with normal and high HbAlc levels in positions 4 and 5, respectively, in the autosampler tray followed by patient samples. Insert one QC specimen after every 12 patient samples, alternating between normal and high levels. Place a QC specimen at the end of the run.

### c. Instrument Setup for the Diamat Analyzer System

(1) Set the Diamat Analyzer parameters as shown in Table 1.

Table 1
Instrument Settings for the Diamat Analyzer

| Parameter | Setting |
|---|---|
| Wavelength | Sample: 415 ± 3 nm<br>Reference: 690 ± 3 nm |
| Column Temperature | 23 °C |
| Injection interval | 8.0 min |
| Injection volume | 20 µL |
| Buffer switching (stepwise) times | 1.7/3.7/5.8 min |
| Upper/Lower Pressure Limits | 20/120 kg/cm$^3$ |
| Signal Mode | Peak Area |

(2) Note the number of injections on the injection counter. Change the prefilter if more than 500 injections have

occurred since it was last changed. Record the injection number at which the filter is changed on the Diamat Diary worksheet.

(3) Check levels of buffers 1, 2, and 3 and of the wash solution. Maintain a minimum volume of approximately 300 mL in each bottle. Add more buffers as necessary, checking to ensure matched lot numbers. If it is necessary to switch to a new lot of buffer 1, 2, or 3, all three buffers must be switched, as the buffers are matched to one another (matched buffers have the same color coding on the buffer containers). Record all changes in lot numbers of buffers on the Diamat Diary worksheet and in the Buffers section of the Diamat Equipment Record.

(4) Check thermal printer paper, making sure there is enough for the entire run. Replace the paper roll, if necessary.

(5) Check the level of Diamat waste in the container and empty it into a hazardous waste container, if necessary.

(6) Check all buffer lines for evidence of leakage and tighten any leaking connections or replace tubing as necessary.

(7) Press the POWER button on the operation panel to start the warm-up sequence. (Warm up takes approximately 30 min.)

(8) Check for the presence of condensed water in autosampler wells, which is caused by moderate to high atmospheric humidity levels. Remove water using a filtering flask with Tygon tubing attached to a vacuum.

(9) Make sure the COOL button is depressed on the autosampler control panel.

(10) Verify that the column temperature setting and the actual temperature on the control panel are 23 °C.

(11) Check the date and time on the control panel. If necessary, change the date and time by pressing DATE on the control panel; entering the year, month, day, and time (in 24-hour format); and pressing ENTER.

(12) Press F3, then ENTER on the control panel to print a list of current parameters. Parameters are listed in three files: Parameter, IDT, and TBD. Verify the instrument list with the parameter settings shown in Tables 2, 3, and 4.

Table 2
Parameter File

| Parameter | | Settings |
|---|---|---|
| 1 STARTT | 0 | (100 MS) |
| 2 STPTM | 4500 | (100 MS) |
| 3 OFTIM | 750 | (100 MS) |
| 4 SMPCH | 8 | (100 MS) |
| 5 SSENS | 20.00 | (UV/min) |
| 6 SCNT | 5 | (COUNT) |
| 7 ESENS | 20.00 | (UV/min) |
| 8 ECNT | 5 | (COUNT) |
| 9 SLOPE | 40.00 | (UV/min) |
| 10 MINAR | 2.00 | (MV*SEC) |
| 11 IDTN | 6 | (RECORDS) |
| 12 TBDN | 4 | (RECORDS) |

Table 3
IDT File

| IDTNO | 1.RT | 2.TR | 3.NAME | 4.RF | 5.K |
|---|---|---|---|---|---|
| 1 | 1.90 | 0.40 | A1A | 1.00 | 1.00 |
| 2 | 2.50 | 1.00 | A1B | 1.00 | 1.00 |
| 3 | 3.20 | 0.50 | F | 1.00 | 0.00 |
| 4 | 4.30 | 0.50 | A1C | 1.03 | 1.00 |
| 5 | 6.00 | 0.30 | A0 | 1.00 | 0.00 |
| 6 | 6.50 | 0.30 | S/A0 | 0.00 | 0.00 |

Table 4
TBD File

| TBDNO | 1.TIME | 2.CODE | 3.DATA |
|---|---|---|---|
| 1 | 0.50 | 0 | 5.00 |
| 2 | 3.50 | 3 | 10000.00 |
| 3 | 4.20 | 3 | 2000.00 |
| 4 | 6.00 | 3 | 0.00 |
| 5 | 0.00 | 0 | 0.00 |
| 6 | 0.00 | 0 | 0.00 |

(13) If any parameters need to be changed, use the following procedure:

(a) Press F1 on the control panel twice, followed by the file number of the parameter to be changed (PARAMETER=1, IDT=2, TBD=3).

(b) Press F1, followed by the row number of the parameter to be changed (NOTE: The row number is always 0 if the PARAMETER file is being changed).

(c) Press F1, followed by the item number to be changed. (For the PARAMETER file, the item number corresponds to the row number (1-12). For the other two files, the item number corresponds to the column number at the top of the file.)

(d) Press F1, followed by the new value for the parameter multiplied by 100 (e.g., 3.50 would be entered as 350).

(e) Press F1 four more times, then press ENTER to store the new parameter value. Repeat the above procedures for each parameter to be changed.

(f) Press F3, then ENTER, to list the new parameters and check against the current parameter listing above.

(14) After the pump has been running 15 min or more, check the column pressure indicator on the control panel and record the pressure in the Diamat Diary. (Pressure should be between 20 and 120 $kg/cm^3$.)

(15) After the autosampler cooling unit has been switched on for 30 min or longer, insert the Fluke thermometer probe in the urgent position of the autosampler well. Switch the thermometer on to check the autosampler temperature. (Make sure the thermometer is reading in °C.) The autosampler temperature should be between 6 and 14 °C. Adjust the temperature, if necessary, by turning the temperature adjustment screw on the front of the autosampler well. Turn it clockwise to lower the temperature and counterclockwise to raise the

temperature. Allow the autosampler 10 min to come to its new temperature, then check the temperature again. Record both the autosampler temperature and the room temperature in the Diamat Diary.

(16) After 30 min warmup, the Diamat pump will shut off, and the STANDBY indicator on the Operation Panel will light. The instrument is now ready to begin analysis.

d. **Operation**

(1) Press the 1ST button on the Operation Panel, then "1" and ENTER. Press the LAST button, the number corresponding to the position of the last specimen in the autosampler, then ENTER. Press the SAMP button, then "1" and ENTER. Press START. After the instrument goes through a 4-min warmup, begin the analysis.

(2) Disregard the first reference standard chromatogram. If the %HbA1c value of the second reference standard is outside the assigned value by ±0.2%, adjust the HbA1c response factor (RF) parameter in the IDT using the following procedure. Check the current RF factor from the parameter list printed previously (IDT file, row 4, column 4). The RF parameter affects the %HbA1c value by approximately 0.1% HbA1c for every 0.02 increase or decrease in RF value. Adjust the RF using the procedure outlined in section 8.c.11. of this manual. Press the REPRINT button on the operation panel to reprint the chromatogram for the second reference standard with the adjusted %HbA1c value. If necessary, repeat the adjustment of the RF factor until the reference standard value reads within ±0.2%HbA1c of its assigned value.

(3) If the value of the reference standard in position 3 is within 0.2% of its assigned target, proceed with the run.

(4) If the reference standard in position 3 is outside the 0.2% range, repeat the calibration procedure. Change the FIRST and SAMPLE numbers back to "1" to restart the calibration.

(5) If the reference standard in position 3 is still outside the 0.2% range limit, repeat the calibration process using a new reference standard. *Do not analyze patient samples until calibrators read within 0.2% HBA1c of their assigned value*.

(6) Check chromatograms from normal and abnormal QC specimens in positions 4 and 5. If %HbA1c values for both QC specimens fall within their 2SD ranges, the run can proceed. If either QC specimen falls outside its 2SD range, repeat the calibration procedure. If the %HbA1c value for a QC specimen is still outside 2SD, repeat the calibration procedure using a fresh QC specimen. Do not analyze patient samples until the %HBA1c values for both QC specimens read within their 2SD ranges. If the new QC specimen remains outside 2SD limits, notify the supervisor immediately.

(7) Note the retention time of the HbA1c peak. If the time is less than 4.2 min or greater than 4.6 min, flow rate adjustment may be necessary. (See Section 8.f.1.d. of this manual.) Repeat the calibration procedure if the flow rate is adjusted.

(8) When the last specimen has been analyzed, the instrument will go through a 15 min wash, then shut down automatically.

(9) After the run is completed, mark the sample ID numbers on the individual chromatograms. Match the position of each vial in the autosampler tray to the sample number printed at the top of each chromatogram.

(10) Examine individual chromatograms: after an initial injection peak, each should show five peaks identified as A1A, A1B, F, A1c, and Ao. All peaks should be clearly resolved, and no troughs between peaks should extend below reference baseline.

(11) Do not report %HbA1c values for samples in which troughs extend below the reference baseline and reassay samples.

(12) Do not report results for any samples with total Ao peak areas less than 2000.00 (Hb concentration too low) or more than 8000.00 (Hb concentration too high). Reanalyze these specimens. Dilute the specimens during sample preparation if necessary.

(13) Mark any samples that show a deterioration peak (extra peak between the A1c and Ao peaks usually designated as p4 or p5) as "XPK." Reanalyze the sample by the alternative standardized affinity method and

report the standardized affinity result.

(14) Samples with heterozygous hemoglobin S present will show an additional peak after the Ao peak designated as S/Ao. Label the chromatogram as "HbS" and report the %HbA1c value for these specimens.

(15) Samples with heterozygous HbC present will show an extra peak after the Ao with a time >7.0 min which is not fully resolved. Run the samples by the alternative standardized affinity method (label chromatogram "HbC") and reanalyze the sample immediately after the HbC sample.

(16) Report any other abnormal chromatograms to the supervisor for further investigation.

e. **Recording of Data**

(1) Quality Control Data
Record all quality control data for each run on the Diamat Diary worksheet. Also record the UMC batch and sample numbers and the NHANES shipper and box numbers of samples run. Calculate and record mean and range values for each control and record these values in designated blanks on the Diamat Diary worksheet.

(2) Analytical Results
Use the "NHANES III Blood Vial Transmittal Sheet" to record specimen results. Record %HbA1c results, matching sequential UMC ID numbers on chromatograms with corresponding numbers on the transmittal sheet. If the chromatogram shows a deterioration peak (XPK), mark XPK-AFF in the notes section on the transmittal sheet and do not record a %HbA1c result. Do not record %HbA1c results for any samples to be repeated. Record "HbS" in the notes field for samples that demonstrate the presence of heterozygous HbS, and record %HbA1c results. Record "HbC" in the notes field for samples that show the presence of heterozygous HbC, and do not record a %HbA1c result.

(3) Enter the data into a DBASE III file using a custom-written program (HANES.EXE). To minimize data entry error, obtain the specimen information directly from the ioutput.txt file provided by the NHANES mobile unit.

(4) During the data entry process, check the lab accession number to ensure it matches the correct NHANES specimen ID. After all data has been entered, generate a data report using the HANES.EXE program.

(5) Save the data file on the data diskette under both DBASE (DT#####.DBF) and ASCII (DT#####.TXT) formats. The prefix DT identifies the file as a data-containing file, and ##### identifies the NHANES shipment number provided by the NHANES mobile unit.

(6) Use the "LABQC" program provided by NHANES to enter the quality control data. Save two LABQC files, one labelled MOmmddyy.DBF and one labelled MOmmddyy.TXT onto the data diskette. The prefix MO identifies the UMC laboratory; mmddyy is the month, day, and year the QC data is entered.

(7) Give copies of the transmittal sheet, data report, daily assay worksheet, and data diskette to the supervisor. After the data is checked and corrected, a back-up copy of the diskette and lab report are made. The original diskette and report are sent to NCHS in an antistatic floppy-disk mailer. The back-up diskette and report are kept on file at the Diabetes Diagnostic Laboratory at the UMC.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Diamat Analyzer System

(a) Record all maintenance in equipment record.

(b) Column prefilters. Change every 500 injections or whenever unusual increase in column pressure is observed. At least five prefilters should be on hand at all times.

(c) Analytical column. Change after 4500 injections or whenever signs of column deterioration (increase in column pressure, loss of peak resolution) are evident. The column may be reversed one time to extend its life. Keep at least two spare columns available at all times.

(d) Column flow rate. Check weekly. To check the flow rate, allow the Diamat to run a minimum of 10 min

into the warmup cycle. Remove the waste line coming from the detector to the waste container and place it in a 10-mL graduated cylinder while simultaneously starting the timer. After exactly 10 min, remove the line from the cylinder, measure the volume in mL, and divide by 10 to obtain the flow rate in mL/minute. The flow rate should measure 0.97±0.02 mL/min. If necessary, adjust the flow rate by turning the micrometer located at the rear of the pump assembly (the numbers on micrometer represent the percentage of the maximum pump stroke). Each 1.0% increase or decrease in the pump stroke results in an approximate 0.05-mL increase or decrease in the flow rate. After adjusting the flow rate, pump the buffer for 10 min and recheck the flow rate.

(e) Thermal printer paper. Replace as necessary. Keep at least one box (10 rolls) available at all times. Reorder when the last box is opened.

(f) Plunger seals and bushings. Change annually or whenever evidence of pump leakage is observed. Keep at least two spare seals and two pairs of spare bushings on hand at all times.

(g) Autosampler XYZ assembly. Check the motion of the autosampler needle assembly monthly by placing a sample in position 48 of the autosampler. Enter 1ST and LAST as 48 on the operation panel, pressing ENTER after each entry, and then press START. Observe the motion of the needle assembly as it moves toward position 48. It should be smooth with no evidence of sticking or inhibition of movement. The needle should descend smoothly into the sample vial, draw the sample, and then ascend smoothly. After this occurs, return the assembly to wash station. After the sample is injected, discard the excess sample and lower the needle into the wash station for a complete wash cycle. Clean and lubricate shafts and pulleys with LPS-1 lubricant every 6 months or whenever evidence of inhibited movement of the XYZ assembly is observed.

(h) Injector valve assembly. Replace with a new or rebuilt injector valve annually. Check micro-switches for proper operation by observing the motion of the injector valve arm during and immediately after sample injection. The arm should move approximately one-quarter turn during the injection, and then return to its home position after the injection.

(i) Thermal printer. Check the motion of the thermal print head daily. Clean and lubricate the rails and drive shaft with LPS-1 lubricant every 6 months or whenever evidence of inhibited motion of the print head is observed.

(j) Detector. Flush with 20% S/P Micro Wash detergent and rinse with distilled water annually.

(k) Autosampler syringe assembly. Clean and lubricate the rods and lead screw with LPS-1 lubricant every 6 months; replace syringe if it is leaking.

(l) Overrun switches. Each two are located on the X, Y, and Z axes of the syringe assembly. Check for proper operation annually by pressing each switch with the Diamat in standby mode and observing the overrun LED located on the control panel. If the LED does not illuminate when the switch is pressed, replace the switch.

(2) Diamat Autodilutor. Calibrate every 6 months. Check the syringes every 6 months for leakage and replace them if necessary. Record all maintenance in the Equipment Record.

(3) Jouan model 4.12 Centrifuge. Check the brushes, RPMs, temperature, and timer quarterly. Replace the brushes or have the instrument serviced if necessary. Clean and regrease the centrifuge buckets quarterly. Record all maintenance in the Centrifuge Maintenance Record.

g. **Calculations**

(1) All calculations are performed by the Diamat Analyzer. The area of each peak is calculated by the integrator, and %Peak Area is calculated by using the following formula:

$$\% \text{ Peak Area} = \text{Area}_N \div \text{Area(A1a+A1b+A1c+Ao+F)} \times 100$$

Thus, %HbA1c is calculated as follows:

$$\%HbA1c = Area_{A1c} \div Area(A1a+A1b+A1c+Ao+F) \times 100$$

Note that the area of each peak is multiplied by its own response factor (9).

(2) Retest any specimens with %HbA1c values less than 4.0% or greater than 11.0% for verification. Values that are outside 10% CV when compared to the original value should be reported to the supervisor.

## 9. REPORTABLE RANGE OF RESULTS

HbA1c results are reported throughout the range of 2.0% to 20.0%. Results above 11.0% or below 4.0% are repeated for verification prior to being reported.

## 10. QUALITY CONTROL (QC) PROCEDURES

Two types of quality control systems are used in this method: 1) "batch QC" specimens that are placed in each run, and 2) "sample QC" specimens (5% of the total specimens) that are randomly selected from each run and analyzed in another run. If the coefficient of variation between duplicates is greater than 10%, the specimen is reanalyzed and the chromatograms, instrument, and QC data from both the original and duplicate runs are investigated before the results from the original run are reported.

The batch QC consist of two levels of frozen whole blood controls. Values are in the low-normal (4.6%) and high (10.2%) ranges. Daily means and ranges are calculated from 20 interassay determinations. QC limits are established by calculating 95% (2SD) and 99% (3SD) confidence limits for both daily means and daily ranges for each control.

Table 5 shows the precision and accuracy as demonstrated by representative pools used during this survey.

Table 5
Precision and Accuracy

| Pool | Mean | 95% limits | 99% limits | Runs | CV (%) | 95% range | 99% range |
|---|---|---|---|---|---|---|---|
| WB5 | 4.6 | 4.4-4.8 | 4.3-4.9 | 20 | 1.73 | 0.3 | 0.4 |
| WB6 | 10.2 | 9.8-10.6 | 9.6-10.8 | 20 | 1.57 | 0.3 | 0.4 |

Two types of long-term QC charts are used. The first type of chart plots the mean values for each run. The system is declared "out of control" if any of the following conditions occur:

- The mean from a single run for a single control falls outside the 99% confidence limits.
- The means from a single run for both controls fall outside the 95% confidence limits.
- The means from eight successive runs for a single control fall either all above or all below the mean line.

The second type of QC chart plots the range values for each run. The system is declared "out of control" if any of the following conditions occur:

- The range from a single run for a single control falls above the upper 99% confidence limits.
- The ranges from a single run for both controls fall above the upper 95% confidence limits.
- The ranges from eight successive runs for a single control fall above the mean line.

If a run is declared "out of control," all patient samples from that run are retested in other runs. Additionally, the instrument, standard, and controls are investigated to determine the cause of the problem before further analysis occurs.

The UMC laboratory participates in an external QC program sponsored by the College of American Pathologists (CAP). A lyophilized blood specimen is received quarterly from CAP, reconstituted, and analyzed. Results are submitted to CAP, where the %HbA1c value is compared with consensus mean, low, and high %HbA1c values obtained from participating laboratories in using the same assay method. A copy of the CAP report is received by the UMC laboratory and is reviewed and approved by the supervisor.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. If the system is declared "out of control" for any of the reasons listed in Section 10 of this manual, take the following steps:

(1) Examine chromatograms from the run for any abnormalities (e.g., peaks coming off the column too quickly or too slowly, poor resolution of peaks, extra peaks). Peaks coming off the column too quickly or too slowly may indicate leakage or a need to adjust the flow rate. Poor resolution of peaks may indicate the need to reverse or change the analytical column. For other problems, consult the supervisor for appropriate corrective action.

(2) Prepare fresh standards and controls and analyze them.

(3) Reanalyze fresh dilutions of all samples in another run.

b. If the above steps do not correct the "out of control" condition, consult the supervisor for further corrective actions. Do not report values for runs that are not in control.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

a. Patients with hemolytic anemias may exhibit decreased HbA1c values because of a shortened red cell life span. Patients with polycythemia or postsplenectomy may exhibit increased HbA1c values because of a somewhat lengthened red cell life span (5,10).

b. Hemoglobin F values less than 2.0% have no effect on HbA1c results because HbF is completely resolved as a separate peak. Hemoglobin F values above 2.0% affect the resolution of the HbA1c peak, resulting in a decrease in apparent HbA1c values. Any sample for which the chromatogram shows an HbF value greater than 2.0% should be analyzed by using the alternative standardized affinity method.

c. Elevated hemoglobin $A_2$ levels (above 1.5%) may result in slightly decreased HbA1c values since $HBA_2$ co-chromatographs with HbAo and is thus included in the total hemoglobin calculation (5).

d. The presence of hemoglobin C interferes with this test as it does not fully resolve within the 8 min injection interval. Any specimens demonstrating the presence of HbC are run by using the standardized affinity method. Any samples adjacent to a specimen containing HbC are reassayed because of the possible carryover of HbC on the column.

e. Hemoglobin S in its heterozygous state does not interfere with this assay because its retention time is longer than that of HbAo and its peak area is not included as part of the total area calculation. In its rare homozygous form, hemoglobin S contains no HbA, therefore, this test is not recommended (5).

f. Test results are not affected by lipemic or hemolyzed samples.

**13. REFERENCE RANGES (NORMAL VALUES)**

a. The normal range for the HbA1c test was established in-house. The mean %HbA1c value for 194 nondiabetic subjects collected from throughout the continental United States was 4.9%, with a 95% range of 4.1 to 5.7%. Subjects were all under 40 years old and had confirmed fasting blood sugar levels < 120 mg/dL (9).

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable for this procedure.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should be refrigerated at 4-8 °C prior to preparation. Specimens are returned to 4-8 °C immediately after preparation and frozen at ≤-70 °C after results are processed.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

If the Diamat Analyzer system fails, specimens are to be analyzed by the alternative standardized affinity

chromatography method until the system resumes operation.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Files on the UMC mainframe system (backed up on diskettes) are used for specimen tracking. Hard copies of all original transmittal sheets and results are also kept on site. Only NHANES ID numbers, and the age, and sex of the individual are known to the laboratory. NHANES does not provide other personal identification information to the laboratory, in order to protect the confidentiality of subject participants. Excess NHANES III hemolysate specimens are discarded by autoclaving after data reporting has been completed.

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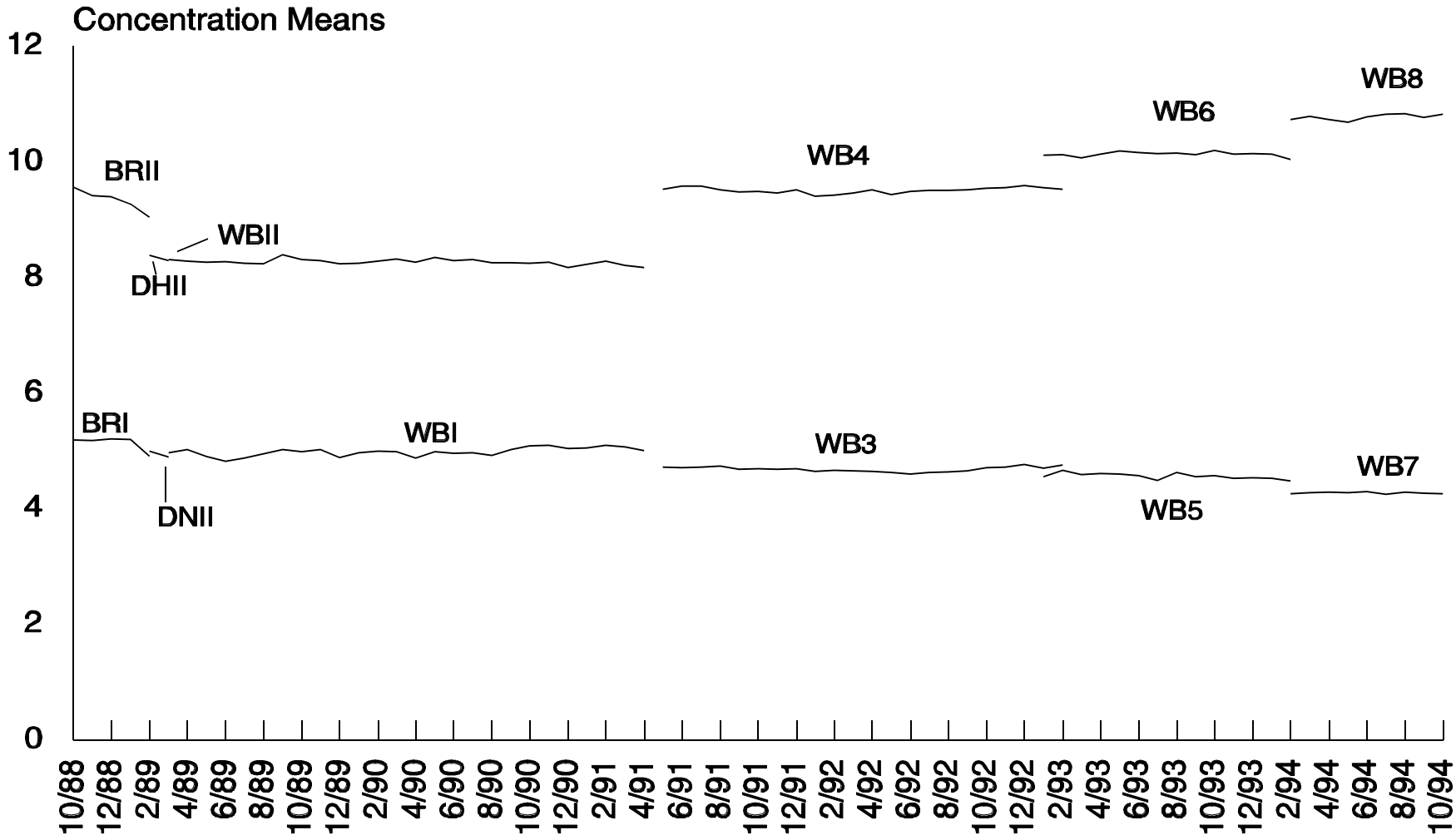
SUMMARY STATISTICS FOR
GLYCATED HEMOGLOBIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| BRI | 10/21/88 - 02/24/89 | 5.1732 | 0.15959 | 3.08485 | 224 |
| BRII | 10/21/88 - 02/24/89 | 9.3567 | 0.21751 | 2.32461 | 224 |
| DHII | 02/02/89 - 03/17/89 | 8.3345 | 0.11897 | 1.42748 | 55 |
| DNII | 02/02/89 - 03/17/89 | 4.9417 | 0.12114 | 2.45142 | 60 |
| WBI | 03/01/89 - 04/29/91 | 4.9946 | 0.15504 | 3.10426 | 1085 |
| WBII | 03/01/89 - 04/29/91 | 8.2691 | 0.15027 | 1.81726 | 948 |
| WB3 | 05/01/91 - 01/25/93 | 4.6841 | 0.10477 | 2.23666 | 831 |
| WB4 | 05/01/91 - 01/25/93 | 9.4989 | 0.14969 | 1.57581 | 645 |
| WB5 | 01/26/93 - 02/14/94 | 4.5705 | 0.10479 | 2.29275 | 601 |
| WB6 | 01/26/93 - 02/14/94 | 10.1304 | 0.12661 | 1.24977 | 510 |
| WB7 | 02/15/94 - 10/17/94 | 4.2765 | 0.08583 | 2.00694 | 357 |
| WB8 | 02/15/94 - 10/17/94 | 10.7707 | 0.11850 | 1.10018 | 352 |

# Glycated Hemoglobin Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Human C-peptide is a peptide chain 31 amino acids in length. Metabolically inert, it originates in the pancreatic beta cells as a by-product of the enzymatic cleavage of proinsulin to insulin (1,2).

The C-peptide radioimmunoassay (RIA) is a 3-day, batch, sequential-saturation method with two incubations (3). Antibody-bound proinsulin is removed by polyethylene glycol (PEG) precipitation (4,5). Supernates from PEG-precipitated samples, controls, and standards are incubated with a fixed amount of C-peptide antiserum. During this incubation, the unlabeled C-peptide binds with the antibody. A fixed amount of $^{125}$I C-peptide is added. During the second incubation, the $^{125}$I C-peptide binds to available antibody sites to which unlabeled C-peptide has not bound. The result is a mixture of labeled and unlabeled antibody:C-peptide complexes. The bound and unbound antibody fractions are separated by precipitating the antibody:C-peptide complexes with 95% ethanol and then centrifuging and aspirating the supernate. The radioactivity of the bound portion is counted in a gamma counter. The amount of radioactivity present in the precipitated antibody complexes is inversely proportional to the amount of unlabeled C-peptide. C-peptide concentrations are interpolated from a calibration curve constructed by plotting the percent bound of each standard versus its concentration.

Serum C-peptide is useful for determining pancreatic beta cell activity and, in the presence of anti-insulin antibodies, indirectly monitoring insulin secretion (6).

## 2. SAFETY PRECAUTIONS

Wear eye protection, gloves and a laboratory coat when handling all human blood specimens. Dispose of all plastic tips, gloves, and contaminated assay items in biohazard waste containers. Discard all disposable glassware into a sharps waste container. All contaminated waste is collected and disposed of bi-weekly by the University of Missouri-Columbia (UMC) hazardous waste management personnel.

Each $^{125}$I-labelled C-peptide kit (1500 tubes) contains approximately 25 µCi radioactivity. Wear a laboratory coat, eye protection, and gloves while handling all radioactive materials. Wear a film badge which monitors radioisotope dosage on the lapel during the RIA procedures. Survey the work area for contamination monthly. Discard liquid and solid radioactive waste into their properly labelled containers. The University of Missouri health physics/environmental health personnel collect and dispose of these containers twice a week.

Protect all work surfaces with disposable absorbent benchtop paper, which is discarded into biohazard waste containers weekly, or whenever blood contamination occurs. Wipe all work surfaces with 10% sodium hypochlorite (bleach) solution weekly.

Dispose of all radioactive waste in properly labelled radioactive waste containers. Dispose of all biological sample and diluted specimens in a biohazard waste container at the end of the analysis. Smoking, eating, or drinking is not permitted in areas where radioactive materials are being handled.

Thimerosol (merthiolate) is a highly toxic mercury-containing compound. Use special care when handling and disposing of thimerosol. Do not breathe dust. Dispose of buffers containing thimerosol in special waste containers.

Material safety data sheets (MSDSs) for thimerosol, 95% ethanol, and sodium hypochlorite are available through the University of Missouri, Columbia (UMC).

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Integrity of specimen and analytical data generated by this method is maintained by the following protocol:

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e. whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. A backup copy is made of the KOUTPUT.TXT file onto a 3½" floppy diskette and given a file name of "SP#####.DBF." The prefix SP identifies the file as an incoming file containing sample information. ##### identifies the unique shipment ID number.

c. Each specimen vial is given a unique sequential UMC lab accession number, which is recorded next to the NCHS ID number on the transmittal sheet.

d. All data and quality control (QC) files are stored on the UMC mainframe computer system that uses the CMS operating system. Backup copies of all files are also kept on high-density 5¼" diskettes.

e. Specimen tracking and system maintenance records are kept on flow sheets in the "NHANES III FLOW LOG" notebook.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Random blood samples, samples from fasting subjects, or samples used for 2-hour post-oral glucose tolerance tests (OGTT) are collected for C-peptide analysis in accordance with NHANES sample collection criteria. Each type of specimen is identified by a unique vial identification number ("6" and "6A" for fasting, "6B" for 2-hour post-OGTT and "6*" for random samples).

b. The specimens are 1.5 mL serum without anticoagulants or preservatives.

c. The optimal volume for this test is 1.5 mL and the minimum is 0.5 mL.

d. Three to 5 mL whole blood is collected in vacuum tubes (red-top clot tube or serum-separator tubes). Specimens are allowed to clot at 20-25 °C for 15 to 30 min, and are then centrifuged at 1500 x g for 10 min. Serum is transferred to a 2-mL polypropylene screw-top vial and frozen at ≤-70 °C. Frozen samples are sent in batches in styrofoam insulated shipping containers with dry ice to the UMC Diabetes Diagnostic Laboratory weekly via an overnight courier.

e. C-peptide is stable for at least 1 month at ≤-20 °C and 1 year at ≤-70 °C. Specimens should be processed within 1 hour of collection. Upon receipt, the processing laboratory will store the specimens in a ≤-70 °C freezer until analysis. Repeated freeze/thawing, except as needed for analysis, should be avoided.

f. Criteria for sample rejection are insufficient volume or being thawed upon arrival. These conditions are noted on the transmittal sheet.

g. No analyses are performed on specimens that do not meet acceptance criteria. The reasons are noted on the transmittal sheet.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Models LB 2111 and LB 2104 gamma counters, with multiple 1-⅛ x 1-¼" crystals (Berthold, Nashua, NH). Efficiencies: approximately 75% for $^{125}$I using plastic tubes. Measuring ranges: up to 250,000 CPM per detector.

(2) Refrigerated centrifuge model GR4-22 (Jouan, Winchester, VA). Temperature range: -8 °C to 60 °C. Temperature accuracy: <± 2 °C. Maximum RPM: 8,000. Timer range: 0-99 min in 1 min increments.

(3) Electronic balance model AT200 (Mettler, Hightstown, NJ).

(4) Maxi-Mix model I (Thermolyne, Dubuque, IA).

(5) Jenco microcomputer-based bench pH meter, model 6071 (Markson Science, Houston, TX); temperature range: 0 to 100.0 °C, pH range: -2.00 to 16.00, pH accuracy: ±0.1%.

(6) Eppendorf tip ejector fixed-volume pipettors, 50-, 100-, 200-, 300-µL volume (Fisher Scientific, St. Louis. MO).

(7) Eppendorf repeater pipet, range from 10 µL to 5 mL, precision to 0.1% (Fisher Scientific).

(8) Combitips for Eppendorf repeater with adapter, 2.5-mL tip graduated in 50-µL increments (Fisher Scientific).

(9) Combitips for Eppendorf repeater with adapter, 5-mL tip graduated in 100-µL increments (Fisher Scientific).

(10) Combitips for Eppendorf repeater with adapter, 12.5-mL tip graduated in 250-µL increments (Fisher Scientific).

(11) Cornwall repeating dispenser, 2- and 5-mL (Fisher Scientific).

(12) Pipetman adjustable pipet, 200-1000 µL (Rainin Instruments, Woburn, MA).

(13) Milli-Q Plus ultrapure water system (Millipore, Bedford, MA).

b. **Other Materials**

(1) Bovine serum albumin, processed to reduce interference of insulin in RIA assay, pH 7 (Sigma Chemical Co., St. Louis, MO).

(2) Sodium phosphate, monobasic, monohydrate, $NaH_2PO_4.H_2O$, ACS reagent grade (Sigma Chemical Co.).

(3) Sodium phosphate, dibasic anhydrous, $Na_2HPO_4$, ACS reagent grade (Sigma Chemical Co.).

(4) Polyethylene glycol, avg. mol. wt: 8,000 (Sigma Chemical Co.).

(5) Sodium chloride, NaCl, >99.5% (purify by titration) (Sigma Chemical Co.).

(6) Thimerosol (sodium ethylmercurithiosalicylate), approx. 98% pure by HPLC (Sigma Chemical Co.).

(7) 95% ethanol, USP, 190 proof, reagent quality, (Midwest Grain Product Company, MO, or any acceptable commercial source). Do not prepare from absolute ethanol, which is liable to contain traces of benzene and toluene that may be detrimental to the assay. Store at 20-25 $^{o}$C.

(8) M1221 antibody -- lyophilized antihuman C-peptide serum. Antisynthetic human C-peptide guinea pig serum M1221. (Novo Nordisk, BiosPacific, Inc. Emeryville, CA). The vial contains 1.7 mL of freeze-dried serum M1221 diluted (1:180) in 0.1% FAM. When the antibody is stored at $\leq$-20 $^{o}$C, it will remain stable for 1 year.

(9) $^{125}$I human tyr-C-peptide solution -- lyophilized $^{125}$I C-peptide (Novo Nordisk, BiosPacific, Inc.). Each vial contains $^{125}$I-labelled synthetic human C-peptide lyophilized from 1 mL of a solution containing 0.08 M TRIS, 0.02 N HCL, 0.2 M NaCl, 1% human albumin, and aprotinin (60 KIU/mL), pH 8.5. The C-peptide content per vial is 25 pmol. Radioactivity per vial (on the day of iodination) is approximately 25 µCi.

(10) Lyophilized C-peptide standard (Novo Nordisk). The vial contains 100 pmol of synthetic human C-peptide lyophilized in 1 mL of 6% NaFAM. Store at 4-8 $^{o}$C; freeze-dried C-peptide will be stable for over 1 year.

(11) Bio-Rad Lyphocheck immunoassay, tri-level lyophilized human serum controls (ECS Division, Bio-Rad Laboratories, Anaheim, CA).

(12) Pipet stand (Fisher Scientific, St. Louis, MO).

(13) 12- x 75-mm borosilicate glass culture tubes (any vendor).

(14) Racks for radioimmunoassay tubes (Fisher Scientific).

(15) Waterproof markers, for labelling tubes (any vendor).

(16) Graduated cylinders, 100-, 250-, 1000-mL in volume (Fisher Scientific).

(17) Class A volumetric flasks, 50-, 100-, 250-, 500-, and 1000-mL (Fisher Scientific).

(18) Class A 10.0-mL volumetric pipet, graduated at 1/10 mL to tip (Fisher Scientific).

(19) Class A 5.0-mL volumetric pipet (Fisher Scientific).

(20) Reusable glass beakers, accurate to ± 5%, 50- to 1000-mL in various sizes (Fisher Scientific).

(21) Disposable gloves (any vendor).

(22) 12-well plastic rack for gamma counter (Berthold, Nashua, NH).

(23) Berthold multicalibrator matched $^{125}I$ sources (Berthold).

(24) Aluminum foil (any vendor).

(25) Absorbent benchtop paper (any vendor).

(26) Bleach (sodium hypochlorite) (any vendor).

(27) Computer printout paper (any vendor).

(28) Calculator, TI60X (Texas Instruments Co., Dallas, TX).

c. **Reagent Preparation** (7,8)

The shelf life of all reagents is 1 month at 4-8 °C. Prepare all buffers with Type I reagent-grade water using volumetric pipets and flasks.

Measure all chemicals using an electronic analytical balance. Transfer chemicals to flasks by washing out the weighing boat with the buffer appropriate to the reagent. After bringing the solutions to volume, add a magnetic stir bar and stir until all solute is dissolved.

(1) 0.1% Phosphate Albumin Merthiolate (FAM)
0.04 mol/L phosphate buffer, containing 0.1 g/dL of bovine albumin, used for dilution of antiserum and $^{125}I$.
4.603 g $Na_2HPO_4$ (anhydrous, dibasic)
1.05 g $NaH_2PO_4.H_2O$ (monohydrate, monobasic)
1.00 g Bovine serum albumin
0.24 g Thimerosal(*Caution! Highly Toxic Chemical!*)
Dilute reagents to volume with distilled water in a 1-L flask. Verify that the pH is between 7.3 and 7.5. If the pH falls outside the limits, prepare a new batch of buffer. Store at 4-8 °C. Prepare a fresh batch monthly.

(2) 6% NaFAM
0.1% FAM, containing 6 g/dL bovine serum albumin to simulate serum protein matrix, and NaCl to make it isotonic; used for the preparation of standard solution and dilutions of serum.
3.00 g NaCl
29.5 g Bovine serum albumin
Dilute reagents to volume with 0.1% FAM in a 500-mL flask. Dissolve albumin slowly with gentle stirring to avoid foaming. Store at 4-8 °C for 2 days or less. Store at ≤-70 °C for extended stability of at least 6 months.

(3) 0.1% NaFAM
0.1% FAM containing NaCl to render it isotonic; used to dilute $^{125}I$ to make the working solution.
0.6 g NaCl
Dilute to volume with 0.1% FAM in a 100-mL flask. Store at 4-8 °C.

(4) 25% Polyethylene Glycol [$H(OCH_2CH_2)_nOH$]
Used for precipitating and removing the antibody-bound proinsulin.
Dilute to volume with distilled water in a 100-mL flask. Store at 4-8 °C. Prepare at least 1 day before use.

(5) Wash Solution
(*Caution! Flammable Material!*)
Prepare on the day of use; mix well.
160 mL 95% Ethanol
3 mL 0.1% FAM
27 mL distilled $H_2O$

(6) M1221 Antibody

(a) Stock Antibody Solution
Reconstitute the contents of one vial of M1221 antibody serum by adding 1700 µL distilled water using a calibrated micropipettor; mix well. Dispense 200-µL aliquots into polypropylene screw-capped storage vials and store at ≤-70 °C. Stable for many years. If more than one vial of antibody are used, pool all vials together prior to aliquotting for storage.

(b) Working Antibody Solution
Dilute 200 µL stock solution with 19.8 mL 0.1% FAM to make a 1:18,000 working dilution.

(7) $^{125}$I Human Tyr-C-peptide Solution

(a) $^{125}$I C-peptide Stock Solution
Within 1 week of receiving the labelled material, dissolve the vial contents in 900 µL distilled water using a precalibrated Pipetman pipet. Transfer 100-µL aliquots each of the stock solution into nine polypropylene storage tubes and store at ≤-70 °C. The stock solution is stable for 4 months from the date of iodination. If more than one vial of isotopes is used, pool and mix all vials together prior to aliquotting for storage.

(b) $^{125}$I C-peptide Working Solution

Use a 30-mL volumetric pipet to transfer 0.1% FAM into a 50-mL flask. Remove 100 µL FAM with a precalibrated pipet. Add 100 µL $^{125}$I C-peptide stock solution and mix well to achieve a 1:300 (v/v) working solution of the tracer.

d. **Standards Preparation**

(1) 100 pmol/mL stock C-peptide solution
Reconstitute three lyophilized vials using 900 µL distilled water per vial with a precalibrated Pipetman pipet. Combine all three vials to yield a total of 2.7 mL 100 pmol/mL stock solution. If the solution is not used immediately, store at ≤-70 °C. Solution is stable for at least 1 year.

(2) Working Standards Prepare each standard with 6% NaFAM as shown in Table 1. Use ONLY class A volumetric pipets and flasks. Transfer 6% NaFAM slowly to avoid foaming.

Transfer 500-µL aliquots of each of these standards except for standard 1.00 pmol/mL (use 400 µL), into labelled polypropylene tubes and store at ≤-70 °C. The standards will remain stable for at least 6 months. Thaw the standards only once, and invert the tubes to mix gently and avoid excessive foaming.

e. **Preparation of Quality Control Materials**

Bio-Rad Lypocheck Immunoassay Tri-level Lyophilized Human Serum Controls
Using a Class A volumetric pipet, reconstitute each vial with 5.0 mL distilled water. Allow each vial to stand for 10 min, then invert it several times to mix contents. (Avoid shaking.) If more than one vial of a control level is reconstituted, pool their contents and mix them together prior to storage. Transfer 750-µL aliquots into polypropylene storage tubes. Cap the tubes tightly and freeze at ≤-70 °C. Thaw and use each aliquot once.

The in-house lot no. 6 (IH6) pool is prepared by collecting one unit each of whole blood (450 mL) from six non-diabetic volunteers at Columbia Blood Donor Center. Serum was separated from clotted whole blood by centrifugation, and screened for HIV and hepatitis B. Pool serum from all six donors and add 500 KIU Trasylol/mL of serum as a preservative. Transfer 750-µL aliquots to polypropylene storage tubes. Cap the tubes tightly and freeze at ≤-70 °C. (Reconstitution of IH6 is not required.) Thaw and use each aliquot once.

Table 1
Preparation of Standards

| Final concentration (pmol/mL) | Volume of 100 pmol/mL standard (mL) | Final vol with 6% FAM (mL) |
|---|---|---|
| 2.00 | 1.0 | 50 |
| 1.00 | 1.0 | 100 |
| **Final concentration (pmol/mL)** | **Volume of 1.00 pmol/mL standard (mL)** | **Final vol with 6% FAM (mL)** |
| 0.50 | 25.0 | 50 |
| 0.30 | 15.0 | 50 |
| 0.20 | 10.0 | 50 |
| 0.10 | 5.0 | 50 |
| 0.05 | 2.5 | 50 |
| 0.03 | 1.5 | 50 |
| 0.00 | 0 | 50 |

If the stock of these controls becomes low, order and prepare another batch in time to analyze it concurrently with the current quality control materials. Use the new controls only after their means and the ranges have been established after 20 characterization runs.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

(1) A calibration curve is constructed by using the linear $B/B_0$ (B=%bound, $B_0$=maximum binding) of standards at 0.00, 0.03, 0.05, 0.10, 0.20, 0.30, 0.50, 1.00, 2.00 pmol/mL plotted against the logit of C-peptide concentrations.

(2) A cubic spline curve option is chosen in the Berthold CREATE PROTOCOL option in the gamma counter software.

(3) The calibration curve is displayed immediately following the standard curve summarization. To verify the mathematical fit, ensure that the smoothing factor is less than 32 for assay acceptance.

(4) Percent $B_0$/TC is used to verify the binding activity of the antibody and labelled $^{125}$I insulin solution, and usually varies between 50% to 65%. If the $B_0$/TC is outside of those limits, notify the supervisor prior to accepting a run.

### b. Verification

New standards are validated with a minimum of three overlapping assays performed with existing standards. When quality control values in all three assays using the new standards meet the criteria for assay acceptance, the new standards are then accepted and used for analysis.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Allow frozen samples, standards, and controls (three levels of Bio-Rad controls and one level of an in-house control) to reach 20-25 $^{o}$C. Invert the vials gently to mix their contents.

(2) The C-peptide RIA is a batch method. Treat standards, controls, and all specimens in the same manner throughout the entire assay procedures.

(3) Label one single 12- x 75-mm borosilicate glass tube for each of the non-specific binding (NSB), standards, controls, and specimens for PEG pre-treatment.

(4) Label a second set of 12- x 75-mm borosilicate glass tubes in an RIA rack in the following sequence: total counts (TC), NSB, 9 levels of standards (all in triplicate), one set of controls, specimens, and another set of controls (all in duplicate).

b. **Sample preparation**

(1) Analyze all fasting serum (vial identified as 6A or 6) without dilution.

(2) Dilute one part of all 2-hour post-oral glucose tolerance test (OGTT) or "random" serum (vial identified as 6B or 6*) with two parts of 6.0% NaFAM using a Eppendorf fixed-volume pipettor.

c. **Procedure**

All single-volume dispensing is done with Eppendorf fixed-volume pipets. Use a new tip for each new specimen. All multiple volume dispensing is done with an Eppendorf 5.0-mL Repipet with Combitip tips graduated in 100-µL increments (i.e., a setting of "1" will dispense a volume of 100 µL; a setting of "3" will dispense a volume of 300 µL).

**Day 1**

(1) Pipet 300 µL of standards, controls, and unknown specimens into their respective borosilicate test tubes.

(2) Use 300 µL of 6.0% NaFAM for NSB.

(3) Add 300 µL **cold** (4-8 °C) 25% PEG to each tube. Vortex the tubes vigorously. Centrifuge all tubes at 2000 x g for 45 min at 4-8 °C.

(4) Within 10 min of the completion of centrifugation, carefully transfer 100 µL supernate into the corresponding RIA tubes.

(5) Add 100 µL of working antiserum (1:18,000) into all tubes except "TC" and "NSB."

(6) Add 100 µL of 0.1% FAM to the NSB tubes.

(7) Vortex all the tubes and cover the rack with aluminum foil. Label the foil cover with the date and the time. Incubate 20 to 24 hours at 4-8 °C.

**Day 2**

(8) Add 100 µL of working $^{125}$I C-peptide into all tubes.

(9) Vortex all the tubes and cover the rack with aluminum foil. Mark the date and the time. Incubate 20 to 24 hours at 4-8 °C.

**Day 3**

(10) Using a Cornwall repeating syringe, add 1.6 mL of 95% ethanol (at 20-25 °C) to all tubes except TC.

(11) Vortex and centrifuge for 10 min at 2000 x g, at 4-8 °C.

(12) Using the vacuum apparatus, aspirate the supernate from all tubes (except TC). Be careful not to disturb the pellet.

(13) Using a Cornwall repeating syringe, add 2 mL wash solution (at 20-25 °C) to all tubes (except TC).

(14) Vortex and centrifuge 10 min at 2000 x g, at 4-8 °C.

(15) Aspirate the supernate and be careful not to disturb the pellet.

(16) Check the background activity of the counting racks by counting the empty racks for 2 min prior to placing any RIA tubes in the racks. Background activity should be less than 100 CPM. **If the background activity exceeds 100 CPM, do not use for the assay.** The rack is set aside or washed with decontaminant detergent until its radioactivity has deteriorated below the limit.

(17) Count the radioactivity in all tubes for 2 min. See Sections 8.d and 8.e for how to set up and operate the Berthold Gamma Counter.

**d. Instrument Setup**

(1) Berthold Multi-Crystal Gamma Counter(9)

(a) An evaluation method must be defined to each protocol number prior to operation. At the MAIN MENU, choose CHANGE DIRECTORY.

(b) Select RIA with the SPACE BAR for the protocol number that is used for RIA C-peptide. Enter it into the DIRECTORY with RETURN.

(c) Create a protocol for C-peptide RIA by choosing CREATE PROTOCOL under the MAIN MENU. Enter the parameters as shown in Table 2.

Table 2
Berthold Gamma Counter Parameters

| Parameter | Setting | Parameter | Setting |
|---|---|---|---|
| Name of test | C-peptide | Units of Concentration | pmol/mL |
| Measurement time | 2.00 min | Standard 1 | 0.03 |
| Isotope | $^{125}$I | Standard 2 | 0.05 |
| Curve fit | Spl-auto | Standard 3 | 0.10 |
| Type of assay | Bound | Standard 4 | 0.20 |
| Replicates/TC | 3 | Standard 5 | 0.30 |
| Replicates/NSB | 3 | Standard 6 | 0.50 |
| Replicates/$B_0$ | 3 | Standard 7 | 1.00 |
| Replicates/Standards | 3 | Standard 8 | 2.00 |
| Replicates/Pat-NSB | 0 | Lower threshold | 0.03 |
| Replicates/Unknowns | 2 | Upper threshold | 2.00 |
| Dilution factor | 1 | Num of QC controls* | 3 |
| Ether interference | Off | NSB/T | Calculated |
| Quality control | Full QC | $B_0$/T | Calculated |
| Number of standards | 8 | Bn/REF | Calculated |
| | | Slope at 50% | Calculated |
| | | ED20 | Calculated |
| | | ED50 | Calculated |
| | | ED80 | Calculated |

* The program allows a maximum of 3 QC samples; for additional controls, specify the number in the assay setup mode.

(2) Jouan Refrigerated Centrifuge Model GR4-22

(a) All controls and indicators are located on the front panel.

(b) Press PROG to program the parameters for various centrifuge conditions. The initial set-up is needed only once for each program.

Table 3
Parameters for Jouan GR4-22 Centrifuge

| Parameter | Setting | Parameter | Setting |
|---|---|---|---|
| Program number | (1-9) | Acceleration Rate | 9 |
| Radius | 172 mm | Brake | 9 |
| Time | 10 min 00 sec | RPM/RCF | RPM |
| Temperature | 4°C | Speed | 3000 RPM |
| Temp Deviation | 0 | Saved Program # | (1-9) |

(c) Press ENTER to move from one parameter to next.

(d) Make changes when necessary. Press ENTER.

(e) Press the program number for SAVE PROG NUMBER.

(f) After the initial set-up has been completed, choose the required centrifuge parameters for RIA procedures by pressing RCL PROG NUMBER.

e. **Operation of the Berthold Gamma Counter**

(1) At the MAIN MENU, select MEASUREMENT UNINTERRUPTED.

(2) Choose the protocol number identified as RIA C-peptide.

(3) The parameters for the Berthold Gamma Counter are shown in Table 4. Press HOME to exit.

(4) After the protocol information is completed, the gamma counter screen displays a graphic of tube sequence. Place the first 12 RIA tubes in a plastic counting rack. Match the tube sequence with the screen display.

(5) Press the red button to initiate the counting. The counting time is indicated on the gamma counter.

(6) At the end of 2 min, a beep will sound for placement of the next 12 RIA tubes in the rack.

(7) Proceed until all tubes have been counted. All data reduction is done automatically.

f. **Recording of Data**

(1) Quality Control Data
All replicate values of quality control data plus all pertinent assay information (i.e., UMC batch ID, NHANES shipper and box numbers, date of analysis, reagent lot #, technician ID, samples ID, etc.) are recorded on the C-peptide Diary Sheet. Calculate the daily mean and range for each control.

(2) Analytical Results
Use the "NHANES III Transmittal Worksheet" to record the C-peptide results. The sequential UMC ID number is used in the assay, and the matching test result is transcribed onto the transmittal worksheet. Record the result for C-peptide in pmol/mL. If a result is below the detection limit of the assay (0.03 pmol/mL), "< 0.03" is noted in the comments field. If a sample is missing or there is not enough sample to run the test, write "NES" (not enough sample) in the comments field. Lipemia and hemolysis are also noted when applicable.

The original transmittal sheet is kept in the NHANES RIA book.

Table 4
Parameters for the Berthold Gamma Counter

| Parameter | | Setting |
|---|---|---|
| Comment | | (NHANES Batch Number) |
| Outlier Rejection | | On |
| Standard Curve Used | | Create new curve |
| Curve Plot | | Linear-log |
| Patient ID File | | not used |
| Auto Incr. Numbers | Start with | (1st Sample ID) |
| Blank lines | | 1 |
| Control Position Setup | Total# | 8 |
| Position of Controls | QC1 | 1 |
| | QC2 | 2 |
| | QC3 | 3 |
| | QC4 | 4 |
| | QC5 | # of samples +5 |
| | QC6 | # of samples +6 |
| | QC7 | # of samples +7 |
| | QC8 | # of samples +8 |

(3) Enter the data in a file using a custom-written DBASE III+ program. To minimize data entry error, obtain the specimen information directly from the KOUTPUT.TXT file provided by the NHANES mobile unit.

(4) During the data entry process, check the lab accession number to ensure it matches the correct NHANES specimen ID.

(5) The data file is saved on the diskette under both DBASE (DT#####.DBF) and ASCII (DT#####.TXT) formats. The prefix DT identifies the file as a data containing file. "#####" identifies the unique shipment ID number.

(6) Use the "LABQC" program provided by NHANES to enter the quality control data. Two LABQC files (one labeled as MOmmddyy.DBF and one labeled as MOmmddyy.TXT) are saved on the same 5¼" diskette as the sample file and sent to NCHS.

(7) Give a copy of the transmittal sheet, the lab report, the daily assay worksheet, and the data file diskette, as well as the original gamma counter printout to the supervisor. After the data are checked and corrected, a backup copy of the diskette and the lab report are prepared. The original diskette and report are sent to NHSC in an anti-static floppy disk mailer. The back-up diskette and report are kept at the Diabetes Diagnostic Laboratory at the UMC.

g. **Replacement and periodic maintenance of key components**

(1) The centrifuge RPMs and timer are checked and the centrifuge buckets are greased quarterly. The brushes are checked semi-annually, and they are changed as needed. Centrifuge maintenance checks are recorded in the Equipment Maintenance Log book.

(2) Perform a monthly calibration program on the Berthold gamma counter using a matching set of multi-calibrator reference sources. Choose CALIBRATION OPTIONS under the gamma counter MAIN MENU. Place the 12 matching $^{125}$I reference sources in the counting rack. Perform INSTRUMENT QC, STANDARDIZATION, and HV ADJUST following the instructions displayed on the screens. Press START, and the calibration program will be performed automatically. A report will be generated listing the efficiency and the background

measurements of all 12 wells. Standardization of all wells is performed via high voltage standardization. The report is kept in the gamma counter log book. If the calibration program fails, notify the supervisor immediately. Clean the air filter for the gamma counter monthly. Record all calibrations and maintenance checks in the equipment maintenance log book.

(3) Calibrate all pipets quarterly using the gravimetric method. The method measures the performance of a pipet using an analytical balance with distilled water. Calculate the accuracy and precision of the pipet at a specified volume using a Lotus 123 spreadsheet program. Return pipets not meeting the accuracy and precision criteria to the manufacturer for repair. Record all calibration results in the calibration log book.

h. **Calculations**

(1) The Berthold gamma counter has full data-reduction capabilities. The calibration curve is obtained with smoothed cubic spline function. The curve plots linear $\%B/B_0$ versus logit of the concentrations where:

$\% B/B_0 = (B - NSB)/(B_0 - NSB)*100$

$B_0$ = Mean Bound Counts at Maximum Binding
B = Mean Bound Counts of Specimen
NSB = Mean Bound Counts of Non-Specific Binding

Berthold data-reduction programs generate a 9-point sigmoidal splined standard curve ($B_0$ included). Control and unknown sample values are obtained from the curve.

The program also provides a smoothing factor that indicates the deviation permitted between measuring points and curve points, or the deviation required for a good curve fit. Generally, the smoothing factor should be between 0.125 and 16. Smoothing factors exceeding 32 require the supervisor's approval before the assay can be accepted.

(2) Freshly iodinated $^{125}$I C-peptide provides approximately 10,000 CPM for total counts (TC). Do not use any labelled materials for which the TC activity has deteriorated below 2,500 CPM.

(3) Percent $B_0$/TC can be used to verify the binding activity between the antiserum and the labelled materials. For M-1221 antiserum, $\%B_0$/TC usually is in the range of 50 to 65%. If it falls outside those limits, notify the supervisor.

(4) Test results are expressed as picomoles of C-peptide per milliliter of serum (pmol/mL). The C-peptide RIA has a low detection limit of 0.03 pmol/mL, and it is linear up to 2 pmol/mL.

(5) Reanalyze any fasting specimen (vials 6A or 6) with a concentration greater than 2 pmol/mL using a diluted specimen. Depending on the concentration, prepare a new specimen using either 1 part of serum with 2 parts of 6% NaFAM (DF3 or dilution factor of 3) or 1 part of serum with 5 parts of 6% NaFAM (DF6). The results output must be multiplied by the appropriate dilution factor (DF) before reporting.

(6) Since the C-peptide values of 2-hour post-OGTT and "random" specimens (vials 6B and 6*) tend to be greater than 2 pmol/mL, dilute the specimens three-fold (DF3) prior to the initial analysis. Multiply the result outputs by 3 to account for the higher dilution. This step will cover specimens with final concentrations from 2 to 6 pmol/mL. Specimens with C-peptide concentrations exceeding 6 pmol/mL will require further dilution (i.e., DF6 or DF10) and reanalysis.

(7) To keep the sample dilution procedure consistent between vial 6 or 6A (DF1 or no dilution) and vials 6B or 6* (DF3), reanalyze any 2-hour or "random" specimens with C-peptide values that are less than 1.5 pmol/mL using the original serum specimen without dilution (DF1). If the final concentration is between 1.5 and 2 pmol/mL, it is reported without re-analysis.

(8) The low detection limit, based on 10 repeat measurements of the zero standard and serial dilution of a sample containing a low C-peptide concentration, is 0.03 pmol/mL. Reanalyze all specimens with C-peptide values less than 0.03 pmol/mL for confirmation and report them as "<0.03."

(9) Duplicate specimen tubes with a coefficient of variance greater than 10% are flagged by the gamma counter

software and the specimen is reanalyzed.

## 9. REPORTABLE RANGE OF RESULTS

Serum C-peptide values are reportable in the range of 0.03 to 2.00 pmol/mL. Values below the detection limit (0.03 pmol/mL) are repeated for verification, and values above 2.00 pmol/mL are reanalyzed at an appropriate dilution factor.

## 10. QUALITY CONTROL (QC) PROCEDURES

Two types of QC systems are used in this analytical method. With the first, "sample precision QC," 5% of specimens are randomly selected and analyzed either within-assay or between-assay for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is repeated. With the second type of QC system, "batch QC" specimens are placed before and after all specimens to be analyzed.

The batch QC pools consist of four levels of control pools that cover the spectrum of C-peptide ranges for both normal and diabetic populations. The bias limit is set at 1 SD or the 67% limit; the warning limit (WL) is the 2 SD or the 95% limit, and the control limit (CL) is the 3 SD or the 99% limit.

The precision and accuracy for a set of controls used for NHANES III are shown in Table 5.

Table 5
Precision and Accuracy

| Pool | Mean | 95% limits | 99% limits | 95% limits, range | 99% limits, range | Runs | Total CV, % |
|---|---|---|---|---|---|---|---|
| L7 | 0.265 | 0.187-0.343 | 0.148-0.382 | 0.107 | 0.134 | 20 | 14.73 |
| L8 | 1.279 | 1.025-1.533 | 0.898-1.660 | 0.243 | 0.301 | 20 | 9.96 |
| L9 | 3.311 | 2.875-3.747 | 2.657-3.965 | 0.728 | 0.933 | 20 | 6.60 |
| IH6 | 1.090 | 0.932-1.248 | 0.853-1.327 | 0.348 | 0.452 | 20 | 7.25 |

After each assay run, all control data are recorded on the daily worksheet. The analysis is accepted or rejected according to the guidelines established by NHANES, with a slight modification on the determination of a trend.

The quality of an assay is assessed by two types of Levey-Jennings quality chart plots. The first chart plots the mean of all the replicate determinations in a run. It is then compared with the target mean, which is the overall mean established by the 20 characterization runs.

a. The NHANES guideline declares a system "out-of-control" if any of the following events occur for any one of the QC materials:

   - The mean from a single control falls outside the 99% confidence limit.
   - The means from two controls fall either above or below the 95% confidence limit.
   - The daily means of one control from eight successive runs (excluding the runs in which the mean is within 1 SD or bias range) falls either all above or all below the center line.

b. The second type of QC chart plots the range of the replicates (the difference between the highest and lowest value of a single control within a run). It is compared with the target range which is the overall mean of daily ranges established by the 20 characteristic runs.

   The NHANES guideline declares a system as "out-of-control" if any of the following events occur for any one of the QC materials:

   - The daily range from a single control falls outside the 99% confidence limit.
   - The daily ranges from two controls fall either above or below the 95% confidence limits.
   - The daily ranges of one control from eight successive runs fall either all above or all below the center line.

If the system is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the cause of the problem before any further analysis of specimens.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

When the system is declared "out of control," take the following steps:

a. Recount the entire run.

b. Troubleshoot the system to locate the probable cause of the problem (i.e., spilled tubes, partial loss of a pellet, pipeting error, problem with one level of standard, etc.). If a cause can be identified and corrected, notify the supervisor. The supervisor will evaluate the situation and determine whether to accept or reject the run.

c. If no obvious cause of a problem can be identified, reject the run and reanalyze all the specimens.

d. Document the problem and any actions taken in the daily worksheet.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

In insulin-treated patients with insulin antibodies, a substantial amount of proinsulin might be bound to the antibodies. To prevent interference of pro-insulin with the C-peptide assay, use a PEG precipitation to remove the antibody-bound proinsulin.

Radioactive substance therapy may introduce inaccuracies in test results.

## 13. REFERENCE RANGES (NORMAL VALUES)

a. NOVO Laboratory reference range for C-peptide immunoreactivity in 20 fasting nondiabetics was 0.24 - 0.72 pmol/mL (mean 0.45 pmol/mL) (8).

b. Reference ranges were established in the Diabetes Diagnostic Laboratory in May of 1991 and are based on results from 31 nonobese, nondiabetic adults (mean age 28.1 years). Volunteers were given 360 calories in a Sustacal Challenge (Mead Johnson) and 1 week later given a 75-gram glucose load (Oral Glucose Tolerance Test). Reference observed ranges for both challenges are shown in Table 6.

Table 6
Mean and Observed Ranges for C-peptide (pmol/mL)

| | | Fasting | 1 hour | 2 hour |
|---|---|---|---|---|
| Sustacal | Mean | 0.578 | 1.697 | 1.096 |
| Sustacal | Range | 0.216-1.016 | 0.534-3.884 | 0.267-2.572 |
| OGTT | Mean | 0.561 | 2.662 | 1.948 |
| OGTT | Range | 0.266-1.079 | 0.888-4.893 | 0.600-3.168 |

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may reach and maintain 20-25 $^{o}$C during analysis. Specimens are returned to $\leq$-70 $^{o}$C storage as soon as the analysis is completed. Repeated freeze/thawing, except as necessary for specimen analysis, should be avoided.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Since the radioimmunoassay is complex and the characteristic of one antibody is very different from that of another, there are no acceptable alternative methods of analysis. If the analytical system fails, all specimens are returned to storage at $\leq$-70 $^{o}$C. The specimens are reanalyzed when the system is back in control.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (eg., electronic - DBASE, floppy disk) should be used to track the specimens. We recommend that records (including specimen data, QC data and shipping information) be maintained for 3 years.

The only means of identifying a specimen should be its unique NCHS or UMC identification number.

Excess serum remaining after analysis is returned to the NCHS repository in Rockville, MD.

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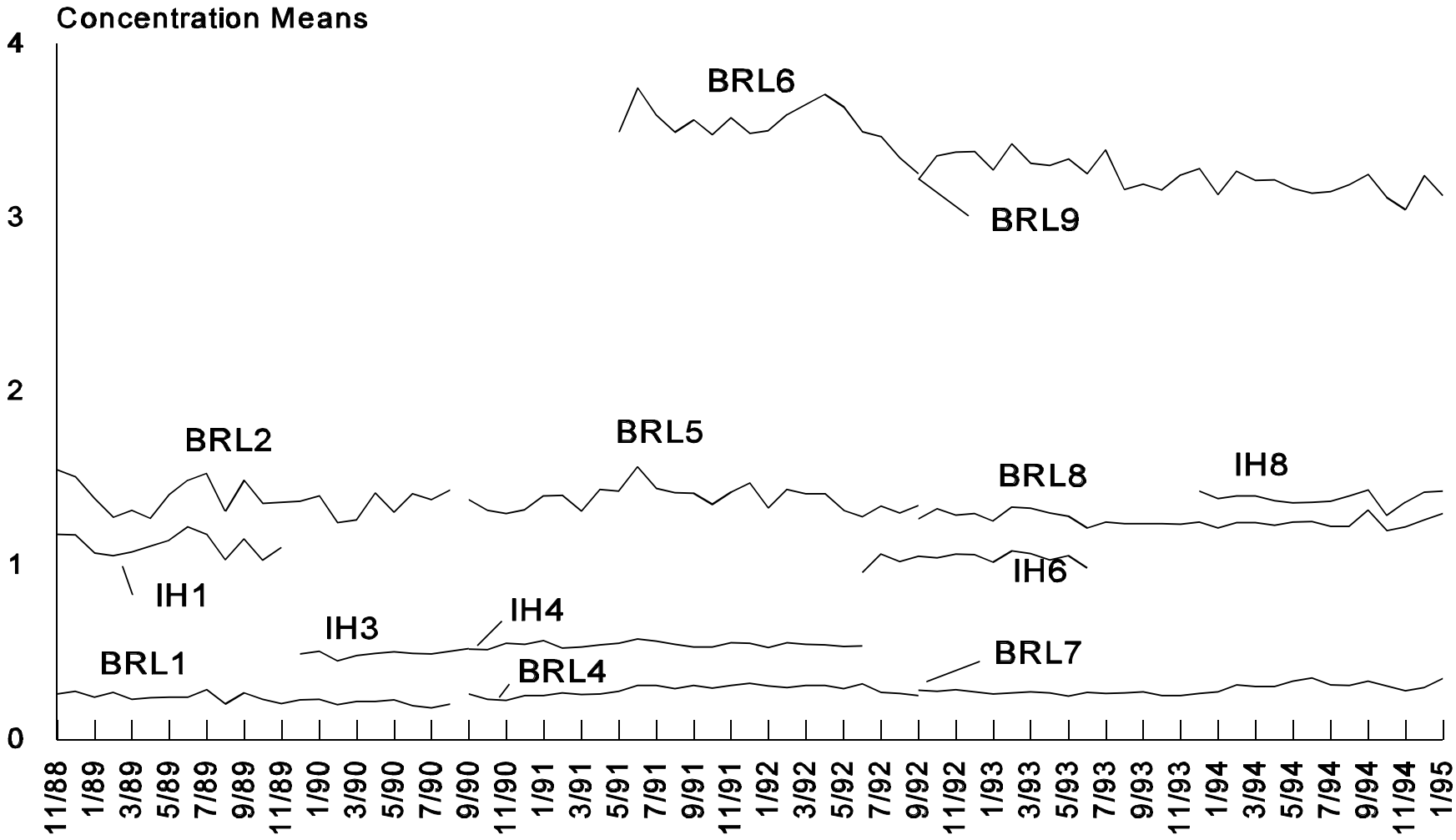
SUMMARY STATISTICS FOR
C-PEPTIDE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| IH1 | 11/15/88 - 11/23/89 | 1.1204 | 0.11562 | 10.3193 | 277 |
| IH3 | 12/13/89 - 10/04/90 | 0.4985 | 0.05162 | 10.3557 | 253 |
| IH4 | 09/27/90 - 06/23/92 | 0.5444 | 0.04980 | 9.1480 | 640 |
| IH6 | 06/25/92 - 06/30/93 | 1.0482 | 0.08584 | 8.1895 | 364 |
| IH8 | 12/30/93 - 01/13/95 | 1.3847 | 0.10878 | 7.8554 | 336 |
| BRL1 | 11/15/88 - 09/28/89 | 0.2309 | 0.04784 | 20.7208 | 565 |
| BRL2 | 11/15/88 - 08/22/90 | 1.3863 | 0.16469 | 11.8802 | 557 |
| BRL4 | 09/09/90 - 09/16/92 | 0.2878 | 0.04787 | 16.6349 | 751 |
| BRL5 | 09/09/90 - 09/16/92 | 1.3824 | 0.13905 | 10.0588 | 748 |
| BRL6 | 05/10/91 - 09/16/92 | 3.5374 | 0.26397 | 7.4625 | 532 |
| BRL7 | 09/23/92 - 01/13/95 | 0.2867 | 0.04307 | 15.0221 | 825 |
| BRL8 | 09/23/92 - 01/13/95 | 1.2611 | 0.10062 | 7.9790 | 828 |
| BRL9 | 09/23/92 - 01/13/95 | 3.2474 | 0.23758 | 7.3158 | 825 |

# C-Peptide Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

### a. Total Cholesterol

Cholesterol is measured enzymatically in serum or plasma in a series of coupled reactions that hydrolyze cholesterol esters and oxidize the 3-OH group of cholesterol (1,2). One of the reaction byproducts, $H_2O_2$, is measured quantitatively in a peroxidase-catalyzed reaction that produces a color. Absorbance is measured at 500 nm. The color intensity is proportional to cholesterol concentration. The reaction sequence is as follows:

$$\text{Cholesterol ester} + H_2O \xrightarrow[\text{hydrolase}]{\text{cholesterol ester}} \text{cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{choles-4-en-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenatone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + H_2O$$

Elevated concentrations of cholesterol increase the risk for coronary heart disease (CHD). Cholesterol is measured to help assess risk status and to follow the progress of treatment designed to lower serum cholesterol concentrations. Desirable cholesterol levels are below 200 mg/dL in adults and below 170 mg/dL in children (3).

### b. Triglycerides

Triglycerides are measured enzymatically in serum or plasma by using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol (1). Glycerol is then oxidized by using glycerol oxidase, and $H_2O_2$, one of the reaction products, is measured quantitatively in a peroxidase-catalyzed reaction that produces a color similar to that for cholesterol. Absorbance is measured at 500 nm. The reaction sequence is as follows:

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol-3-phosphate} + \text{ADP}$$

High levels of serum triglyceride help mark conditions that are associated with increased risk for CHD and peripheral atherosclerosis. Although it is not yet entirely clear whether hypertriglyceridemia constitutes an independent risk factor for atherosclerosis, it is known to increase risk markedly in patients with other risk factors such as low HDL-cholesterol levels or in some groups of patients with elevated apolipoprotein B concentrations. Desirable triglyceride levels are those below 250 mg/dL (3). Triglycerides are also measured because the value is used to calculate low density lipoprotein (LDL)-cholesterol concentrations.

### c. High density lipoprotein (HDL) cholesterol

HDL-cholesterol is measured following the precipitation of the other lipoproteins with a polyanion/divalent cation mixture.

Low serum concentrations of HDL-cholesterol are associated with increased risk for CHD. Coronary risk increases markedly in the concentration range of 30-40 mg/dL. A low HDL-cholesterol concentration is below 35 mg/dL. HDL-cholesterol values are also used in the calculation of LDL-cholesterol.

### d. LDL-cholesterol

Most of the circulating cholesterol is found in three major lipoprotein fractions: very low density lipoproteins (VLDLs), LDLs, and HDLs (5).

$$\text{Total Cholesterol} = (\text{VLDL Chol}) + (\text{LDL Chol}) + (\text{HDL Chol})$$

LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol according to the formula:

$$\text{LDL-cholesterol} = (\text{total chol}) - (\text{HDL chol}) - \frac{\text{TG}}{5}$$

where [TG]÷5 is an estimate of VLDL-cholesterol, and all values are expressed in mg/dL.

LDL carries most of the circulating cholesterol in man, and when elevated, contributes to the development of coronary atherosclerosis. LDL-cholesterol is measured to assess risk for CHD and to follow the progress of patients being treated to lower LDL-cholesterol concentrations. Desirable levels of LDL-cholesterol are below 130 mg/dL in adults and 110 mg/dL in children.

## 2. SAFETY PRECAUTIONS

Treat all serum specimens for analysis as potentially positive for infectious agents, including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eyewear, and lab coat during all steps of this method because of infectious hazards.

Discard all used gloves, vials, pipettes and other items that contact specimens in a biohazard box lined with a red plastic bag. Clean contaminated work areas with sodium hypochlorite (1 part bleach/99 parts water) and dispose of the wipes in a red biohazard bag. The bags are picked up daily by housekeeping for proper disposal. Clean all work benches at the end of each day with 10% sodium hypochlorite, and cover the lab bench with plastic-backed white paper.

Adhere to the CDC Guidelines for Prevention of HIV Infection in Health Care Workers (6).

Material safety data sheets (MSDSs) for the cholesterol reagent, triglyceride reagent, manganese chloride, heparin, dextran sulfate, sodium chloride, sodium bicarbonate and sodium hypochlorite are kept in the Lipid Reference Laboratory.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Analytical data is downloaded from the automated analyzer to a computer and then to computer-generated NHANES logs. When all analyses for specimens are completed and reviewed, the results of the NHANES logs are sent via diskette to NCHS. The QC data is sent upon request to NCHS. Any values that are "panic" or critical values are communicated to NCHS via telephone or FAX.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. A 12-hour fasting specimen should be obtained. Recent food intake exerts little effect on plasma total cholesterol concentration. Plasma triglycerides, however, increase in postprandial plasma to an extent related to the fasting triglyceride levels and the amount of fat intake. This is due to the appearance of chylomicrons in the circulation after an ingestion of a fat-containing meal. Chylomicrons are normally cleared within 9-12 hours, and no chylomicrons should be present after a 12-hour period of fasting (7,8).

b. Serum is the preferred sample type for this assay, although plasma may be used in certain instances. In general, anticoagulants exert osmotic effects in which water leaves the cells and enters the plasma, thus diluting the plasma and lowering the concentrations of nondiffusible components. The magnitude of this effect depends on the anticoagulant used and its concentration. Serum cholesterol and triglyceride concentrations are 3-5% higher in serum than in EDTA plasma, although no significant serum-plasma difference was observed for HDL-cholesterol (9). Thus, the serum concentrations of lipids and lipoproteins probably reflect more accurately the subjects' physiological state at the time of venipuncture. Serum is used for NHANES III.

c. The sample volume required for the total cholesterol measurement is 0.2 mL; for triglycerides 0.2 mL; and for HDL-cholesterol, 2.0 mL. Any remaining sample will be used to perform repeat analyses of total cholesterol, triglyceride, and HDL-cholesterol.

d. The NHANES III study sends polypropylene screw-capped 5.0-mL vials for sample shipment and storage of NHANES III samples. For other studies, a glass serum bottle equipped with a rubber stopper and sealed with an aluminum seal may be used as indicated by the study director.

e. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Serum should be stored at $\leq$-20 $^{o}$C for no longer than 4 weeks. For permanent storage freeze samples at $\leq$-70 $^{o}$C. Total cholesterol and triglyceride will be stable for at least 1 year, possibly longer. HDL-cholesterol should be stable for at least 1 year at this temperature.

f. Hemolysis can interfere with absorbance readings (8). Lipemia can affect the triglyceride measurements by interfering with absorbance measurement (10). When a sample is flagged as turbid by the analyzer, the sample is diluted (1 part sample+1 part saline) and reanalyzed. This is done for samples with triglyceride values exceeding 300 mg/dL and cholesterol values exceeding 400 mg/dL.

g. Any inadequate specimens received by the lab are noted on the log sheet along with the problem codes. If necessary, a call is placed to the originator to alert the Mobile Examination Center (MEC) to the problem. Such problems can include cracked vials, inadequately sealed vials, empty vials, gross hemolysis, and thawed samples.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS, (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Hitachi 704 Analyzer performs cholesterol, triglyceride, and HDL-cholesterol analyses (Boehringer Mannheim Diagnostics, Indianapolis, IN).

Cholesterol and triglycerides are simultaneously measured enzymatically. Triglyceride blanks are measured with the CDC control materials and with the same reagent, but without lipase. It is necessary to run blanks in CDC quality control materials because some of these pools acquire significantly high blanks (50-80 mg/dL) during the preparation of the pools. The instrument analyzes triglycerides from the same sample cups used for cholesterol.

(2) Damon ICE 600 centrifuge (Baxter Scientific Products).

(3) Blood-mixing rotator (Orbitron, Inc., Baekel, IND).

(4) Vortex Genie 2 mixer (Scientific Industries, Bohemia, NY).

(5) Mettler balance (Mettler Instrument Corp., Hightstown, NJ).

### b. Other Materials

(1) Cholesterol High Performance System Pack Reagents, cat. no. N270412 (Boehringer Mannheim Diagnostics, Indianapolis, Indiana). Includes "Preci-cal" calibrator solutions.

(2) Triglycerides/GPO reagent system pack, cat. no. 816370 (Boehringer Mannheim Diagnostics).

(3) Manganese chloride (Sigma Chemical Co., St. Louis, MO).

(4) Heparin (Sigma).

(5) Dextran sulfate (Sigma).

(6) Sodium bicarbonate (Sigma).

(7) Glycerol standard solution (Boehringer Mannheim Diagnostics).

(8) Unesterified cholesterol standard (Boehringer Mannheim Diagnostics).

(9) Cholesterol and triglyceride control pools (Q); HDL-cholesterol control pools (MQ and AQ) (Centers for Disease Control and Prevention (CDC), Atlanta, GA).

(10) 13- X 100-mm tubes, borosilicate, disposable (Baxter Scientific Products, Columbia, MD).

(11) 20-mL glass tube (Research Products International, Mount Prospect, IL).

(12) 1-mL disposable Eppendorf tube (Baxter Scientific Products).

(13) Sodium chloride, NaCl (Sigma Chemical Co., St. Louis, MO).

(14) Disposable, plastic transfer pipet (Baxter Scientific Products).

(15) Sample cups, disposable (Boehringer Mannheim Diagnostics).

(16) Latex gloves (Columbia Diagnostics, Springfield, VA).

(17) SMI fixed-volume pipette, 500-µL, and precision, disposable pipet tips (Baxter Scientific Products).

(18) SMI adjustable-volume pipette, 20- to 100-µL and disposable pipet tips (Baxter Scientific Products).

(19) Small plastic weighing boats (Baxter Scientific Products).

c. **Reagent Preparation**

(1) Cholesterol reagents

The components of Cholesterol High Performance System Pack Reagents include:

(a) Cholesterol reagent (16- x 50-mL)

Reactive ingredients:

- 0.20 mmol 3,4-dichlorophenol
- 0.30 mmol phenol
- 0.05 mmol 4-aminophenazone
- 12.5 U cholesterol oxidase (Nocardia erythropolis; 25 °C)
- 20 U cholesterol esterase (microorganism; 25 °C)
- 10 U peroxidase (horseradish; 25 °C)

Nonreactive ingredients:

- Buffer, stabilizers, preservative

Store the cholesterol system pack reagents at 2-8 °C. For stability of the unopened components, refer to the box or bottle labels for expiration dates.

(b) Working solution (R1)
Reconstitute the contents of one cholesterol reagent vial with exactly 50 mL distilled or deionized water. Mix gently. The R1 working solution is ready for use after 10 min. The solution is stable for 4 weeks at 4-8 °C or 7 days at 20-25 °C when protected from light and contamination by microorganisms.

(2) Triglyceride reagents

(a) Triglyceride reagents
Intended for in vitro diagnostic use. The system pack includes buffer and enzymes that are added on

reconstitution. The reactive ingredient is 0.175 mmol 4-chlorophenol.

(b) Working solution (R1)
Connect one bottle buffer/enzymes to one bottle GPO using one of the enclosed adapters. Mix the solution gently by inversion. Completely dissolve the lyophilisate in the buffer. The R1 working solution is stable for 2 weeks at 4-8 °C or 2 days at 20-25 °C.

(3) HDL-cholesterol reagents

(a) Manganese chloride stock solution (1.12 mol/L)
Store manganese chloride in a tightly closed container to minimize water uptake. Add 22.16 g manganese chloride ($MnCL_2$)·4 $H_2O$ to a 100-mL volumetic flask, dissolve, and dilute to volume with distilled water. Store the solution at 4-8 °C and prepare fresh monthly.

(b) Heparin stock solution, 20,000 USP units/mL (135 mg/mL)
Prepare a solution of 20,000 USP units/mL by diluting heparin solution (40,000 units/mL) with distilled water. The weight concentraiton is approximately 135 mg/mL. Store the solution at 4-8 °C and prepare fresh every 2 weeks.

(c) Combined heparin-MnCl working solution
Prepare a 0.56 M working $MnCl_2$ stock solution to a total volume of 500 mL with distilled water. Prepare the combined reagent by adding 6.0 mL heparin stock solution (135 mg/mL) to 50 mL $MnCl_2$ working solution. The solution is stable at 4-8 °C until the expiration date on the bottle label.

(d) Sodium bicarbonate, $NaHCO_3$, 1 mol/L
Dissolve 8.4 g $NaHCO_3$ in distilled water and dilute to 100 mL. The solution is stable for 6 months when stored at 20-25 °C.

d. **Standards Preparation**

(1) Cholesterol
"Preci-cal" aqueous standard of unesterified cholesterol stabilized in solution with detergents and alcohol. Provided by the manufacturer ready to use.

(2) Triglyceride
Aqueous standard solution of glycerol. Concentration expressed in mmol/L, or in mg/dL expressed as equivalent tristearin concentration. Provided by the manufacturer ready to use.

e. **Preparation of Quality Control Materials**

Cholesterol and triglyceride control pools (Q) and HDL-cholesterol control pools (MQ and AQ) are prepared by CDC. Reference values for each pool are assigned by CDC using CDC reference methods for cholesterol, triglyceride, and HDL-cholesterol. Two Q pools have normal and elevated concentrations to control total cholesterol and triglyceride analyses. MQ pools are used to control cholesterol analysis in the HDL-cholesterol concentration range, and AQ pools are carried through the entire HDL separation and analysis procedure to control HDL-cholesterol analyses.

These pools are also analyzed in duplicate in every analytical run for a maximum of 50 runs. They are used to assess accuracy with respect to CDC reference methods and analytical precision.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

(1) A calibration sequence must be performed to ensure accurate chemistry results on the Hitachi 704. This calibration establishes the calibration factors. The factor converts the electronic response of the instrument into concentration or activity for the constituent being measured.

(2) Analyze a blank sample in duplicate for each parameter to determine the reagent blank absorbance and

establish a baseline. When reagents are added to the blank sample in the reaction cell, the final absorbance readings reflect the absorbance of the reagents. Average the absorbance readings for the two blank samples and store the mean blank absorbance in memory.

(3) Analyze the calibrator in duplicate. Average the absorbance readings and store the mean calibration value in memory. The instrument will calulate the calibration factor.

(4) The instrument retains two sets of calibration data for each test (current and previous). The computer updates the current calibration if the data are acceptable.

(5) A calibration report that contains information on the calibration ID and ABS (absorbance) value is printed by the computer.

(6) The ABS readings are used to establish a linear calibration curve. The slope of the curve is used to calculate results for specimens. Calibration failure occurs automatically when the current ABS readings differ from the usual readings. In the event of a calibration failure, take the following steps: 1) Rerun the calibration curve. If the new curve is satisfactory, continue the continues. 2) If a second failure occurs, analyze a new batch of calibrators. 3) If the curve is still unsatisfactory, contact the BMD service representative. Do not perform analyses until the problem is resolved.

b. **Verification**

In order to verify instrument calibration, analyze the Q Pools in duplicate and compare the results to CDC reference values. Cholesterol and triglyceride results must be within CDC standardization criteria for accuracy and precision and must be within laboratory quality control limits set for the respective analytes/pools. These pools are analyzed in duplicate with each analytical run.

Current CDC standardization criteria are as follows (11):

**Cholesterol:**

Accuracy: $\pm$3% of CDC reference values
CV: $\leq$3%

**Triglycerides**:

Standardization criteria for triglyceride are shown in Table 1.

Table 1
CDC Standardization Criteria for Triglycerides

| Concentration (mg/dL) | Maximum Bias | Maximum Standard Deviation |
|---|---|---|
| 0-88 | ±9 | 7 |
| 89-176 | ±10 | 10 |
| 177-220 | ±11 | 10 |
| $\geq$221 | (0.05)(reference value) | (0.05)(reference value) |

**HDL-cholesterol:**

Standardization criteria for HDL-cholesterol are shown in Table 2.

Table 2
CDC Standardization Criteria for HDL-Cholesterol

| Concentration (mg/dL) | Accuracy | Maximum Standard Deviation |
|---|---|---|
| <40 | ± 10% of reference value | 2.5 |
| 40-60 | ± 10% of reference value | 3.0 |
| >60 | ± 10% of reference value | 3.5 |

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Receipt of samples

When the samples arrive, log them into the laboratory by batch according to the shipping transmittal that accompanies the samples. Note the receipt date and the condition of the samples (OK, thawed, sample missing, etc.,) using the appropriate sample condition code on the transmittal. Transfer the samples to a freezer at ≤-70 °C for storage until they are to be analyzed.

(2) Perform daily maintenance procedures as outlined in Section 8.g.1.

### b. Preparation of Samples

(1) Remove the vials from the freezer and allow them to thaw in an upright position at 20-25 °C. Place the sealed vials on a blood-mixing roller for 30 min at 20-25 °C to ensure complete mixing. Remove the caps from the vials and remove aliquots of the samples for the appropriate tests.

Store specimens at 4-8 °C after they have been thawed and aliquoted. Analysis should be performed within the first 2 days after the specimen thaws and repeat analysis should be performed within 6 days.

(2) Sample preparation for HDL-cholesterol

(a) Add 100 µL of of heparin sulfate-$MnCl_2$ mixture to 1 mL of serum for each sample. The reaction will yield apo-B-containing lipoprotein, precipitate, and soluble HDL-cholesterol

(b) Remove precipitate by centrifuging at 1500 x g for 30 min. Remove the clear supernatant and place it into a 20-mL glass vial.

(c) Place 500 µL of the supernatant and 50 µL sodium bicarbonate into an Eppendorf tube. Vortex intermittently. Let the tubes stand at 20-25 °C for 10 min; then centrifuge at 10,000 x g for 2 min.

(d) Measure HDL-cholesterol in clear supernatant.

### c. Instrument Setup

(1) Cholesterol The instrument settings for cholesterol are shown in Table 3.

Table 3
Instrument Settings for Cholesterol

| Parameter | Setting |
|---|---|
| Temperature | 37 °C |
| Test | [CHOL] |
| Assay Code | [1POINT] : [32]-[0] |
| Sample Volume | [5] |
| R1 Volume | [500][50][NO] |
| R2 Volume | [ 0][50][NO] |
| Wavelength | [700][505] |
| Calib. Method | [LINEAR][0][0] |
| Std (1) Conc.-Pos. | [0]-[1] |
| Std (2) Conc.-Pos. | [___]-[_]* |
| Std (3) Conc.-Pos. | [ 0]-[0] |
| Std (4) Conc.-Pos. | [ 0]-[0] |
| Std (5) Conc.-Pos. | [ 0]-[0] |
| Std (6) Conc.-Pos. | [ 0]-[0] |
| Unit | MG/DL |
| SD Limit | [0,1] |
| Duplicate Limit | [100] |
| Sensitive Limit | [1600] |
| ABS. Limit (INC/DEC) | [0][INC.] |
| Prozone Limit | [0][LOWER] |
| Expected Value | [0]-[239] |
| Instrument Factor | [1.00] |

* ___ denotes user or instrument specific settings

(2) Triglyceride The instrument settings for triglyceride are shown in Table 4.

(3) HDL-cholesterol The instrument settings for HDL-cholesterol are shown in Table 5.

(4) Calibration

The Hitachi 704 analyzer is calibrated at the beginning of each day of use. "Preci-cal" calibrators are used to calibrate the total cholesterol (standard curve of 0, 50, 150 and 400), HDL-cholesterol (standard curve of 0, 50 and 100) and triglyceride (unblanked reference value = 134). Calibrators are obtained from the manufacturer and are used without further preparation.

(a) Full calibration:

If the CRT display is part of the Routine Job Menu, press NEXT or BACK to move to the Calibrator &

Control Test Selection display.

If the CRT display is not within the Routine Job Menu, press ROUTINE, then press 3 ENTER.

(1) The instrument stores operating parameters and calibration data for 2 sets of 20 chemistries. These two sets of chemistries are referred to as PHASE 1 and PHASE 2. The two phases are defined in the channel assignment display.

To indicate which set of chemistries is being used, type the phase number 1 or 2, and press ENTER.

Table 4
Instrument Settings for Triglyceride

| Parameter | Setting |
|---|---|
| Temperature | 37 °C |
| Test | [TRIG] |
| Assay Code | [1POINT] : [32]-[0] |
| Sample Volume | [5] |
| R1 Volume | [500][50][NO] |
| R2 Volume | [ 0][50][NO] |
| Wavelength | [700][505] |
| Calib. Method | [LINEAR][0][0] |
| Std (1) Conc.-Pos. | [0]-[1] |
| Std (2) Conc.-Pos. | [___]*-[2] |
| Std (3) Conc.-Pos. | [ 0]-[0] |
| Std (4) Conc.-Pos. | [ 0]-[0] |
| Std (5) Conc.-Pos. | [ 0]-[0] |
| Std (6) Conc.-Pos. | [ 0]-[0] |
| Unit | MG/DL |
| SD Limit | [0] |
| Duplicate Limit | [130] |
| Sensitive Limit | [1500] |
| ABS. Limit (INC/DEC) | [0][INC.] |
| Prozone Limit | [0][LOWER] |
| Expected Value | [0]-[200] |
| Instrument Factor | [1.00] |

* ___ denotes user or instrument specific settings

Table 5
Instrument Settings for HDL-Cholesterol

| Parameter | Setting |
|---|---|
| Temperature | 37 °C |
| Test | [HDL] |
| Assay Code | [1POINT] : [30]-[0] |
| Sample Volume | [20] |
| R1 Volume | [350][50][NO] |
| R2 Volume | [ 0][50][NO] |
| Wavelength | [700][505] |
| Calib. Method | [LINEAR][0][0] |
| Std (1) Conc.-Pos. | [0.0]-[1] |
| Std (2) Conc.-Pos. | [___]*-[0] |
| Std (3) Conc.-Pos. | [ 0]-[0] |
| Std (4) Conc.-Pos. | [ 0]-[0] |
| Std (5) Conc.-Pos. | [ 0]-[0] |
| Std (6) Conc.-Pos. | [ 0]-[0] |
| Unit | MG/DL |
| SD Limit | [0.1] |
| Duplicate Limit | [100] |
| Sensitive Limit | [2500] |
| ABS. Limit (INC/DEC) | [0][INC.] |
| Prozone Limit | [0][LOWER] |
| Expected Value | [35]-[65] |
| Instrument Factor | [3.00] (or 3.5)** |

* ___ denotes user or instrument specific settings

** Use the "Preci-cal" cholesterol standard (50 mg/dL for calibration of the HDL channel). Do not precipitate the standard. To correct for dilution, enter the instrument factor as [3.0] for the macro assay or [3.5] for the semimicro assay.

(2) Press 1 ENTER to specify "Start-Up" calibration.

(3) Press 1 ENTER to select tests for the blank (saline). If the test selection for the blank is stored in memory, the tests in memory appear at the right margin of the display.

(4) Activate the appropriate test or profile keys for those tests that require a blank calibrator, then press ENTER. (Each test key is activated when its LED is illuminated.) The tests assigned appear at the right margin of the display, and the STANDARD TYPE entry field displays "STD 2-6."

(5) Press the appropriate test keys for those tests that require a standard or standards, then press ENTER. The tests assigned appear at the right margin of the display and the STANDARD TYPE entry field displays "ISE 1,2."

Advance the cursor to the CALIB LOAD LIST entry field. Press 1 ENTER.

(6) Without running the controls, update the system disk with the calibrator test selection as follows:

Advance the cursor to FD READ/WRITE.
Press 2 ENTER.
The CRT displays: "WRITE OK?"
Press 1 ENTER (YES).

*NOTE: It is not absolutely necessary to write calibrator test selection data on the system disk. However, if the laboratory experiences a power failure or electrical "brown-out", this step will prevent permanent loss of test selection information.Wait while the system disk is updated, then proceed to ROUTINE PATIENT TEST SELECTION (Section 2.1.6).*

(b) Blank calibration

If the CRT display shown is within the Routine Job Menu, press NEXT or BACK to move to the Calibrator & Control Test Selection display.

If the CRT display shown is not within the Routine Job Menu, press ROUTINE, then press 3 ENTER.

1) The instrument stores operating parameters and calibration data for two sets of 20 chemistries. These two sets of chemistries are referred to as PHASE 1 and PHASE 2. The two phases are defined in the channel assignment display.

To indicate which set of chemistries is being used, type the phase number 1 or 2, then press ENTER.

2) Press 1 ENTER to specify Start-Up calibration.

3) Press 1 ENTER to select tests for the blank (saline) update. If previous test selections for blanks are stored in memory, the tests in memory will appear at the right margin of the display.

4) Activate the appropriate test or profile keys for those tests requiring a blank update, then press ENTER. (Each test key is activated when its LED is illuminated.) The tests assigned appear at the right margin of the display, and the STANDARD TYPE entry field displays "STD 2-6."

5) Deselect all previously selected tests so only tests are selected for STD 2-6, then press ENTER. No tests should appear at the right margin of the display, and the STANDARD TYPE entry field now displays "ISE 1,2."

5) Press ROUTINE and 4 ENTER, and the Start Conditions screen will appear on the display.

6) Enter the START SAMPLE NO., and request START UP CALIBRATION. Verify that a control interval of $\geq$1 has been selected. (Tests requiring controls were selected in routine job no. 3).

7) Press START to begin the calibration.

e. **Operation of Assay Procedure**

(1) The sample ID numbers for total cholesterol and triglyceride are keyed into the Hitachi 704 analyzer. HDL-cholesterol is analyzed on a separate run.

(2) Using a disposable plastic transfer pipet, place 100 µL of each sample into the disposable sample cups on the instrument in the order of analysis. Analyze the HDL-cholesterol using the clear supernatant from the prepared Eppendorf tube. Place the quality control samples and calibrators into their assigned positions on the instrument and begin the analysis. The results are printed on the Hitachi printout and are also sent to the laboratory computer file.

(3) Inspect the results for the quality control samples. If the run is within control limits, transcribe the results on the NHANES log sheet and enter them into the computer log sheets. If the quality control results exceed control limits, identify the source of the difficulty, correct the problem, and repeat the entire analytical run.

f. **Recording of Data**

(1) Quality control data

When results are down-loaded from the Hitachi 704 analyzer to the Quality Control and NHANES III logs, they are compared with the acceptable ranges by the technician. If a quality control pool is "out of control", the run is repeated. If a specimen exceeds the acceptable values, it is reanalyzed. If the triglyceride test is repeated, the specimen is diluted 1:2. Turbid samples are often performed on dilution for the initial analysis. In extremely turbid samples, it may be necessary to dilute 1:4 or greater.

(2) Analytical results

When analysis of the specimens is complete, the data are reviewed by the data coordinator or by the senior lab technician (preliminary review). After all necessary repeat analyses are completed, the results are then reviewed by the laboratory supervisor, data coordinator, or laboratory director (final review).

The results are compared with ranges determined from the normal population distributions for adults and children. Any specimen whose value is outside the respective range is reanalyzed. The expected ranges for total cholesterol, triglyceride, and HDL-cholesterol are as shown in Table 6.

Table 6
Expected Lipid Ranges in Normal Population (7,12,13)

| Analyte | Normal Range for Children ≤ 12 Years Old (mg/dL) | Normal Range for People ≥ 13 Years Old (mg/dL) |
|---|---|---|
| Cholesterol | 125-250 mg/dL | 140-300 mg/dL |
| Triglyceride | 25-300 mg/dL* | 25-300 mg/dL* |
| HDL-Cholesterol | 25-85 mg/dL | 25-85 mg/dL |

*If the sample is turbid, the triglyceride upper limit is 600 mg/dL and the analysis is repeated on a dilution (1:2). Samples with HDL-cholesterol values less than half the total cholesterol values are reanalyzed for total cholesterol.

g. **Replacement and Periodic Maintenance of Key Components**

(1) Perform the following procedures at the beginning of each work day:

(a) Check the water supply.

(b) Check the Extran supply.

(c) Prepare reagents, controls and calibrators as needed.

(d) Exchange incubation bath water by pressing MAINTENANCE and then pressing 1, ENTER.

(e) Perform a photometer check.

(f) Perform an air purge from the "Start Conditions" display.

(g) Wipe the sample and reagent probes.

(h) Adjust the probes.

(2) Perform the following procedures weekly.

(a) Clean the cell rinse unit nozzles.

(b) Clean the probe/stirrer rinse baths.

(c) Execute CELL BLANK.

(d) Clean the distilled water reservoir.

(e) Clean the inlet water filter.

(f) Clean the stirrer.

(3) Perform the following maintenance procedures monthly.

(a) Replace the reaction cells.

(b) Clean the reaction baths.

(c) Inspect the vacuum reservoir.

(d) Clean the sample disk.

(e) Clean the reagent disk.

(4) Perform the following maintenance procedures quarterly.

(a) Clean the fans and condenser.

(b) Clean the disk drive heads.

(c) Replace the reagent seal.

(d) Replace the pinch tubing.

(5) Perform the following maintenance procedures twice a year.

(a) Replace the sample seals.

(b) Replace the REF and GND electrodes.

h. **Calculations**

(1) Cholesterol and triglyceride concentrations

The Boehringer Mannheim/Hitachi 704 microcomputer uses absorbance measurements to calculate cholesterol and triglyceride concentrations as follows:

$$Cx = K(Ax - Ab) + Cb$$

*Where:*

*Cx = Concentration of sample*
*K = Concentration factor (determined during calibration)*
*Ax = Mean of absorbances of sample + R1 read during cycles 31 and 32*
*Ab = Mean of absorbances of blank +R1 read during cycles 31 and 32*
*Cb = Concentration of blank (STD)*

(2) HDL-cholesterol

The Boehringer Mannheim/Hitachi 704 microcomputer uses changes in absorbance measurements to calculate HDL-cholesterol concentrations as follows:

$$Cx = [K(Ax - Ab) + Cb]IF$$

*Where:*

*Cx = Concentration of sample*
*K = Concentration factor (determined during calibration)*
*Ax = Absorbance of sample + R1 read during cycles 29 and 30*
*Ab = Absorbance of blank + R1 read during cycles 29 and 30*
*Cb = Concentration of blank (STD)*
*IF = Instrument factor (dilution correction)*

**9. REPORTABLE RANGE OF RESULTS** (13)

Serum cholesterol values are reportable in the range of 10 to 700 mg/dL without dilution. If the cholesterol value is >700 mg/dL, the specimen should be diluted (1+1) and reanalyzed.

Serum triglyceride values are reportable in the range of 10 to 1000 mg/dL without dilution. If the triglyceride value is >1000 mg/dL, the specimen should be diluted (1+1) and reanalyzed.

Serum HDL-cholesterol values are reportable in the range of 10 to 200 mg/dL without dilution. If the HDL-cholesterol value is >200 mg/dL, the specimen should be diluted (1+1) and re-analyzed.

**10. QUALITY CONTROL (QC) PROCEDURES**

The Central Laboratory monitors its performance by analyzing quality control materials for which the values are assigned by the Centers for Disease Control and Prevention (CDC) using reference methods. The estimates of analytical error obtained from the analysis of quality control materials represent the error obtained with subject samples, and the control samples are subjected to the same analytical manipulation as subject samples.

Two quality control pools (the "Q" Series -- one with normal and one with elevated concentrations) are used to monitor the analysis of total cholesterol and triglyceride. The precision of lipid and lipoprotein analyses is determined from duplicate analysis of each pool analyzed in each run. The CDC establishes the reference values for these pools using reference methods for cholesterol and triglycerides.

The control limits for each pool are calculated from the average and standard deviations of the daily means and daily ranges of the analyses of the control pools. Temporary control limits for each pool are calculated from the first 20 run days. Permanent control limits are determined after 50 run days and then used until the particular pool is exhausted. Continuity from pool to pool is maintained by conducting at least 20 overlapping runs in which the replacement pool is analyzed concurrently with the current pool. The 20-run temporary limits are established for the replacement pool from this period of overlap. During this period, the acceptability of the analyses is based on values obtained for the current pool; that is, the analyses must be "in control" before the data are accepted and used to establish control limits for the replacement pool.

Limits for the new pool are calculated and evaluated, and control charts are prepared. Care is taken to ensure that data used in the calculations are only from runs that are "in control" (i.e., that meet established quality control criteria). As soon as acceptable temporary limits are established, control is transferred to the replacement pool, and the original pool is no longer analyzed. These procedures are used for both of the two Q pools.

It is important that the data used to calculate control limits be collected during a stable analytical period representative of overall laboratory performance. The daily mean for a control pool is calculated for each run by averaging the two values for the pool:

$$\bar{x} = \frac{\sum \text{of control values}}{\text{number of control values}}$$

The overall mean for the pool X is calculated by summing the individual run means and dividing by the number of runs N:

$$\text{overall mean} = \frac{\sum \text{run means}}{N}$$

N = 20 run days for temporary limits
N = 50 run days for permanent limits

The standard deviation (SS) of the run means is also calculated for the control pool. The basic equation for calculating standard deviation is as follows:

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

The range R for each run is the difference between the highest and the lowest value obtained for the pool in that run: Because duplicates are used, the range is the difference between duplicates. The average range R for a series of runs is

$$R = X_{high} - X_{low}$$

calculated by dividing the sum of the ranges for the series by the number of runs:

$$\bar{R} = \frac{\sum R}{N}$$

Again, N=20 for temporary limits and N=50 for permanent limits.

The control limits (99%) for the means chart are calculated as follows and are rounded to the nearest whole number:

$$\text{Lower control limit} = \text{overall mean} - 3SD$$

The warning (95%) limits for the X chart for control pools are calculated rounded to nearest whole number as follows:

$$\text{Upper warning limit} = \text{overall mean} + 2SD$$

$$\text{lower warning limit} = \text{overall mean} - 2SD$$

The limits on X are evaluated.

The limits used for the R chart are calculated by multiplying R for the pool by the following probability factors appropriate for duplicate analyses:

$$\text{Range control limit} = 3.27\bar{R}$$

$$\text{Range warning limit} = 2.46\bar{R}$$

The lower limit for the range chart is zero because there is no negative range.

Before the control chart can be used for quality control, it is reviewed to determine that the data have been collected during a stable analytical period. The chart is examined for outliers, for periods of questionable or unstable performance, and for evidence of excessive bias. An outlier will distort the 99% limits if incorporated into the final calculations. An outlier is considered to be any value of X >3SD limits or any value of R which exceeds the upper control limit for R. These values are eliminated as are values from any questionable period of performance. The values of $\bar{x}$, SD, and the control limits are recalculated, and the charts are evaluated again.

When values from at least 20 acceptable runs are used for the final calculations, the control charts are constructed according to the criteria listed below. If there are not 20 acceptable runs after unacceptable data have been eliminated continue analyzing the pool until at least 20 acceptable runs have been completed.

The criteria used in the control laboratory were those that served as guidelines for the Lipid Research Clinics Program and are designed to minimize both bias and variability. As used in this manual, the bias of the total cholesterol, triglyceride, or HDL-cholesterol measurement is the difference between that measurement and the CDC reference value (RV) for the pool. The bias is calculated as the algebraic difference between the overall mean and the CDC RV.

Construct X and R control charts for each pool on the same piece of graph paper. Draw the overall mean line across the entire sheet and the 2SD and 3SD limit lines parallel to the overall mean line. Draw and label lines to represent the CDC RV. Draw the limit lines and a line to represent R on the R chart. Enter the daily mean and range values on the appropriate chart. The chart should be kept current; the values should be plotted daily. Keep detailed notes on factors affecting the quality of analyses (personnel changes, reagent problems, changes in instrument components, etc.).

- Values for X that are outside the 3SD limit or values of R that are outside the R + 3.27R limit indicate the run is out of control. If it is, the run must be repeated. On the average, one in 100 runs is out of control during normal stable operation. A value outside the 95% limits but not the 99% limits is interpreted as an indication of possible trouble.
- For runs consistently out of control, check the expiration date of the calibrator material and standards. Consult the Hitachi 704 troubleshooting guide and repeat calibration.

If one QC pool fails to maintain the specified ranges, regardless of whether the calibrators are within range, the run is declared "out of control" and the analyses are repeated. Any decisions to override to this rule must be made by the lab director, lab supervisor, or quality control manager.
The precision of the NHANES III data derived from the CDC Q pools for total cholesterol and triglyceride and the MQ and AQ pools for HDL-cholesterol is shown in Tables 7 and 8.

Table 7
Within Run Variability

| | Cholesterol | | Triglyceride | | HDL-Cholesterol | |
|---|---|---|---|---|---|---|
| POOL | Q20 | Q17 | Q20 | Q17 | MQ8 | AQ13 |
| n | 100 | 64 | 99 | 100 | 186 | 187 |
| Mean | 261 | 166 | 196 | 104 | 50 | 50 |
| SD | 4.07 | 2.32 | 5.01 | 3.14 | 0.76 | 3.84 |
| CV (%) | 1.56 | 1.40 | 2.56 | 3.02 | 1.54 | 7.73 |

Table 8
Total Pool Variability

| | Cholesterol | | Triglyceride | | HDL-Cholesterol | |
|---|---|---|---|---|---|---|
| Pool | Q20 | Q17 | Q20 | Q17 | MQ8 | AQ13 |
| n | 100 | 64 | 99 | 100 | 186 | 186 |
| Mean | 213 | 166 | 196 | 104 | 50 | 50 |
| SD | 2.67 | 2.20 | 4.86 | 3.00 | 0.72 | 1.24 |
| CV (%) | 1.25 | 1.33 | 2.48 | 2.89 | 1.46 | 2.49 |

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more quality control samples fall outside the ±2 SD range or a within-run control sample shifts ≥2 SD from its previous value, then take the following steps:

a. Determine the type of errors occurring (random, systematic, or both) based on the control rules being violated.

b. Refer to the BMD troubleshooting guide to inspect the components of the method or instrument that can cause the type of error observed.

c. Correct the problem, then reanalyze the participant's samples and control samples, testing for statistical control by the same procedure.

d. Consult the quality control manager and laboratory director for any decision to report data when there is a lack of statistical control. For NHANES III analyses, no data are taken from "out of control" runs.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. **Cholesterol and Triglyceride**

(1) Hemoglobin: No interference from ≤200 mg/dL.

(2) Lipemia: No interference unless lipemia is marked.

(3) Bilirubin: No interference from ≤12 mg/dL.

(4) The Trinder color reaction is unaffected by common interfering substances such as uric acid, creatinine, and glutathione.

(5) Drug interferences (taken from documentation provided by the manufacturer):

(a) Therapeutic amounts of 38 drugs showed no interference with this procedure.

(b) A two-fold toxic dose of L-methyldopa reduced recovery by 50%.

(c) Noramidopyrine reduced recovery by 20%.

(d) A 10-fold therapeutic concentration of ascorbic acid reduced the recovery of cholesterol by 5%.

b. **HDL-Cholesterol**

If the supernatant is not clear, complete precipitation of LDL and VLDL has not occurred and will result in falsely elevated HDL-cholesterol values. Samples from participants with certain hyperlipidemias and dysproteinemias may not be precipitated adequately.

## 13. REFERENCE RANGES (NORMAL VALUES) (13)

Table 9
Expected Values

| Analyte | Desirable values (mg/dL) | Borderline high (mg/dL) | High (mg/dL) | Low (mg/dL) |
|---|---|---|---|---|
| Cholesterol | <200 | 200-239 | >240 | |
| Triglycerides | <250 | 250-500 | >500 | |
| HDL-Cholesterol | 25-80 | | ≥60 | ≤35 |

Table 10
Reference Ranges for Men

| Age (years) | Triglycerides (mg/dL) | Cholesterol (mg/dL) | HDL-Cholesterol (mg/dL) |
|---|---|---|---|
| 0-7 | 30-75 | 130-195 | 40-70 |
| 8-9 | 25-90 | 125-185 | 40-75 |
| 10-11 | 30-105 | 130-210 | 35-75 |
| 12-13 | 35-110 | 120-200 | 35-75 |
| 14-15 | 30-130 | 115-200 | 35-70 |
| 16-17 | 40-140 | 120-190 | 30-60 |
| 18-19 | 35-145 | 105-195 | 30-60 |
| 20-24 | 45-165 | 120-210 | 30-65 |
| 25-29 | 45-205 | 130-235 | 30-65 |
| 30-34 | 45-255 | 140-260 | 30-65 |
| 35-39 | 50-315 | 145-265 | 30-65 |
| 40-44 | 55-320 | 150-260 | 25-65 |
| 45-49 | 55-280 | 165-275 | 30-65 |
| 50-54 | 65-315 | 155-275 | 30-65 |
| ≥55 | 60-260 | 160-275 | 30-70 |
| Cord Blood | 5-65 | 50-95 | 20-55 |

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**(14)

The results as established by NCHS are as follows:

Total cholesterol >400 mg/dL
Triglyceride >500 mg/dL

There is no critical call value for HDL-cholesterol.

Whenever critical call results are obtained for cholesterol and triglyceride, the sample is reanalyzed immediately to confirm the results.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach and be kept at 20-25 °C during analysis, but should be returned to 4-8 °C for storage. After a run is accepted, samples are frozen at -70 °C for long-term storage.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

The use of alternative methods is inappropriate. If the analytical system fails, store samples at ≤-20 °C is recommended until the system is restored to functionality. If long-term interruption (greater than 4 weeks) is anticipated, store samples at ≤-70 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Upon confirmation of the results, the sample information is either called or FAXed to NCHS. (FAXes are used more commonly as the FAX itself acts as documentation.)

Table 11
Reference Ranges for Women

| Age (years) | Triglycerides (mg/dL) | Cholesterol (mg/dL) | HDL-Cholesterol (mg/dL) |
|---|---|---|---|
| 0-7 | 25-140 | 130-190 | 35-70 |
| 8-9 | 30-115 | 130-195 | 35-75 |
| 10-11 | 35-130 | 125-200 | 35-75 |
| 12-13 | 40-105 | 130-210 | 40-70 |
| 14-15 | 35-125 | 115-200 | 35-70 |
| 16-17 | 40-120 | 120-200 | 35-75 |
| 18-19 | 40-145 | 105-225 | 35-75 |
| 20-24 | 40-125 | 110-220 | 30-80 |
| 25-29 | 40-130 | 130-220 | 35-80 |
| 30-34 | 40-140 | 130-220 | 40-75 |
| 35-39 | 40-175 | 140-250 | 35-80 |
| 40-44 | 45-180 | 145-255 | 35-85 |
| 45-49 | 45-190 | 145-265 | 35-85 |
| 50-54 | 50-215 | 165-290 | 35-90 |
| ≥55 | 55-280 | 175-295 | 35-85 |
| Cord Blood | 5-65 | 50-95 | 20-55 |

(These data were obtained from the Lipid Research Clinics Prevalence Studies Data Book and are rounded to the nearest 5 mg/dL.)

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record-keeping means (electronic and log accession books) are used to track specimens. All records, including related QC data, are maintained for a minimum of 5 years, in electronic and hardcopy format. Only numerical identifiers are used to identify participants.

Residual NHANES III specimens are sent to the NCHS specimen repository in Bethesda, MD, for long-term storage.

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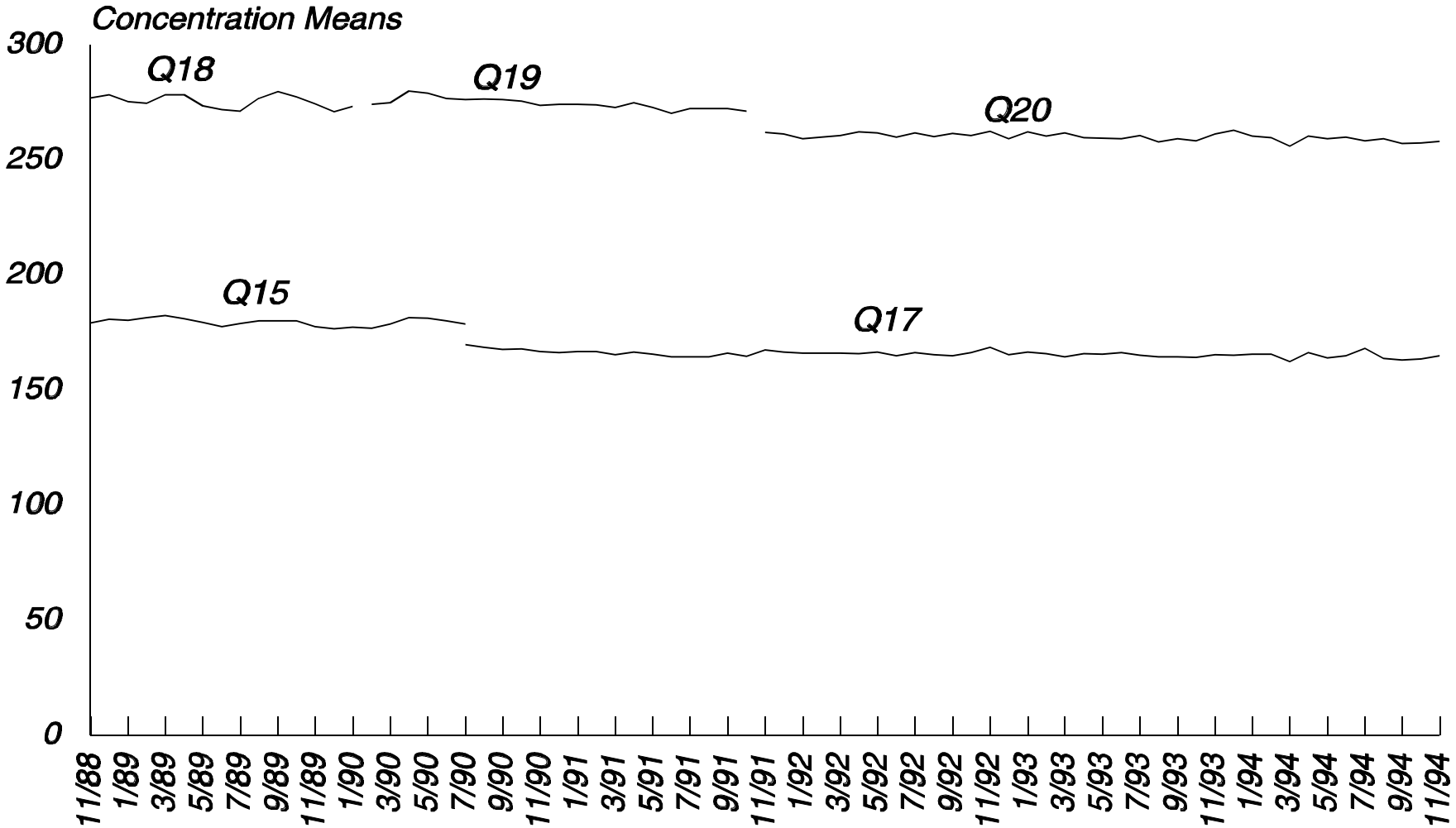
SUMMARY STATISTICS FOR
TOTAL CHOLESTEROL
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| Q15 | 11/23/88 - 07/06/90 | 179.368 | 4.19553 | 2.33906 | 391 |
| Q17 | 07/17/90 - 11/30/94 | 165.724 | 2.25397 | 1.36008 | 812 |
| Q18 | 11/23/88 - 01/29/90 | 274.844 | 7.05672 | 2.56753 | 270 |
| Q19 | 02/06/90 - 10/28/91 | 274.692 | 4.62844 | 1.68496 | 370 |
| Q20 | 11/14/91 - 11/30/94 | 259.988 | 3.39675 | 1.30650 | 566 |

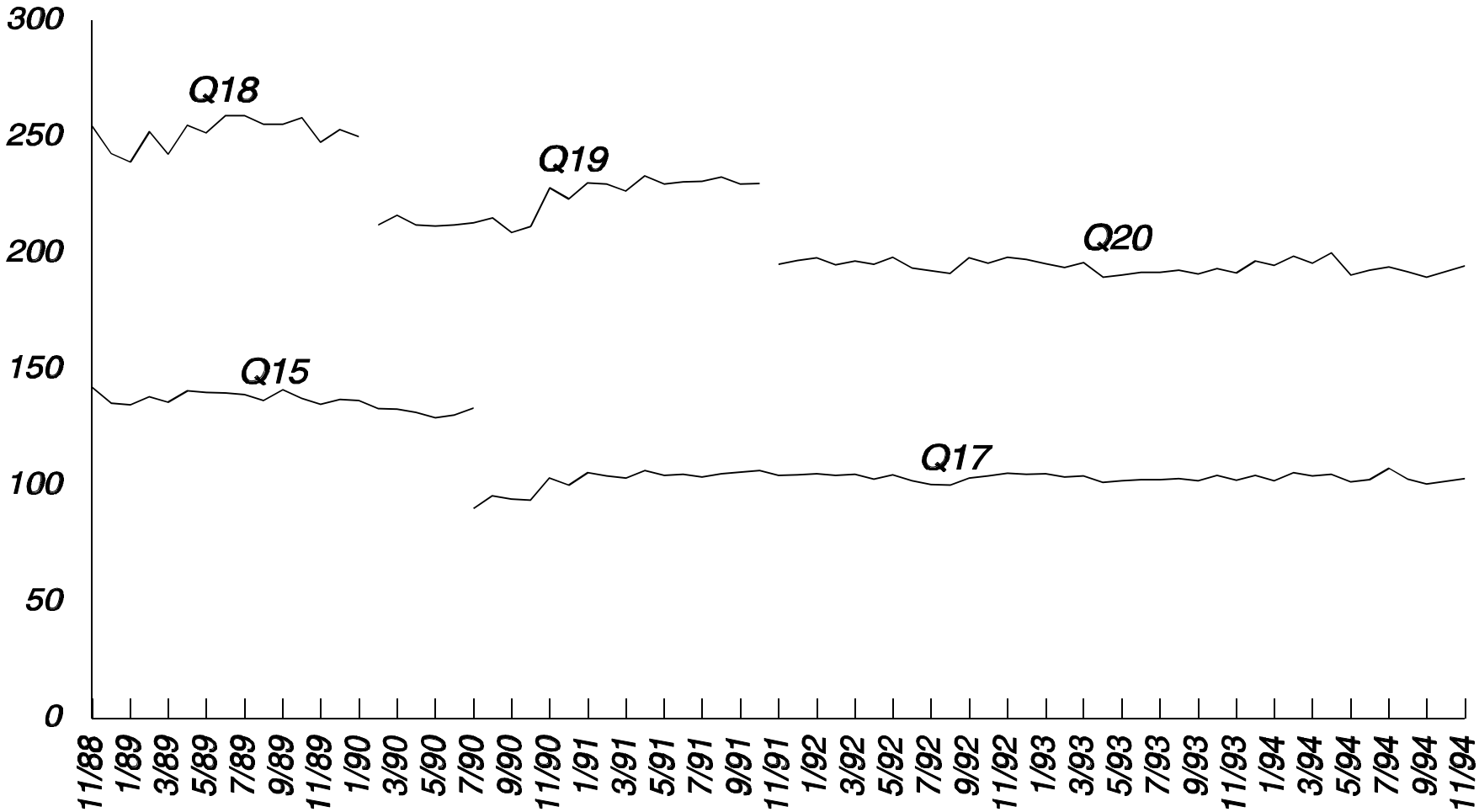
# Total Cholesterol Monthly Means



SUMMARY STATISTICS FOR
TRIGLYCERIDES
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| Q15 | 11/23/88 - 07/06/90 | 136.582 | 6.3596 | 4.65625 | 490 |
| Q17 | 07/17/90 - 11/30/94 | 103.045 | 3.7090 | 3.59945 | 806 |
| Q18 | 11/23/88 - 01/29/90 | 251.621 | 12.8224 | 5.09593 | 393 |
| Q19 | 02/06/90 - 10/28/91 | 222.009 | 9.7526 | 4.39290 | 334 |
| Q20 | 11/19/91 - 11/30/94 | 194.333 | 4.9041 | 2.52356 | 570 |

# Triglycerides Monthly Means

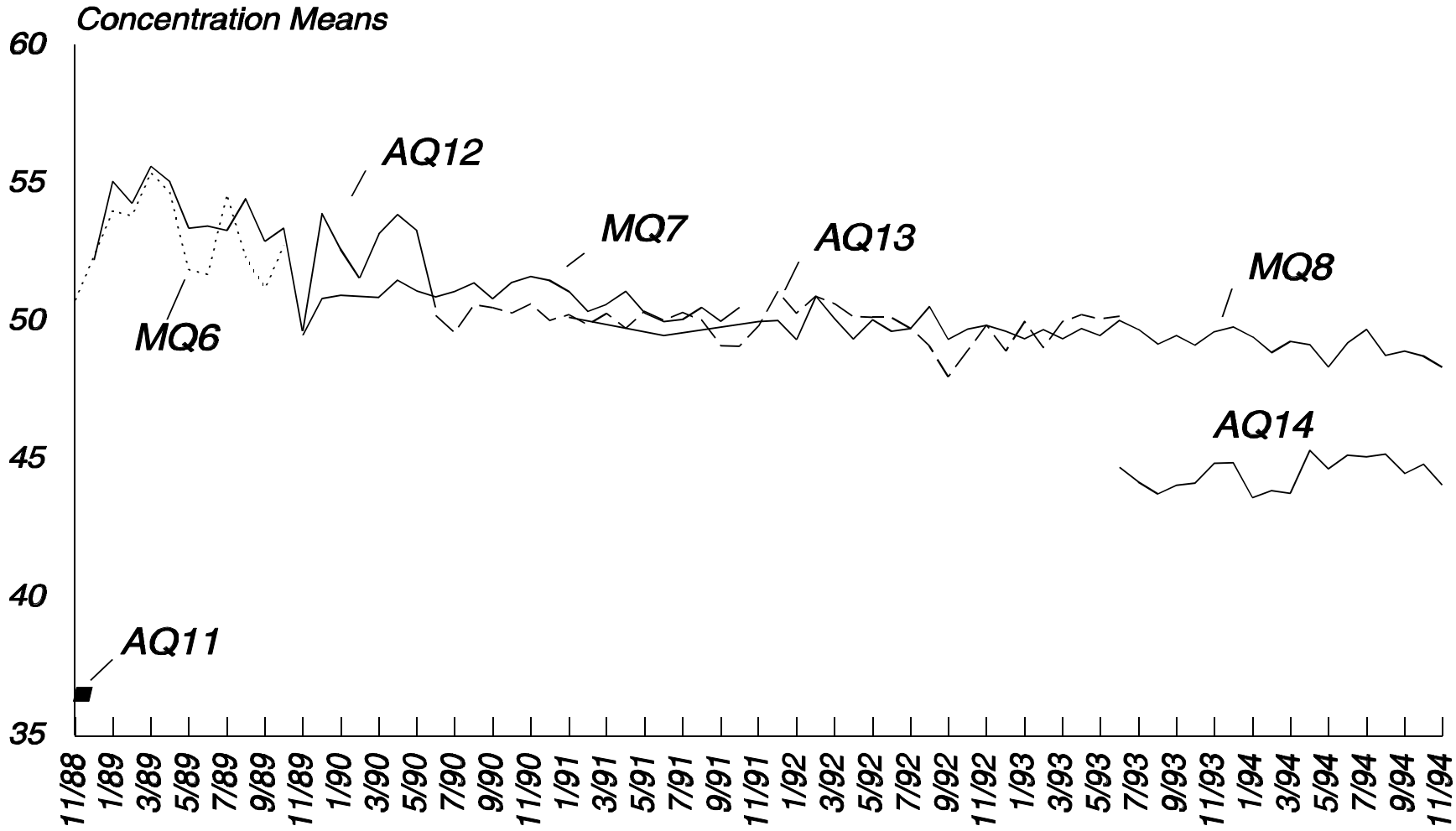



*NOTE: No samples for Triglycerides were analyzed during the month of 10/94.*

SUMMARY STATISTICS FOR
HDL-CHOLESTEROL
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| AQ11 | 11/28/88 - 11/29/88 | 36.5000 | 2.12132 | 5.81184 | 10 |
| AQ12 | 12/13/88 - 06/07/90 | 53.3975 | 2.92541 | 5.47854 | 322 |
| AQ13 | 06/12/90 - 06/09/93 | 50.0580 | 1.24214 | 2.48140 | 552 |
| AQ14 | 06/10/93 - 11/30/94 | 44.4708 | 1.11249 | 2.50162 | 240 |
| MQ6 | 11/28/88 - 10/12/89 | 52.9946 | 2.71830 | 5.12939 | 186 |
| MQ7 | 11/14/89 - 10/23/91 | 50.8389 | 1.24592 | 2.45072 | 416 |
| MQ8 | 11/20/91 - 11/30/94 | 49.5596 | 0.97641 | 1.97017 | 570 |

# HDL-Cholesterol Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This method quantifies C-reactive protein (CRP) by latex-enhanced nephelometry. Particle-enhanced assays are based on the reaction between an analyte present in human body fluids and the corresponding antigen or antibody bound to polystyrene particles. For the quantification of CRP, particles consisting of a polystyrene core and a hydrophilic shell are used in order to link anti-CRP antibodies covalently.

A dilute solution of test sample is mixed with latex particles coated with rabbit anti-CRP antibodies. CRP present in the test sample will form an antigen-antibody complex with the latex particles. An accelerator reagent containing detergent is added to the reaction mixture to enhance complex binding. This accelerator reagent has had rabbit serum added prior to use to prevent or to minimize falsely elevated CRP caused by potential anti-rabbit antibody in the specimens.

Light scattering, measured by a nephelometric procedure after 6 min, is proportional to the concentration of the analyte present in the sample. An automatic blank subtraction is performed. CRP concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve. These assays are performed on a Behring Nephelometer for quantitative protein determination.

The clinical usefulness of quantitative CRP determinations has been demonstrated for various indications. In response to an inflammatory stimulus, a rise of CRP may be detected within 6 to 10 hours, and it may increase by as much as 4000-fold at the peak of the acute phase response (1-3).

Elevated values can be found among people with certain diseases, i.e., inflammatory states of organic diseases, rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis and Crohn's disease; in diagnosis and therapy of infections in neonates, post-surgical complications, and in premature rupture of membranes or prediction of chorioamnionitis; differential diagnosis of pyelophritis versus cystitis, bacterial versus viral infections, necrotizing pancreatitis versus edematous interstitial pancreatitis; and suspected renal allograft rejection (4-7).

C-reactive protein has been of increasing interest because of the current availability of quantitative assays. CRP has been called the classical acute-phase reactant; in contrast to the erythrocyte sedimentation rate (ESR), it provides a direct measurement of a serum protein that rises and falls rapidly in response to acute inflammation and/or tissue destruction. As a result, although CRP is still a nonspecific indicator, increasing numbers of investigators advocate its quantification for early detection of bacterial infections in a wide variety of clinical settings and for following disease activity and therapy in a number of chronic diseases (e.g., rheumatoid arthritis and inflammatory bowel disease).

## 2. SAFETY PRECAUTIONS

Consider all samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses when handling all human blood products and infectious viruses. Place disposable plastic, glass, paper, and gloves that contact blood in a biohazard bag or discard pan to be autoclaved. Use disposable plastic-backed paper for all work surfaces. Disinfect all work surfaces with bleach solution. Dispose of all biological samples and diluted specimens in a biohazard bag at the end of the analysis.

Material safety data sheets for glycine, sodium chloride, sodium hydroxide, Tween 20, sodium hypochlorite, and sodium azide are kept in the Immunology Division, University of Washington (UW).

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit arrives with a corresponding transmittal sheet and an ASCII data file on a 5¼" floppy diskette (output.txt). The ASCII file containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. The original NHANES disk is copied on two other floppy disks and in the database. The file from the original disk is uploaded into the database in preparation for reporting results.

c. After the data is calculated and the final values are approved for release by the reviewing supervisor, the data entry clerk transcribes the results to the NHANES III disk by a data entry system.

d. The CRP results are entered onto the original NHANES diskette by a data entry system. The disks and hard copies are sent to the National Center for Health Statistics (NCHS).

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Fresh or frozen human serum samples are acceptable.

c. Blood should be collected aseptically and the serum separated by standard laboratory techniques. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers.

d. The requested sample volume for the assay is 1.0 mL, and the minimum sample volume is 0.3 mL.

e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Once specimens are received, freeze them at ≤-20 °C until time for analysis. Refreeze residual serum at ≤-20 °C.

g. Contamination or introduced particulate matter can lead to erroneous results.

h. Avoid repeat freeze/thaw cycles.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Behring Nephelometer Analyzer System (BNA) with a 2-channel, 2-valve dilutor with one 2500-µL and one 250-µL syringe, 840 nm±25 wavelength analyzer and terminal equipped with a 16/32 bit processor, 1-Mbyte memory, 10-Mbyte Winchester drive, cat. no. OVCl11 (Behring Diagnostics Inc., Somerville, NJ).

The instrument is fully automated. The analyzer includes a dilutor with transfer arm, rack station, buffer compartment, cuvette rotor, cuvette washing device, and optical system. The terminal includes a CPU, Winchester drive, disk drive, monitor, keyboard bar code reader, six serial RS 232C interfaces, and an EPSON LX-80 or HP ThinkJet printer.

The CRP assay parameter settings for the BNA instrument are shown in Table 1.

(2) Centrifuge, model 340400 (Beckman Instruments, Fullerton, CA).

(3) Vortex mixer, model M16715 (Thermodyne, Dubuque, Iowa).

(4) Computer (Sunquest Information System, Tucson, AZ).

b. **Materials**

(1) Transfer pipets, cat. no. #7524 (Becton-Dickinson, Franklin Lakes, NJ).

(2) NA Latex CRP Kit, cat. no. #OUSV 04/05 or OUSV 10/11 (Behring Diagnostics, Inc.).

(3) Calibrator 7, cat. no. #84403 (INCStar, Stillwater, MN).

Table 1
BNA Parameter Settings for Test #56

| Parameter | Setting |
|---|---|
| Protein Name | CRP |
| Sample Dilution* | 1:400 |
| Sample Volume | 50 µL |
| Antiserum/Latex R. | 40 µL |
| N/NA Suppl.Reag./N Acc** | 10 |
| Reaction Buffer for Sample<br>Reaction Buffer for Reagent | 60 µL N Diluent<br>60 µL N Diluent |
| Standard No. | 7 |
| No. of Standard Points | 7 |
| Standard Dilutions | 1:40-1:2560 |
| Measuring Range***(mg/dL)<br>(Converted to mg/dL unless ind. otherwise) | 0.25-16 mg/dL |

* Automatic sample predilution with N Diluent
** N Accelerator are components of the corresponding combipacks.

(4) Rabbit Serum, cat. no. # S-2632 (Sigma Chemical Company, St. Louis, MO).

(5) Chemicon CRP (Chemicon, Temecula, CA).

(6) Gibco normal human serum (NHS) (Gibco, Gaithersburg, MD).

(7) CAP Reference Serum Protein Standard (College of American Pathologists, Northfield, IL).

(8) 10-mL tubes, cat. no. 2059 (Becton-Dickinson, Lincoln Park, NJ).

(9) Conical-bottom tubes, cat. no. 72.699 (Sarstedt, Hayward, CA).

(10) Filters, cat. no. 6010-2400 (Ahlstrom, Mt. Holly Springs, PA).

(11) 0.22-µm filter, cat. no. SLGS0250S (Millipore Corporation, Bedford, MA).

(12) Latex gloves, disposable (Johnson & Johnson, Arlington, TX).

(13) Pipettors and disposable tips, 100-µL and 1000-µL (Oxford Labware, San Francisco, CA).

(14) 240 x 5-sample cups, cat. no. OVCM31 (Behring Diagnostics Inc.).

(15) 9 x 5-cuvette segments, cat. no. OVCN11(Behring Diagnostics Inc.).

(16) Distilled water (University of Washington, Seattle, WA).

c. **Reagent Preparation**

All reagents except for the rabbit antiserum are provided as part of the NA Latex CRP Kit, cat. no. OUSV (Behring Diagnostics, Inc.).

(1) N CRP Reagent
Each vial contains a lyophilized polystyrene particles coated with antibodies from a rabbit anti-human-CRP serum and 0.1% sodium azide ($NaN_3$) as a preservative. Reconstitute with distilled water to the volume indicated on the vial. The reagent is ready to use 15 min after dissolution and is stable for 7 days after reconstitution; store at 4-8 °C. Avoid freezing.

(2) N Reaction Buffer
The buffer contains PBS with polyethylene glycol and 0.1% sodium azide ($NaN_3$). It is stable until the manufacturer's expiration date. Store at 4-8 °C.

(3) N Diluent
The diluent is supplied ready-to-use and contains PBS with 0.1% $NaN_3$. It is stable until the manufacturer's expiration date. Store at 4-8 °C.

(4) Rabbit Serum (5-mL vial)
Reconstitute with 2.5 mL distilled water. Swirl gently, let stand 15 min, and freeze in aliquots at ≤-70 °C. The serum is stable until the manufacturer's expiration date. Store at 4-8 °C.

(5) N ASL/CRP Accelerator
Supplied ready-to-use, this accelerator contains a solution of detergents and 0.1% $NaN_3$. It is stable until manufacturer's expiration date. Store at 4-8 °C.

(6) Working N ASL/CRP Accelerator
Add 200 µL reconstituted rabbit serum to each 4 mL of accelerator. Swirl gently to mix; let stand at least 1 hour, preferably overnight. Filter using a Millipore 0.22-µm filter. This solution is stable until expiration date on the accelerator bottle. Store at 4-8 °C. Note: Refilter using the Millipore 0.22-µm filter each day before use.

d. **Standards Preparation**

N CRP standard

This is lyophilized human serum containing high CRP concentrations and 0.1% sodium azide. Reconstitute with 1.0 mL of distilled water. the standard is ready to use 15 min after dissolution and is stable for 7 days after reconstitution when stored at 2-8 °C. **DO NOT FREEZE**. The standard is diluted 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560 by the instrument. These dilutions must be used within 4 hours of preparation.

The standard was prepared by Behring Diagnostics and standardized against the WHO International Reference Preparation (IRP) of C-reactive protein serum, available from the National Institute of Biological Standards and Controls, London, UK. This material is an internationally recognized source of purified human C-reactive protein.

e. **Preparation of Quality Control Materials**

Two levels of control materials are prepared in the Immunology Division from normal and/or pooled patient serum and are run with each assay.

Analyze new pools for at least 20 runs in parallel with the current control. Prepare in sufficient quantity to provide serum samples for 2 years. For the "borderline" control, dilute Calibrator 7 with normal human serum 1:12.7. Prior to aliquoting and defining, test the stock once for approximate value and adjust if necessary. Mix the pool well and sterile-filter it. For the high control, use Chemicon CRP to spike Gibco normal human serum. Mix, spin, and filter the resulting pool.

Divide the stock control material into 10-mL tubes containing a volume for a 3-4 month supply and label with 'I #' and freeze at ≤-85 °C. As needed, thaw a stock control tube and divide into approximately 250-µL aliquots to be stored for a maximum of 3-4 months at ≤-85 °C. Thaw and use one aliquot of control material for each run.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

Light scattering is measured with an automatic blank substraction. CRP concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve.

This method results in a linearized 7-point (including zero) standard curve with a direct relationship of measured light scatter to concentration of C-reactive protein in the serum sample. Serum results are expressed as mg/dL.

A valid standard curve for the CRP assay must be stored in the BNA memory before sample results can be quantified. Reference curves must be determined daily. The instrument will notify the operator if a specific reference curve is valid or expired.

### b. Verification

(1) The instruments used to read BNA assay results are equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the quality control manual, the entire series is invalidated.

(2) The CAP Reference Serum Protein Standard (CAP RPSP) is run annually to verify system calibration. The standard (lot no. 3) consists of 1.62-1.81 mg/dL purified human C-reactive protein. This correlation is reverified annually. Over the course of the project, CAP RPSP lot nos. 2-4 have been used.

(3) The CRP standard is prepared by Behring Diagnostics and standardized against the WHO International Reference Preparation(IRP) of C-reactive protein serum, available from the National Institute of Biological Standards and Controls, UK. This material is an internationally recognized source of purified human C-reactive protein.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Bring all reagents and patient specimens to 20-25 $^{o}$C before use.

(2) Check the levels of buffer and diluent. Transfer straws from water beaker into appropriate containers of diluent, buffer, and wash solution. Check the paper supply.

(3) Check the waste line.

(4) Turn on the power for the BNA and printer.

(5) Change flow cells and stir disks weekly.

(6) Clean flow cells and probes.

(7) Prime the system by checking the pipettor syringes, wash stations, and flow cells.

(8) Set up the worklist.

(9) Run the worklist.

### b. Sample Preparation

(1) Bring test specimens and controls to 20-25 $^{o}$C.

(2) Centrifuge each diluted sample for 15 min at 3000 rpm.

c. **Instrument Setup of Behring Nephelometric Analyser (BNA)**

(1) From the JOB LIST menu select 1-JOB LIST INPUT.

(2) The monitor will display the job list input. At identification prompt, enter the sample numbers for the samples.

(3) At the remark prompt, either enter a number (1-32) to call a remark previously stored, (e.g., "hemolysis"), or leave blank and press ENTER.

(4) Press T to switch to single tests.

(5) Type "56" for test number and ENTER.

(6) Press ENTER to end job list input.

(7) The monitor will display the sample serum entered in an abbreviated form. Press K to correct any information entered previously.

(8) The screen prompts "Loading of BNA is prepared; Continue?" Press Y and ENTER.

(9) Load the sample cassette and press ENTER.

(10) Load the dilution cassette and press ENTER.

(11) Load the anti-sera cassette and press ENTER.

(12) Press Y or ENTER to begin measurement.

d. **Operation of Assay Procedure**

(1) Bring all reagents to 20-25 $^{o}$C prior to use.

(2) Carefully pipet 200 µL of samples into the appropriate position of the sample tray.

(3) From the main menu select program 3-CALIBRATION and press ENTER.

(4) Select subprogram 3.1 REFERENCE CURVES and press ENTER. If valid reference curves are stored for each type of assay in the job list, return to the main menu.

(5) Select MAIN MENU program 1-JOB LIST and press ENTER.

(6) Compile the job list for the samples.

(7) Once the JOB LIST has been entered, load sample cups and vials of reagents into the positions on the BNA rack transport indicated on the BNA monitor.

(8) Once reagents and samples are in position, begin the measurements.

e. **Recording of Data**

(1) Quality Control Data

(a) As new stock control is prepared, define a new control range using MAQ in the laboratory data computer (Sunquest) under INDEX 0 and a reasonable arbitrary control range. Append TEMP to the control lot number name.

(b) Log in control (FUNCTION RE) under location IMMQC- so it will appear on the worksheet. Use the date of 1/1 as both the collect and the receive date so it will appear first on the worksheet, and append the comment: RESULT UNDER 'I____,0' to alert the technologists performing the assay that there is a new

control to be checked out and to tell them where to enter the results.

(c) After 20 parallel runs, use the data from the Levey-Jenning chart to assign a permanent mean and standard deviation.

(d) Using the function MAQ, modify the control range. Remove TEMP from the lot name and add the date of range calculation. Enter the same number of decimal places as used when reporting results. If the control is to be used right away, print a Levey-Jennings chart for the current control (FUNCTION QC OPTION L) and advance the index of the new control to "1." Leave the printout, dated with the date of advancement on the supervisor's desk. DO NOT ADVANCE A CONTROL LOT UNLESS ALL ASSAYS USING THIS CONTROL ARE ALSO READY TO BE ADVANCED.

(e) Delete the IMMQC log-in on the worksheet, either by deletion (FUNCTION RE) or by entering XRQR as a result.

(f) New controls in the computer are defined by using function "MAQ". All control information is kept in the University of Washington computer only. Enter the following information:

i. Method code
ii. Control code (I__)
iii. Test code. (Note: Some tests have tests codes that apply only to control tests, usually because the computer performs some calculation to that test which should not be applied to the control. All code names will be listed with the methods. Generally QC-specific test names will end in "C."
iv. Control data: mean and standard deviation (SD), or "accept" range.
- if qualitative controls: mean = 0 (neg), or mean=1 (pos).
- if mean and SD are entered, enter '2' at range prompt (2 SD's will be used to set the range.)

(2) General information

(a) Index number:

Index 0 = lot number under evaluation

Index 1 = lot in use

Index $\geq$2 = previous lots

(b) The aliquot label should include the date of preparation and a letter indicating sequential aliquot. (Examples: 9/90-A for the first time this control is aliquoted, 9/90-B for the second time. Record the label on the QC record sheet.

(c) The lot name should include an identifying name, the date the control was prepared (month and year), and information about the control range (temp or date of calculation or recalculation).

NOTE: controls used in many assays (e.g., pool) and qualitative assays (1/0) do not need information about the control range attached to the lot name.

Results are entered into the Sunquest computer and are evaluated by Westgard rules at each entry. From the main menu select 5-CONTROLS-DAILY RESULTS. Any violations of control specifications is displayed.

(3) Analytical Results

Results are automatically calculated and printed as the tests are completed. From the main menu, select 4, followed by 3-LAB JOURNAL for a list of all sample results measured. Any repeat measurements, special dilutions, or results exceeding the measuring range of a determination are

displayed.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Behring Nephelometer Analysis System

Check the following functions as part of regular maintenance as directed in the BNA operator's manual.

(a) Dismantle and assemble the cuvette rotor; replace the cuvettes.

(b) Prime the tubing system and the syringes.

(c) Prime the cuvette washing device.

(d) Replace the syringes.

(e) Replace the tubings.

(f) Replace the stirrer.

(g) Disinfect instrument parts.

(2) Pipettors

All micro-pipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

The Behring instrument has full data-reduction capabilities. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve. The protein concentration is calculated automatically by the instrument from the corresponding reference curve. A blank is automatically subtracted.

This method results in a linearized 7-point (including zero) standard curve with a direct relationship of measured light scatter to concentration of C-reactive protein in the serum sample. Serum results are expressed as mg/dL.

A valid standard curve for the CRP assay to be run must be stored in the BNA memory before sample results can be quantified. Reference curves must be determined daily. The instrument will notify the operator if a specific reference curve is valid or expired.

Repeat testing on all specimens with results >1.0 mg/dL. Use the repeat measurement menu to rerun controls and high patients. For results greater than 10 mg/dL, "repeat measurement" using 1:4000 dilution instead of the default 1:2000 dilution.

## 9. REPORTABLE RANGE OF RESULTS

Report values $\geq$0.3 to the nearest 0.1 mg/dL.

Report values <0.3 as <0.3 mg/dL.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years. The method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-the-art. The primary standard used was prepared by Behring Diagnostics and standardized against the WHO International Reference Preparation (IRP) of C-reactive

protein serum, available from the National Institute of Biological Standards and Controls, UK. This material is an internationally recognized source of purified human C-reactive protein. The diluted standards are run daily. Estimates of imprecision can be generated from long-term quality control pool results.

Bench quality controls are used in this analytical method. Bench quality control specimens are inserted by the analyst two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

The bench controls are prepared in sufficient quantity to provide serum samples for all the assays for 2 years. Ranges are established after 20 parallel runs with previously established controls. Ranges are established by using the formulas for statistical calculations for geometric data. The quality control pools comprise two levels of concentration spanning the borderline and high ranges for C-reactive protein.

After the standards and bench quality control materials are analyzed (at the beginning of an analytical run), the long-term quality control charts for each control material are consulted to determine if the system is "in control." Two types of charts are used. The first chart plots the means of the duplicate determinations and compares them with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs). The system is out of control if any of the following events occur for any one of the quality control materials:

- The mean from a single pool falls outside the 99% confidence limits.
- The means from two pools fall either both above or both below the 95% confidence limits.
- The means from eight successive runs for one pool fall either all above or all below the center line.

The second type of quality control chart plots the range of the duplicate determinations and compares them with the 95% and 99% limits as well as with the center line. The system is out of control if any of the following events occur for any one of the quality control materials:

- The range from a single pool falls above the 99% limit.
- The ranges from two pools fall above the 95% limit.
- The ranges from eight successive runs fall above the center line.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the run is declared "out of control", the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any results are released. Consult with the supervisor for appropriate actions.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Avoid contamination of assay reagents and disposables by particulate matter, especially dust and lint. Cover all reagents immediately after use.

b. Markedly increased serum lipids can interfere with nephelometric determinations. If such interference is suspected, centrifuge samples before assaying.

c. Nonspecific reactions could occur with individuals sensitive to rabbit proteins including rabbit IgG-reactive rheumatoid factors. This has been minimized by the addition of rabbit serum to the accelerator in the test system.

d. Clear serum samples are recommended for analysis. Avoid lipemic, icteric, or hemolyzed samples when assaying for c-reactive protein, if possible.

e. Results obtained using plasma (EDTA, citrate, and heparinized) do not correlate well with those obtained using serum.

f. Sample results >10.0 mg/dL should be remeasured at a higher dilution because the assay may not be linear above 10.0 mg/dL.

## 13. REFERENCE RANGES (NORMAL VALUES)

The reference range was determined to be 0-1 mg/dL in normal healthy adults by in-house testing of serum from over 300 patients from March 1990 to April 1990. NOTE: while the trend of CRP response during inflammatory processes is predictable, the degree of change varies from person to person, and without baseline measurements, it can be difficult to interpret. For example, undiagnosed disease processes may be contributing to an observed acute-phase response. Pregnancy or the use of intrauterine devices or hormonal contraceptives may also raise CRP concentrations. "Normal" values should be used only as a guide by the physician and must be interpreted together with other clinical signs and symptoms.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable to this procedure.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should be maintained at 20-25 ºC during testing. After testing, the samples are stored at ≤-85 °C.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods of analysis. Specimens may be stored at 4-8 °C for no longer than 8 days. Otherwise, specimens should be stored at ≤-85 °C until the system is returned to functionality.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this procedure.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping should be used for tracking specimens. The instrument results menu includes current daily results as well as stored test results. An additional subprogram initiates a print-out of results in tabular form. Only numerical identifiers should be used.

The original NHANES III disk is copied onto two backup floppy disks and onto the hard drive of the IBM computer. After the results are entered into the database, the disks are stored in three separate locations.

The residual serum is stored at ≤-85 °C for 1 year after analysis; then it is returned to the NHANES Repository in Rockville, MD, for long-term storage.

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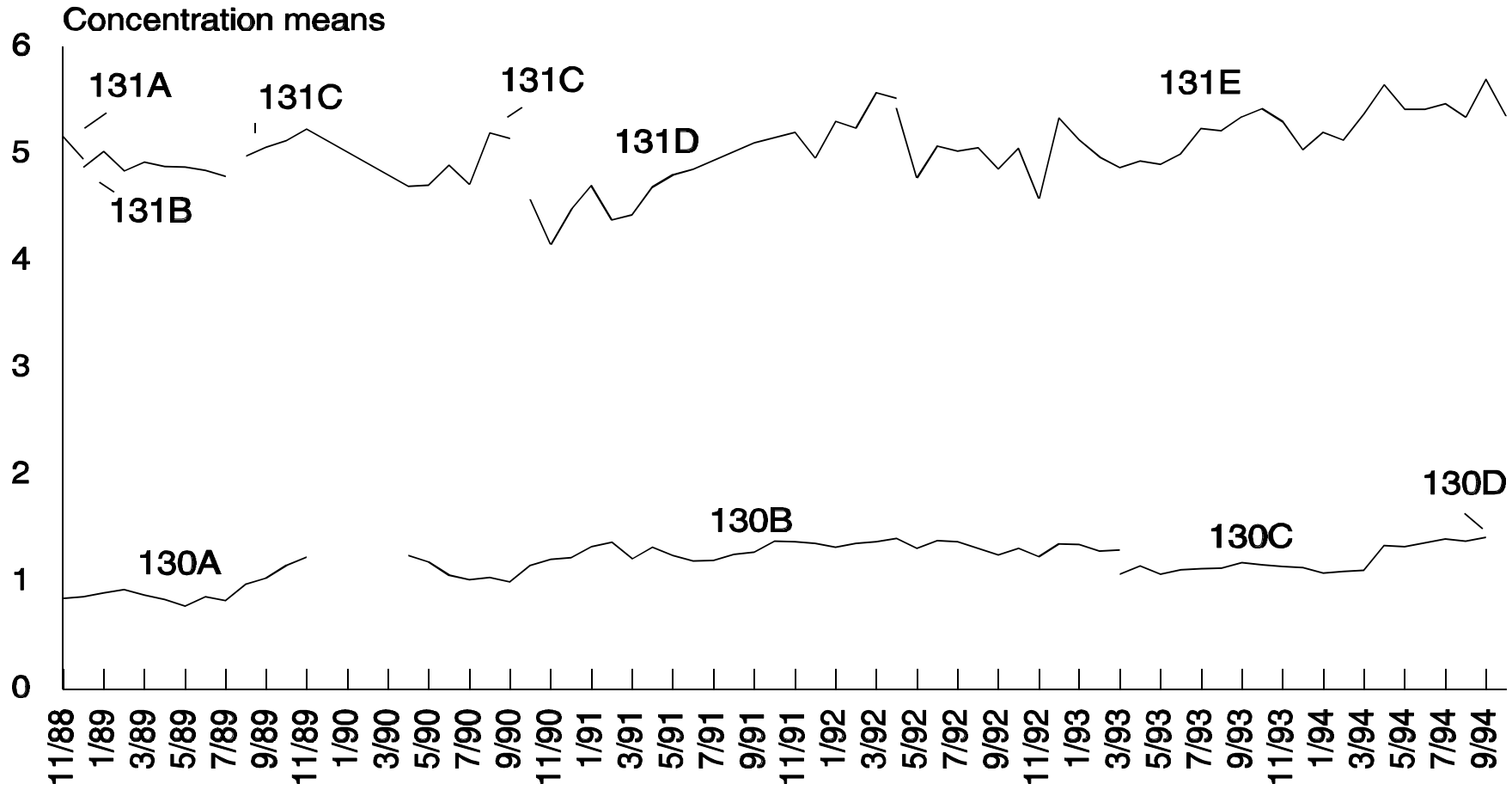
SUMMARY STATISTICS FOR
C-REACTIVE PROTEIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 130A | 11/18/88 - 11/13/89 | 0.91881 | 0.14760 | 16.0646 | 436 |
| 130B | 04/03/90 - 03/10/93 | 1.24907 | 0.14638 | 11.7189 | 378 |
| 130C | 03/11/93 - 09/30/94 | 1.19291 | 0.13862 | 11.6206 | 134 |
| 130D | 10/01/94 - 10/19/94 | 1.35000 | 0.05477 | 4.0572 | 6 |
| 131A | 11/21/88 - 12/08/88 | 5.08880 | 0.16517 | 3.2457 | 50 |
| 131B | 12/14/88 - 08/01/89 | 4.88057 | 0.22640 | 4.6389 | 265 |
| 131C | 08/02/89 - 09/30/90 | 4.94688 | 0.31053 | 6.2773 | 237 |
| 131D | 10/01/90 - 04/15/92 | 4.75859 | 0.47708 | 10.0256 | 149 |
| 131E | 04/16/92 - 10/19/94 | 5.15991 | 0.32304 | 6.2607 | 217 |

# C-Reactive Protein Monthly Means




**Testing of NHANES specimins for C-Reactive Protein was not conducted for the period 12/89 - 3/90.**

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Rheumatoid factors are auto-antibodies directed against the Fc portion of human IgG. Rheumatoid factors can belong to the IgG, IgA, or IgM classes of immunoglobulins (1). IgM rheumatoid factor is the most easily detected by laboratory methods, namely the latex fixation test, because of the five reactive sites on IgM. Latex particles are coated with IgG, which serves as the antigen. If an IgM rheumatoid factor is present in serum, its five reactive sites react with the IgG molecules on several latex particles, drawing them together to produce visible agglutination. RF results were reported from the Singer-Plotz latex agglutination test (2).

Rheumatoid factors are present in serum in the majority of people with rheumatoid arthritis, but are not unique to this disease. They are also present in patients with systemic lupus erythematosus, Sjogren's syndrome, scleroderma, polymyositis, and chronic infectious diseases such as leprosy, tuberculosis, and subacute bacterial endocarditis. They may also occur in some people with hypergammaglobulinemia, liver disease, and sarcoidosis or syphilis. Three percent of the normal population have rheumatoid factors. This figure increases for elderly patients; their titers, however, are usually low (3).

Specimens for rheumatoid factor (RF) analysis are initially screened by using latex-enhanced nephelometry. Particle-enhanced assays are based on the reaction between an analyte present in human body fluids and the corresponding antigen or antibody bound to polystyrene particles. For the quantification of rheumatoid factors, particles consisting of a polystyrene core and a hydrophilic shell are used for absorptive binding on IgG.

Light scattering, measured by a nephelometric procedure after 6 min, is proportional to the concentration of the analyte present in the sample. An automatic blank substraction is performed. RF concentrations are calculated using a calibration curve. Data reduction of the signals is performed using a storable logit-log function for the calibration curve. These assays are performed on the Behring Nephelometer for quantitative protein determination. Nephelometric results are used for the purpose of general titering indications and are never reported as final results.

For quantitation, all samples having nephelometric measurements >13 IU/mL are titered in a 12-tube titer sequence in the Singer-Plotz latex agglutination procedure to determine reportable results. All samples with concentrations $\leq$13 IU/mL are run at 1:20 and 1:400 in the same procedure (4).

## 2. SAFETY PRECAUTIONS

Consider all serum samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses when handling all human blood products and infectious virus. Place all disposable plastic, glass, and paper such as pipet tips or gloves that contacts blood in a biohazard bag or discard pan to be autoclaved. Use disposable plastic backed paper for all work surfaces. Disinfect all work surfaces with bleach solution. Dispose of all biological samples and diluted specimens in a biohazard bag at the end of the analysis.

Material safety data sheets (MSDS) for glycine, sodium chloride, sodium hydroxide, Tween-20, sodium hypochlorite, and sodium azide are kept in the Immunology Division, University of Washington (UW).

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit arrives with a corresponding transmittal sheet and an ASCII data file on a 5¼" floppy diskette (output.txt). The ASCII file containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. The data on the NHANES III disks are copied into a database.

c. After the data is calculated and the final values are approved by the reviewing supervisor for release, the results are transcribed by the data entry clerk to the NHANES III disk by a data entry system.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Fresh serum samples are preferable.

c. Blood should be collected aseptically and the serum separated by standard laboratory techniques. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers.

d. Preferred sample volume is 1.0 mL, and the minimum sample volume for the assay is 0.3 mL.

e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Once received, specimens should be frozen at ≤-20 °C until time for analysis. Residual serum is refrozen at ≤-20 °C.

g. Contamination or introduced particulate matter can lead to erroneous results.

h. Repeat freeze/thaw cycles should be avoided.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Screening test -- Latex-enhanced nephelometry

(a) Behring Nephelometer Analyzer System (BNA) with 2-channel, 2-valve dilutor with one 2500-µL and one 250-µL syringe, 840 nm±25 wavelength analyzer and terminal equipped with a 16/32 bit processor, 1-Mbyte memory, 10-Mbyte Winchester drive, order no. OVCl11 (Behring Diagnostics Inc., Somerville, NJ). Set the instrument parameters as shown in Table 1.

The instrument is fully automated. The analyzer includes a dilutor with transfer arm, rack station, buffer compartment, cuvette rotor, cuvette washing device, and optical system. The terminal (BNT) includes a CPU, Winchester drive, disk drive, monitor, keyboard bar code reader, six serial RS 232C interfaces and EPSON LX-80 or HP ThinkJet printer.

(b) Blue M waterbath, model MW11ZOA-1, 56 °C, (Magni-Whirl, Blue Island, IL).

(c) Refrigerated centrifuge, model CT422 (Jouan, Winchester, VA).

(2) Singer-Plotz tube titration latex agglutination

This is a manual procedure with no major instrumentation used.

b. **Other Materials**

(1) N Latex RF Kit, cat. no. OUUA10/11 (Behring Diagnostics Inc.).

(2) Transfer pipets, cat. no. 7524 (Becton-Dickinson, Franklin Lakes, NJ).

(3) 13- x 100-mm test tubes, cat. no. 60825-571 (VWR, Seattle, WA).

(4) Distilled water (University of Washington, Seattle, WA).

(5) Kimax volumetric flask, cat. no. 2963-560 (VWR).

(6) 240- x 5-sample cups, cat. no. OVCM31 (Behring Diagnostics Inc.).

Table 1
Parameters for BNA Instrument

| Parameter | Setting |
|---|---|
| Test no. | 54 |
| Protein name | RF |
| Sample dilution* | 1:20 |
| Sample volume | 30 µL |
| Antiserum/Latex R. | 40 µL |
| N/NA Suppl.Reag./N Acc** | 0 |
| Reaction buffer for sample<br>Reaction buffer for reagent | 60 µL N Diluent<br>60 µL N Diluent |
| Standard no. | 5 |
| No. of standard points | 6 |
| Standard dilutions | 1:2.5-1:80 |
| Measuring range (mg/dL)<br>Converted to mg/dL unless ind. otherwise | 20-640 IU/mL |

* Automatic sample predilution with N Diluent
**N Accelerators are components of the corresponding combipacks.

(7) 9- x 5-cuvette segments, cat. no. OVCN11 (Behring Diagnostics Inc.).

(8) Bacto-latex particles, 0.81-µ, cat. no. #3102-57 (VWR).

(9) Sodium hypochlorite (bleach) solution (any vendor).

(10) Latex gloves, disposable (Johnson & Johnson, Arlington, TX).

(11) Pipettors and disposable tips, 100 µL and 1000 µL (Oxford Labware, San Francisco, CA).

(12) Parafilm (American National Can, Greenwich, CT).

(13) Glycine, cat. no. 4059-00 (JT Baker, Phillipsburg, NJ).

(14) Sodium chloride (NaCl), cat. no. 3624.05 (JT Baker).

(15) Sodium hydroxide (NaOH), cat. no. 3722.05 (JT Baker).

(16) Tween-20, polyoxyethylenesorbitan monolaurate, (Sigma Chemical, St Louis, MO).

c. **Reagent Preparation**

All reagents except the wash solution are supplied by Behring Diagnostics as part of the N Latex CRP Kit.

(1) Screening test -- latex-enhanced nephelometry

(a) N RF Reagent
Two 3-mL vials of lyophilized polystyrene particles coated with human gamma-globulin, and preserved with 0.1 g/dL sodium azide. Reconstitute with the volume of distilled water listed on the label and allow to stand at 20-25 °C for a minimum of 15 min. Swirl gently before use. Store at 4-8 °C.

(b) Wash solution
Dilute Tween-20 1:4 (1 volume + 3 volumes) with distilled water, then add 4 drops of the dilution to 1 L of distilled water.

(c) N RF anti-sera
Serum contains lyophilized polystyrene particles which are coated with human gamma globulin.

(d) N Diluent
Diluent contains phosphate buffered saline (PBS) with 0.1% sodium azide ($NaN_3$). Stable until manufacturer's expiration date.

(2) Singer-Plotz tube titration latex agglutination

(a) Glycine buffer ($NH_2CH_2COOH$)
0.2 mol/L, pH 8.2. Mix 30 g glycine, 40 g NaCl and 10 mL 1-N NaOH. Dissolve in a smaller quantity of distilled water in a 4-L flask. Adjust pH to 8.2, then bring to volume. Stable for 1 year from date of preparation.

(b) Fraction II (gamma globulin)
Weigh out 0.5 g lyophilized Human Cohn Fraction II (ICN, Cleveland, OH). Place in a flask with 95 mL glycine buffer and mix thoroughly. Seal with Parafilm and leave in refrigerator for 5 days, mixing periodically. When dissolved, place in a 100-mL volumetric flask and bring to volume with glycine buffer. Store only the aliquot in current use in the refrigerator. The 25-mL aliquots are stable 1 year from the date of preparation when frozen at <-85 °C.

d. **Standards Preparation**

(1) Screening test -- latex-enhanced nephelometry

The standard material is a mixture of human sera with a high rheumatoid factor. It is supplied in a lyophilized form with sodium azide (maximum 1 g/L) added. Four 1-mL vials are supplied with the kit. Reconstitute each vial with 1.0 mL distilled water, allow it to stand for 15 min, and mix the contents thoroughly. If the entire kit in not used in one run, store at 4-8 °C for 1 week. Do not use the standard if it is turbid.

The standard was prepared by Behring Diagnostics and standardized against the WHO International Reference Preparation (IRP) of rheumatoid arthritis serum, available from the National Institute of Biological Standards and Controls, UK. The WHO IRP material is an internationally recognized source of purified human rheumatoid factor.

The standard dilutions are diluted 1:2.5, 1:5, 1:10, 1:20, 1:40, and 1:80 by the instrument. These dilutions must be used within 4 hours of preparation.

e. **Preparation of Quality Control Materials**

Analyze new pools for at least 20 runs in parallel with the current control. Prepare in sufficient quantity to provide serum samples for 2 years. Prior to aliquoting and establishing target values, test the stock once for approximate value, and adjust if necessary. Obtain each concentration level from nonlipemic pooled patient serum, mix well, and filter. Divide the stock control material into tubes containing a volume for a 3-4 month supply and labeled with 'I #' and freeze at
≤-85 °C. As needed, thaw a stock control tube and aliquot approximately 130 µL into conical-bottom vials. Freeze at ≤-85 °C for a maximum of 3-4 months. Thaw one vial of each control for each run.

(1) Screening test -- latex-enhanced nephelometry

Low and high controls (University of Washington).

Two controls are prepared in Immunology from normal and/or pooled patient serum and are run with each BNA screening assay. The quality control pools comprise two levels of concentration spanning the low and high ranges.

(2) Singer-Plotz tube titration latex agglutination

Negative, low positive, and high positive controls (University of Washington).

Three controls were prepared from pooled patient serum and run with each tube agglutination assay. The quality control pools for the tube agglutination assay comprise three levels of concentration spanning the negative, low positive and high positive ranges for rheumatoid factors. Acceptable criteria for the tube-titer controls are shown in Table 2.

Table 2
Tube-Titer Control Results

| **Control** | Results |
|---|---|
| **Negative control** | no visible agglutination |
| Low positive control | 1:160-1:320 titer |
| High positive control | 1:2560-1:10240 titer |

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

(1) Screening test -- latex-enhanced nephelometry

Light scatter is measured with an automatic blank subtraction. RF concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit log function for the calibration curve.

This method results in a linearized 6-point (including zero) standard curve with a direct relationship of measured light scatter to concentration of rheumatoid factors in the serum sample. Serum results are expressed as IU/mL.

A valid standard curve for the RF assay to be run must be stored in the BNT memory before sample results can be quantified. Reference curves must be determined daily. The instrument will notify the operator whether a specific reference curve is valid or expired.

The instrument used to read BNA assay results is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the quality control manual, the entire series is invalidated.

(2) Singer-Plotz tube titration latex agglutination

No calibration curve is generated by the user as part of this assay method.

### b. Verification

(1) Screening test -- Latex-enhanced nephelometry

RF calibration standards are prepared by Behring Diagnostics and standardized against the WHO International Reference Preparation (IRP) of rheumatoid arthritis serum, available from the National Institute of Biological Standards and Controls, London, UK. The diluted standards are run daily. This material is an internationally recognized source of purified human rheumatoid factor.

(2) Singer-Plotz tube titration latex agglutination

Tube agglutination is a manual method involving a visual check for agglutination in the tube.

The UW Immunology Laboratory is externally monitored and certified by the College of American Pathologists, an interlaboratory comparison (proficiency testing) system designed to compare laboratory performance with those of other laboratories.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Screening test -- latex-enhanced nephelometry

(a) Bring test specimens, controls, and reagents to 20-25 °C.

(b) Check to see if sufficient volumes of buffer, diluent, and washing solution are present in the proper color-coded positions in the analyzer buffer station, and replenish if necessary. Place color-coded tubing with level sensors into the proper bottles, route tubing through the slit provided, and close the compartment lid.

(c) Turn on power for the BNA and printer. The main power switch is located on the left side of the BNA. The operator program will automatically proceed to the MAIN MENU programs.

(d) Check the waste line.

(e) Change flow cells and stir disks (weekly).

(f) Clean flow cells and probes.

(g) Prime the system by checking pipettor syringes, wash stations, and flow cells.

(2) Singer-Plotz tube titration latex agglutination

(a) Bring controls and reagents to 20-25 °C.

(b) Spin the Fraction II reagent for 1 hour at 1800 rpm (800 X G).

(c) Prepare the Fraction II-latex reagent as follows:

(i) Count all tubes to be tested. Use Table 3 to determine the volumes of glycine buffer, Fraction II, and latex particles to be used (1.0 mL reagent is required for each test).

Table 3
Fraction II-Latex Reagent Preparation

| Total # of tubes to be tested | 50 | 75 | 100 | 125 | 150 | 175 | 200 |
|---|---|---|---|---|---|---|---|
| Glycine Buffer (mL) | 47 | 70.5 | 94 | 117.5 | 141 | 164.5 | 188 |
| Fraction II (mL) | 2 | 3.0 | 4 | 5.0 | 6 | 7.0 | 8 |
| Latex Particles (mL) | 1 | 1.5 | 2 | 2.5 | 3 | 3.5 | 4 |

(ii) Pour glycine buffer into an Erlenmeyer flask.

(iii) Add latex particles using a volumetric pipette. Mix thoroughly.

(iv) Slowly add Fraction II while swirling the flask. Use reagent for one run only, and discard any residual solution.

b. **Sample preparation**

(1) Screening test -- latex-enhanced nephelometry

Set up work list.

(2) Singer-Plotz tube titration latex agglutination

(a) Set up controls and unknowns as follows:

(i) Negative control

(ii) Low positive control

(iii) High positive control

(iv) Unknowns

(b) Use one 13- x 100-mm test tube for each control or patient sample.

(c) Pipette 1.9 mL of 0.1 mol/L glycine buffer (pH 8.2) into each tube.

(d) Add 0.1 mL each of the controls and participant sample.

c. **Instrument setup**

(1) Screening test -- latex-enhanced nephelometry

(a) Behring Nephelometric Analyser (BNA)

(i) From the JOB LIST menu, select 1-JOB LIST INPUT.

(ii) The monitor will display JOB LIST input. At the identification prompt, enter the sample number for each sample, one after the other.

(iii) At the prompt, either enter a number (1-32) to call a remark previously stored (e.g., "hemolysis") or leave blank and press ENTER.

(iv) Press T to switch to single tests.

(v) Type the test number "RF 40" and ENTER.

(vi) Press ENTER to end job list input.

(vii) The monitor will display the serum sample entered in an abbreviated form. Press "K" to correct any information entered previously.

(viii) The screen prompts "Loading of BNA is prepared; Continue?" Press "Y" and ENTER.

(ix) Load the sample cassette and press ENTER.

(x) Load the dilution cassette and press ENTER.

(xi) Load the anti-sera cassette and press ENTER.

(xii) Press "Y" or ENTER to begin measurement.

(2) Singer-Plotz tube titration latex agglutination

Tube agglutination is a manual method involving a visual check for agglutination in the tube.

d. **Operation of Assay Procedure**

(1) Screening test -- Latex-enhanced nephelometry

(a) Run a sample for RF analysis on the same sample cup as for C-reactive protein and run appropriate controls on the Behring Nephelometric Analyser.

(i) From the MAIN MENU, select program 3-CALIBRATION and press ENTER.

(ii) Select subprogram 3.1-REFERENCE CURVES and press ENTER. If valid reference curves are stored for each type of assay in the job list, return to the MAIN MENU.

(iii) Select program 4-CONTROLS and press ENTER.

(iv) Select subprogram 4.2-INPUT and press ENTER. The screen will show three additional subprograms, which allow the operator to select the specific controls, number of sample determinations between control assays, control lot numbers, protein constituents, and assigned values.

(v) Select the MAIN MENU program 1-JOB LIST and press ENTER.

(vi) Compile the JOB LIST for the samples.

(vii) Once the JOB LIST has been entered, load sample cups and vials of reagents into the positions on the BNA rack transport indicated on the BNA monitor.

(viii) Once reagents and samples are in position, begin the measurements.

(b) The original 1:20 dilution and a 1:400 dilution of all samples <13 IU/mL are run as tube tests. All values >13 IU/mL are titered for 12 tubes in the agglutination assay. The negative and both positive controls are also titered for 12 tubes.

(2) Singer-Plotz tube titration latex agglutination

(a) Incubate dilutions at 56 °C for 30 min to inactivate serum complement.

(b) While sera are inactivating, label tubes for the controls as follows:

(i) Negative control 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12.

(ii) Low-positive control 2-2, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 2-11, 2-12.

(iii) High-positive control 3-2, 3-3, 3-4, 3-5, 3-6,3-7, 3-8, 3-9, 3-10, 3-11, 3-12.

(c) To check for prozone (suboptimal reactivity due to a disproportionately high amount of reagent upon initial addition, resulting in erroneously low apparent concentration), make a 1:400 dilution of all the negative unknowns (those ≤13 IU/mL by the BNA) by adding 0.05 mL of the 1:20 dilution to 0.95 mL of the glycine buffer. Then, run both the original 1:20 dilution and the 1:400 dilution as tube tests. Only tube titer results are to be reported for the NHANES III samples.

(d) Remove 0.90 mL from all negative 1:20 dilutions. A negative measurement has been defined as ≤13 IU/mL on the basis of a comparative study performed at the University of Washington with at least 300 patient samples.

(e) Label additional tubes for samples >13 IU/mL by BNA 4-2, 4-3, 4-4, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 4-11, 4-12, etc.

(f) Pipette 1.0 mL of glycine buffer into all but the 1:20 dilution tube (tube 1).

(g) Titer controls and unknowns using a 1.0-mL pipette. Discard 1.0 mL from the last tube in each titration

set.

(h) Dispense 1.0 mL Fraction II-latex reagent to all tubes.

(i) Incubate all tubes for 2 hours at 56 °C.

(j) Remove the tubes from the water bath and spin for 4 min at 2000 rpm.

(k) Store the tubes at 4-8 °C.

(l) Read the agglutination titer and report the last titer with visible agglutination. Tubes are read by vigorously tapping them and looking for agglutination with oblique lighting. Use an intense light source and a dark background. With a finger behind the tube, look for agglutination and opacity of particle suspension. Compare the last positive tube with a known negative to confirm the endpoint.

e. **Recording of Data**

(1) Quality Control Data

(a) As new stock control is prepared, define a new control range using MAQ Sunquest laboratory data computer. Set a reasonable arbitrary control range. Append TEMP to the control lot number name.

(b) Log in control (function RE) under location IMMQC-, so it will appear on the worksheet. Use the date of 1/1 as both the collect and the receive date so it will appear first on the worksheet, and append the comment: "result under 'I____,0'" to alert the technologists performing the assay that there is a new control to be checked out, and to tell them where to enter the results.

(c) After 20 parallel runs, use the data from the Levey-Jennings chart to assign a permanent mean and standard deviation.

(d) Using the function MAQ, modify the control range. Remove TEMP from the lot name, adding the date of range calculation. In general, enter the same number of decimal places as used when reporting results. If the control is to be used immediately, print a Levey-Jennings chart for the current control (function QC option L) and advance the index of the new control to 1. Leave the printout, dated with the date of advancement on the supervisor's desk. *Do not advance a control lot unless all assays in which this lot is used are also ready to be advanced.*

(e) Delete the IMMQC log-in on the worksheet by using FUNCTION RE.

(f) Defining new controls in the computer is done using function MAQ. All control information is kept in the University of Washington computer only.

(g) Enter the following information:

(i) Method code.

(ii) Control code (I__).

(iii) Test code. Note: Some tests have test codes that apply only to control tests. This is usually because the computer performs some calculation for that test that should not be applied to the control. All code names will be listed with the methods. Generally QC-specific test names will end in 'C'.

(iv) Control data: mean and standard deviation (SD) or "accept" range.

- if qualitative controls: mean = 0 (negative), or mean=1 (positive), SD = 0.1.
- if mean and SD are entered, enter '2' at range prompt (2 SD's will be used to set the range.)

(h) General information

(i) Index number:
Index 0 = lot number under evaluation
Index 1 = lot in use
Index ≥2 = previous lots

(ii) The aliquot label should include the date of preparation and a letter indicating which aliquoting sequence (e.g., 9/90-A for the first time this control is aliquoted, 9/90-B for the second time). Record the label on the QC record sheet.

(iii) The lot name should include an identifying name, the date the control was prepared (month and year), and information about the control range (temperature or date of calculation or recalculation).

(2) Analytical Results

The nephelometry results are not reported to NCHS. The test results from the tube agglutination assay are added to the NHANES III disk by a data entry system.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Behring Nephelometric Analyser (BNA) Instrument

(a) Dismantling and assembling the BNA cuvette rotor; replacing the cuvettes.

Excessive cuvette blank values require a replacement of the cuvettes and consequently a dismantling of the cuvette rotor. Starting with the MAIN MENU, select program 6-MAINTENANCE/USER SERVICE followed by 1-CUVETTES, instruction 4-CHANGE CUVETTES. After undoing the knobs and lifting the rotor, replace the individual cuvette segments in accordance with their position. After reassembling press ENTER for automatic measurement of the cuvette blank values.

(b) Priming the tubing system and the syringes.

After the initial staartup, prime the syringes and the tubing connections of the dilutor when replacing the dilutor syringes, discovering bubbles in the tubing system, replacing a buffer, or disinfecting the tubing system. From the MAIN MENU, select program 6-MAINTENANCE/USER SERVICE followed by 3-DILUTOR; depending on which syringe or tube is used, select 1-PRIME WITH DILUENT or 2-PRIME WITH BUFFER. Enter the number of rinse cycles and repeat until the system is filled and without any bubbles.

(c) Priming the cuvette washing device.

Prime the cuvette washing device before the initial startup, disinfection, or if the washing solution bottle needs replacing. From the MAIN MENU, select program 6-MAINTENANCE/USER SERVICE followed by 2-WASHING DEVICE, and instruction 1-PRIME WASH DEVICE. After replacing the wash solution, select 3, and perform 30 rinse cycles.

(d) Replacing the syringes.

From the MAIN MENU, select program 6-MAINTENANCE/USER SERVICE followed by 3-DILUTOR, and instruction 3-REPLACE SYRINGES. After installing the syringes, press ENTER. The program returns to subprogram DILUTOR and automatically rinses the syringes.

(e) Replacing the tubing.

If the tubing has buckled, blocked, or has air bubbles after repeated rinsing, the tube connection must be replaced. After adding new tubing, rinse the tubing with buffer or diluent depending on the tubing that is replaced.

(f) Replacing the stirrer.

Replace if damaged or if it runs 'untrue'. From the MAIN MENU select program 6-MAINTENANCE/USER SERVICE followed by 5-TEST STIRRER.

(h) Disinfect instrument parts as outlined in the operator's manual.

(2) Pipettors

All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) Screening test -- latex-enhanced nephelometry

The Behring instrument has full data-reduction capabilities. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve. The protein concentration is calculated automatically by the instrument from the corresponding reference curve. A blank is automatically subtracted.

This method results in a linearized 6-point (including zero) standard curve with a direct relationship of measured light scatter to concentration of rheumatoid factors in the serum sample. Serum results are expressed as IU/mL.

A valid standard curve for the RF assay to be run must be stored in the BNA memory before sample results can be quantified. Reference curves must be determined daily. The instrument will notify the operator if a specific reference curve is valid or expired.

(2) Singer-Plotz tube titration latex agglutination

Positive tests from tube titer are reported to the last dilution showing visible agglutination.

h. **Special Procedure Notes**

(1) Complete reconstitution of the reagents is important. Allow reconstituted reagents to stand at 20-25 °C for a minimum of 15 min: Failure to do so can cause inaccurate results.

(2) The dilutions of the RF standard must be used within 4 hours of preparation.

## 9. REPORTABLE RANGE OF RESULTS

Final reports express results as negative or positive with titer for the presence of rheumatoid factors in the sample.

Positive tests are reported to the last dilution showing visible agglutination.

Serum titered because the BNA result was >13 IU/mL but <30 IU/mL can be reported as negative if the tube titer is negative. Serum that has a BNA result >30 IU/mL and a negative tube titer must be repeated on the following run before it can be reported.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years. The method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-the-art. For nephelometry, the primary standard used was prepared by Behring Diagnostics and standardized against the WHO International Reference Preparation (IRP) of rheumatoid arthritis serum, available from the National Institute of Biological Standards and Controls, UK. Estimates of imprecision can be generated from long-term QC pool results.

Bench quality control materials are used in this analytical method. Bench quality control specimens are inserted by the

analyst two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

The bench controls are prepared in sufficient quantity to provide serum samples for all the assays for 2 years. Ranges are established after 20 parallel runs with previously established controls. Ranges are established by using the formulas for statistical calculations for geometric data. The QC pools for the tube agglutination assay comprise three levels of concentration spanning the negative, low-positive, and high-positive ranges for rheumatoid factors. The QC pools for the BNA assay comprise two levels of concentration spanning the low and high ranges.

After the standards and bench QC materials are analyzed (at the beginning of an analytical run), the long-term QC charts for each control material are consulted to determine if the system is "in control." Two types of charts are used. The first chart plots the means of the duplicate determinations and compares them with the 95% and 99% confidence limits as well as with the center line (the overall mean of the characterization runs). The system is out of control if any of the following events occur for any one of the QC materials:

- The mean from a single pool falls outside the 99% confidence limits.
- The means from two pools fall either above or below the 95% confidence limits.
- The means from eight successive runs for one pool fall either all above or all below the center line.

The second type of QC chart plots the range of the duplicate determinations and compares them with the 95% and 99% limits as well as to the center line. The system is out of control if any of the following events occur for any one of the quality control materials:

- The range from a single pool falls above the 99% limit.
- The ranges from two pools fall above the 95% limit.
- The ranges from eight successive runs fall above the center line.

If the run is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any analysis of specimens occurs.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Consult with the supervisor for appropriate actions. For either analysis, if the run is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any results are released.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

(a) Screening test -- latex-enhanced nephelometry

(1) Heavily lipemic or turbid samples should be cleared by filtration before assaying. Microbial contamination may denature proteins in the sample.

(2) Some samples may show an increase in RF levels when higher sample dilutions are assayed. The cause of this phenomenon is unknown, but may relate to "masking" of molecules, which is eliminated when the RF concentration is reduced by dilution.

(3) Light scatter from sources other than the analyte reaction must be minimized for optimal performance. Be sure that the black-tinted covering of the rotor assembly is in place.

(4) Heat inactivation of the serum is not necessary since the complement factors do not interfere with the test; however, positive samples must be inactivated for follow-up tube titration.

(b) Singer-Plotz tube titration latex agglutination

Initial 1:20 serum dilution for the tube agglutination testing is heat-activated.

## 13. REFERENCE RANGES (NORMAL VALUES)

Three percent of the normal population have rheumatoid factors present in their serum, and this figure increases for the elderly.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable to this procedure.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may reach or maintain 20-25 °C during analysis. Otherwise, they are stored at ≤-85 °C.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no acceptable alternative methods of analysis. Specimens may be stored at 4-8 °C for no longer than 8 days. Otherwise, specimens should be stored at ≤-85 °C until the system is restored to functionality.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this procedure.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping should be used for tracking specimens. The instrument results menu includes current daily results as well as stored test results. An additional subprogram initiates a print-out of results in tabular form. Only numerical identifiers should be used.

The original NHANES III disk is copied onto two backup floppy disks and onto the hard drive of IBM computer. After the results are entered into the database, the disks are stored in three separate locations.

Residual serum samples are stored at ≤-85 °C for 1 year after analysis and are then shipped to the NCHS serum repository in Rockville, MD, for long-term storage.

## REFERENCES

1. Singer J, Plotz C. The latex fixation test. I: Application to the diagnosis of rheumatoid arthritis. Am J Med 1956;21:888-92.

2. Rose NR, Friedman H. Manual of Clinical Immunology, American Society for Microbiology, ASM, Washington, (DC):1976.

3. Singer J. The latex fixation test in rheumatic diseases. Am J Med 1961;31:766-79.

4. Quality Control for Immunologic Tests. Atlanta: Center for Disease Control,1979 (HEW Publication No. 79-8376).

## ACKNOWLEDGMENTS

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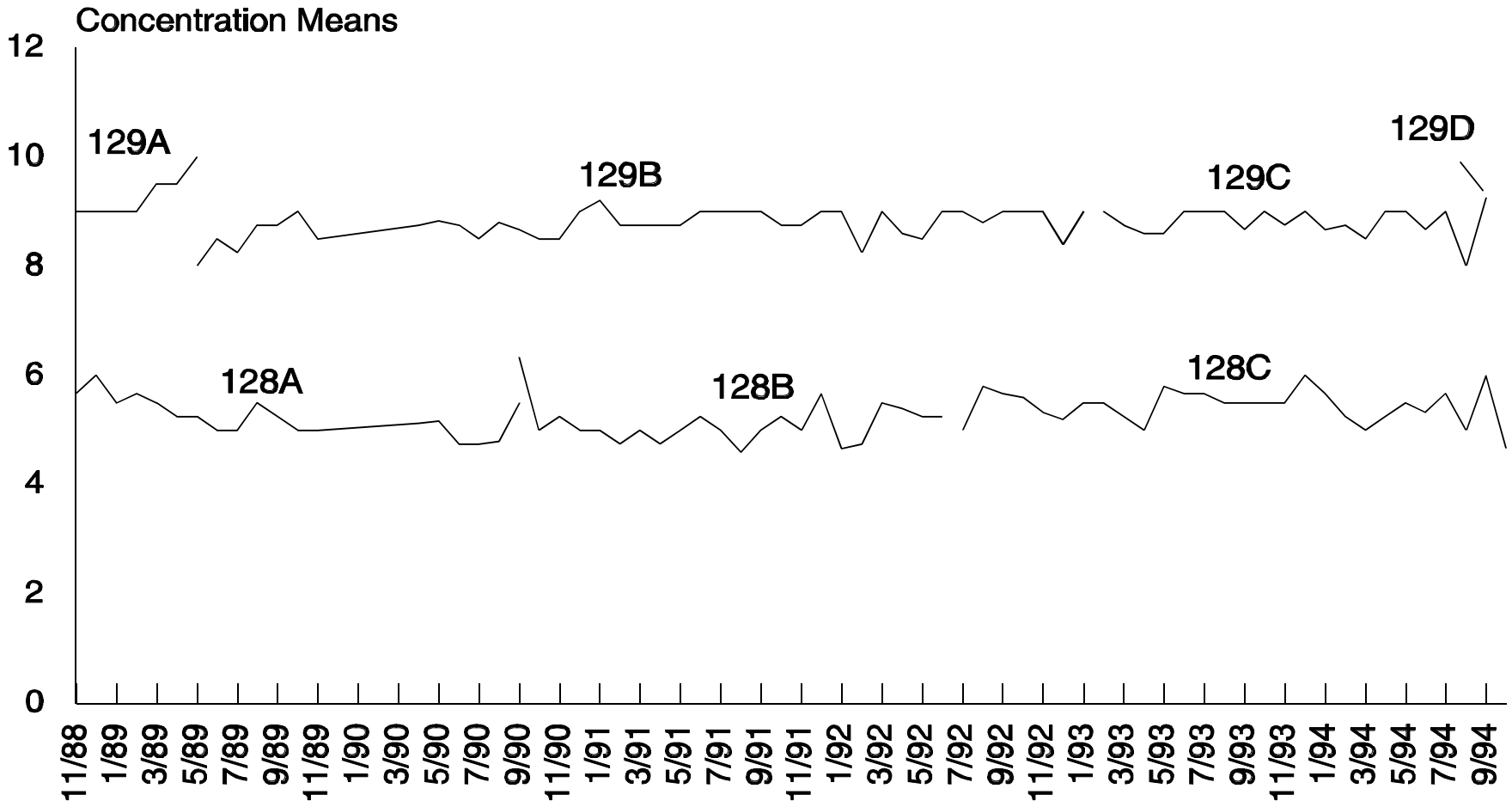
SUMMARY STATISTICS FOR
RHEUMATOID FACTOR
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 128A | 11/01/88 - 09/15/90 | 5.20513 | 0.56658 | 10.8850 | 78 |
| 128B | 09/16/90 - 06/30/92 | 5.11111 | 0.65192 | 12.7550 | 81 |
| 128C | 07/01/92 - 10/20/94 | 5.44340 | 0.53595 | 9.8459 | 106 |
| 129A | 11/01/88 - 05/10/89 | 9.26087 | 0.54082 | 5.8399 | 23 |
| 129B | 05/11/89 - 02/03/93 | 8.75949 | 0.54633 | 6.2370 | 158 |
| 129C | 02/04/93 - 09/30/94 | 8.79747 | 0.51589 | 5.8641 | 79 |
| 129D | 10/01/94 - 10/20/94 | 9.33333 | 0.57735 | 6.1859 | 3 |

# Rheumatoid Factor Monthly Means




**Testing of NHANES specimens for rheumatoid factor was not conducted for the period 12/89 - 3/90.**

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The 22 analytes described in this method constitute the routine biochemistry profile. The analyses are performed with a Hitachi Model 737 multichannel analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN) Each analyte is described separately within each pertinent section of this document. NOTE: Glucose, cholesterol, and triglycerides were analyzed as part of this profile, but the results do not replace the formalized reference methods data from NHANES III samples analyzed at other institutions.

### a. Alanine Aminotransferase (ALT)

α-Ketoglutarate reacts with L-alanine in the presence of ALT to form L-glutamate plus pyruvate. The pyruvate is used in the indicator reaction for a kinetic determination of the reduced form of nicotinamide adenine dinucleotide (NADH) consumption. The International Federation of Clinical Chemistry (IFCC) has now recommended standardized procedures for ALT determination, including 1) optimization of substrate concentrations, 2) the use of Tris buffers, 3) preincubation of a combined buffer and serum solution to allow side reactions with NADH to occur, 4) substrate start (α-ketoglutarate), and 5) optimal pyridoxal phosphate activation.

As a group, the transaminases catalyze the interconversion of amino acids and α-keto acids by transferring the amino groups. The enzyme ALT been found to be in highest concentration in the liver, with decreasing concentrations found in kidney, heart, skeletal muscle, pancreas, spleen, and lung tissue. Alanine aminotransferase measurements are used in the diagnosis and treatment of certain liver diseases (e.g., viral hepatitis and cirrhosis) and heart diseases. Elevated levels of the transaminases can indicate myocardial infarction, hepatic disease, muscular dystrophy, or organ damage. Serum elevations of ALT activity are rarely observed except in parenchymal liver disease, since ALT is a more liver-specific enzyme than asparate aminotransferase (AST) (1).

### b. Albumin

At the reaction pH, the bromcresol purple (BCP) in the Boehringer Manneheim Diagnostics (BMD) albumin system reagent binds selectively with albumin. This reaction is based on a modification of a method described by Doumas (4). Although BCP is structurally similar to the conventional bromcresol green (BCG), its pH color change interval is higher (5.2 - 6.8) than the color change interval for BCG (3.8 - 5.4), thus reducing the number of weak electrostatic dye/protein interactions. The BCP system eliminates many of the nonspecific reactions with other serum proteins as a result of the increased pH. In addition, the use of a sample blank eliminates background spectral interferences not completely removed by bichromatic analyses.

Albumin constitutes about 60% of the total serum protein in normal, healthy individuals. Unlike most of the other serum proteins, albumin serves a number of functions which include transporting large insoluble organic anions (e.g., long chain fatty acids and bilirubin), binding toxic heavy metal ions, transporting excess quantities of poorly soluble hormones (e.g., cortisol, aldosterone, and thyroxine), maintaining serum osmotic pressure, and providing a reserve store of protein. Albumin measurements are used in the diagnosis and treatment of numerous diseases primarily involving the liver or kidneys (2).

### c. Alkaline Phosphatase (ALP)

In the presence of magnesium ions, p-nitrophenylphosphate is hydrolyzed by phosphatases to phosphate and p-nitrophenol. The rate of p-nitrophenol liberation is proportional to the ALP activity and can be measured photometrically.

Increased ALP activity is associated with two groups of diseases: those affecting liver function and those involving osteoblastic activity in the bones. In hepatic disease, an increase in ALP activity is generally accepted as an indication of biliary obstruction. An increase in serum phosphatase activity is associated with primary hyperparathyroidism, secondary hyperparathyroidism owing to chronic renal disease, rickets, and osteitis deformans juvenilia due to vitamin D deficiency and malabsorption or renal tubular dystrophies. Increased levels of ALP are also associated with Von Recklinghausen's disease with bone involvement and malignant infiltrations of bone. Low levels are associated with hyperthyroidism, and with the rare condition of idiopathic hypophosphatasia associated with rickets and the excretion of excess phosphatidyl ethanolamine in the urine (3).

### d. Aspartate Aminotransferase (AST)

α-Ketoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The

indicator reaction uses the oxaloacetate for a kinetic determination of NADH consumption. The International Federation of Clinical Chemistry (IFCC) has now recommended standardized procedures for ALT determination, including 1) optimization of substrate concentrations, 2) the use of Tris buffers, 3) preincubation of a combined buffer and serum solution to allow side reactions with NADH to occur, 4) substrate start ($\alpha$-ketoglutarate), and 5) optimal pyridoxal phosphate activation.

As a group, the transaminases catalyze the interconversion of amino acids and $\alpha$-keto acids by transferring the amino groups. The enzyme AST has been demonstrated in every animal and human tissue studied. Although the enzyme is most active in the heart muscle, significant activity has also been seen in the brain, liver, gastric mucosa, adipose tissue, skeletal muscle, and kidneys of humans. AST measurements are used in the diagnosis and treatment of certain types of liver and heart disease. AST is present in both the cytoplasm and mitochondria of cells. In cases involving mild tissue injury, the predominant form of serum AST is from the cytoplasm, with smaller amounts from the mitochondria. Severe tissue damage results in more of the mitochondrial enzyme being released. Elevated levels of the transaminases can signal myocardial infarction, hepatic disease, muscular dystrophy, or organ damage (4).

e. **Bicarbonate ($HCO_3$)**

Bicarbonate reacts with phosphoenolpyruvate (PEP) in the presence of PEPC to produce oxaloacetate and phosphate. This reaction occurs in conjunction with the transfer of a hydrogen ion from NADH to oxaloacetate using MDH. The resultant formation of NAD causes a decrease in absorbance in the UV range (320-400 nm). The change in absorbance is directly proportional to the concentration of bicarbonate in the sample being assayed.

Bicarbonate is the second largest fraction of the anions in plasma. Included in this fraction are the bicarbonate ($HCO_3^-$) and carbonate ($CO_3^{-2}$) ions and the carbamino compounds. At the pH of blood, the ratio of carbonate to bicarbonate is 1:1000. The carbamino compounds are also present, but are generally not mentioned specifically. The bicarbonate content of serum or plasma is a significant indicator of electrolyte dispersion and anion deficit. Together with pH determination, bicarbonate measurements are used in the diagnosis and treatment of numerous potentially serious disorders associated with acid-base imbalance in the respiratory and metabolic systems (5).

f. **Blood Urea Nitrogen (BUN)**

Urea is hydrolyzed by urease to form $CO_2$ and ammonia. The ammonia formed then reacts with $\alpha$-ketoglutarate and NADH in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and $NAD^+$. The decrease in absorbance due to consumption of NADH is measured kinetically.

Urea is synthesized in the liver from ammonia produced as a result of deamination of amino acids. This biosynthetic pathway is the human body's chief means of excreting surplus nitrogen. BUN measurements are used in the diagnosis of certain renal and metabolic diseases. The determination of serum urea nitrogen is the most widely used test for the evaluation of kidney function. The test is frequently requested in conjunction with the serum creatinine test for the differential diagnosis of prerenal, renal, and postrenal uremia. High BUN levels are associated with impaired renal function, increased protein catabolism, nephritis, intestinal obstruction, urinary obstruction, metallic poisoning, cardiac failure, peritonitis, dehydration, malignancy, pneumonia, surgical shock, Addison's disease, and uremia. Low BUN levels are associated with amyloidosis, acute liver disease, pregnancy, and nephrosis. Normal variations are observed according to a person's age and sex, the time of day, and diet, particularly protein intake (6).

g. **Calcium**

Calcium reacts with o-cresolphthalein complexone in the presence of 8-hydroxyquinoline-5-sulfonic acid to form a purple complex. The intensity of the final reaction color is proportional to the amount of calcium in the specimen.

Elevated total serum calcium levels are associated with idiopathic hypercalcemia, vitamin D intoxication, hyperparathyroidism, sarcoidosis, pneumocystic carinii pneumonia and blue diaper syndrome. Low calcium levels are associated with hypoparathyroidism, pseudohypoparathyroidism, chronic renal failure, rickets, infantile tetany, and steroid therapy (7).

h. **Cholesterol**

All cholesterol esters present in serum or plasma are hydrolyzed quantitatively into free cholesterol and fatty acids

by microbial cholesterol esterase. In the presence of oxygen, free cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3-one. The $H_2O_2$ reacts in the presence of peroxidase (POD) with phenol and 4-aminophenazone to form an o-quinone imine dye. The intensity of the color is proportional to the cholesterol concentration and is measured photometrically.

An elevated cholesterol level is associated with diabetes, nephrosis, hypothyroidism, biliary obstruction, and those rare cases of idiopathic hypercholesterolemia and hyperlipemia; low levels are associated with hyperthyroidism, hepatitis, and sometimes severe anemia or infection (8).

i. **Creatinine**

This method, which uses the Jaffe reaction, is based on the work of Popper, Seeling, and Wuest. In an alkaline medium, creatinine forms a yellow-orange-colored complex with picric acid. The rate of color formation is proportional to the concentration of creatinine present and may be measured photometrically.

Creatinine measurement serves as a test for normal glomerular filtration. Elevated levels are associated with acute and chronic renal insufficiency and urinary tract obstruction. Levels below 0.6 mg/dL are of no significance (9).

j. **Gamma Glutamyltransaminase (γ-GT)**

In this rate method, L-γ-glutamyl-3-carboxy-4-nitroanilide is used as a substrate and glycylglycine as a acceptor. The rate at which 5-amino-2-nitrobenzoate is liberated is proportional to γ-GT activity and is measured by an increase in absorbance.

γ-GT measurement is principally used to diagnose and monitor hepatobiliary disease. It is currently the most sensitive enzymatic indicator of liver disease, with normal values rarely found in the presence of hepatic disease. It is also used as a sensitive screening test for occult alcoholism. Elevated levels are found in patients who chronically take drugs such as phenobarbital and phenytoin (10).

k. **Glucose**

Hexokinase catalyzes the phosphorylation of glucose by adenosine triphosphate (ATP). G-6-PD is oxidized to 6-phosphogluconate in the presence of NAD by the enzyme glucose-6-phosphate dehydrogenase. No other carbohydrate is oxidized.

The glucose hexokinase method, based on the work of Schmidt, Peterson, and Young, has long been recognized as the most specific method for the determination of glucose. Glucose measurements are used in the diagnosis and treatment of pancreatic islet cell carcinoma and of carbohydrate metabolism disorders, including diabetes mellitus, neonatal hypoglycemia, and idiopathic hypoglycemia (11).

l. **Iron**

Iron ($Fe^{3+}$) is separated from transferrin by means of guanidinium chloride in the weakly acidic pH range and reduced to $Fe^{2+}$ with ascorbic acid. $Fe^{2+}$ then forms a colored complex with ferrozine.

Ingested iron is absorbed primarily from the intestinal tract and is temporarily stored in the mucosal cells as $Fe_n^{3+}$ - ferritin, a complex of ferric hydroxide-ferric phosphate attached to the protein apoferritin. On demand, iron is released from the mucosal cells into the blood as $Fe_2^{3+}$-transferrin in equilibrium with a very small amount of free $Fe^{3+}$. Transferrin is the plasma iron transport protein that binds iron strongly at physiological pH levels.

Iron (non-heme) measurements are used in the diagnosis and treatment of diseases such as iron deficiency anemia, chronic renal disease, and hemochromatosis (a disease associated with widespread deposit in the tissues of two iron-containing pigments, hemosiderin and hemofuscin, and characterized by pigmentation of the skin) (12).

m. **Lactate Dehydrogenase (LDH)**

This enzyme converts lactate and NADH to pyruvate and NADH respectively. The rate at which NADH is formed is determined by the rate of absorbance and is directly proportional to enzyme activity.

LDH measurements are used in the diagnosis and treatment of liver diseases such as acute viral hepatitis,

cirrhosis, and metastatic carcinoma of the liver; cardiac diseases such as myocardial infarction; and tumors of the lungs or kidneys (13).

n. **Phosphorus**

Inorganic phosphorus reacts with ammonium molybdate in an acidic solution to form ammonium phosphomolybdate with a formula of $(NH_4)_3[PO_4(MoO_3)_{12}]$. The ammonium phosphomolybdate is quantified in the ultraviolet range (340 nm), through the use of a sample-blanked endpoint method.

More than 80% of the body's phosphorus is present in the bones as calcium phosphate. The remainder is involved in the intermediary metabolism of carbohydrates and is a component of such physiologically important substances as phospholipids, nucleic acids, and ATP. Phosphorus is present in blood as inorganic and organic phosphates, nearly all the latter residing in the erythrocytes. The small amount of extracellular organic phosphate exists almost exclusively in the form of phospholipid; the remainder of serum phosphorus is present as inorganic phosphate.

There is a reciprocal relationship between serum calcium and inorganic phosphorus. Any increase in the level of inorganic phosphorus causes a decrease in the calcium level by a mechanism not clearly understood. Hyperphosphatemia is associated with vitamin D hypervitaminosis, hypoparathyroidism, and renal failure. Hypophosphatemia is associated with rickets, hyperparathyroidism, and Fanconi syndrome.

Measurements of inorganic phosphorus are used in the diagnosis and treatment of various disorders, including parathyroid gland and kidney diseases and vitamin D imbalance (14).

o. **Sodium, Potassium, and Chloride**

An ion-selective electrode (ISE) makes use of the unique properties of certain membrane materials to develop an electrical potential (electromotive force, EMF) for the measurement of ions in solution. The electrode has a selective membrane in contact with both the test solution and an internal filling solution. The internal filling solution contains the test ion at a fixed concentration. Because of the particular nature of the membrane, the test ions will closely associate with the membrane on each side. The membrane EMF is determined by the difference between the ion concentration in the test solution and that in the internal filling solution. The EMF develops according to the Nernst equation for a specific ion in solution:

[1] $E = E_0 + RT/nf \times \ln (f \times Ct/f \times Ci)$

Where:

- E = electrode EMF
- $E_0$ = standard EMF
- R = constant
- T = temperature
- n = charge of ion
- F = Faraday's constant
- ln = natural logarithm (base e)
- f = activity coefficient
- Ct = ion concentration in test solution
- Ci = ion concentration in internal filling solution

For sodium, potassium, and chloride, which all carry a single charge, R,T,n, and f are combined into a single value referred to as the slope (S). For determinations on the Hitachi 737 ISE module, where the sample is diluted 1:31, the ionic strength (and therefore the activity coefficient) is essentially constant. The concentration of the test ion in the internal filling solution is also constant. These constants may be combined into the $E_0$ term. The value of $E_0$ is also specific for the type of reference electrode used. Equation [1] can be rewritten to reflect these conditions:

[2] $E = E'_0 + S \times \ln(Ct)$

The complete measurement system for a particular ion includes the ISE, a reference electrode, and electronic circuits to measure and process the EMF to give the test ion concentration. The direct-liquid-junction type reference electrode renews the reference electrode solution before and after sample measurement. The electromotive force is then measured to prevent drift.

The type of ISE used on the ISE Module is classified as the liquid/liquid junction type. The sodium and potassium electrodes are based on neutral carriers, and the chloride electrode is based on an ion exchanger.

Sodium is the major cation of extracellular fluid. It plays a central role in the maintenance of the normal distribution of water and the osmotic pressure in the various fluid compartments. Hyponatremia (low serum sodium level) is associated with a variety of conditions, including severe polyuria, metabolic acidosis, Addison's disease, diarrhea, and renal tubular disease. Hypernatremia (increased serum sodium level) is associated with Cushing's syndrome, severe dehydration due to primary water loss, certain types of brain injury, diabetic coma after therapy with insulin, and excess treatment with sodium salts.

Potassium is the major intracellular cation. Hypokalemia (low serum potassium level) is associated with body potassium deficiency, excessive potassium loss caused by prolonged diarrhea or prolonged periods of vomiting and increased secretion of mineralocorticosteroids. Hyperkalemia (increased serum potassium level) is associated with oliguria, anuria, and urinary obstruction.

Chloride is the major extracellular anion. Low serum chloride values are associated with salt-losing nephritis, Addisonian crisis, prolonged vomiting, and metabolic acidosis caused by excessive production or diminished excretion of acids. High serum chloride values are associated with dehydration and conditions causing decreased renal blood flow, such as congestive heart failure (15).

p. **Total Bilirubin**

Total bilirubin is coupled with diazonium salt DPD (2,5-dichlorophenyldiazonium tetrafluoroborate) in a strongly acidic medium (pH 1-2). The intensity of the color of the azobilirubin produced is proportional to the total bilirubin concentration and can be measured photometrically.

Bilirubin is an organic compound formed by the reticuloendothelial system during the normal and abnormal destruction of red blood cells. Elevated levels are associated with hemolytic jaundice, paroxysmal hemoglobinuria, pernicious anemia, polycythemia, icterus neonatorum, internal hemorrhage, acute hemolytic anemia, malaria, and septicemia. Low bilirubin levels are associated with aplastic anemia, and certain types of secondary anemia resulting from toxic therapy for carcinoma and chronic nephritis (16).

q. **Total Protein**

In alkaline solution, a colored chelate forms between cupric ions and compounds containing at least two $-CONH_2$, $-CSNH_2$, $-CH_2NH_2$ or similar groups, joined directly or through a carbon or nitrogen atom. In proteins, the chelate is formed between one cupric ion and about six nearby peptide bonds. The intensity of the color is proportional to the total number of peptide bonds undergoing reaction and thus to the total amount of protein present. This is similar to the biuret reaction. Although compounds undergoing the biuret reaction give colors ranging from pink to purple, the violet colors given by serum albumins and globulins are essentially the same. Peptides of low molecular weight are present in serum, but their concentration is too low to cause interference.

Serum proteins perform a number of different functions in the body. In addition to being major structural components of cells, proteins are involved in transport, enzymatic catalysis, homeostatic control, hormonal regulation, blood coagulation, immunity, growth and repair, and heredity. Total protein measurements are used in the diagnosis and treatment of a variety of diseases involving the liver, kidney, or bone marrow, as well as other metabolic or nutritional disorders (17).

r. **Triglycerides**

This method uses microbial lipase to promote rapid and complete hydrolysis of triglycerides to glycerol with subsequent oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The peroxide reacts with 4-aminophenazone and 4-chlorophenol in a Trinder reaction to a colorimetric endpoint.

Triglyceride measurements are used in the diagnosis of diabetes mellitus, nephrosis, liver obstruction, and other diseases involving lipid metabolism and various endocrine disorders and in the teatment of patients with these diseases (18).

s. **Uric Acid**

Uric acid is oxidized by the specific enzyme uricase to form allantoin and $H_2O_2$. The $H_2O_2$ reacts with 2,4,6-tribromo-3-hydroxybenzoic acid (TBHB) and 4-aminophenazone in the presence of peroxidase to form quinone-imine dye and hydrogen bromide (HBr). The intensity of the red color is proportional to the uric acid concentration.

Uric acid measurements are used in the diagnosis and treatment of numerous renal and metabolic disorders, including renal failure, gout, leukemia, psoriasis, starvation or other wasting conditions and in the treatment of patients receiving cytotoxic drugs (19).

## 2. SPECIAL SAFETY PRECAUTIONS

Wear gloves, scrubs, laboratory coats, and face shields while handling all human blood products. Dispose of all biological samples and diluted specimens in a biohazard container at the end of the analysis. Place all disposable plastic, glass, and paper (pipet tips, Hitachi analyzer cups, tubes, gloves, etc.) that contact blood in the biohazard container located at the work sites. These containers will be used until they are 75% full, they then will be sealed, labeled, and transported to a biohazard storage facility until their removal by commercial contractor. Wipe down all work surfaces with 10% sodium hypochlorite solution when work is finished. Waste reagents from the Hitachi 737 and all control serum samples are considered a source of infectious material and must be treated with the same degree of caution as a high-risk specimen.

Material Safety Data Sheets (MSDS) for BCP chromogen; magnesium-L-aspartate; 2-amino-2-methyl-1-propanol buffer; magnesium; NADH/LDH; Tris/L-alanine buffer solution; α-ketoglutarate solution; NADH; magnesium acetate; and phosphoenolpyruvate (PEP) buffer solution; bicarbonate diluent; PEPC/MDH, detergent/HCl solution; 2,5-dichlorophenyldiazonium tetrafluoroborate (DPD); GLDH/NADH/α-ketoglutarate; urease substrate; o-cresolphthalein complexone/acetate buffer; solution contains 3,4-dichlorophenol; phenol; 4-aminophenazone; solution of cholesterol oxidase, cholesterol esterase, and horseradish; sodium hydroxide; picric acid; glycylglycine; Tris buffer; L-γ-glutamyl-3-carboxy-4-nitroanilide; sodium chloride; sodium chloride/sulfuric acid; ammonium molybdate; solution containing sodium hydroxide, potassium sodium tartrate and potassium iodide; solution containing cupric sulfate, sodium hydroxide, potassium sodium tartrate and potassium iodide; boric acid; solution containing boric acid, sodium chloride, sodium bicarbonate, and potassium phosphate; potassium chloride; ATP/enzymes; buffer/4-chlorophenol; phosphate buffer/TBHB; solution of uricase/4-aminophenazone, phosphate buffer, and sodium hypochlorite are located adjacent to the BMD HITACHI 737 in the WSRC clinical laboratory.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transferred data from a printed copy of the output filer and storing data in multiple computer systems. Data files containing the date, analytical run ID, specimen analytical results by specimen ID, and method code are stored in archive files in the Hitachi 737 main computer system in an ASCII format. Files are downloaded from the Hitachi 737 to the host computer (CompuAdd 386) via an RS232 port. The data are stored in two files: 1) the H_737.DBF, which contains all data received from the Hitachi 737 and includes all participant demographic data and analytical results, and 2) the H_TABLE.DBF file, which contains the names of the tests, their respective Hitachi 737 test code numbers, and the date and time the samples were entered into the Hitachi 737 workstation. An output file, created by selecting fields from the NHANES files, is downloaded to a 5¼" HD diskette. An ASCII file of the data, created and copied to a 5¼" HD diskette, is sent to NCHS. The file is also copied onto another CompAadd 386 in the laboratory administration area.

b. Routine backup procedures include: 1) weekly backup of hard disks and 2) archival of data on a 3½" HD floppy diskette. Floppy diskettes containing sensitive data are stored in locked cabinets.

c. Documentation for system maintenance is contained in hard copies of data recorded, as well as in files on the local tape drives used for archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Use a nonhemolyzed specimen from a fasting subject.

b. Specimen type: serum or plasma with EDTA, heparin, citrate, or fluoride anticoagulants. Do not use oxalate.

Separate serum or plasma from cells within 1 hour of collection.

c. The optimal amount of specimen is 1.0 mL serum; the minimum is 0.5 mL serum or plasma.

d. Acceptable containers for collection include 10- or 15-mL red-top or serum-separator Vacutainer tubes. Store serum in 2.0-mL Nalge tubes.

e. Specimens should be refrigerated if not used immediately. Specimens stored longer than 24 hours should be frozen at ≤-20 °C. Specimen stability has been demonstrated for 1 year at ≤-20 °C.

f. The criteria for unacceptable specimens are low volume (<0.25 mL), hemolysis, improper labeling, and prolonged contact of serum or plasma with cells.

g. Specimen handling conditions are outlined in the White Sands Clinical Laboratory's *Collection Procedures and Specimen Requirements Manual* located in all sections of the laboratory and available to clients upon request. Collection, transport, and special requirements are discussed. In general, serum specimens from NCHS collection sites are transported on dry ice and stored at ≤-70 °C until analysis. Residual samples should be refrozen at ≤-70 °C.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Hitachi 737 automated analyzer (Boehringer Mannheim Diagnostics (BMD), Indianapolis, IN). The analyzer includes a tungsten-halogen lamp, DMS 16-bit computer with 1MB RAM and 20 MB hard disk, remote computer workstation, and Okidata Microline 320 9-pin printer.

(2) Sealpette variable-volume micropipets: 2-20, 20-200, and 200-1000 μL volumes (Cole Scientific, Moorpark, CA).

(3) Tek Pro Tek-tator V variable rotator (Baxter Healthcare, Valencia, NC).

(4) Pipet-aid (Drummond Scientific Co., Broomall, PA).

(5) Fisher hematology mixer (Fisher Scientific, Pittsburgh, PA).

### b. Other Materials

(1) Reagents (BMD).

(2) BMD Precical calibrators (BMD).

(3) BMD Precitrol normal and abnormal human assayed control serum (BMD).

(4) Dade Monitrol levels I & II unassayed quality control serum (Dade, Miami, FL). This is a human serum-based quality control material that has been assayed in our laboratory against an assayed quality control serum.

(5) Physiological saline, 0.9% (Ricca Chemical, Arlington, TX). Must contain no additives or preservatives.

(6) 3.0-mL, class A volumetric pipets (any vendor).

(7) Conical-bottom 2.0-mL polystyrene autosampler cups (BMD).

(8) Ultrapure water, Barnstead E-pure water purification system with a resistivity of >16 megohm-cm (Culligan

Water Systems, Alamogordo, NM).

(9) 3.5-mL Beral polypropylene transfer pipettes (Sarstedt, Newton, NC).

(10) Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).

(11) Clean-room vinyl gloves (Baxter Healthcare).

(12) Biohazard autoclave bags (Curtin-Matheson Scientific, Inc., Atlanta, GA).

(13) Bleach (10% sodium hypochlorite solution) (any vendor).

(14) 9½" x 11" 20-lb white computer paper (Viking Office Products, Irving, TX).

c. **Reagent Preparation**

(1) ALT

(a) Reagent 1 (R1) working solution
Transfer 24 tablets of NADH/LDH to 600 mL Tris/L-alanine buffer solution, pH 7.3. Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 4-8 °C for 4 weeks.

(b) Reagent 2 (R2) working solution
Use α-ketoglutarate solution, supplied "ready to use". Store capped at 4-8 °C until the expiration date on the package.

(2) Albumin

(a) Reagent 1 (R1) working solution
Contains buffer, preservatives, and surfactants, supplied "ready to use." Transfer to an analyzer bottle. Store at 4-8 °C until the expiration date on the package.

(b) Reagent 2 (R2) working solution
Contains 526 μmol/L BCP, buffer, preservatives, and surfactants, supplied ready to use. Transfer the contents of BCP chromogen to an analyzer bottle. Store at 4-8 °C until the expiration date on the package.

(3) ALP

(a) Reagent 1 (R1) working solution
Using a funnel, transfer 24 tablets of magnesium-L-aspartate to 600 mL 2-amino-2-methyl-1-propanol buffer solution, pH 10.5. Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 4-8 °C until the expiration date on the package.

*NOTE: A $CO_2$ "Scrubber" may be used on the R1 reagent bottle to control absorption of atmospheric carbon dioxide.*

(b) Reagent 2 (R2) working solution
Transfer 6 tablets of magnesium and 36 tablets of p-nitrophenylphosphate into 150 mL 2-amino-2-methyl-1-propanol buffer, pH 10.5. Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 4-8 °C until expiration date on package.

(4) AST

(a) Reagent 1 (R1) working solution
Transfer 24 tablets of NADH/LDH/MDH to 600 mL Tris/L-aspartate buffer solution, pH 7.8. Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 4-8 °C for 4 weeks.

(b) Reagent 2 (R2) working solution
Use α-ketoglutarate solution, supplied "ready to use." Store capped at 4-8 °C until the expiration date

on the package.

(5) Bicarbonate ($HCO_3$)

(a) Reagent 1 (R1) working solution
Contains NADH, magnesium acetate, and PEP in a pH 7.5 buffer solution. Add 300 mL deionized $CO_2$-free water to R1. Mix gently to dissolve. Store at 4-8 °C until the expiration date on the package. When sample-blanked absorbance is less than 10000, prepare fresh R1 working solution.

(b) Reagent 2 (R2) working solution
Connect one bottle of bicarbonate diluent to one bottle of PEPC/MDH using one of the adapters. Mix by gentle inversion. Store at 4-8 °C until the expiration date on the package. Prepare fresh R2 when quality control (QC) materials fail to conform to acceptable guidelines.

(6) BUN

(a) Reagent 1 (R1) working solution
Reconstitute the contents of one bottle of GLDH/NADH/α-ketoglutarate with 600 mL ammonia-free deionized water. Mix gently to dissolve. Stable at 4-8 °C until the expiration date on the package when protected from light and from contamination by microorganisms. Discard any solution with visible microbial growth, or when controls demonstrate shifts or trends.

(b) Reagent 2 (R2) working solution
Reconstitute the contents of one bottle of urease substrate with 300 mL ammonia-free deionized water. Mix gently to dissolve. Upon reconstitution, the R2 working solution will form small bubbles, which clear on standing. Stable at 4-8 °C until the expiration date on the package when protected from contamination by microorganisms. Discard any solution with visible microbial growth, or when controls demonstrate shifts or trends.

(7) Calcium

(a) Reagent 1 (R1) working solution
Use contents of the blank reagent, supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(b) Reagent 2 (R2) working solution
Use contents of the o-cresolphthalein complexone/acetate buffer reagent, supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(8) Cholesterol

Reagent 1 (R1) working solution
Contains 3,4-dichlorophenol, phenol, 4-aminophenazone, cholesterol oxidase, cholesterol esterase, and horseradish. Reconstitute the contents of the cholesterol reagent with 600 mL deionized water. Mix gently. The working solution is ready for use after 10 min. Store at 4-8 °C until the expiration date on the package.

(9) Creatinine

(a) Reagent 1 (R1) working solution
Use one bottle of NaOH, supplied "ready to use." Stable until the expiration date on the package if stored capped at 4-8 °C.

(b) Reagent 2 (R2) working solution
Use one bottle of picric acid, supplied ready to use. Stable until the expiration date on the package if stored capped at 4-8 °C.

(10) γ-GT

(a) Reagent 1 (R1) working solution
Transfer 6 tablets of glycylglycine to 600 mL Tris buffer solution, pH 8.25. Swirl gently to dissolve.

Aliquot into clean analyzer bottles and store capped at 4-8 °C for 3 months.

(b) Reagent 2 (R2) working solution
Use the tweezers to transfer 12 tablets of L-γ-glutamyl-3-carboxy-4-nitroanilide into 108 mL diluent. Swirl gently to dissolve. Aliquot into clean analyzer bottles and store capped at 4-8 °C for 3 weeks.

(11) Glucose

(a) Reagent 1 (R1) working solution
Reconstitute the contents of one bottle of sodium chloride (serum blank) with 600 mL deionized water. Mix gently. Store at 4-8 °C until the expiration date on the package.

(b) Reagent 2 (R2) working solution
Reconstitute the contents of one bottle of glucose/HK with 300 mL deionized water. Mix gently. Stable 2 weeks when stored at 4-8 °C.

(12) Iron

(a) Reagent 1 (R1) working solution
Add 10 level measuring spoons (provided) of granulated ascorbic acid into 500 mL buffer and allow to dissolve. The solution is ready for use after 10 min. Mix gently. Stable for 4 weeks when stored at 4-8 °C.

(b) Reagent 2 (R2) working solution
Use contents of one bottle of chromogen, supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(13) LDH

(a) Reagent 1 (R1) working solution
Reconstitute the contents of Tris-(hydroxymethyl)-aminomethane/lithium L-lactate/potassium chloride with 300 mL deionized water. Swirl gently to dissolve. After contents have dissolved, add an additional 300 mL deionized water. Mix gently. Stable 14 days when stored at 4-8 °C.

(b) Reagent 2 (R2) working solution
Reconstitute the contents of NAD with 100 mL deionized water. Mix gently. Stable for 7 days when stored capped at 4-8 °C.

(14) Phosphorus

(a) Reagent 1 (R1) working solution
Use one bottle of sodium chloride/sulfuric acid (phosphorus blank), supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(b) Reagent 2 (R2) working solution
Use ammonium molybdate, supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(15) Sodium, Potassium, and Chloride

(a) ISE diluent
Contains 650 mmol/L boric acid. Add 1800 mL of deionized water to one bottle of ISE diluent. Mix thoroughly by inversion. Store at 20-25 °C until the expiration date on the package.

(b) ISE internal reference solution
Contains 650 mmol/L boric acid, 32.3 mmol/L sodium chloride, 12.9 mmol/L sodium bicarbonate, and 1.6 mmol/L potassium phosphate. Add 1800 mL of deionized water to one bottle of ISE internal reference solution. Store at 20-25 °C until the expiration date on the package.

(c) Reference electrode internal solution
Contains 1 N KCl. Use reagent as provided. Stable until the expiration date on the package.

(16) Total Bilirubin

Reagent 1 (R1) working solution
Using the adapter, connect one bottle of detergent/HCl solution to one bottle of DPD. Mix by gentle inversion. Completely dissolve the lyophilizate in the buffer. Store at 4-8 °C until the expiration date on the package or until controls demonstrate any deterioration.

(17) Total Protein

(a) Reagent 1 (R1) working solution
Contains sodium hydroxide, potassium and sodium tartrate, and potassium iodide. Use contents of blank, supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(b) Reagent 2 (R2) working solution
Contains cupric sulfate, sodium hydroxide, potassium sodium tartrate, and potassium iodide. Use the bottle of biuret, supplied "ready to use." Store at 4-8 °C until the expiration date on the package.

(18) Triglycerides

Reagent 1 (R1) working solution
Using an adapter connect ATP/enzymes to buffer/4-chlorophenol. Mix by gentle inversion to completely dissolve the lyophilizate. Stable for 2 weeks when stored at 4-8 °C.

(19) Uric acid

(a) Reagent 1 (R1) working solution
Reconstitute contents of phosphate buffer/TBHB with 600 mL deionized water. Dissolve by gently swirling. Stable 8 weeks when stored at 4-8 °C and protected from light.

(b) Reagent 2 (R2) working solution
Transfer 15 brown mottled tablets of uricase/4-aminophenazone to 150 mL of phosphate buffer. Stable 8 weeks when stored at 4-8 °C and protected from light.

d. **Standards Preparation**

(1) Precical diluent (Sodium bicarbonate diluent only.)
Store unopened diluent at 4-8 °C or 20-25 °C until the expiration date on the vial. Opened diluent is stable for 45 days after the vial is opened when stored tightly capped at 4-8 °C.

(2) BMD Precical calibrator serum
A human serum with added chemicals, human and animal tissue extracts, and preservatives. Constituent concentrations are specific for each lot used. Store unopened Precical calibrator serum at 4-8 °C until the expiration date on the vial.

(a) Bring Precical diluent to 20-25 °C before use.

(b) Remove Precical calibrator serum from 4-8 °C storage. Tap the calibrator serum bottle lightly to dislodge the lyophilized material.

(c) Using a volumetric pipette, transfer exactly 3.0 mL of Precical diluent into a bottle of Precical calibrator serum. Do not mix lot numbers of diluent and calibrator serum. Do not pour diluent directly into the calibrator serum vial.

(d) Invert the bottle containing the calibrator serum several times to cover all inside surfaces of the bottle. Immediately place the calibrator serum on a mechanical rotator for 10 min.

(e) After 10 min, remove the calibrator serum from the mechanical rotator and store at 4-8 °C for 30-60 min

prior to first use. Visually inspect the calibrator serum for total dissolution before use.

(f) Store the reconstituted calibrator serum at 4-8 °C between each use. Invert the bottles of serum gently before each use to ensure total homogeneity.

(g) Reconstituted calibrator serum is stable for 3 days when stored securely stoppered and protected.

(4) Calibration standards

(a) Using a disposable Beral pipette, dispense 500 μL saline solution into a Hitachi sample cup, and place the cup in barcoded tube 990001.

(b) Using another Beral pipette, dispense 500 μL Precical calibrator into a Hitachi sample cup and place in barcoded tube 990004.

(c) Using additional clean Beral pipettes, dispense 500 μL Precitrol normal, Precitrol abnormal, Monitrol I, and Monitrol II quality control serum into separate Hitachi sample cups. Place the cups in barcoded tubes 991003, 991004, 991001, and 991002, respectively.

(d) Place all barcoded tubes on the Hitachi 737 sample wheel starting at position #1 with the barcodes facing towards the center. Place the calibration standards on the disk before control samples. This will ensure that the instrument is calibrated prior to the control sample analysis. In the case of photometric, linear chemistries, place the saline solution before the calibration samples.

(e) At the computer terminal, request RTN 2 "CALIBRATION SELECTION" by pressing the function key F2. Move the cursor to item #1 and type M ENTER. Move the cursor to item #2 and continue to press ENTER until the cursor is in front of the parameter name, type "12," and press ENTER. Move the cursor to item #3, type "Y," and press ENTER. Move the cursor to item #4, type "Y," and press ENTER. The cursor will move to bottom of computer screen.

(f) Press the function key F3 to order controls for the run. The Control Select Menu will appear on the screen. In item #1, type "M" and press ENTER. In item #2, deselect any tests not matching selected controls from item #1. Ensure that all 4 controls are selected for each parameter. Type the number of control groups in item 3 followed by ENTER. Type "Y" and press ENTER in item #4. Type "Y" and press ENTER in item #5.

(g) The cursor will move to the "HOME" position. Press F7 to monitor the run.

c. **Preparation of Quality Control Materials**

The quality control materials are commercial preparations of human serum with added human and animal tissue extracts and preservatives. The constituent concentrations are specific for each lot.

(1) Reconstitute Precitrol normal and abnormal control serum as follows:

(a) Bring all vials of control serum and diluent to 20-25 °C before reconstitution.

(b) Tap the control serum bottle lightly to dislodge the lyophilized material.

(c) Using a volumetric pipette, transfer exactly 3.0 mL of the appropriate diluent into a bottle of the control serum. Do not mix lot numbers of diluent and controls. Do not pour diluent directly into the control serum vial.

(d) Invert bottles several times and place them on a mechanical rotator for 10 min.

(e) After 10 min, remove bottles of control serum from the rotator and store the bottles at 4-8 °C for 60 min prior to use.

(f) Store reconstituted control serum at 4-8 °C between each use. Invert the bottles gently before each use to ensure total homogeneity.

(2) Reconstitute Dade Monitrol levels I & II unassayed control serum as follows:

(a) Bring all vials of control serum and diluent to 20-25 °C before reconstitution.

(b) Tap the vials gently to dislodge lyophilized cake. Remove the cap and rubber stopper.

(c) Using a volumetric pipette, reconstitute the contents of each vial with 10.0 mL of the lot number of carbonate diluent provided.

(d) Recap the vial and let it stand at 20-25 °C for 10 min, then swirl the contents gently to mix.

(e) Invert the vial and continue to swirl and mix the contents intermittently until they are completely dissolved.

(f) Store reconstituted control serum at 4-8 °C between each use. Invert the vial gently before each use to ensure total homogeneity.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

Endpoint/endpoint with sample blank, kinetic, and ISE are the three calibration curves generated by this instrument.

(1) Endpoint/endpoint with sample blank

This instrument generates an endpoint/endpoint with sample blank calibration curve for albumin, bicarbonate ($HCO_3$), total bilirubin, calcium, cholesterol, glucose, iron, phosphorus, total protein, triglycerides, and uric acid parameters.

(a) A calibration sequence must be performed to ensure accurate chemistry results on the Hitachi 737. This calibration establishes the calibration factors. The factors are then used to convert the electronic response of the instrument into concentration or activity for the constituent being measured.

(b) To determine the reagent blank absorbance and thus establish a baseline for each test, analyze the blank sample in duplicate for each requested test. When reagents are added to the blank sample in the reaction cell, the final absorbance readings reflect the absorbance of the reagents. The absorbance readings for the two blank samples are averaged and the mean blank absorbance thus determined is stored in memory.

(c) The calibrator is analyzed in duplicate. The absorbance readings are averaged and the mean calibration value thus determined is stored in memory. A calibration factor is then calculated by the computer.

(d) The computer retains two sets of calibration data for each test (current and previous). The computer updates the current calibration if the data are acceptable.

(e) A calibration report is then printed by the computer. It contains information on the calibration ID, the set point, the ABS or MV reading, the factor calibrated from the curve, and the sensitivity. It also prints the previous calibration and calculates a ratio. The "ratio" column is calculated by dividing the previous factor by the current results. This number gives the operator a quick indication of the stability of the calibration analysis for each channel.

(f) The calibration for bicarbonate is different from other parameters requiring an endpoint with a sample blank. To calculate the calibration for bicarbonate, do the following:

i. Select SYS 5,1 and change line 4 to 1. Use the director password to leave SYS 5.

ii. Select SYS 9,1 and change line 7 to read, Blank conc = 0, Calib. conc. = 33.6, and Factor = 0.

iii. Select MTN 6/UTILITY and change line 12 to YES. Then select RTN 1/STARTUP and update the reagent volumes for the fresh bicarbonate reagent and define bicarbonate priming. Select

MTN2/TEST & MAINTENANCE and initiate a FULL bicarbonate prime.

iv. Select RTN 2/CALIBRATION SELECTION and request bicarbonate calibration. Make note of the appropriate calibration requisition numbers before leaving the display. Select RTN 5/CONTROL SELECTION and request assayed controls to be run following bicarbonate calibration.

v. Pour approximate 1 mL of each of the following into individual sample cups: physiological saline, bicarbonate calibrator, Precitrol normal and Precitrol abnormal control serum. Place each sample cup in the appropriate bar-coded tube and place the tubes in the appropriate position on the sample disk. Initiate a run by typing "RUN 1 1."

vi. When calibration is complete, press F4 to request a calibration report. Ensure that the sample blanked absorbance (ABS or MV) in the current calibration column on the calibration report is >10,000.

vii. Compute and record (on the worksheet) the "true" factor (should $\cong$-95 ± 20%) on the worksheet. Use the following guidelines to compute the true factor:

(1) Multiply sensitivity by 0.001 to convert it from mAbs to Abs.

(2) Divide 1 by the converted sensitivity to obtain the true factor.

viii Ensure that control results are within 5% of the expected values on control package inserts.

ix. Pour a fresh aliquot of bicarbonate calibrator and repeat steps 5 through 9 two more times. All three runs must be performed within 4 hours.

x. Calculate the mean value for the three factor values on the worksheet. Then perform the precision check outlined under "VERIFICATION."

(2) Kinetic

This instrument generates a kinetic calibration curve for alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine, gamma glutamyl transaminase, and lactate dehydrogenase.

(a) A calibration sequence must be performed to ensure accurate chemistry results on the Hitachi 737. This calibration establishes the calibration factors. These factors in turn are used to convert the electronic response of the instrument into concentration or activity for the constituent being measured.

(b) To determine the reagent blank absorbance and thus establish a baseline for each test, analyze the blank sample in duplicate for each requested test. When reagents are added to the blank sample in the reaction cell, the final absorbance readings reflect the absorbance of the reagents. The absorbance readings for the two blank samples are printed and used in the factor calculation.

(c) The calibration factor K for this parameter assay is established according to the following formula when the instrument is installed by a Boehringer Mannheim Diagnostics representative.

$$K = \frac{\text{total reaction volume} \times 100}{\text{extinction coefficient} \times \text{lightpath (cm)} \times \text{specimen vol.}}$$

(d) The factor is then typed into the "factor (fixed)" column in system 9, 12. Because this factor remains constant for this instrument, no recalibration is required. This factor is monitored with the QC program and a daily zero point calibration against saline.

(e) A calibration report must then be requested from the computer, which contains information on the calibration ID, set point, ABS or MV reading, factor setting, and the sensitivity. It also prints the previous calibration and calculates a ratio. The "ratio" column is calculated by dividing the previous factor by the current results. This number gives the operator a quick indication of the stability of the calibration analysis for each channel.

(3) ISE

This instrument generates an ISE calibration curve for sodium, potassium, and chloride.

(a) The ISE module may be calibrated with the chemistry channels. The calibration requires the use of high and low standard solutions to determine the slope factor, and a serum-based calibrator to adjust the ISE calibration for differences between the response of aqueous standards and the response of serum.

(b) An internal reference solution is measured during calibration and between each sample to correct the calibration for drift between calibrations.

(c) The electromotive force of the internal reference solution must fall within the following ranges:

$Na^+$ : -90 to -10 mV
$K^+$ : -90 to -10 mV
$Cl^-$ : 80 to 160 mVs

(d) The values must also fall between the EMF values for the low standard and the high standard.

(e) The slope values must fall within the following ranges:

$Na^+$ : 32.0 to 68.0 mV/decade
$K^+$ : 32.0 to 68.0 mV/decade
$Cl^-$ : -35 to -68 mV/decade

(f) A calibration report is then printed by the computer; it contains information on the calibration ID, the set point, the ABS or MV reading, the factor calibrated from the curve, and the sensitivity. It also prints the previous calibration and calculates a ratio. The "ratio" column is calculated by dividing the previous factor by the current results. This number gives the operator a quick indication of the stability of the calibration analysis for each channel.

b. **Verification**

Calibration verification is accomplished every 6 months by analyzing split samples of calibrators, QC materials, and unknown samples on parallel instruments.

An in-house comparison of this method on the Hitachi 737, with the Hitachi 705 method was used as a reference resulted in the linear regression statistics shown in Table 1.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) For information regarding the range of linearity and how to handle results outside this range, refer to the calculations section of this document (Section 8.g.).

(2) Allow frozen blood specimens, quality control serum, and calibration serum to reach 20-25 °C and mix by inversion for 10 sec.

(3) Prepare a sufficient number of barcoded tubes for the samples being tested.

b. **Sample Preparation**

(1) Store specimens at 4-8 °C until analysis.

(2) Dispense 300 μL of each specimen into an analyzer cup in the appropriately barcoded analyzer tube.

(3) Place all barcoded tubes on the Hitachi 737 sample wheel starting at position #1 with the barcodes facing center. Ensure that the instrument is calibrated and verified before starting the unknown sample analysis.

Table 1
Comparison of Hitachi 737 Method with Hitachi 705 Method

| Analyte | Slope | Correlation Coefficient |
|---|---|---|
| ALT | y = 1.00x - 1.35 | 0.999 |
| Albumin | y = 0.992x + 0.057 | 0.999 |
| ALP | y = 1.009x - 5.33 | 0.999 |
| AST | y = 0.983x - 1.73 | 0.999 |
| Bicarbonate | y = 0.992x = 0.86 | 0.998 |
| BUN | y = 0.983x + 0.14 | 0.999 |
| Calcium | y = 1.04x - 0.20 | 0.992 |
| Cholesterol | y = 0.973x + 1.63 | 0.999 |
| Creatinine | y = 1.047x - 0.064 | 1.000 |
| γ-GT | y = 1.015x - 0.22 | 0.999 |
| Glucose | y = 0.990x + 1.15 | 1.000 |
| Iron | y = 1.076x + 12.2 | 0.994 |
| LDH | y = 0.999x + 0.03 | 0.999 |
| Phosphorous | y = 1.012x + 0.120 | 0.999 |
| Sodium, Potassium, and Chloride | Sodium: y = 0.91x + 13.1<br>Potassium: y = 1.09x - 0.30<br>Chloride: y = 0.961x + 3.3 | Sodium: 0.994<br>Potassium: 0.997<br>Chloride: 0.993 |
| Total Bilirubin | y = 1.043x + 0.08 | 0.999 |
| Total protein | y = 0.995x + 0.06 | 0.999 |
| Triglycerides | y = 1.018x + 10.0 | 0.999 |
| Uric acid | y = 0.978x + 0.18 | 0.998 |

c. **Instrument Setup for the Hitachi 737 Chemistry Analyzer**

(1) Set the parameters for the Hitachi 737 as shown in Tables 2a and 2b.

(2) Turn on the water by opening the valve on the Barnstead unit.

(3) Power up the Hitachi 737 analyzer by turning the main circuit breaker ON. The computer will boot the operating program. Allow 30 min for the waterbath on the instrument and ISE chamber to reach 37 °C and the mechanical devices to perform a synchronization. The message "INITIALIZATION" remains displayed in the upper portion of the CRT monitor until all mechanical systems are synchronized. When the initial state is complete, the message "INITIALIZATION" is replaced with the message "STANDBY."

(4) Request RTN 1, "START-UP" by depressing the F1 key. The screen will display reagent volumes and the number of tests remaining for each chemical profile. Determine if sufficient reagent is available for calibration and the scheduled run. Prepare any needed reagents, place them in the in appropriate channel in the reagent compartment, and update new reagent volumes on RTN 1 by inputting the new volume.

(5) Depress the HOME key, type "24," and press ENTER. This is the "PRIME" field. Type 5 and press ENTER. Depress the HOME key.

Table 2a
Instrument Parameters for Hitachi 737

| Parameter | Abumin, SYS 9,6 | ALP, SYS 9,12 | ALT, SYS 9,8 | AST, SYS 9,7 | Bicarbonate, SYS 9,1 | T. Bilirubin, SYS 9,13 | BUN SYS 9,20 | Calcium SYS 9,10 | Cholest. SYS 9,14 | Creatine, SYS 9,4 |
|---|---|---|---|---|---|---|---|---|---|---|
| Test Name | ALB BCP | ALP | ALT | AST | HCO3 | TBIL | BUN | CA | CHOL | CREA |
| Assay Code | ENDP-07-20 | RATE-11-20 | RATE-11-20 | RATE-11-20 | ENDP-07-20 | ENDP-12 | RATE-12-16 | ENDP-07-20 | ENDP-20 | RATE-11-14 |
| Sample Vol (µL) | 3 | 6 | 10 | 10 | 3 | 6 | 4 | 5 | 3 | 10 |
| R1 Volume (µL) | 250 | 250 | 250 | 250 | 250 | 250 | 320 | 250 | 300 | 250 |
| R2 Volume (µL) | 150 | 50 | 50 | 50 | 100 | 0 | 80 | 100 | 0 | 50 |
| Wavelength 1 (nm) | 600 | 415 | 340 | 340 | 376 | 570 | 340 | 570 | 505 | 505 |
| Wavelength 2 (nm) | 700 | 660 | 415 | 415 | 415 | 600 | 376 | 660 | 700 | 570 |
| Comp Limit | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Calib. Req. 1 | 01 | 01 | 01 | 01 | 01 | 01 | 01 | 01 | 01 | 01 |
| Calib. Req. 2 | 04 | -- | -- | -- | -- | 04 | 04 | 04 | 04 | 04 |
| Calib. Req. 3 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Calib. Req. 4 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Calib. ID 1 | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline |
| Calib. ID 2 | 04 Calib | -- | -- | -- | -- | 04 Calib | 04 Calib | 04 Calib | 04 Calib | 04 Calib |
| Calib. ID 3 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Calib. ID 4 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Conc. 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Conc. 2 | assigned val. | -- | -- | -- | -- | assigned val. | assigned val. | assigned val. | assigned val. | assigned val. |
| Conc. 3 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Conc. 4 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Equation no. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Factor (Fixed) | 0 | 3336 | -5320 | -5320 | -100 | 0 | 0 | 0 | 0 | 0 |
| Unit Factor | 1.00 | 1.00 | 1.00 | 1.00 | 100 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| ABS LImit (Rate) | 0 | 12000 | 6000 | 4500 | 0 | 0 | 5000 | 0 | 0 | 4500 |
| INC/DEC | INC | INC | DEC | DEC | DEC | INC | DEC | INC | INC | INC |

Table 2b
Instrument Parameters for Hitachi 737

| Parameter | γ-GT, SYS 9,17 | Glucose, SUS 9,2 | Iron, SYS 9,19 | LDH, SYS 9.11 | Phosphorus, SYS 9,9 | Tot. Protein SYS 9,5 | Na, K, Cl, SYS 9,21; 9,22; 9,23 | | | Triglycerides, SYS 9,15 | UA, SYS 9,18 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Test Name | GGT | GLU BCP | IRON | LD | PHOS | TPRO | Na | K | Cl | TRIG | UA |
| Assay Code | RATE-11-20 | ENDP-08-20 | ENDP-07-20 | RATE-14-20 | ENDP-07-20 | ENDP-07-20 | ISEN | ISEK | ISEC | ENDP-20 | ENDP-20 |
| Sample Vol (µL) | 8 | 3 | 15 | 9 | 5 | 3 | 20 | 20 | 20 | 3 | 8 |
| R1 Volume (µL) | 300 | 250 | 250 | 250 | 250 | 250 | --- | --- | --- | 300 | 300 |
| R2 Volume (µL) | 60 | 250 | 50 | 100 | 100 | 100 | --- | --- | --- | 0 | 60 |
| Wavelength 1 (nm) | 415 | 340 | 570 | 340 | 340 | 570 | --- | --- | --- | 505 | 505 |
| Wavelength 2 (nm) | 660 | 660 | 700 | 376 | 376 | 700 | --- | --- | --- | 700 | 660 |
| Comp Limit | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 5.0 | 5.0 | 0.0 | 0.0 |
| Calib. Req. 1 | 01 | 01 | 01 | 01 | 01 | 01 | 02 | | | 01 | 01 |
| Calib. Req. 2 | -- | 04 | 04 | -- | 04 | 04 | 03 | | | 04 | 04 |
| Calib. Req. 3 | -- | -- | 05 | -- | -- | -- | --- | | | -- | -- |
| Calib. Req. 4 | -- | -- | -- | -- | -- | -- | 04 | | | -- | -- |
| Calib. ID 1 | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 01 Saline | 02 ISE low | | | 01 Saline | 01 Saline |
| Calib. ID 2 | -- | 04 Calib | 04 Calib | -- | 04 Calib | 04 Calib | 03 ISE high | | | 04 Calib | 04 Calib |
| Calib. ID 3 | -- | -- | 05 Sal | -- | -- | -- | --- Int. Ref. | | | -- | -- |
| Calib. ID 4 | -- | -- | -- | -- | -- | -- | 04 Calib | | | -- | -- |
| Conc. 1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 120 | 3.0 | 80 | 0.0 | 0.0 |
| Conc. 2 | -- | assigned val. | assigned val. | -- | assigned val. | assigned val. | 160 | 7.0 | 120 | assigned val. | assigned val. |
| Conc. 3 | -- | -- | 0.0 | -- | -- | -- | 140 | 5.0 | 100 | -- | -- |
| Conc. 4 | -- | -- | -- | -- | -- | -- | assigned val. | | | -- | -- |
| Equation no. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Factor (Fixed) | 7749 | 0 | 0 | 10128 | 0 | 0 | -- | -- | -- | 0 | 0 |
| Unit Factor | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| ABS LImit (Rate) | 5500 | 0 | 0 | 12000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| INC/DEC | INC | INC | INC | INC | INC | INC | -- | -- | -- | INC | INC |

(6) The operator must now request a start-up report. Type "S T A" and press ENTER in the "COMMAND?" field in RTN 1. The system will initiate a system function check. Quickly review the report and verify the current photometer and temperature conditions as well as the programmed system parameters.

(7) If a problem is detected at this point, the supervisor must be notified for technical assistance.

(8) To initiate priming of reagents, request MTN 2, "TEST MAINTENANCE." Select Job No. 9, type "Y," and press ENTER in item 2.

(9) Request RTN 2, "CALIBRATION SELECTION" by pressing the function key F2. Type "M" and press ENTER at the cursor prompt. Type "Y" and press ENTER in item 3. Type "Y" and press ENTER in item 4.

(10) Request RTN 5, "CONTROL SELECTION" by pressing the function key F3. Type M and press ENTER at the cursor prompt. Type "1" and press ENTER in item 3. Type "Y" and press ENTER in item 4. Type "Y" and press ENTER in item 5.

(11) Enter the demographic data on the specimen donor into the computer. Request RTN 4 by pressing the function key F5. Enter information from the NHANES III transmittal form submitted with specimens.

(12) Obtain a work pending list by pressing the function key F6.

d. **Operation of Assay Procedure**

(1) Request RTN 3, "MONITOR RUN" by pressing F7. Type "RUN 1 1" and press ENTER.

(2) After all calibration and control material has been analyzed, request a calibration report by pressing the F4 function key. If the channel has been calibrated, proceed to control verification. If the channel did not calibrate, the channel should be recalibrated.

(3) Verify the calibration by printing a control report. Request RTN 14, "REPORT SELECTION." Depress the HOME key, type "5," and press ENTER. Check that the quality control materials are within the specified limits and that no shifts or trends are present.

(4) If the values observed for the control materials are "in control," proceed with the analysis of the NHANES III specimens.

(5) Load all barcoded specimens on the Hitachi 737 assay tray with the barcoded label facing the center of the tray.

(6) At the computer workstation, request RTN 3 "MONITOR RUN," by pressing function key F7. Type "RUN 1 1."

e. **Recording of Data**

(1) Quality Control Data

The quality control data are automatically stored on the hard disk daily. At the end of each day, request all control data accumulated during the instrument operation period by requesting RTN 14 and moving the cursor to item 4, "DAILY QC." Select ID Code 01, and Request: Y. The report will be printed on two-part carbonless computer paper. This report must be given to a computer analyst for entry into the QC data table. At the end of each month, print out Levy-Jennings charts, means, 2 SD ranges and %CVs. To print the report, request RTN 14 and move the cursor to item 7, "CUMULATIVE QC." Select ID Code 01, Test: Alb, and Request: Y. Repeat these steps for each of the controls: 02, 03, and 04. This report must be posted on the QC board in the laboratory for one month and then placed in a bound QC book for archival.

The quality control data are automatically stored on the computer in the QC program. Results are printed daily, and Levey-Jennings charts are printed monthly. These charts are included in the quarterly report to NCHS.

(2) Analytical Results

Results which are collated by the Hitachi 737 computer system include 1) participant demographic information, 2) names of tests performed, 3) units for each parameter, 4) normal ranges, 5) result obtained, 6) any flags

pertaining to those results (high or low), and 7) the results depicted graphically. To obtain a report of the participant results, the supervisor must first review the data by requesting REV 5, "Participant REVIEW." If the supervisor decides that any of the results are unacceptable, the operator must perform a rerun of the necessary parameters. If the results are accepted by the supervisor as being accurate, she must select item #62, type "Y," and press ENTER. The supervisor's PASS command is required to exit REV 5 if the review function has been exercised. Request a printout of the participant report by pressing the function key F10.

Give the form to the computer analyst so that results can be verified against the ASCII file, which is printed from the host computer system. After verifying the results, the computer analyst will transfer the ASCII file to a 5¼" HD diskette and send the diskette with the results to the NHANES coordinator. A printout of the ASCII file of the results will be filed in the study notebook.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Clean the dispensor nozzles daily and the reagent lines monthly with a 10% sodium hypochlorite solution. If a QC or calibration problem occurs, clean the lines and nozzles as part of the problem-solving procedure.

(2) Clean the reaction cells daily. Maintain a complete set of spare cells so that replacements can be made when a cell breaks.

(3) Take photometer lamp readings daily and record the results in the Maintenance Log. Maintain spare lamps so that a replacement lamp can be installed if readings significantly change.

(4) Maintain spare ISE cartridges and reference and ground electrodes so that these can be replaced when problems occur with the ISE channel.

g. **Calculations**

This instrument performs separate calculations for each assay type. The four assay types calculated by the Hitachi are endpoint with sample blank, endpoint, kinetic, and ISE. Specimen analysis must be repeated when results are outside the ±2 SD range. Duplicates must agree within 10%.

(1) Assay type: endpoint with sample blank

The analyzer computer uses absorbance measurements to calculate albumin, bicarbonate ($HCO_3$), calcium, glucose, iron, phosphorus, and total protein concentrations as follows:

Cx = K(Ax - Ab) + Cb where:

Cx = concentration of sample
K = concentration factor (determined during calibration)
Ax = absorbance of sample + R1 + R2, read during cycle 20, minus absorbance of sample + R1, read during cycle 07
Ab = absorbance of blank + R1 + R2, read during cycle 20, minus absorbance of blank + R1, read during cycle 07
Cb = concentration of blank (CALIB 1 SALINE)

Ax and Ab are corrected for reagent/sample volume by:
d = (sample vol. + R1)/(sample vol. + R1 + R2)

(a) Albumin

The albumin method is linear up to 10.0 g/dL.

When reanalyzing any specimen with a concentration greater than 10 g/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1.0 g/dL. Results below the detection limit are reported as <1.0 g/dL, and the specimen is reassayed

with a microprotein assay.

(b) Bicarbonate ($HCO_3$)

The method is linear up to 50.0 mmol/L.

When reanalyzing any specimen with a concentration greater than 50 mmol/L, prepare a twofold (1+1) dilution of the specimen with deionized carbon dioxide-free water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 5 mmol/L. Results below the detection limit are reported as <5 mmol/L.

(c) Calcium

The method is linear up to 20.0 mg/dL.

When reanalyzing any specimen with a concentration greater than 20 mg/dL, prepare a twofold (1+1) dilution of the specimen with physiological saline. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1.0 mg/dL. Results below the detection limit are reported as <1.0 mg/dL.

(d) Glucose

The method is linear up to 1000 mg/dL.

When reanalyzing any specimen with a concentration greater than 1000 mg/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1.0 mg/dL. Results below the detection limit are reported as <1.0 mg/dL.

(e) Iron

The method is linear up to 1000 $\mu$g/dL.

When reanalyzing any specimen with a concentration greater than 1000 $\mu$g/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1.0 $\mu$g/dL. Results below the detection limit are reported as <1.0 $\mu$g/dL.

(f) Phosphorus

The method is linear up to 20.0 mg/dL.

When reanalyzing any specimen with a concentration greater than 20 mg/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 0.5 mg/dL. Results below the detection limit are reported as <0.5 mg/dL.

(g) Total protein

The method is linear up to 15.0 g/dL.

When reanalyzing any specimen with a concentration greater than 15 g/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 0.5 g/dL. Results below the detection limit are reported as <0.5 g/dL, and the specimen is reassayed with a microprotein assay.

(2) Assay type: Endpoint

The analyzer computer uses absorbance measurements to calculate total bilirubin, cholesterol, triglycerides, and uric acid parameters concentration as follows:

$C_x = K(A_x - A_b) + C_b$ where:

Cx = concentration of sample
K = concentration factor (determined during calibration)
Ax = absorbance of sample + R1 read during cycle 12
Ab = absorbance of blank + R1 read during cycle 12
Cb = concentration of blank (CALIB 1 SALINE)

(a) Total bilirubin

The method is linear up to 30.0 mg/dL.

When reanalyzing any specimen with a concentration greater than 30.0 mg/dL, prepare a twofold (1+1) dilution of the specimen with saline. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 0.1 mg/dL. Results below the detection limit are reported as <0.1 mg/dL.

(b) Cholesterol

The method is linear up to 10.0 g/dL.

When reanalyzing any specimen with a concentration greater than 700 mg/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 10.0 mg/dL.

(c) Triglycerides

The method is linear up to 1000 mg/dL.

When reanalyzing any specimen with a concentration greater than 1000 mg/dL, prepare a twofold (1+1) dilution of the specimen with distilled deionized water. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 10 mg/dL. Results below the detection limit are reported as <10 mg/dL.

(d) Uric acid

The method is linear up to 20.0 mg/dL.

When reanalyzing any specimen with a concentration greater than 20 mg/dL, prepare a twofold (1+1) dilution of the specimen with physiological saline. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1.0 mg/dL. Results below the detection limit are reported as <1.0 mg/dL.

(3) Assay type: Kinetic

The analyzer computer uses absorbance measurements to calculate alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), BUN, creatinine, gamma glutamyl transaminase (γ-GT), and lactate dehydrogenase (LDH) concentrations as follows:

$C_x = K(\triangle A_x - \triangle A_b) + C_b$ where:

Cx = activity of sample
K = factor for determining enzyme activity, established for each kinetic assay during installation
△Ax = change in absorbance per minute of sample + R1 + R2 during cycles 11 through 20
△Ab = change in absorbance per minute of blank + R1 + R2 during cycles 11 through 20, minus absorbance of blank + R1 read during cycle 07
Cb = Concentration of blank (CALIB 1 SALINE).

(a) Alkaline phosphatase (ALP)

The method is linear up to 1000 U/L.

When reanalyzing any specimen with a concentration greater than 1000 U/L, prepare a 10-fold (1+9) dilution of the specimen with saline.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 2 U/L. Results below the detection limit are reported as <2 U/L.

(b) Alanine aminotransferase (ALT)

The method is linear up to 400 U/L.

When reanalyzing any specimen with a concentration greater than 400 U/L, prepare a 10-fold (1+9) dilution of the specimen with saline.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1 U/L. Results below the detection limit are reported as <1 U/L.

(c) Aspartate aminotransferase (AST)

The method is linear up to 800 U/L.

When reanalyzing any specimen with a concentration greater than 800 U/L, prepare a 10-fold (1+9) dilution of the specimen with saline. The results must then be multiplied by 10 to account for this dilution.

The minimum detection limit, based on linear a regression curve of certified material analyzed 20 times, is 1 U/L. Results below the detection limit are reported as <1 U/L.

(d) Blood urea nitrogen (BUN)

The method is linear up to 150 mg/dL.

When reanalyzing any specimen with a concentration greater than 150 mg/dL, prepare a twofold (1+1) dilution of the specimen with physiological saline. The results must then be multiplied by 2 to account for this dilution.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 1.0 mg/dL. Results below the detection limit are reported as <1.0 mg/dL.

(e) Creatinine

The method is linear up to 25.0 mg/dL.

When reanalyzing any specimen with a concentration greater than 25.0 mg/dL, prepare a twofold (1+1) dilution of the specimen with saline.

The minimum detection limit, based a on linear regression curve of certified material analyzed 20 times, is 0.1 mg/dL. Results below 0.6 mg/dL are of no significance and can be reported as <0.1 mg/dL.

(f) Gamma glutamyl transaminase (γ-GT)

The method is linear up to 1200 U/L.

When reanalyzing any specimen with a concentration greater than 1200 U/L, prepare a 10-fold (1+9) dilution of the specimen with saline.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 2 U/L. Results below the detection limit are reported as <2 U/L.

(g) Lactate dehydrogenase (LDH)

The method is linear up to 1000 U/L.

When reanalyzing any specimen with a concentration greater than 1000 U/L, prepare a 10-fold (1+9) dilution of the specimen with saline.

The minimum detection limit, based on a linear regression curve of certified material analyzed 20 times, is 2 U/L. Results below the detection limit are reported as <2 U/L.

(4) Assay type: ISE

The analyzer computer uses changes in EMF to determine ion concentrations. The slope is determined by using ISE standard solutions, and a compensation coefficient is determined by using Precical calibrator serum. The concentrations of $Na^+$, $K^+$, and $Cl^-$ in the test samples are determined by measuring the EMFs and calculating the results from the following equation:

$C_x = (C_{IRS} \times 10^{\triangle Ex/S}) + K$ where:

$C_x$ = concentration of test ion
K = compensation factor
$C_{IRS}$ = concentration of internal reference solution as determined during calibration
$\triangle E_x$ = EMF of test sample - EMF of internal reference solution
S = slope

The slope, S, is calculated from the following equation:

$$S = \frac{E_H - E_L}{\log (a_H/a_L)} = \text{mV/decade where:}$$

$E_L$ = EMF of low standard for each electrolyte
$E_H$ = EMF of high standard for each electrolyte
$a_H$ = input value of the high standard concentration
$a_L$ = input value of the low standard concentration

The concentration of the internal reference solution is established from the following equation:

$C_{IRS} = a_L \times 10^{(EIRS-EL2)/S}$ where:

$a_L$ = input value of the low standard concentration
$E_{IRS}$ = EMF of internal reference solution
$E_L$ = EMF of low standard for each electrolyte
S = slope

The compensation factor, K, is calculated from the following equation:

K = assigned value (calib) - calculated value (calib)

Sodium, potassium, and chloride

The method is linear up to 180 mmol/L for $Na^+$, 10.0 mmol/L for $K^+$, and 140 mmol/L for $Cl^-$.

The minimum detection limits, based on linear regression curve of certified material analyzed 20 times, is 10 mmol/L $Na^+$, 1.5 mmol/L $K^+$, and 10 mmol/L $Cl^-$. Results below the detection limit are reported as less than the minimum detection limit (as outlined above).

**9. REPORTABLE RANGE OF RESULTS**

a. **ALT**. Serum ALT values are reportable in the range 1-400 U/L without dilution. If the ALT value is >400 U/L, the specimen should be diluted 10-fold (1+9) and reanalyzed. The results must then be multiplied by 10 to account for this dilution.

b. **Albumin**. Serum albumin values are reportable in the range 1.0-10.0 g/dL without dilution. If a serum albumin value is <1.0 g/dL, an alternate albumin method must be used which detects micro amounts of albumin. If the albumin value is >10.0 g/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

c. **ALP**. Serum ALP values are reportable in the range 2-1000 U/L without dilution. If the value is >1000 U/L, the specimen should be diluted 10-fold (1+9) and reanalyzed. The results must then be multiplied by 10 to account for this dilution.

d. **AST**. Serum AST values are reportable in the range 1-800 U/L without dilution. If the AST value is >800 U/L, the specimen should be diluted 10-fold (1+9) and reanalyzed. The results must then be multiplied by 10 to account for this dilution.

e. **Bicarbonate (HCO3)**. Serum bicarbonate values are reportable in the range 5-50 mmol/L without dilution. If the bicarbonate value is >50 mmol/L, the specimen should be diluted twofold (1+1) and reanalyzed.

f. **BUN**. Serum BUN values are reportable in the range 1.0-150 mg/dL without dilution. If the BUN value is >150 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

g. **Calcium**. Serum calcium values are reportable in the range 1.0-20.0 mg/dL without dilution. If the value is >20.0 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

h. **Cholesterol**. Serum cholesterol values are reportable in the range 10-700 mg/dL without dilution. If the cholesterol value is >700 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

i. **Creatinine**. Serum creatinine values are reportable in the range 0.5-25.0 mg/dL without dilution. If the value is >25.0 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed. The results must then be multiplied by 2 to account for this dilution.

j. **γ-GT**. Serum GGT values are reportable in the range 2-1200 U/L without dilution. If the value is >1200 U/L, the specimen should be diluted 10-fold (1+9) and reanalyzed. The results must then be multiplied by 10 to account for this dilution.

k. **Glucose**. Serum glucose values are reportable in the range 1.0-1000 mg/dL without dilution. If the value is >1000 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

l. **Iron**. Serum iron values are reportable in the range 1.0-1000 μg/dL without dilution. If the iron value is >1000 μg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

m. **LDH**. Serum LDH values are reportable in the range 2-1000 U/L without dilution. If the value is >1000 U/L, the specimen should be diluted 10-fold (1+9) and reanalyzed. The results must then be multiplied by 10 to account for this dilution.

n. **Phosphorus**. Serum phosphorus values are reportable in the range 0.5-20.0 mg/dL without dilution. If the value is >20.0 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

o. **Sodium, Potassium, and Chloride**. Serum sodium values are reportable in the range 80-180 mmol/L, potassium values in the range 1.5-10.0 mmol/L and chloride values in the range of 60-140 mmol/L without dilution.

p. **Total Bilirubin**. Serum total bilirubin values are reportable in the range 0.1-30.0 mg/dL without dilution. If the total bilirubin value is >30.0 mg/dL, the specimen should be diluted (1+1) and re-analyzed.

q. **Total Protein**. Serum total protein values are reportable in the range 0.5-15.0 g/dL without dilution. If the value is <0.5 g/dL an alternate method capable of detecting micrograms of protein must be used. If the value is >15.0 g/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

r. **Triglycerides**. Serum triglyceride values are reportable in the range 10-1000 mg/dL without dilution. If the value is >1000 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

s. **Uric acid**. Serum uric acid values are reportable in the range 1.0-20.0 mg/dL without dilution. If the uric acid value is >20.0 mg/dL, the specimen should be diluted twofold (1+1) and reanalyzed.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol is in compliance with Clinical Laboratory Improvement Act of 1988 (CLIA) and the College of American Pathologists (CAP) guidelines. The Hitachi analyzer has proven to be accurate, precise, and reliable. Precision studies (Table 3) were generated from the use of a long-term lot number of quality control material.

Three types of quality control material are used to establish the White Sands Research Center quality control program: (1) a normal and abnormal level of quality control material assayed by the BMD, (2) a normal and abnormal level of in-house quality control material, which will have a moving 2 SD range as determined by assay within the laboratory on its instrument and compared nationwide with the 2SD range of peers, and (3) the CAP Proficiency Survey. All levels of this parameter are assessed by taking these samples through the complete analytical process. The data from these materials are used to estimate methodological imprecision, shifts, and trends.

Two levels of unassayed control materials are used. A particular lot of each level is purchased from Dade Scientific in a sufficient quantity to last a minimum of 2 years. The daily data points from the results generated on the Hitachi 737 are stored on the hard disk in the QC program, and are printed at the end of each month. This program generates a monthly mean, SD, %CV, and number of data points that were evaluated. At the end of each month, the laboratory submits this summary to the Dade computer center and the center calculates a monthly mean, SD, and %CV for the peer group which consists of laboratories using the same method and lot number of control material for each analyte. The Standard Deviation Index (SDI) is used to compare results obtained by the members of the peer group. The SDI indicates where the laboratory stands with respect to the other members of the group and the group mean. This material is useful in assessing within-run and run-to-run shifts and trends in the data. One set of normal and one set of abnormal unassayed controls are analyzed according to the method protocol at the beginning and at two 4-hour intervals during the analyzer run period.

The assayed quality control materials are used to form a normal and an abnormal level of each analyte for the Hitachi 737, manufactured by BMD. These materials are lyophilized preparations of pooled human serum with added chemicals, human and animal tissue extract and preservatives. The controls are assayed by the BMD which establishes concentrations for each lot and determines the mean and ±2 SD ranges for each parameter. One set of assayed controls is analyzed at the beginning of the analyzer run period to verify calibration and as an accuracy check. If the inventory of these materials becomes low, another lot should be ordered in time to analyze it concurrently with the lot currently in use so that a bridge may be formed between the materials.

Table 3
Precision For Hitachi 737 Analyzer

| Analyte/Pool | Units | N | Within run mean | 95% limits, within run | 99% limits, within run | CV, %, within run | Mean, total | 95% limits, total | 99% limits, total | CV, %, total |
|---|---|---|---|---|---|---|---|---|---|---|
| ALT/A | U/L | 20 | 32 | 31-33 | 30-34 | 3.5 | 32 | 31-33 | 30-35 | 3.8 |
| ALT/B | U/L | 20 | 179 | 177-182 | 174-184 | 1.4 | 179 | 175-183 | 171-187 | 2.2 |
| ALT/C | U/L | 20 | 344 | 340-347 | 337-350 | 1.0 | 344 | 334-354 | 324-363 | 2.8 |
| Albumin/A | g/dL | 20 | 2.5 | 2.47-2.53 | 2.44-2.56 | 1.2 | 2.5 | 2.45-2.55 | 2.40-2.60 | 1.8 |
| Albumin/B | g/dL | 20 | 4.2 | 4.16-4.24 | 4.12-4.28 | 0.9 | 4.2 | 4.13-4.27 | 4.06-4.34 | 1.7 |
| Albumin/C | g/dL | 20 | 7.5 | 7.44-7.56 | 7.38-7.62 | 0.9 | 7.5 | 7.38-7.62 | 7.26-7.74 | 1.6 |
| ALP/A | U/L | 20 | 117 | 116-118 | 115-119 | 0.9 | 117 | 114-120 | 110-124 | 2.9 |
| ALP/B | U/L | 20 | 304 | 303-305 | 302-306 | 0.4 | 304 | 294-314 | 285-323 | 1.4 |
| ALP/C | U/L | 20 | 782 | 776-788 | 770-794 | 0.7 | 782 | 764-800 | 746-818 | 2.3 |
| AST/A | U/L | 20 | 27 | 27-28 | 25-29 | 2.7 | 27 | 26-28 | 25-29 | 3.7 |
| AST/B | U/L | 20 | 146 | 145-148 | 143-149 | 1.0 | 146 | 143-149 | 140-152 | 2.1 |
| AST/C | U/L | 20 | 394 | 390-398 | 386-401 | 0.9 | 394 | 385-403 | 376-412 | 2.3 |
| Bicarbonate/A | mmol/L | 20 | 16.4 | 15.9-16.9 | 15.4-17.4 | 3.0 | 16.4 | 15.6-17.2 | 14.9-17.9 | 4.7 |
| Bicarbonate/B | mmol/L | 20 | 21.8 | 21.3-22.3 | 20.8-22.8 | 2.3 | 21.8 | 21.0-22.6 | 20.2-23.4 | 3.6 |
| Bicarbonate/C | mmol/L | 20 | 34.0 | 33.6-34.4 | 33.2-34.8 | 1.1 | 34.0 | 33.2-34.8 | 32.4-35.6 | 2.3 |
| BUN/A | mg/dL | 20 | 13.5 | 13.2-13.8 | 12.9-14.0 | 2.0 | 13.5 | 13.1-13.9 | 12.7-14.3 | 2.8 |
| BUN/B | mg/dL | 20 | 38.6 | 38.3-38.9 | 38.0-39.2 | 0.8 | 38.6 | 38.1-39.1 | 37.7-39.5 | 1.2 |
| BUN/C | mg/dL | 20 | 95.6 | 95.0-96.2 | 94.4-96.8 | 0.6 | 95.6 | 94.7-96.5 | 93.9-97.3 | 0.9 |
| Calcium/A | mg/dL | 20 | 9.8 | 9.71-9.89 | 9.62-9.98 | 1.0 | 9.8 | 9.66-9.94 | 9.52-10.08 | 1.4 |
| Calcium/B | mg/dL | 20 | 13.8 | 13.7-13.9 | 13.6-14.0 | 0.6 | 13.8 | 13.7-13.9 | 13.5-14.1 | 1.0 |
| Cholesterol/A | mg/dL | 20 | 105 | 81-107 | 79-110 | 2.3 | 105 | 102-108 | 99-111 | 2.9 |
| Cholesterol/B | mg/dL | 20 | 109 | 107-111 | 104-114 | 2.2 | 109 | 106-112 | 103-115 | 2.8 |
| Cholesterol/C | mg/dL | 20 | 181 | 179-184 | 176-186 | 1.4 | 181 | 178-185 | 174-188 | 2.0 |
| Creatinine/A | mg/dL | 20 | 1.7 | 1.67-1.73 | 1.64-1.76 | 1.8 | 1.7 | 1.65-1.75 | 1.60-1.80 | 2.7 |
| Creatinine/B | mg/dL | 20 | 3.5 | 3.46-3.54 | 3.42-3.58 | 1.1 | 3.5 | 3.42-3.50 | 3.34-3.66 | 2.1 |

| Analyte/Pool | Units | N | Within run mean | 95% limits, within run | 99% limits, within run | CV, %, within run | Mean, total | 95% limits, total | 99% limits, total | CV, %, total |
|---|---|---|---|---|---|---|---|---|---|---|
| Creatinine/C | mg/dL | 20 | 4.4 | 4.35-4.45 | 4.30-4.50 | 1.2 | 4.4 | 4.31-4.50 | 4.22-4.66 | 2.0 |
| γ-GT/A | U/L | 20 | 60.2 | 58.7-61.7 | 57.2-63.2 | 2.5 | 60.2 | 58.2-62.2 | 56.2-64.2 | 3.4 |
| γ-GT/B | U/L | 20 | 111.3 | 109.3-113.3 | 107.3-115.3 | 1.8 | 111.3 | 108.8-113.8 | 106.3-116.3 | 2.3 |
| γ-GT/C | U/L | 20 | 190.5 | 188.5-192.5 | 186.5-194.5 | 1.1 | 190.5 | 187.4-193.6 | 184.3-196.7 | 1.6 |
| Glucose/A | mg/dL | 20 | 68.2 | 67.7-68.7 | 67.1-69.3 | 0.8 | 68.2 | 67.5-68.9 | 66.8-69.6 | 1.0 |
| Glucose/B | mg/dL | 20 | 121.3 | 119.2-123.4 | 120.3-122.3 | 0.8 | 121.3 | 119.8-122.8 | 118.3-124.3 | 1.2 |
| Glucose/C | mg/dL | 20 | 287.3 | 285.0-289.6 | 282.8-291.8 | 0.8 | 287.3 | 284.6-290.0 | 281.9-292.7 | 0.9 |
| Iron/A | µg/dL | 20 | 67.9 | 65.4-70.4 | 62.9-72.9 | 3.7 | 67.9 | 63.5-72.3 | 59.1-76.7 | 6.5 |
| Iron/B | µg/dL | 20 | 145.9 | 143.3-148.6 | 140.6-151.2 | 1.8 | 145.9 | 141.6-150.2 | 137.4-154.4 | 2.9 |
| Iron/C | µg/dL | 20 | 318.6 | 315.9-321.3 | 313.2-324.0 | 0.8 | 318.6 | 311.0-326.2 | 303.5-333.7 | 2.4 |
| LDH/A | U/L | 20 | 152.1 | 150.9-153.3 | 149.7-154.5 | 0.8 | 152.1 | 149.7-154.6 | 147.1-157.0 | 1.6 |
| LDH/B | U/L | 20 | 206.2 | 204.7-207.7 | 203.3-209.1 | 0.7 | 206.2 | 204.5-207.9 | 202.7-209.7 | 0.8 |
| LDH/C | U/L | 20 | 428.4 | 426.2-430.6 | 424.1-432.7 | 0.5 | 428.4 | 424.1-432.4 | 419.9-436.5 | 1.0 |
| Phosphorus/A | mg/dL | 20 | 2.8 | 2.77-2.83 | 2.74-2.86 | 1.0 | 2.8 | 2.72-2.88 | 2.64-2.96 | 2.8 |
| Phosphorus/B | mg/dL | 20 | 4.6 | 4.55-4.65 | 4.50-4.70 | 1.0 | 4.6 | 4.51-4.69 | 4.42-4.78 | 1.9 |
| Phosphorus/C | mg/dL | 20 | 8.3 | 8.25-8.35 | 8.20-8.40 | 0.6 | 8.3 | 8.15-8.45 | 8.00-8.60 | 1.8 |
| Sodium/A | mmol/L | 20 | 129.6 | 128.3-130.1 | 125.9-131.0 | 0.6 | 129.6 | 128.4-130.8 | 127.2-132.9 | 0.9 |
| Sodium/B | mmol/L | 20 | 146.4 | 145.4-147.4 | 144.3-148.5 | 0.7 | 146.4 | 144.3-148.5 | 142.3-150.5 | 1.4 |
| Sodium/C | mmol/L | 20 | 166.5 | 165.5-167.6 | 164.4-168.6 | 0.6 | 166.5 | 164.7-168.3 | 163.0-170.0 | 1.1 |
| Potassium/A | mmol/L | 20 | 4.72 | 4.67-4.77 | 4.62-4.82 | 1.0 | 4.72 | 4.62-4.82 | 4.52-4.92 | 2.1 |
| Potassium/B | mmol/L | 20 | 6.94 | 6.86-7.02 | 6.78-7.10 | 1.1 | 6.94 | 6.94-7.09 | 6.64-7.24 | 2.2 |
| Chloride/A | mmol/L | 20 | 101.6 | 100.4-102.8 | 99.1-104.1 | 1.2 | 101.6 | 99.7-103.5 | 97.8-105.4 | 1.9 |
| Chloride/B | mmol/L | 20 | 112.1 | 110.4-113.8 | 108.6-115.6 | 1.6 | 112.1 | 109.4-114.8 | 106.4-117.4 | 2.4 |
| Total Bilirubin/A | mg/dL | 20 | 1.19 | 1.17-1.21 | 1.15-1.23 | 2.0 | 1.19 | 1.15-1.23 | 1.11-1.27 | 3.1 |
| Total Bilirubin/B | mg/dL | 20 | 2.94 | 2.91-2.97 | 2.88-3.00 | 0.9 | 2.94 | 2.84-2.99 | 2.79-3.04 | 1.6 |
| Total Bilirubin/C | mg/dL | 20 | 6.62 | 6.48-6.76 | 6.34-6.90 | 1.1 | 6.62 | 6.48-6.76 | 6.34-6.90 | 2.1 |

| Analyte/Pool | Units | N | Within run mean | 95% limits, within run | 99% limits, within run | CV, %, within run | Mean, total | 95% limits, total | 99% limits, total | CV, %, total |
|---|---|---|---|---|---|---|---|---|---|---|
| Total Protein/A | g/dL | 20 | 5.2 | 5.11-5.29 | 5.02-5.38 | 1.8 | 5.2 | 5.08-5.32 | 4.96-5.44 | 2.4 |
| Total Protein/B | g/dL | 20 | 6.3 | 6.18-6.42 | 6.06-6.54 | 1.8 | 6.3 | 6.16-6.44 | 6.02-6.58 | 2.2 |
| Total Protein/C | g/dL | 20 | 5.1 | 5.00-5.20 | 4.90-5.30 | 1.9 | 5.1 | 4.98-5.22 | 4.86-5.34 | 2.4 |
| Triglycerides/A | mg/dL | 20 | 74.5 | 72.6-76.4 | 70.7-78.3 | 2.6 | 74.5 | 71.6-77.4 | 68.7-80.3 | 3.8 |
| Triglycerides/B | mg/dL | 20 | 116.5 | 114.7-118.3 | 112.9-120.1 | 1.8 | 116.5 | 113.5-119.5 | 110.5-122.5 | 2.6 |
| Triglycerides/C | mg/dL | 20 | 156.6 | 154.7-158.5 | 152.8-160.4 | 1.2 | 156.6 | 153.4-159.8 | 150.2-163.0 | 2.0 |
| Uric Acid/A | mg/dL | 20 | 3.46 | 3.44-3.48 | 3.42-3.50 | 0.7 | 3.46 | 3.42-3.50 | 3.38-3.54 | 1.3 |
| Uric Acid/B | mg/dL | 20 | 7.08 | 7.02-7.14 | 6.96-7.20 | 0.8 | 7.08 | 6.99-7.17 | 6.90-7.26 | 1.2 |
| Uric Acid/C | mg/dL | 20 | 11.52 | 11.42-11.62 | 11.24-11.80 | 0.4 | 11.52 | 11.42-11.62 | 11.24-11.80 | 1.2 |

After the standards and the assayed and unassayed quality control materials are analyzed, the 30-day Levey-Jennings control chart (which is stored in the Hitachi 737 computer system) is consulted to determine if the system is "in control." The system is out of control if any of the following events occur for any one of the parameters:

a. Test the control data using the 2 SD rule. Accept the run when all controls are within 2SD of the mean. Report participants' results. When at least one control observation is more than 2SD from the mean, hold the participants' results and inspect the control data further, using additional Westgard control rules.

b. Inspect control data within the run.

- Reject the run when one observation is more than 3SD from the mean. Do not report participants' results.
- Reject the run when two control observations are more than 2 SD on the same side of the mean. Do not report participants' results.
- Reject the run when the range of one control observation is more than 2SD above the mean and that of another is more than 2SD below the mean. Do not report participants' results.

c. Inspect control data across runs.

- Reject the run when two consecutive control results are more than 2SD on the same side of the mean. Do not report participants' results.
- Reject the run when four consecutive control observations fall more than 1SD on the same side of the mean. Do not report participants' results.
- Reject the run when three conseutive control observations fall more than 1SD on the same side of the mean. Do not report participants' results.
- Reject the run when 10 consecutive observations fall on the same side of the mean. Do not report participants' results.
- Reject the run when 9 consecutive observations of the same control material fall on the same side of the mean. Do not report participants' results.

d. Accept the run when none of the rules indicate a lack of statistical control. Report participants' results.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more quality control samples fall outside the ±2 SD range or a within-run control sample shifts 2 SD or more from its previous value, then take the following steps:

a. Determine the type of errors occurring (random, systematic, or both) on the basis of the control rules being violated.
b. Refer to the BMD troubleshooting guide to determine the elements of the method or the components of the instrument that can cause the type of error observed.

c. Correct the problem, then reanalyze the participants' samples and control samples, testing for statistical control by the same procedure.

d. Consult the chemistry technical supervisor for any decision to report data when there is a lack of statistical control. The chemistry technical supervisor may make a decision to report data when there is a lack of statistical control in the following situations:

(1) The control problem can be due to the control materials themselves.

(2) The control problem has resulted from an isolated event that would not have affected the rest of the run.

(3) The control problem occurs in a concentration range that is different from the concentration range of the participants' samples. The method is in-control in the range of the participants' samples.

(4) The size of the analytical error is judged to be small relative to the medical usefulness requirements.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS** (54-55)

a. **ALT**. Bilirubin does not affect test results. Hemolysis causes elevated results, and lipemia may cause absorbance flagging as a result of an absorbance increase. The effects of various drugs on ALT activity should be taken into consideration in the case of participants receiving large doses of drugs.

b. **Albumin**. The absorptivity of the dye-albumin complex differs for albumin obtained from different species. Therefore materials for the standardization control of test results must be human in origin. Bilirubin, hemolysis, and lipemia do not affect test results.

c. **ALP**. Bilirubin and lipemia do not affect test results. Hemolysis causes significant negative interference at 200 mg/dL. Phosphatases catalyze the hydrolysis of a great number of phosphoric esters of which p-nitrophenylphosphate has proven to be the most convenient. The interaction with other esterases present in the specimen cannot be excluded. It is postulated that under the conditions of the assay, with a pH of 10.5, the measured activity is mainly related to ALP.

d. **AST**. Bilirubin does not affect test results. Hemolysis causes elevated results, and lipemia may cause absorbance flagging as a result of an absorbance increase. The effects of various drugs on AST activity should be taken into consideration in the case of participants receiving large doses of drugs.

e. **Bicarbonate**. Bilirubin, lipemia, and hemolysis have no effect on this method.

f. **BUN**. Bilirubin and hemolysis do not affect test results. Highly lipemic samples may cause absorbance flags; if that occurs, the sample must be diluted.

g. **Calcium**. Bilirubin, hemolysis, and lipemia cause no significant interference; however, extremely turbid samples may exhibit a negative interference. Oxalate, citrate, and EDTA anticoagulants interfere by binding calcium.

h. **Cholesterol**. Bilirubin causes a significant negative interference at 15 mg/dL. Hemolysis, lipemia, uric acid, creatinine, and glutathione cause no significant interference. A two-fold toxic dose of $\alpha$-methyldopa lowered recovery by 50%. Noramidopyrine lowered recovery by 20%, and a 10-fold therapeutic concentration of ascorbic acid lowered cholesterol recovery by 5%.

i. **Creatinine**. Bilirubin causes a significant interference at 15 mg/dL. Hemolysis and lipemia do not affect test results.

j. **$\gamma$-GT**. Bilirubin and lipemia do not affect test results. Hemolysis causes significant negative interference at 100 mg/dL.

k. **Glucose**. Bilirubin, hemolysis, and lipemia do not affect test results.

m. **Iron**. Bilirubin, lipemia, and atypical gammaglobulins do not affect test results. Hemoglobin levels of 31-199 mg/dL produce significant negative interference. Hemolyzed serum with abnormally low iron values may produce negative results. Hemoglobin levels >400 mg/dL cause significant positive interference. Deferoxamine-bound serum iron does not react in the test, resulting in falsely lowered values.

m. **LDH**. Bilirubin, hemolysis, and lipemia do not affect test results.

n. **Phosphorus**. Bilirubin and lipemia do not affect test results. RBC contamination will elevate results. The presence of monoclonal immunoglobulins in the serum may give erroneous results. Use of an alternative method is recommended.

o. **Sodium, Potassium, and Chloride**. Bilirubin, hemolysis, and lipemia do not affect test results.

p. **Total Bilirubin**.

(1) Urea and creatinine do not interfere; however, bilirubin may react with other metabolites whose levels are elevated in uremic serum.

(2) At indican levels twice those found in dialysis patients, no significant bias (<+0.2 mg/dL bilirubin) is observed when 570 nm is used as the primary measuring wavelength.

(3) Elevated VLDL levels (triglyceride concentrations of at least 1000 mg/dL) produce a positive bias of approximately 0.3 mg/dL bilirubin. Intralipid at a concentration of approximately 60 mg/dL will produce a positive bias of 0.5 mg/dL at normal bilirubin levels.

(4) Typical hemolysis (<200 mg/dL hemoglobin) will produce less than +0.2 mg/dL bias at normal bilirubin levels and less than -0.7 mg/dL bias at elevated bilirubin levels (e.g., neonatal).

q. **Total Protein**. Bilirubin and lipemia do not affect test results. Hemolysis will elevate results.

r. **Triglycerides**. Bilirubin and lipemia do not affect test results. Occasionally, extremely elevated triglyceride levels (>3000 mg/dL) have been found to give a "normal" result. To ensure accurate results, dilute grossly lipemic serum with 1 part serum to 4 parts saline and multiply the result by 5. Hemolysis causes a significant positive interference at 400 mg/dL.

s. **Uric acid**. Bilirubin levels >7.5 mg/dL cause significant negative interference. Hemoglobin levels >200 mg/dL cause significant positive interference. Lipid levels >750 mg/dL cause significant positive interference. Ascorbic acid causes no interference up to 5 mg/dL. Therapeutic levels of dipyrone give falsely low results. Uricase reacts specifically with uric acid. Other purine derivatives can inhibit the uric acid reaction.

## 13. REFERENCE RANGES (NORMAL VALUES)

a. **ALT**. The normal range for serum ALT by this method, as determined by BMD for the Hitachi 737, is ≤40 U/L (37 °C) among males and ≤31 U/L (37 °C) among females.

b. **Albumin**. The normal range for serum albumin by this method, as determined by BMD for the Hitachi 737, is 3.4-5.0 gm/dL (conventional units) or 34-50 g/L (SI Units).

Physiological factors are capable of modifying the levels of albumin in healthy individuals. Levels are subject to seasonal variation, tending to be lower in summer than in winter. Transient increases are found 11-12 hours after strenuous exercise. Dietary factors play a role in these normal value ranges. Premature infants may show higher levels.

c. **ALP**. The normal range for serum ALP by this method, as determined by BMD for the Hitachi 737, is 117-390 U/L (37 °C) for children (3-15 years) and 39-117 U/L (37 °C) for adults.

d. **AST**. The normal range for serum AST by this method, as determined by BMD for the Hitachi 737, is ≤37 U/L (37 °C) for males and ≤31 U/L (37 °C) for females.

e. **Bicarbonate ($HCO_3$)**. The normal range for serum bicarbonate by this method, as determined by BMD for the Hitachi 737, is 23.0-29.0 mmol/L.

f. **BUN**. The normal range for serum BUN by this method, as determined by BMD for the Hitachi 737, is 6-19 mg/dL (conventional units) or 2.14-6.78 mmol/L urea (SI Units).

g. **Calcium**. The normal range for serum calcium by this method, as determined by BMD for the Hitachi 737, is 8.4-10.2 mg/dL (conventional units) or 2.1-2.6 mmol/L (SI Units).

h. **Cholesterol**. According to the National Cholesterol Education Program, the desirable blood cholesterol level is <200 mg/dL, the borderline-high blood cholesterol level is 200-239 mg/dL, and the high blood cholesterol level is ≥240 mg/dL.

i. **Creatinine**. The normal range for serum creatinine by this method, as determined by BMD for the Hitachi 737, is 0.6-1.1 mg/dL for males and 0.5-0.9 mg/dL for females.

j. **γ-GT**. The normal range for serum GGT by this method, as determined by BMD for the Hitachi 737, is 11-51 U/L (37 °C) for males and 7-33 U/L (37 °C) for females.

k. **Glucose**. The normal range for serum glucose by this method, as determined by BMD for the Hitachi 737, is 70-105 mg/dL (conventional units) or 3.8-5.8 mmol/L (SI Units).

l. **Iron**. The normal range for serum iron by this method, as determined by BMD for the Hitachi 737, is 30-160 $\mu$g/dL (conventional units) or 5.4-28.6 $\mu$mol/L (SI Units) for females and 45-160 $\mu$g/dL (conventional units) or 8.1-28.6 $\mu$mol/L (SI Units) for males.

m. **LDH**. The normal range for serum LDH by this method, as determined by BMD for the Hitachi 737, is 118-273 U/L (37 °C) for males and 122-220 U/L (37 °C) for females.

n. **Phosphorus**. The normal range for serum phosphorus by this method, as determined by BMD for the Hitachi 737, is 2.7-4.5 mg/dL (conventional units) or 0.87-1.45 mmol/L (SI Units).

o. **Sodium, Potassium, and Chloride**. The normal range by this method, as determined by BMD for the Hitachi 737, is 133-145 mmol/L for sodium, 3.3-5.1 mmol/L for potassium, and 96-108 mmol/L for chloride.

p. **Total Bilirubin**. The normal range for total bilirubin by this method, as determined by BMD for the Hitachi 737, is up to 1.0 mg/dL for adults and children. Ranges for neonates are as follows:
- 24 hrs: 1.0-6.0 mg/dL for premature neonates and 2.0-6.0 mg/dL for full term neonates.
- 48 hrs: 6.0-8.0 mg/dL for premature neonates and 6.0-7.0 mg/dL for full term neonates.
- 3-5 days: 10-15 mg/dL for premature neonates and 4.0-12.0 mg/dL for full term neonates.

q. **Total Protein**. The normal range for serum total protein by this method, as determined by BMD for the Hitachi 737, is 6.5-8.0 gm/dL (conventional units) or 65-80 g/L (SI Units).

Physiological factors are capable of modifying the levels of protein in healthy individuals. Levels are subject to seasonal variation, tending to be lower in summer than in winter. Transient increases are found 11-12 hours after strenuous exercise. Dietary factors play a role in these normal value ranges. Premature infants may show higher levels.

r. **Triglycerides**. The normal range for serum triglycerides by this method, as determined by BMD for the Hitachi 737, is $\geq$200 mg/dL (conventional units) or $\geq$ 2.26 mmol/L (SI Units).

s. **Uric acid**. The normal range for serum uric acid by this method, as determined by BMD for the Hitachi 737, is 3.4-7.0 mg/dL (conventional units) or 0.2-0.41 mmol/L (SI Units) for males and 2.4-5.7 mg/dL (conventional units) or 0.14-0.34 mmol/L (SI Units) for females.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Results requiring notification of the NHANES Coordinator or medical intervention are shown in Table 4.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may reach and maintain 20-25 °C during analysis, but they should be returned to 4-8 °C storage as soon as possible. After a run is accepted, samples should be returned to $\leq$-70 °C for long-term storage.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

The analysis for these parameters can be performed on the Roche Cobas Bio in the event of a malfunction of the Hitachi 737. The Cobas may be used as a substitute for the Hitachi 737 until functionality of the Hitachi can be restored. In addition, the Hitachi 704 can be used as a backup instrument to the Hitachi 737. The same reagents, controls and calibrators are used for all three instruments.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

White Sands will report the results to the NCHS Coordinator by telephone or fax. The NCHS physician will notify the participant's medical provider.

Table 4
Critical Call Results

| Analyte | Units | Result Ranges to be Reported to NHANES III Coordinator | Result Ranges Requiring Medical Intervention |
|---|---|---|---|
| ALT (1) | U/L | ≥129 | ≥400 |
| Albumin (2) | gm/dL | -- | <1.5 |
| ALP (3) | U/L | -- | >500 |
| AST (4) | U/L | ≥120 | ≥400 |
| Bicarbonate (5) | mmol/L | -- | <10 or >40 |
| BUN (6) | mg/dL | -- | >50 |
| Calcium (7) | mg/dL | <7.0 or >12.0 | <6.0 |
| Cholesterol (8) | mg/dL | -- | >240 |
| Creatinine (9) | mg/dL | -- | >3.0 |
| γ-GT (10) | U/L | -- | >400 |
| Glucose (11) | mg/dL | ≥140 | <40 or >375 |
| Iron (12) | µg/dL | -- | <30 or >250 |
| LDH (13) | U/L | -- | >1000 |
| Phosphorus (14) | mg/dL | -- | <1.0 or >10.0 |
| Sodium (15) | mmol/L | <122 or >154 | <120 or >150 |
| Potassium (15) | mmol/L | <2.9 or >5.4 | <2.5 or >6.0 |
| Chloride (15) | mmol/L | -- | <80 or >120 |
| Total Bilirubin (16) | mg/dL | -- | >5.0 |
| Total Protein (17) | g/dL | -- | <4.0 or >9.0 |
| Triglycerides (18) | mg/dL | -- | >200 |
| Uric acid (19) | mg/dL | -- | >12 |

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (electronic and log accession books) are used to track specimens. All records, including related QC data, are maintained for a minimum of 7 years, in electronic and hardcopy format. Only numerical identifiers are used to identify participants, with all related personal identifiers being maintained by the NCHS coordinator to safeguard confidentiality.

**REFERENCES**

1. Procedural insert: Hitachi 737 ALT/IFCC. Indianapolis: Boehringer Mannheim Corporation, 1991.

2. Procedural insert: Hitachi 737 Albumin/BCP. Indianapolis: Boehringer Mannheim Corporation, 1991.

3. Procedural insert: Hitachi 737 Alk Phos/AMP. Indianapolis: Boehringer Mannheim Corporation, 1992.

4. Procedural insert: Hitachi 737 AST/IFCC. Indianapolis: Boehringer Mannheim Corporation, 1991.

5. Procedural insert: Hitachi 737 Bicarbonate. Indianapolis: Boehringer Mannheim Corporation, 1991.

6. Procedural insert: Hitachi 737 BUN. Indianapolis: Boehringer Mannheim Corporation, 1990.

7. Procedural insert: Hitachi 737 Calcium/CAPS Boehringer Mannheim Corporation, 1990.

8. Procedural insert: Hitachi 737 Cholesterol HP. Indianapolis: Boehringer Mannheim Corporation, 1991.

9. Procedural insert: Hitachi 737 Creatinine. Indianapolis: Boehringer Mannheim Corporation, 1990.

10. Procedural insert: Hitachi 737 γ-GT. Indianapolis: Boehringer Mannheim Corporation, 1990.

11. Procedural insert: Hitachi 737 Glucose/HK. Indianapolis: Boehringer Mannheim Corporation, 1991.

12. Procedural insert: Hitachi 737 Iron. Indianapolis: Boehringer Mannheim Corporation, 1991.

13. Procedural insert: Hitachi 737 LDH. Indianapolis: Boehringer Mannheim Corporation, 1990.

14. Procedural insert: Hitachi 737 Phosphorus. Indianapolis: Boehringer Mannheim Corporation, 1991.

15. Procedural insert: Hitachi 737 ISE/Na,K,Cl. Indianapolis: Boehringer Mannheim Corporation, 1991.

16. Procedural insert: Hitachi 737 Bilirubin/DPD. Indianapolis: Boehringer Mannheim Corporation, 1991.

17. Procedural insert: Hitachi 737 Total Protein/Biuret. Indianapolis: Boehringer Mannheim Corporation, 1991.

18. Procedural insert: Hitachi 737 Triglycerides/GPO. Indianapolis: Boehringer Mannheim Corporation, 1991.

19. Procedural insert: Hitachi 737 Uric Acid/PAP. Indianapolis: Boehringer Mannheim Corporation, 1991.

**Additional Sources**

Glick MR, and Ryder KW. IN: Interferographs: Users Guide to Interferences in Clinical Chemistry Instruments, Indianapolis, Science Enterprises, Inc., 1987. p. 132.

Parameter Procedural inserts: Hitachi 737. Indianapolis: Boehringer Mannheim Corporation, 1991.

Procedural insert: Hitachi 737. Indianapolis: Boehringer Mannheim Corporation, Indianapolis, IN. IN: National Health and Nutrition Examination Survey III procedure manual update September, 1989. Primate Research Institute, New Mexico State University, Hollomon AFB, NM. p. 19-65.

**ACKNOWLEDGMENTS**

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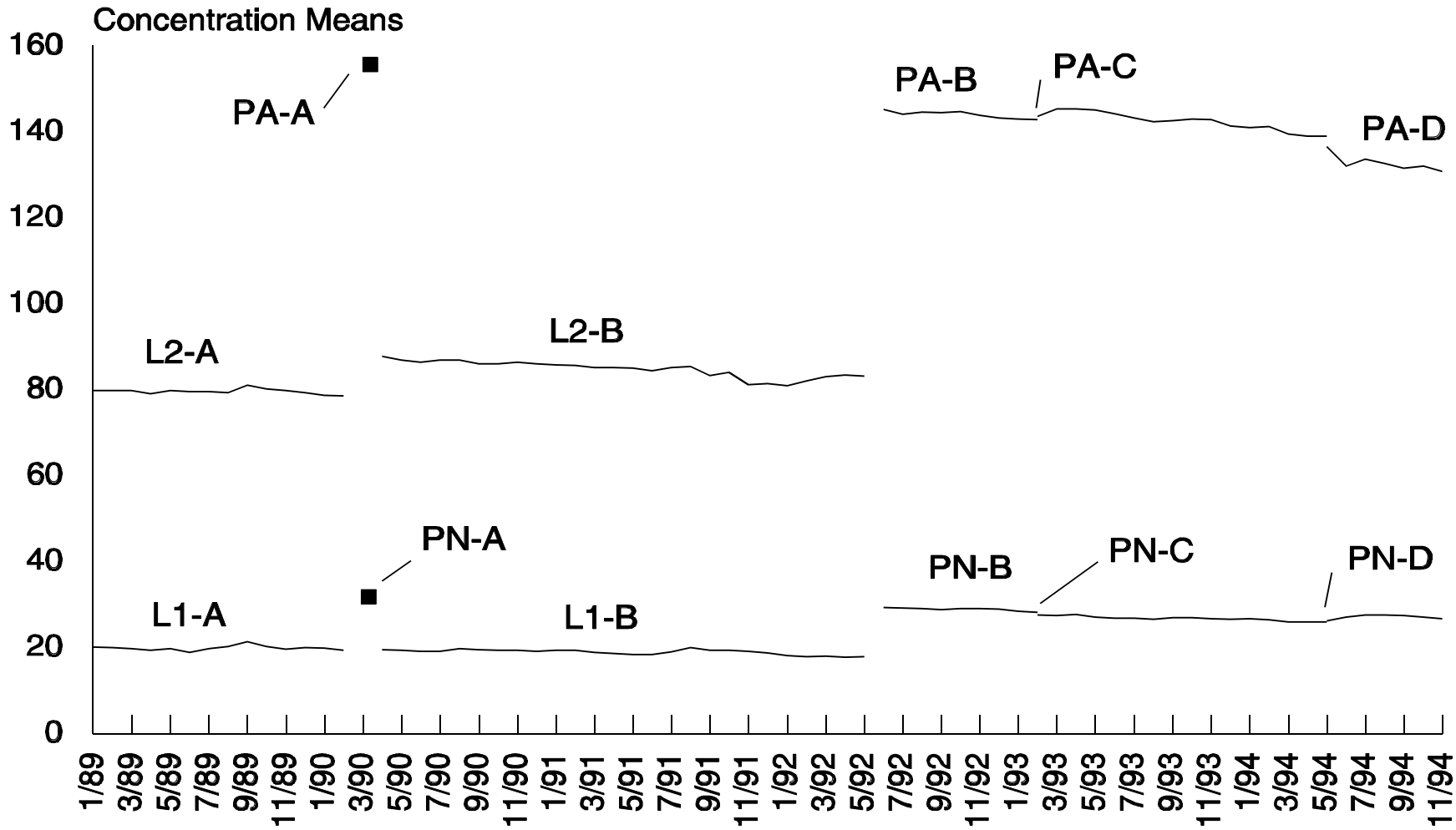
SUMMARY STATISTICS FOR
ALANINE AMINOTRANSFERASE (ALT)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 19.971 | 0.56491 | 2.82858 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 19.019 | 0.61710 | 3.24462 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 79.636 | 0.62093 | 0.77971 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 84.812 | 1.91715 | 2.26048 | 26 |
| PA-A | 03/31/90 | 155.000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 144.044 | 0.83981 | 0.58302 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 142.450 | 2.11880 | 1.48740 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 132.786 | 1.86764 | 1.40651 | 7 |
| PN-A | 03/31/90 | 31.000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 29.000 | 0.37417 | 1.29023 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 26.838 | 0.54757 | 2.04032 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 27.186 | 0.49473 | 1.81983 | 7 |

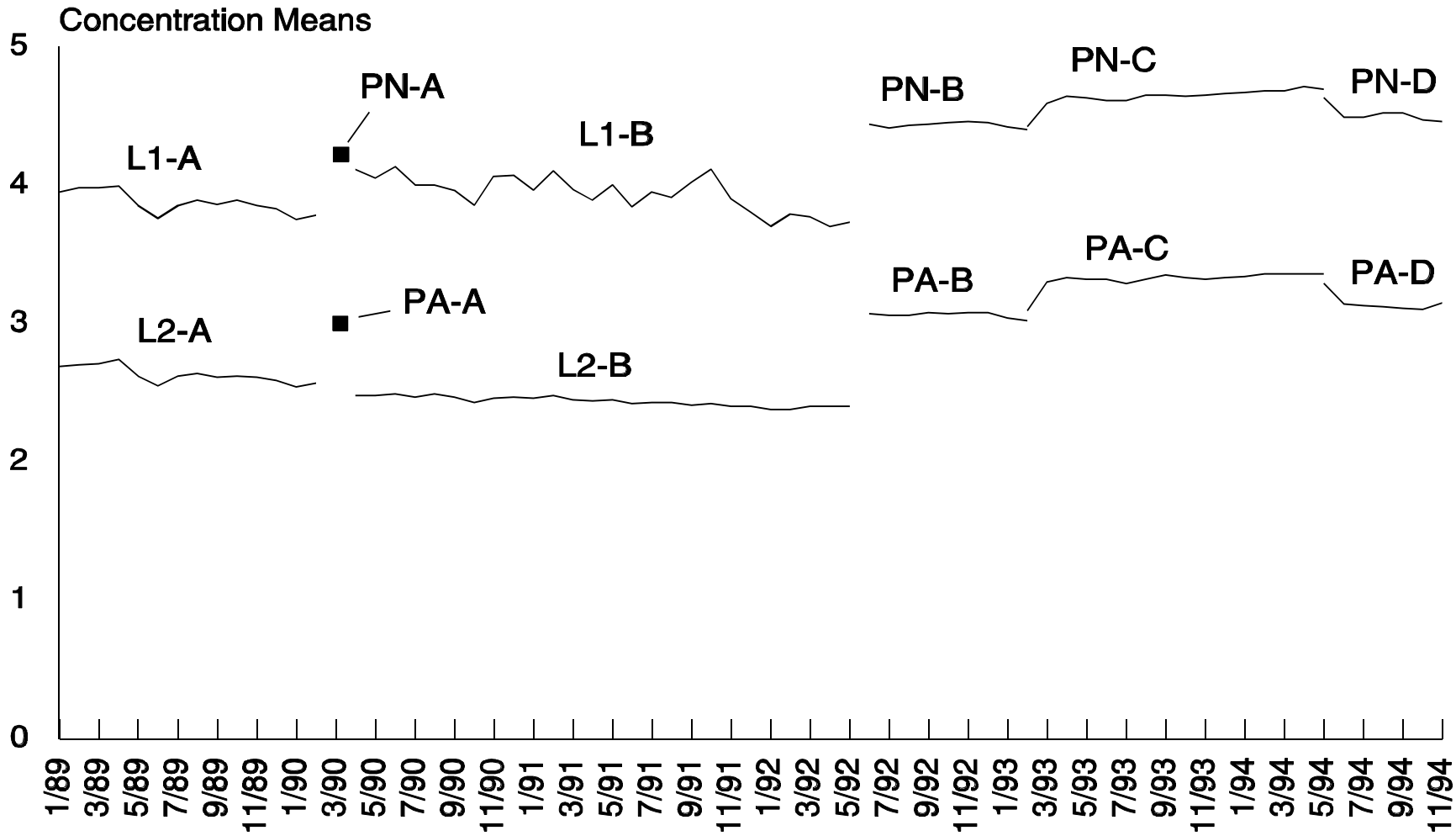
# Alanine Aminotransferase (ALT) Monthly Means



SUMMARY STATISTICS FOR
ALBUMIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 3.87214 | 0.08002 | 2.06648 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 3.93731 | 0.13193 | 3.35066 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 2.62929 | 0.06070 | 2.30848 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 2.43808 | 0.03499 | 1.43506 | 26 |
| PA-A | 03/31/90 | 3.00000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 3.06222 | 0.02048 | 0.66881 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 3.31750 | 0.06434 | 1.93950 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 3.14857 | 0.06466 | 2.05364 | 7 |
| PN-A | 03/31/90 | 4.20000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 4.43333 | 0.02000 | 0.45113 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 4.63625 | 0.06571 | 1.41740 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 4.51143 | 0.05699 | 1.26319 | 7 |

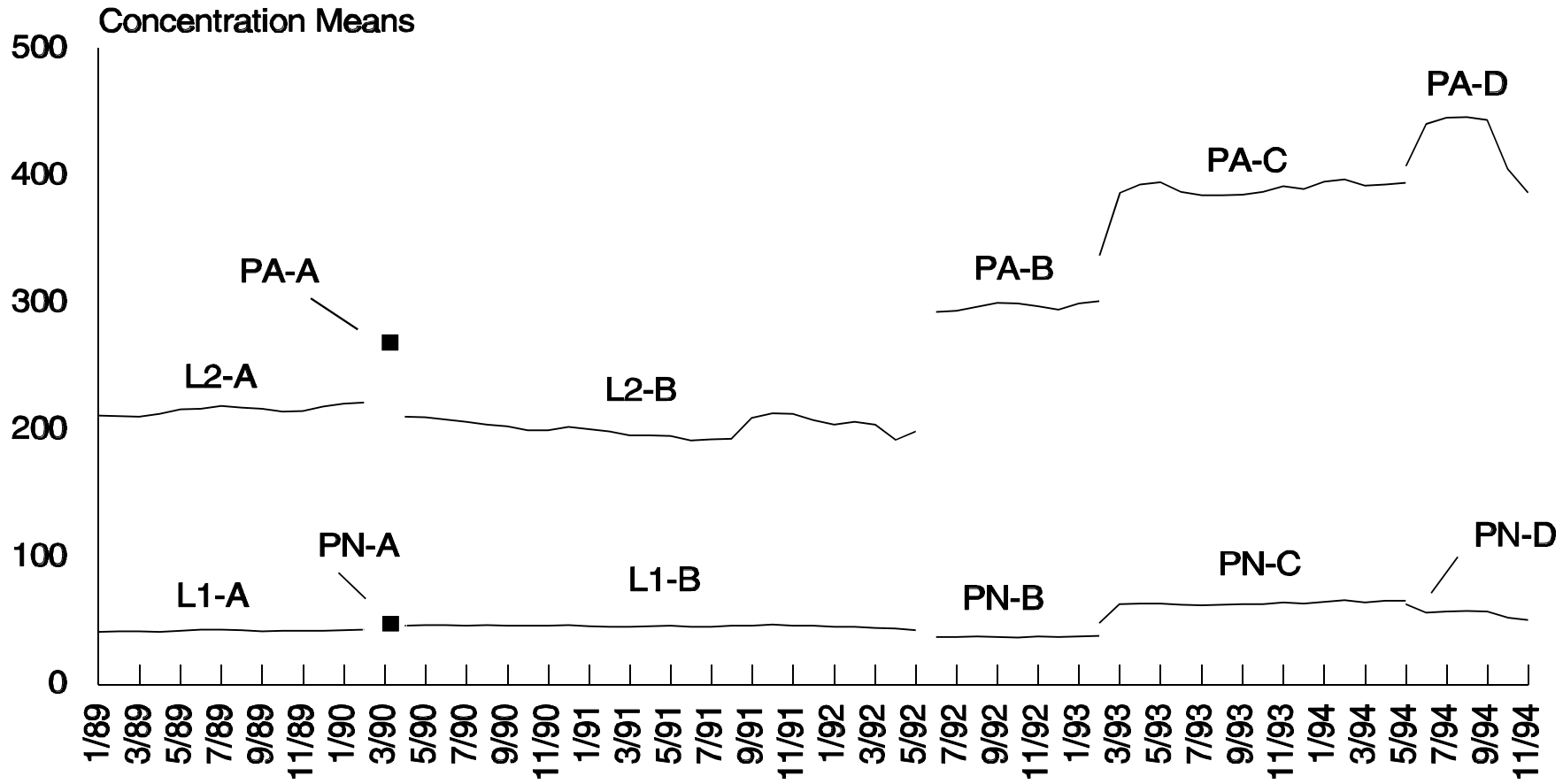
# Albumin Monthly Means



SUMMARY STATISTICS FOR
ALKALINE PHOSPHATASE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 42.321 | 0.6204 | 1.46591 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 45.942 | 0.9597 | 2.08883 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 216.136 | 3.6138 | 1.67200 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 202.396 | 6.5244 | 3.22357 | 26 |
| PA-A | 03/31/90 | 266.000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 297.511 | 3.0726 | 1.03278 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 387.213 | 14.0453 | 3.62730 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 424.986 | 24.7945 | 5.83418 | 7 |
| PN-A | 03/31/90 | 47.000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 37.656 | 0.3167 | 0.84096 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 63.200 | 4.1239 | 6.52518 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 56.657 | 4.0529 | 7.15342 | 7 |

# Alkaline Phosphatase Monthly Means

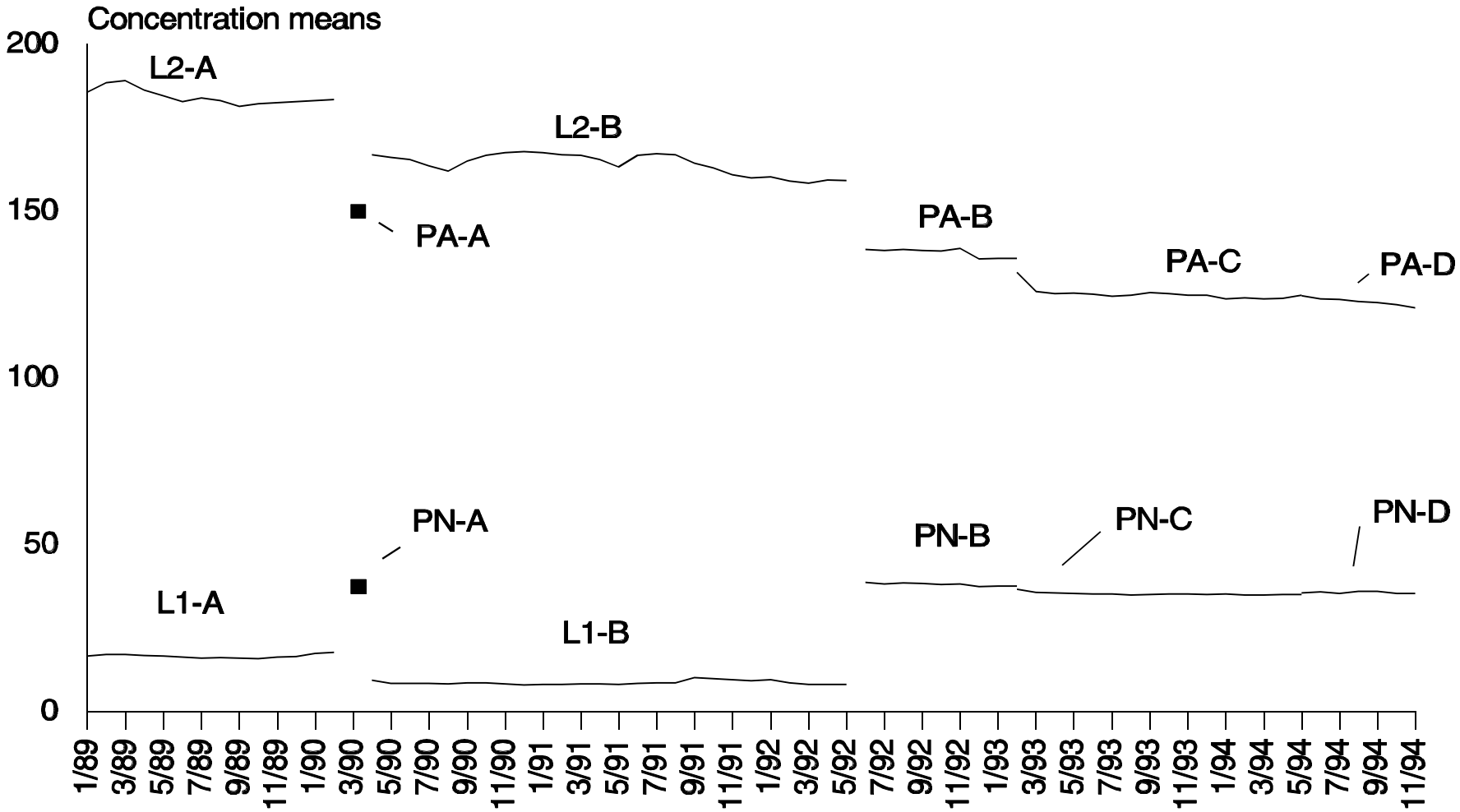



NOTE: The downward shift of pool PA-D after 9/94 is the result of a change in the parameter factor suggested by the manufacturer. All subsequent external quality control performance was acceptable.

SUMMARY STATISTICS FOR
ASPARTATE AMINOTRANSFERASE (AST)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 16.736 | 0.56242 | 3.36061 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 8.762 | 0.60271 | 6.87906 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 184.279 | 2.33079 | 1.26482 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 164.138 | 3.17970 | 1.93720 | 26 |
| PA-A | 03/31/90 | 149.000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 137.578 | 1.30171 | 0.94616 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 125.250 | 1.81255 | 1.44714 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 122.986 | 1.19782 | 0.97395 | 7 |
| PN-A | 03/31/90 | 37.000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 38.211 | 0.44845 | 1.17362 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 35.419 | 0.42617 | 1.20325 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 35.814 | 0.27946 | 0.78029 | 7 |

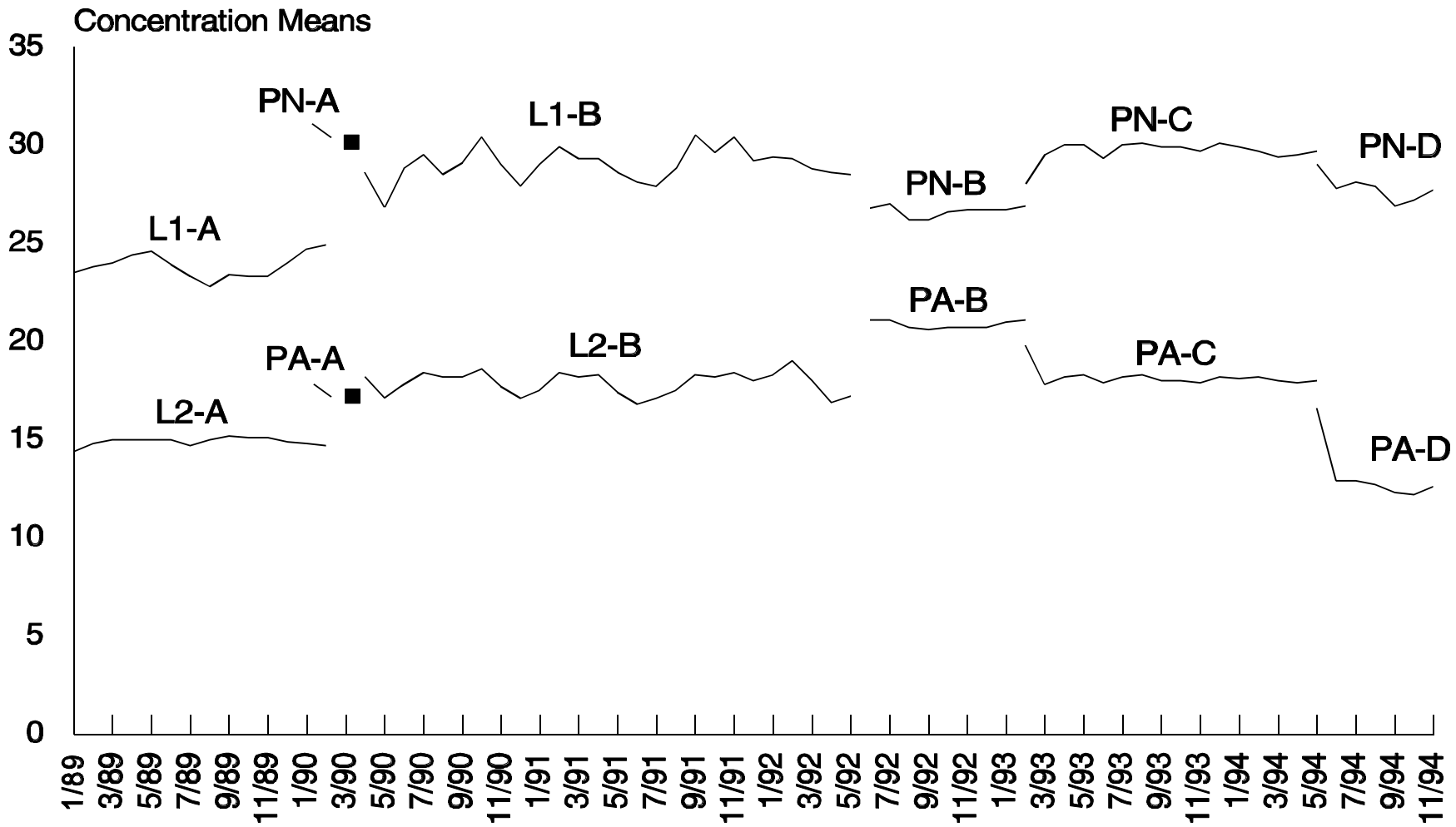
# Aspartate Aminotransferase (AST) Monthly Means



SUMMARY STATISTICS FOR
BICARBONATE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 23.8500 | 0.62481 | 2.6197 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 28.9923 | 0.83038 | 2.8642 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 14.9071 | 0.20926 | 1.4038 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 17.8769 | 0.58741 | 3.2858 | 26 |
| PA-A | 03/31/90 | 17.0000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 20.8556 | 0.21279 | 1.0203 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 18.1750 | 0.45971 | 2.5294 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 13.1714 | 1.53592 | 11.6610 | 7 |
| PN-A | 03/31/90 | 30.0000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 26.6444 | 0.27889 | 1.0467 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 29.6688 | 0.51084 | 1.7218 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 27.8000 | 0.67330 | 2.4219 | 7 |

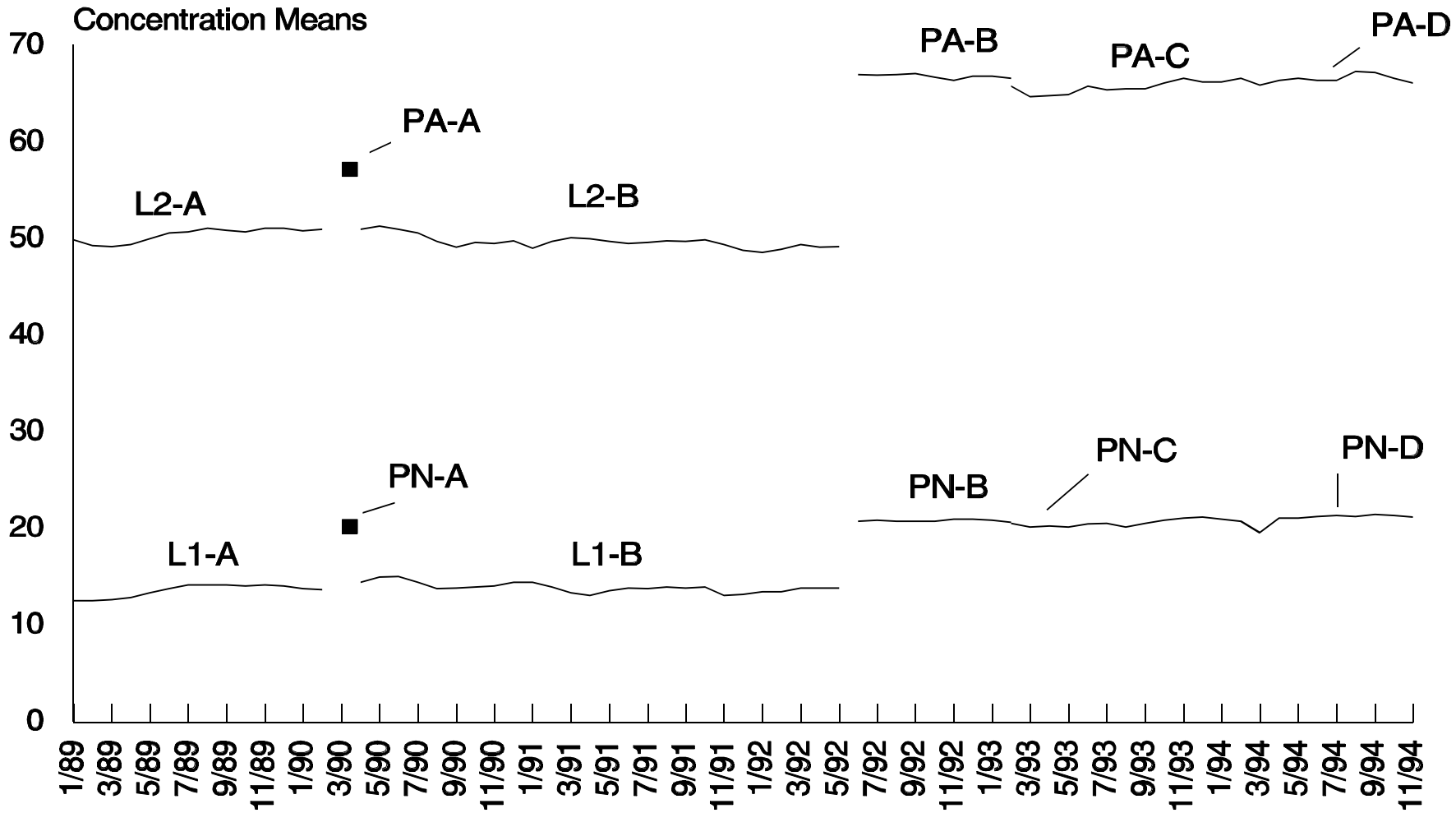
# Bicarbonate Monthly Means



SUMMARY STATISTICS FOR
BLOOD UREA NITROGEN (BUN)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 13.6071 | 0.64267 | 4.72302 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 13.9462 | 0.51243 | 3.67435 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 50.4143 | 0.70804 | 1.40444 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 49.6923 | 0.67701 | 1.36240 | 26 |
| PA-A | 03/31/90 | 57.0000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 66.8111 | 0.22048 | 0.33000 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 65.8125 | 0.63548 | 0.96559 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 66.6571 | 0.43916 | 0.65883 | 7 |
| PN-A | 03/31/90 | 20.0000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 20.8556 | 0.10138 | 0.48610 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 20.6250 | 0.44497 | 2.15744 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 21.3143 | 0.13452 | 0.63112 | 7 |

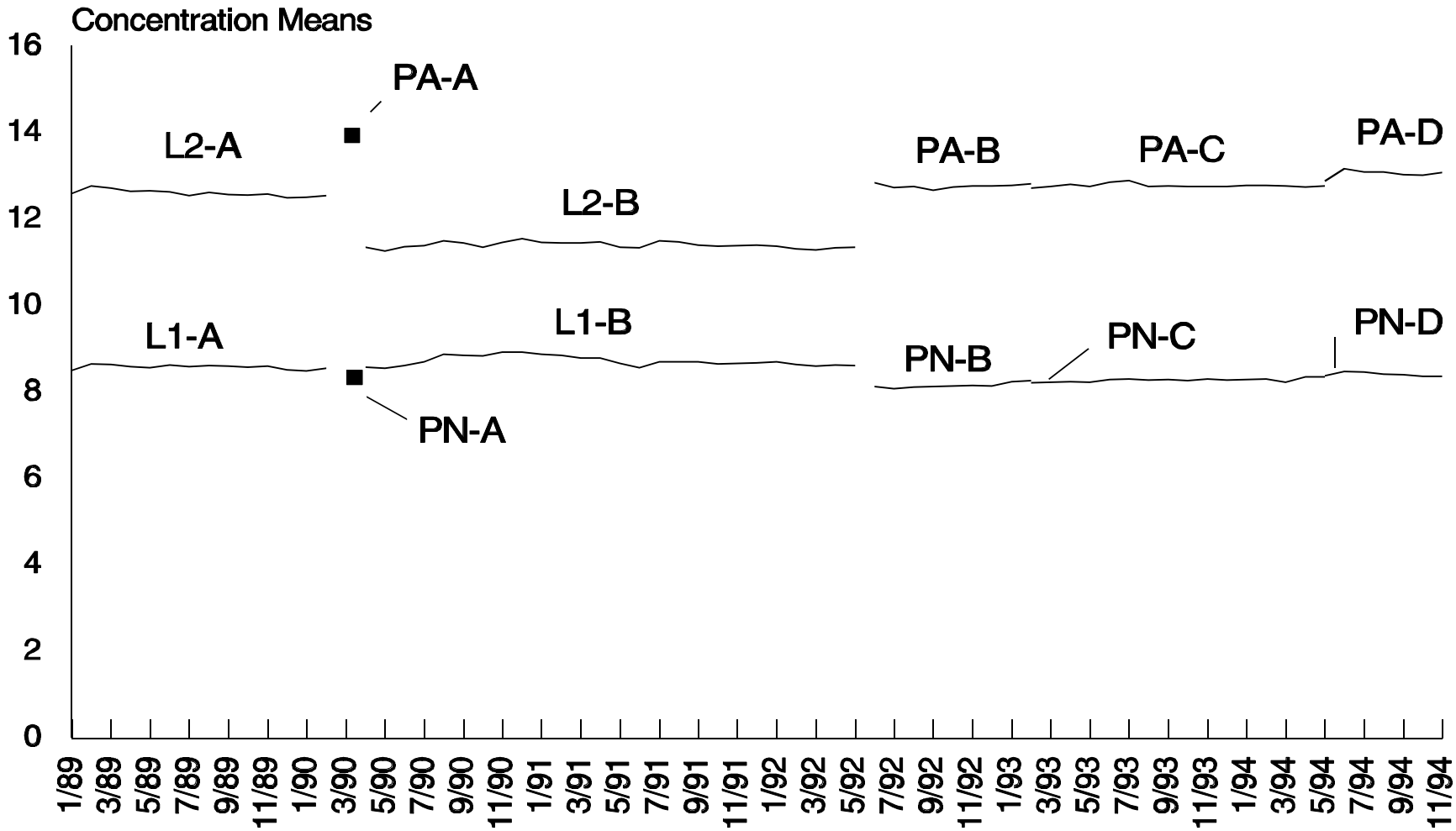
# Blood Urea Nitrogen (BUN) Monthly Means



SUMMARY STATISTICS FOR
CALCIUM
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 8.5836 | 0.05002 | 0.58270 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 8.7238 | 0.11493 | 1.31741 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 12.6064 | 0.07672 | 0.60859 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 11.4046 | 0.07212 | 0.63241 | 26 |
| PA-A | 03/31/90 | 13.9000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 12.7678 | 0.04893 | 0.38325 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 12.7788 | 0.04209 | 0.32938 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 13.0543 | 0.09163 | 0.70188 | 7 |
| PN-A | 03/31/90 | 8.3000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 8.1600 | 0.06021 | 0.73784 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 8.2825 | 0.04008 | 0.48395 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 8.4143 | 0.04577 | 0.54400 | 7 |

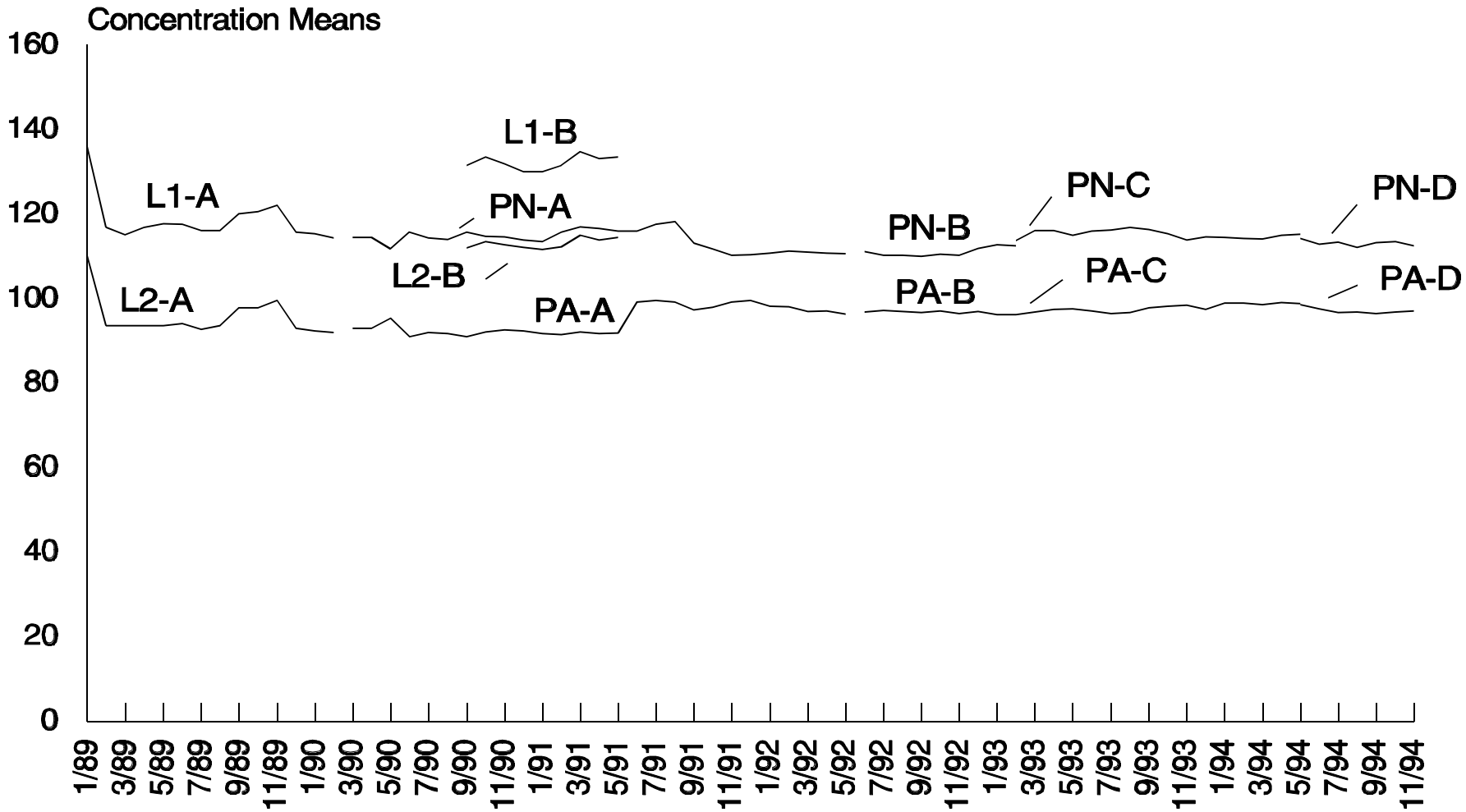
# Calcium Monthly Means



SUMMARY STATISTICS FOR
CHLORIDE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 118.671 | 5.51698 | 4.64895 | 14 |
| L1-B | 09/30/90 - 05/31/91 | 132.259 | 1.63881 | 1.23910 | 9 |
| L2-A | 01/31/89 - 02/28/90 | 95.601 | 4.81213 | 5.03357 | 14 |
| L2-B | 09/30/90 - 05/31/91 | 113.130 | 1.20283 | 1.06323 | 9 |
| PA-A | 03/31/90 - 05/31/92 | 94.948 | 3.21942 | 3.39073 | 27 |
| PA-B | 06/30/92 - 02/09/93 | 96.814 | 0.36743 | 0.37952 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 97.859 | 0.94220 | 0.96281 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 97.196 | 0.72675 | 0.74772 | 7 |
| PN-A | 03/31/90 - 05/31/92 | 113.917 | 2.41910 | 2.12357 | 27 |
| PN-B | 06/30/92 - 02/09/93 | 111.123 | 1.07436 | 0.96682 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 115.258 | 0.99344 | 0.86193 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 113.200 | 0.71335 | 0.63017 | 7 |

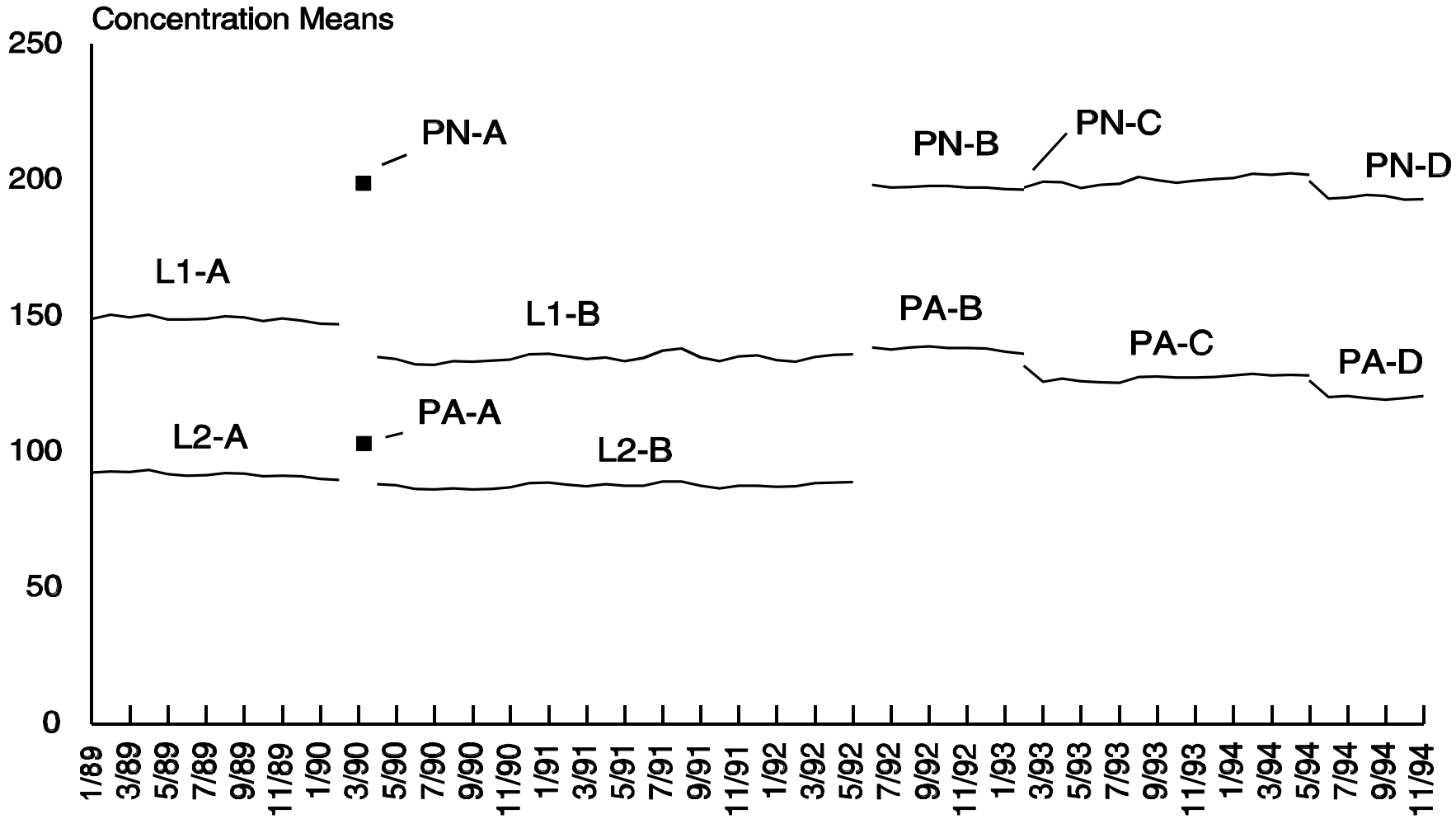
# Chloride Monthly Means



SUMMARY STATISTICS FOR
HITACHI CHOLESTEROL
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 149.036 | 1.04852 | 0.70354 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 134.731 | 1.45156 | 1.07738 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 91.814 | 1.03244 | 1.12449 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 87.773 | 0.91414 | 1.04148 | 26 |
| PA-A | 03/31/90 | 103.000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 138.033 | 0.92195 | 0.66792 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 127.675 | 1.51899 | 1.18973 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 121.029 | 2.41296 | 1.99371 | 7 |
| PN-A | 03/31/90 | 199.000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 197.556 | 0.58547 | 0.29636 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 200.150 | 1.69823 | 0.84848 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 194.543 | 2.35928 | 1.21273 | 7 |

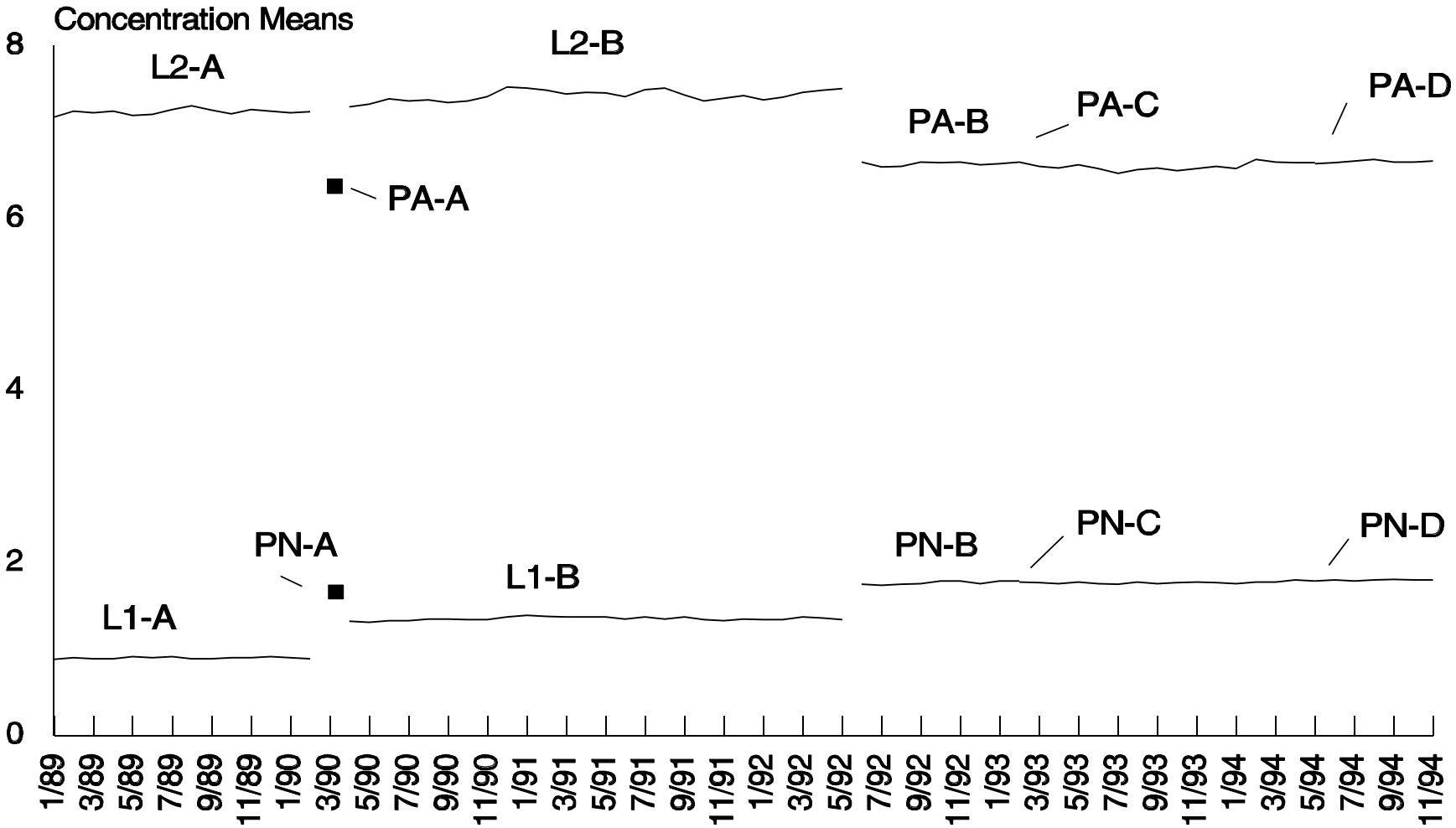
# Hitachi Cholesterol Monthly Means



SUMMARY STATISTICS FOR
CREATININE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 0.89714 | 0.009139 | 1.01865 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 1.35115 | 0.019663 | 1.45524 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 7.23071 | 0.032925 | 0.45535 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 7.41962 | 0.063717 | 0.85876 | 26 |
| PA-A | 03/31/90 | 6.30000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 6.63111 | 0.023154 | 0.34917 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 6.59875 | 0.043951 | 0.66605 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 6.65286 | 0.016036 | 0.24103 | 7 |
| PN-A | 03/31/90 | 1.60000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 1.76889 | 0.020883 | 1.18059 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 1.77313 | 0.013022 | 0.73443 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 1.79857 | 0.006901 | 0.38367 | 7 |

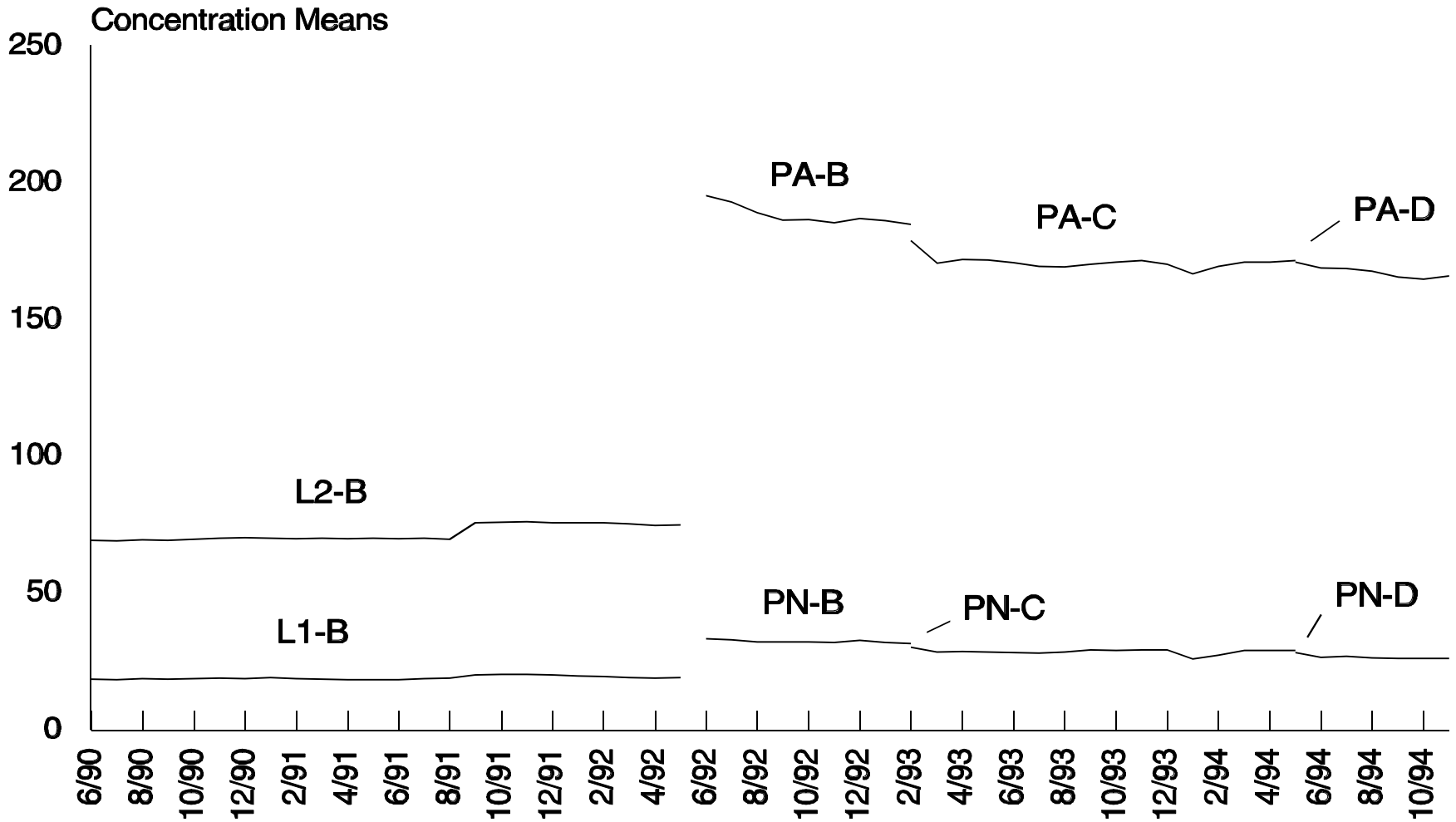
# Creatinine Monthly Means



SUMMARY STATISTICS FOR
GAMMA GLUTAMYL TRANSFERASE (GGT)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-B | 06/30/90 - 05/31/92 | 19.125 | 0.60523 | 3.1646 | 24 |
| L2-B | 06/30/90 - 05/31/92 | 72.021 | 2.91249 | 4.0440 | 24 |
| PA-B | 06/30/92 - 02/09/93 | 188.189 | 3.62093 | 1.9241 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 170.944 | 2.43227 | 1.4229 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 167.486 | 2.21015 | 1.3196 | 7 |
| PN-B | 06/30/92 - 02/09/93 | 32.400 | 0.56347 | 1.7391 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 28.719 | 0.96209 | 3.3501 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 26.714 | 0.74258 | 2.7797 | 7 |

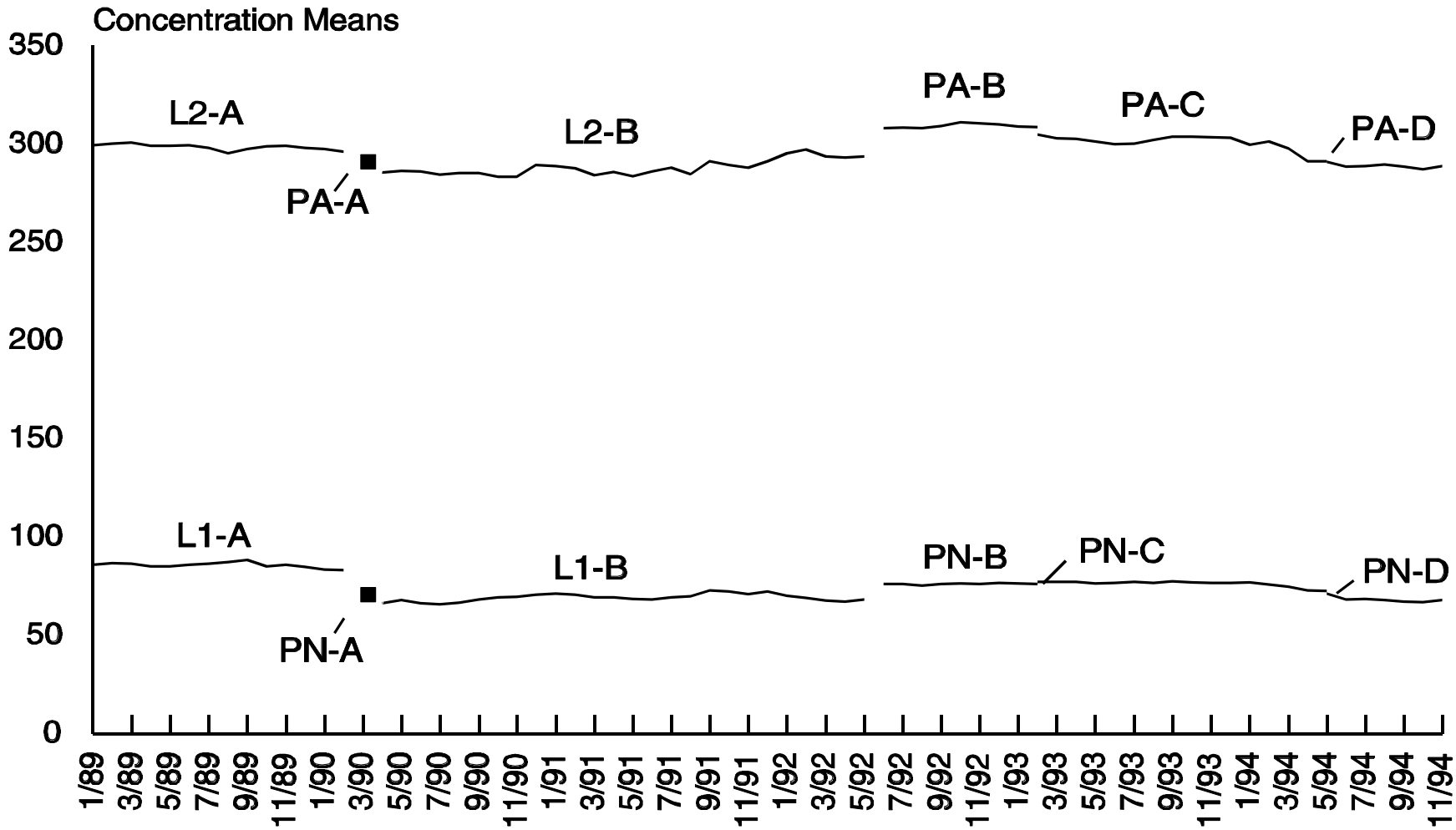
# Gamma Glutamyl Transferase (GGT) Monthly Means



SUMMARY STATISTICS FOR
HITACHI GLUCOSE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 85.864 | 1.42322 | 1.65752 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 69.331 | 1.92328 | 2.77407 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 298.614 | 1.51295 | 0.50666 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 288.208 | 3.92794 | 1.36289 | 26 |
| PA-A | 03/31/90 | 290.000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 309.300 | 1.10000 | 0.35564 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 300.669 | 4.08880 | 1.35990 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 289.029 | 1.17716 | 0.40728 | 7 |
| PN-A | 03/31/90 | 69.000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 76.311 | 0.39826 | 0.52189 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 76.369 | 1.49609 | 1.95904 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 68.414 | 1.43577 | 2.09864 | 7 |

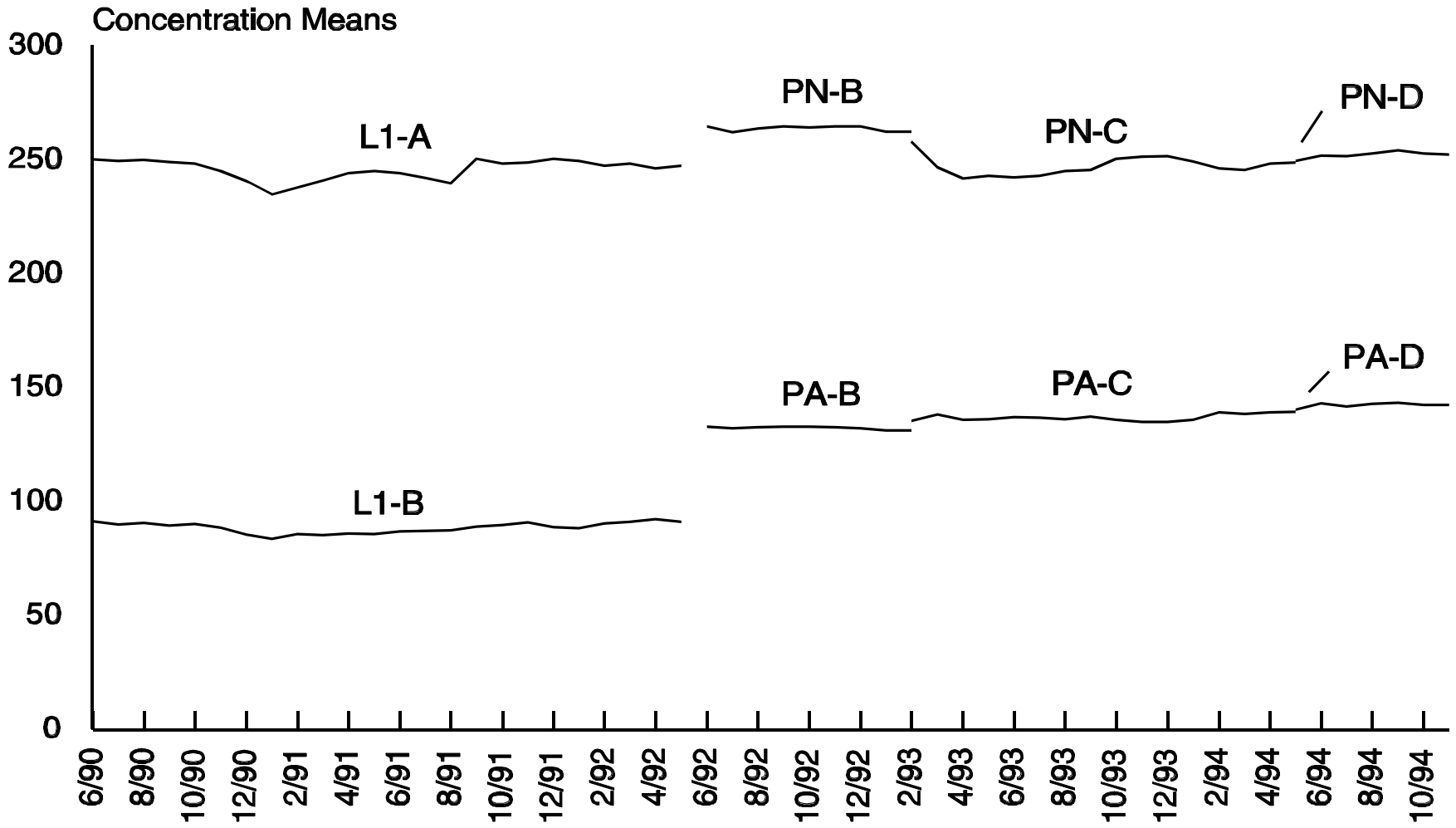
# Hitachi Glucose Monthly Means



SUMMARY STATISTICS FOR
HITACHI IRON
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-B | 06/30/90 - 05/31/92 | 88.671 | 2.37953 | 2.68355 | 24 |
| L2-B | 06/30/90 - 05/31/92 | 245.754 | 4.37969 | 1.78214 | 24 |
| PA-B | 06/30/92 - 02/09/93 | 132.344 | 0.62472 | 0.47204 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 136.963 | 1.54656 | 1.12918 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 142.371 | 1.02910 | 0.72283 | 7 |
| PN-B | 06/30/92 - 02/09/93 | 263.700 | 1.20208 | 0.45585 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 247.300 | 4.20682 | 1.70110 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 252.214 | 1.45193 | 0.57567 | 7 |

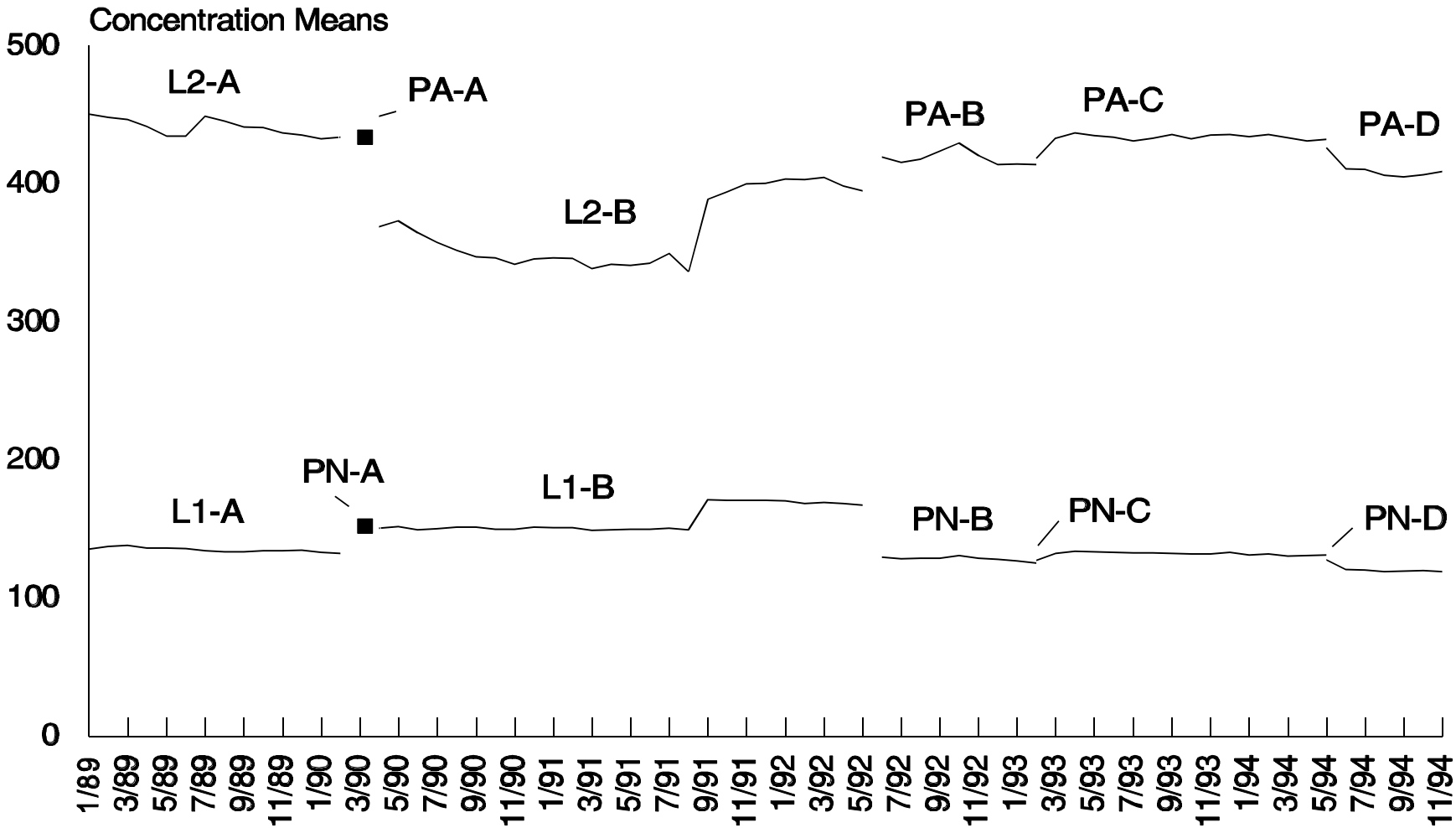
# Hitachi Iron Monthly Means



SUMMARY STATISTICS FOR
LACTATE DEHYDROGENASE (LDH)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 135.257 | 1.6928 | 1.25157 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 157.354 | 9.4530 | 6.00746 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 441.079 | 6.2509 | 1.41719 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 366.750 | 25.5601 | 6.96934 | 26 |
| PA-A | 03/31/90 | 434.000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 419.022 | 5.2851 | 1.26129 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 433.206 | 4.2905 | 0.99041 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 410.943 | 7.1243 | 1.73366 | 7 |
| PN-A | 03/31/90 | 150.000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 128.767 | 1.6055 | 1.24680 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 132.225 | 1.5776 | 1.19308 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 121.257 | 3.1230 | 2.57548 | 7 |

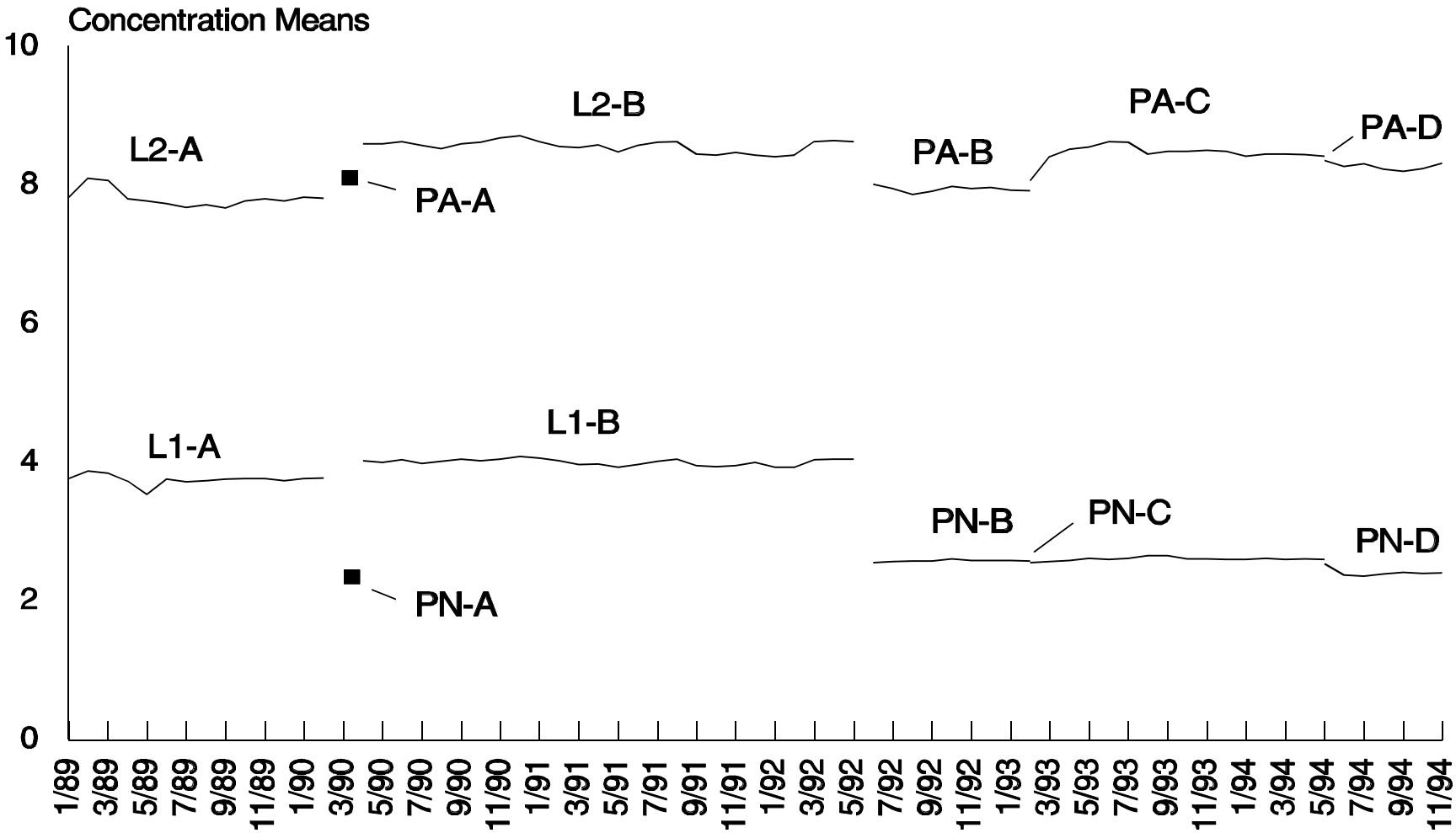
# Lactate Dehydrogenase (LDH) Monthly Means



SUMMARY STATISTICS FOR
PHOSPHORUS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 3.75571 | 0.07583 | 2.01893 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 4.00692 | 0.04680 | 1.16796 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 7.81000 | 0.12655 | 1.62038 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 8.56423 | 0.08510 | 0.99362 | 26 |
| PA-A | 03/31/90 | 8.10000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 7.94333 | 0.04153 | 0.52287 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 8.46188 | 0.12566 | 1.48497 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 8.27571 | 0.05682 | 0.68659 | 7 |
| PN-A | 03/31/90 | 2.30000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 2.58333 | 0.01414 | 0.54744 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 2.60813 | 0.02613 | 1.00197 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 2.41429 | 0.05884 | 2.43707 | 7 |

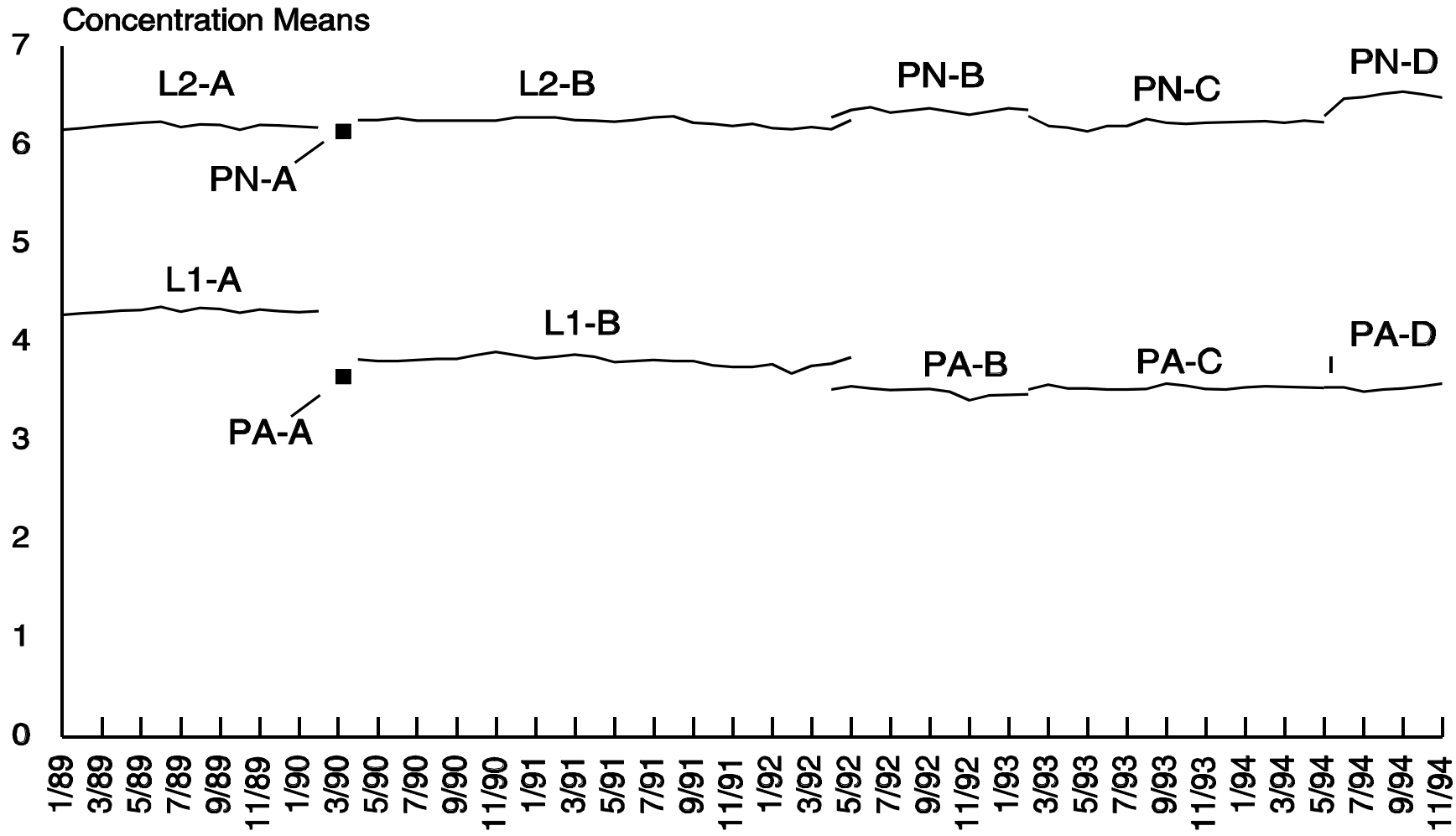
# Phosphorus Monthly Means



SUMMARY STATISTICS FOR
POTASSIUM
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 4.31671 | 0.02203 | 0.51033 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 3.81277 | 0.04734 | 1.24157 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 6.19314 | 0.02332 | 0.37655 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 6.23904 | 0.03785 | 0.60661 | 26 |
| PA-A | 03/31/90 | 3.65000 | - | - | 1 |
| PA-B | 04/30/92 - 02/09/93 | 3.49764 | 0.04154 | 1.18776 | 11 |
| PA-C | 02/28/93 - 05/17/94 | 3.53944 | 0.01791 | 0.50614 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 3.53800 | 0.02510 | 0.70943 | 7 |
| PN-A | 03/31/90 | 6.12000 | - | - | 1 |
| PN-B | 04/30/92 - 02/09/93 | 6.34500 | 0.03008 | 0.47402 | 11 |
| PN-C | 02/28/93 - 05/17/94 | 6.22081 | 0.03608 | 0.58002 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 6.47214 | 0.08374 | 1.29380 | 7 |

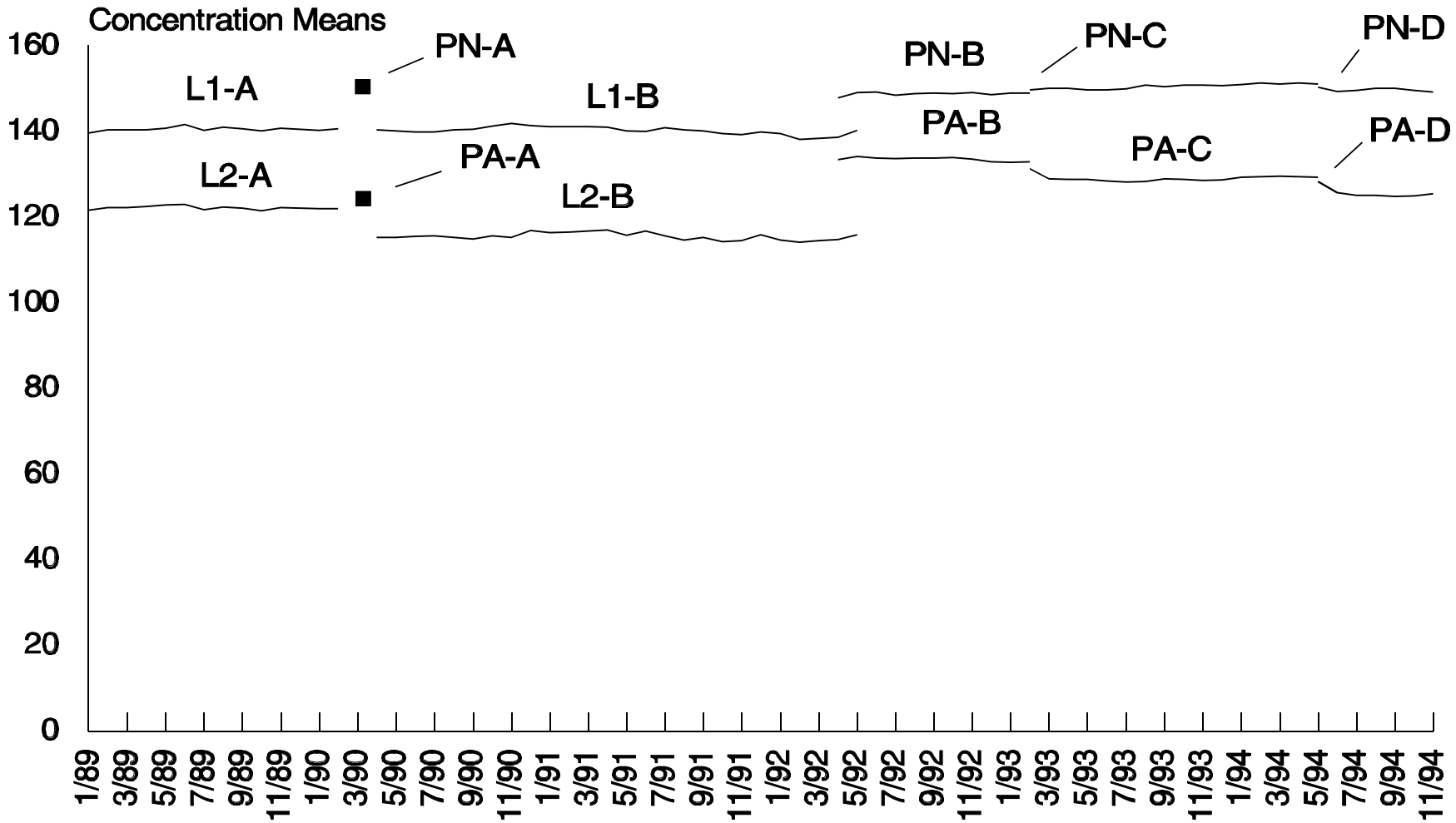
# Potassium Monthly Means



SUMMARY STATISTICS FOR
SODIUM
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 140.530 | 0.46965 | 0.33420 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 140.239 | 0.93300 | 0.66529 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 122.179 | 0.43305 | 0.35444 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 115.121 | 0.83409 | 0.72202 | 26 |
| PA-A | 03/31/90 | 123.900 | - | - | 1 |
| PA-B | 04/30/92 - 02/09/93 | 133.555 | 0.45851 | 0.34331 | 11 |
| PA-C | 02/28/93 - 05/17/94 | 129.068 | 0.73168 | 0.56690 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 125.693 | 1.24060 | 0.98701 | 7 |
| PN-A | 03/31/90 | 150.400 | - | - | 1 |
| PN-B | 04/30/92 - 02/09/93 | 148.810 | 0.37505 | 0.25203 | 11 |
| PN-C | 02/28/93 - 05/17/94 | 150.557 | 0.59122 | 0.39269 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 149.791 | 0.45028 | 0.30060 | 7 |

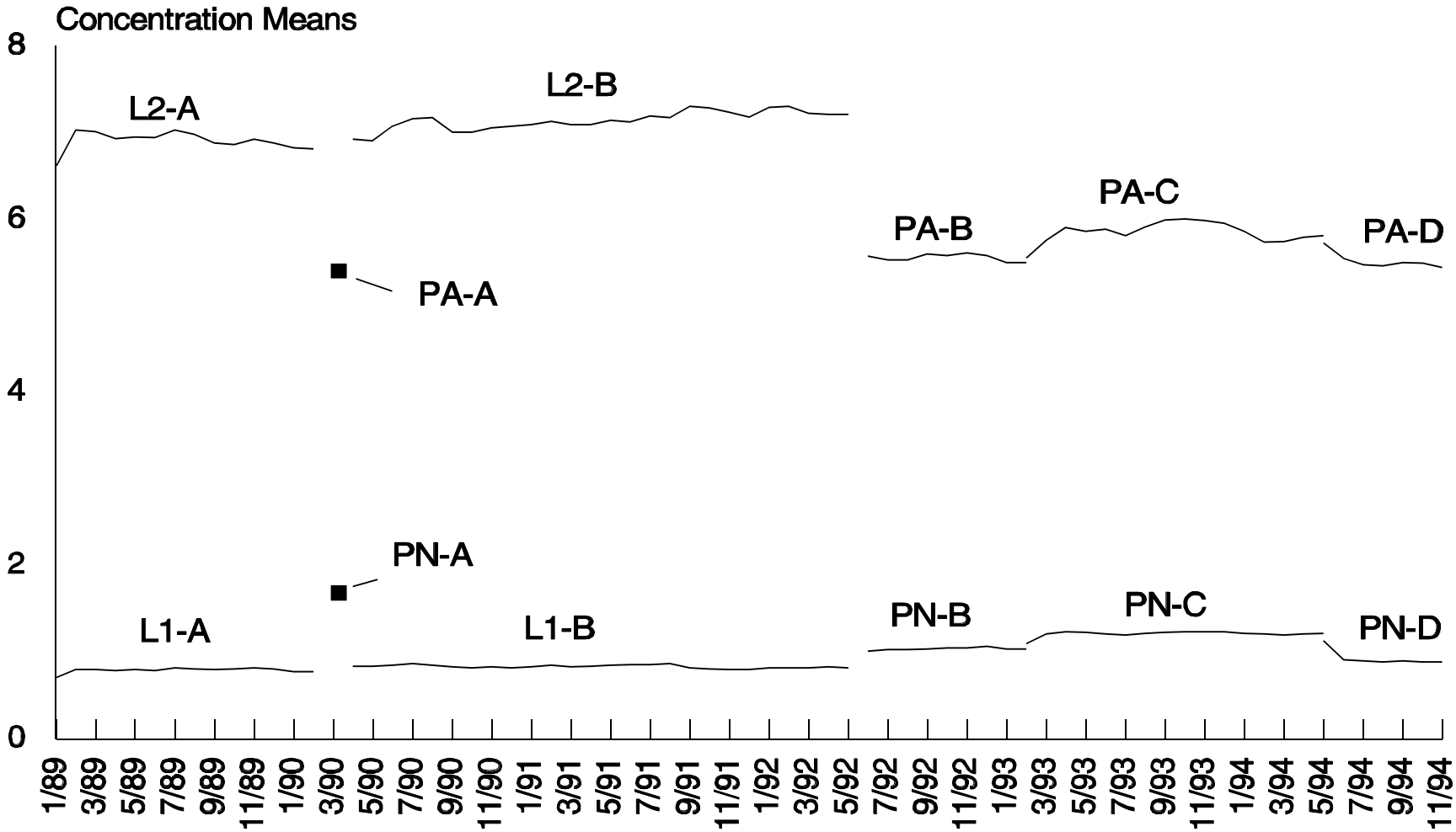
# Sodium Monthly Means



SUMMARY STATISTICS FOR
TOTAL BILIRUBIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 0.79429 | 0.02378 | 3.44663 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 0.83385 | 0.01941 | 2.32736 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 6.90357 | 0.11043 | 1.59955 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 7.13769 | 0.10860 | 1.52153 | 26 |
| PA-A | 03/31/90 | 5.40000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 5.55556 | 0.04157 | 0.74820 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 5.84438 | 0.11804 | 2.01968 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 5.51857 | 0.09547 | 1.72995 | 7 |
| PN-A | 03/31/90 | 1.60000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 1.04000 | 0.01658 | 1.59453 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 1.21375 | 0.03344 | 2.75522 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 0.93000 | 0.08851 | 9.51679 | 7 |

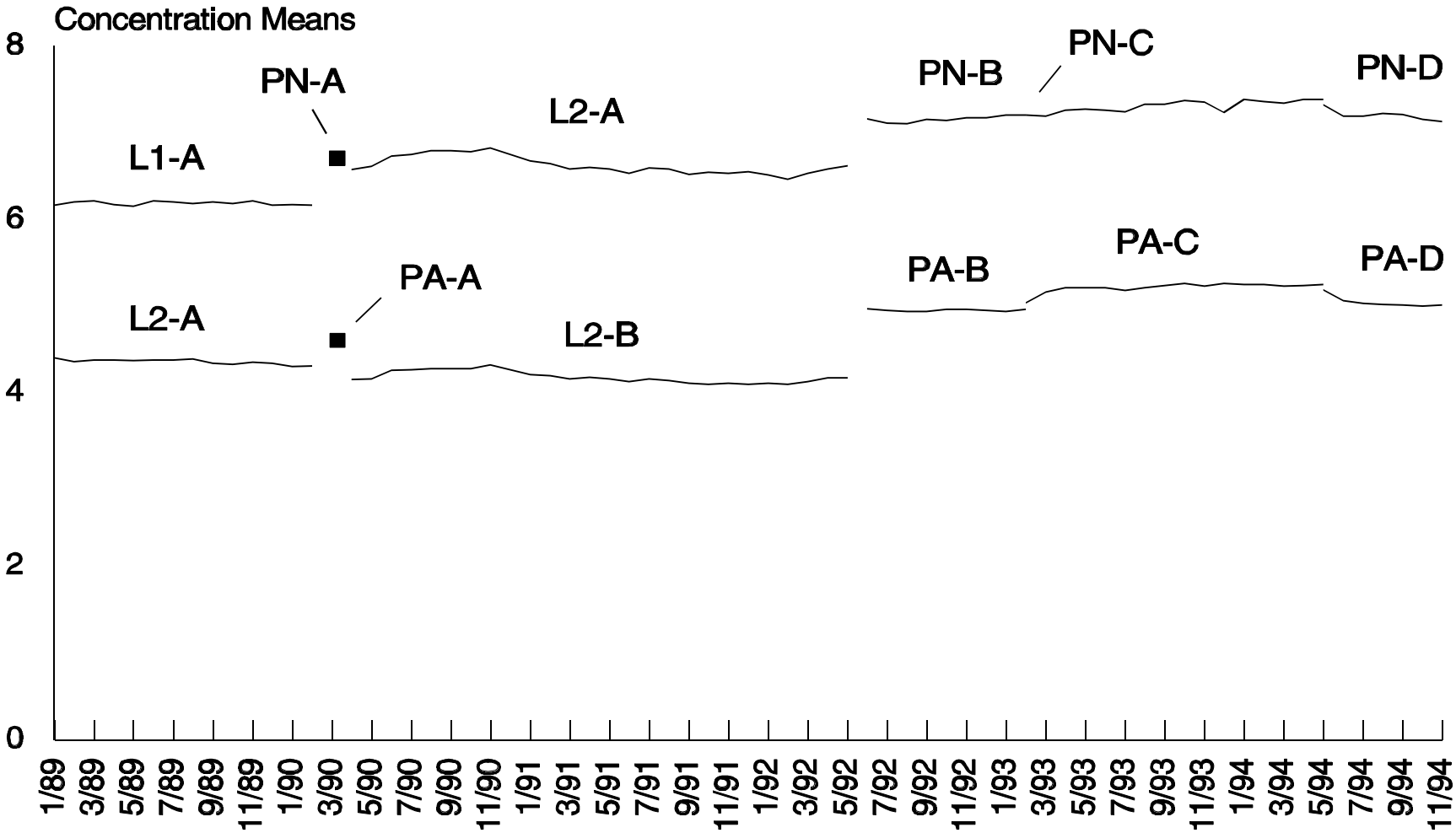
# Total Bilirubin Monthly Means



SUMMARY STATISTICS FOR
TOTAL PROTEIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 6.18286 | 0.02164 | 0.34994 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 6.62308 | 0.10306 | 1.55613 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 4.35786 | 0.03043 | 0.69822 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 4.18115 | 0.06831 | 1.63382 | 26 |
| PA-A | 03/31/90 | 4.60000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 4.95222 | 0.01093 | 0.22069 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 5.21438 | 0.05440 | 1.04331 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 5.04571 | 0.06655 | 1.31889 | 7 |
| PN-A | 03/31/90 | 6.70000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 7.15556 | 0.03504 | 0.48968 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 7.30438 | 0.06733 | 0.92173 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 7.20143 | 0.06122 | 0.85008 | 7 |

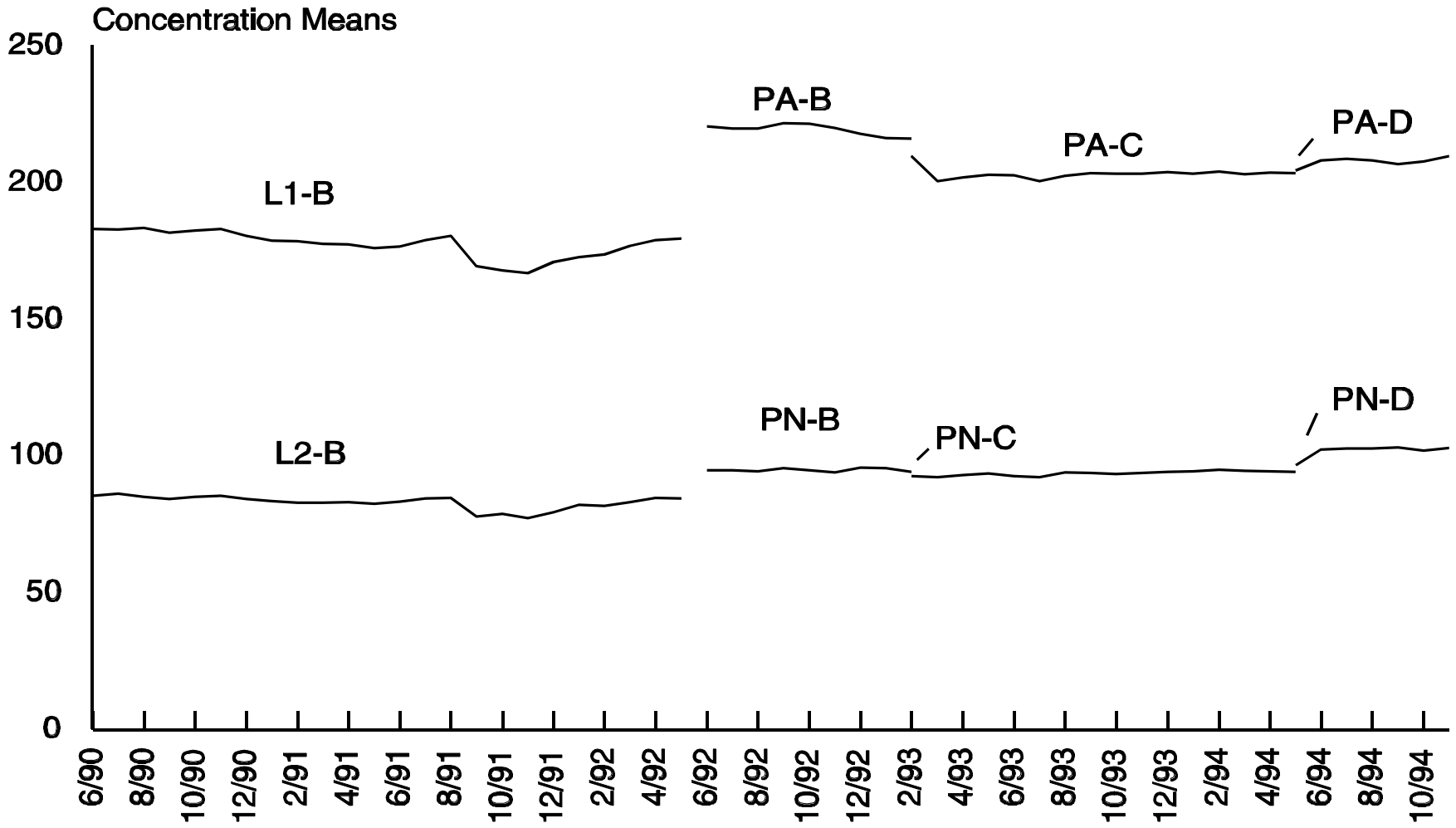
# Total Protein Monthly Means



SUMMARY STATISTICS FOR
HITACHI TRIGLYCERIDES
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-B | 06/30/90 - 05/31/92 | 177.438 | 4.87690 | 2.74852 | 24 |
| L2-B | 06/30/90 - 05/31/92 | 83.097 | 2.39984 | 2.88800 | 24 |
| PA-B | 06/30/92 - 02/09/93 | 219.289 | 2.12864 | 0.97070 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 203.256 | 2.00963 | 0.98872 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 207.671 | 1.64085 | 0.79012 | 7 |
| PN-B | 06/30/92 - 02/09/93 | 94.922 | 0.62205 | 0.65532 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 93.650 | 0.86872 | 0.92762 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 101.857 | 2.39364 | 2.35000 | 7 |

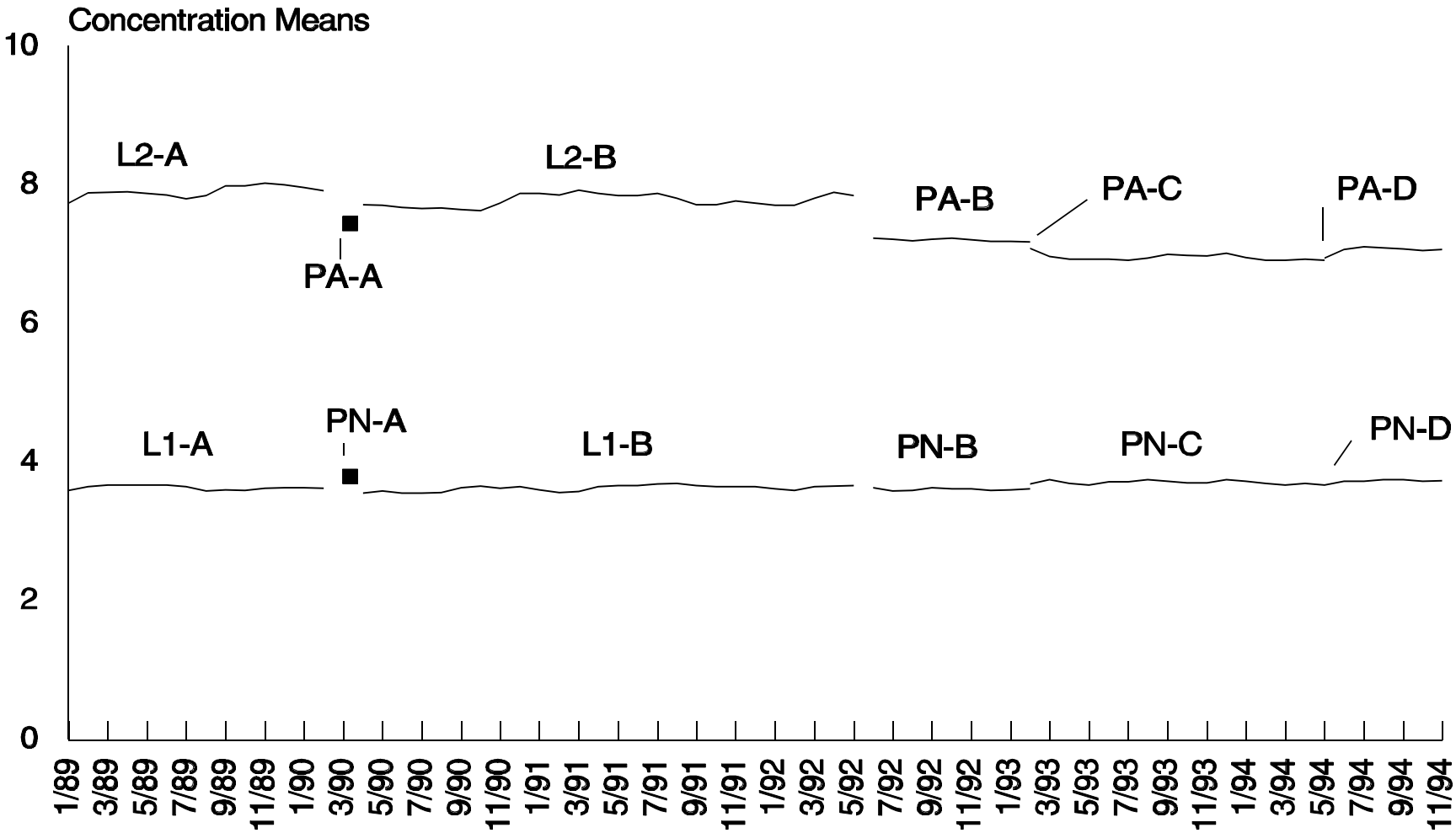
# Hitachi Triglycerides Monthly Means



SUMMARY STATISTICS FOR
URIC ACID
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1-A | 01/31/89 - 02/28/90 | 3.64000 | 0.032106 | 0.88202 | 14 |
| L1-B | 04/30/90 - 05/31/92 | 3.63012 | 0.043234 | 1.19097 | 26 |
| L2-A | 01/31/89 - 02/28/90 | 7.91000 | 0.082927 | 1.04838 | 14 |
| L2-B | 04/30/90 - 05/31/92 | 7.77731 | 0.090402 | 1.16238 | 26 |
| PA-A | 03/31/90 | 7.40000 | - | - | 1 |
| PA-B | 06/30/92 - 02/09/93 | 7.21000 | 0.021794 | 0.30228 | 9 |
| PA-C | 02/28/93 - 05/17/94 | 6.96000 | 0.047329 | 0.68001 | 16 |
| PA-D | 05/31/94 - 11/30/94 | 7.06286 | 0.052825 | 0.74793 | 7 |
| PN-A | 03/31/90 | 3.80000 | - | - | 1 |
| PN-B | 06/30/92 - 02/09/93 | 3.61556 | 0.017401 | 0.48127 | 9 |
| PN-C | 02/28/93 - 05/17/94 | 3.71250 | 0.024631 | 0.66345 | 16 |
| PN-D | 05/31/94 - 11/30/94 | 3.73000 | 0.023805 | 0.63820 | 7 |

# Uric Acid Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Protective immunity against Clostridium tetani depends on the development of neutralizing antibodies directed against the tetanus toxin. This is achieved by active immunization with tetanus toxoid (TT), obtained by chemical inactivation of the toxin. The inactivation destroys the binding site of the toxin, but the antigenic sites are not affected by the treatment. Therefore, when TT is used by itself or as part of the Diphtheria-Tetanus-Pertussis (DTP) vaccine, the immunized individual will form antibodies directed to the antigenic sites of the toxoid. These antibodies react with identical sites in the toxin and prevent its binding to the receptors in the nervous tissue, which induce the effects of the toxin. Since active immunization of infants with tetanus toxoid was adopted as part of the routine postnatal care in most developed and developing countries in the 1950s, the incidence of tetanus and diphtheria decreased significantly world wide (1,2). Currently, the incidence of tetanus in the United States is about 100 cases/year (0.02 cases/100,000 US population). There is an increasing population of unimmunized or improperly immunized individuals among the very old and some groups of preschool children. This is particularly true among immigrants from undeveloped countries. In these segments of the population, there is fertile ground for a recrudescence of both diphtheria and tetanus. This recrudescence mostly affects older individuals who have lost immunity or were never immunized. The recommended criteria for tetanus immunization calls for universal inoculation with tetanus toxoid doses at 2-3 months, 6 months, and 1 year, and routine boosters every 10 years (3).

The quantitation of specific antibodies is unquestionably the best approach to test for effective protection after immunization with tetanus or diphtheria toxoids. Although healthy, asymptomatic children may show lack of response to either or both toxoids, the assay of antibodies after vaccination is not routinely done because it is not perceived to be cost effective. However, quantitation of tetanus antibodies in the population has been used as a survey tool to evaluate the general population's immunization status. It is also useful in confirming suspected cases of tetanus or diptheria (4).

A variety of assays for antibodies to tetanus and diphtheria toxoids have been introduced. The biological protection assay, introduced by the pioneers in the area, is still the most widely used assay. Other methods include passive hemagglutination assays, precipitation assays, radioimmunoassays, and enzymeimmunoassays. The Medical University of South Carolina began by developing a solid-phase immunoassay using TT immobilized in agarose beads (5). Because the antigenic portion of the toxin and toxoid are identical, TT can be used as the antigenic substrate for the immunoassay, and serum with known amounts of neutralizing antibodies expressed in Units can be used for calibration of the assay. The method used in NHANES III was developed later.

The tetanus method used in NHANES III is a solid-phase ELISA in which purified TT is used to coat the microassay plates (5). After adequate washing and blocking of unreacted sites, calibration standards and unknowns are added to the TT-coated plates. The binding of antibody to immobilized TT is directly proportional to the antibody concentration. Serial dilutions of human hyper-tet, a commercial preparation of gamma globulin with known tetanus antibody concentration, are used to calibrate the assay. The amount of antibody is quantitated by adding an identical dilution of a peroxidase-conjugated polyvalent antiserum reactive with all classes of human antibodies. After washing the wells, a substrate is added which degrades peroxidase and acquires a dark green color. The intensity of the color is directly proportional to the amount of peroxidase-labeled anti-human immunoglobulin remaining bound to the plate and to the amount of tetanus antibody bound to the immobilized toxoid, and is quantitated in a colorimeter. A calibration curve is established by using the readings of the calibration standards, and concentrations of antibody in the unknowns are derived from this calibration curve. This EIA is relatively simple to perform and shows remarkable reproducibility with day-to-day run and within-run coefficients of variations of 11.2% and 7.3%, respectively. Because the method has adequate sensitivity to measure samples with low concentrations of antibody (0.032 U/ml), the lower limit of detection is 0.001 U/ml.

Normal limits for antitetanus toxin antibodies have not been established. The levels of antitoxin antibodies depend upon a variety of factors, including genetic predisposition, immunization, and frequency of boosting. As a rule, the very young and very old have the lowest levels, and females have lower levels than males.

Tetanus is an infectious disease, manifested by tonic muscle spasms and hyperreflexia caused by exotoxin elaborated by the sporulated form of the causative bacteria, Clostridium tetani. The disease is the result of the elaboration of the potent tetanus toxin, or tetanospasm, at the site of the injury, usually a minor penetrating wound. The incubation period is 3-21 days, usually within 14 days. The case fatality ratio was approximately 65% from 1950-1967 (3).

## 2. SAFETY PRECAUTIONS

Consider all serum samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe Universal Precautions. Wear gloves, lab coat, and safety glasses or face shield while handling all human blood products. Place all disposable plastic, glass, and paper that have been contaminated with blood, such as pipette tips, microtiter plates, and gloves, in a biohazard autoclave bag. Dispose of all biological samples and diluted specimens in a biohazard autoclave bag at the end of the analysis. These bags should be kept in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% sodium hypochlorite solution when work is finished.

Material Safety Data Sheets (MSDSs) for sodium bicarbonate, sodium carbonate, sodium chloride, potassium chloride, sodium phosphate, potassium phosphate monobasic, citric acid monohydrate, hyrogen peroxide, and 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] are accessible through the Medical University of South Carolina's mainframe computer. In addition, the university's Office of Occupational Safety and Health maintains a file of MSDS copies, which can be provided upon request.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data and storing data in multiple computer systems.

b. Data are received from NHANES on 5 1/4" DS/DD diskettes. The diskette contents are downloaded onto the local hard drive, and the results from the assay are transcribed onto the report sheets and then entered into the files. All entries are proofread and then the files are copied back onto the original diskettes. The diskettes are mailed with their corresponding report sheets to the National Center for Health Statistics (NCHS). The local hard drive is backed up on archival 5 1/4" diskettes. In addition, report sheet copies and data-file hard copies are kept chronologically in log books and stored separately from the diskettes. Furthermore, the initial raw data files produced by the Molecular Devices Vmax microplate reader are also archived on separate 5 1/4" diskettes, labeled, and stored.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Specimen type: serum. Serum specimens may be collected in regular red-top or serum-separator Vacutainers.

c. The optimal amount of specimen is 1.0 mL, the minimum is 150 µL (0.15 mL).

d. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

e. Specimen stability has been demonstrated for at least 1 year at $\leq$-20 °C.

f. All specimens of sufficient volume are accepted. Any deviations from normal serum, (e.g., lipemic serum), will be entered on the report sheets and computer files. However, all samples will be run as received.

g. Specimen characteristics that may compromise test results are marked "chylomicronemia" (serum forms creamy layer on top) or marked "hemolysis" (serum is dark red).

h. Locally collected serum specimens should be transported and stored at 4-8 °C. NHANES specimens are shipped frozen on dry ice, by overnight carrier.

i. Once specimens are received, they should be frozen at $\leq$-70 °C until time for analysis. Residual serum is refrozen at $\leq$-70 °C. Samples thawed and refrozen several times are not compromised.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) V-max kinetic microplate reader. The Vmax has a range of 0.000 to 4.000 OD (400-750 nm). It reads within 10 sec with a resolution of 0.001 OD. In the range of sample values (between 0.1 and 0.8 OD), the Vmax absorbance range is 0.000 to 2.000 OD with an accuracy of ≤1.0% and ±0.010 OD with a precision (repeatability) of ≤1.0% and ±0.005 OD (Molecular Devices, Menlo Park, CA).

(2) Incubator, 38 °C (Corning Glassworks, Corning, NY).

(3) Vacuum system with Drummond 8-channel manifold (Drummond Scientific Company, Broomall, PA).

(4) Labindustries 8-channel multiple manifold with repetitive dispenser and tubing (Labindustries, Berkley, CA).

(5) Oxford 8-channel model 8800 pipetter and replacement syringes (Oxford Labware, St. Louis, MO).

(6) Eppendorf adjustable automatic dispenser, 250-µL to 12.5-mL (Brinkmann Instruments, Westbury, NY).

(7) Titertek 50- to 200-µL 8-channel micropipetter (ElKay Labsystems, Marlboro, MA).

(8) Magnetic stirrer (Corning Glassworks).

(9) Hot plate (Corning Glassworks).

(10) Analytical balance (Baxter Scientific Products, McGaw Park, IL) and Mettler Balance, EH Sargent (Mettler Toledo, Greifensee, Switzerland).

(11) pH meter (Baxter Scientific Products).

(12) pH electrode (Orion Research, Boston, MA).

(13) Vortex Genie mixer (Fisher Scientific, Fairlawn, NJ).

### b. Materials

(1) Glassware: 1000-, 500-, and 200-mL bottles for storing solutions; 1000-, 400-, 250-, 150-, 100- and 50-mL beakers; 1000-, 500-, 250-, 100- and 50-mL graduated cylinders; 4000- and 500-mL flasks (Corning Glassworks).

(2) Plastic weigh boats (Baxter Scientific Products).

(3) Precision disposable pipet tips, 1000-µL and 200-µL capacity (Brinkmann Instruments).

(4) ELISA 96-well flat-bottom plates, cat. no. 011-010-3350 (Dynatech, Baton Rouge, LA).

(5) Dialysis tubing ( Baxter Scientific Products).

(6) Pasteur capillary pipettes (Costar Corporation, Cambridge, MA).

(7) pH standard buffers, 4.0, 7.0, and 9.0 (Baxter Scientific Products).

(8) 2 N sodium hydroxide (NaOH) (Fisher Scientific).

(9) 5-mL polystyrene test tubes (Sarstedt, Newton, NC).

(10) Eppendorf tapered capped tubes (Brinkmann Instruments).

(11) Latex gloves (any vendor).

(12) Kimwipes (Kimberly-Clark Corp., Roswell, GA).

(13) Sodium bicarbonate ($NaHCO_3$), cat. no. S-263 (Fisher Scientific).

(14) Sodium carbonate ($Na_2CO_3$), cat. no. S-233 (Fisher Scientific).

(15) Sodium chloride (NaCl), cat. no. S-9625 (Sigma Chemical, St. Louis, MO).

(16) Potassium chloride (KCl), cat. no. F-217 (Fisher Scientific).

(17) Sodium phosphate ($Na_2HP0_4$), cat. no. S-374 (Fisher Scientific).

(18) Potassium phosphate monobasic ($KH_2P0_4$), cat. no. P-285 (Fisher Scientific).

(19) Bovine serum albumin, 5 g/dL, cat. no. A-7906; 1 g/dL cat. no. A-7906, (Sigma Chemical).

(20) Tween-20 (Polyoxyethylenesorbitan monolaurate), cat. no. P-1379 (Sigma Chemical).

(21) Citric acid monohydrate ($(HO_2CCH_2)_2C(OH)(CO_2H).H_2O$) cat. no. A-104 (Fisher Scientific).

(22) 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)diammonium salt], cat. no. 102946 (Boehringer Mannheim, Indianapolis, IN).

(23) Hydrogen peroxide ($H_2O_2$), cat. no. H-323 (Fisher Scientific).

(24) Tetanus toxoid refined concentrate (Wyeth Laboratories, Great Valley, PA).

(25) Peroxidase-conjugated rabbit anti-human IgG (heavy- and light-chain specific, Cappel cat. no. 3201-0082, (Oregon Teknika, West Chester, PA).

(26) Tetanus immune globulin (human), USP, Hyper-Tet, cat. no. 614-86, (Cutter Biological, Cutter Laboratories, Inc., Berkeley, CA).

(27) 2 N hydrochloric acid (HCl) (Curtin Matheson Scientific, Inc. Houston, TX).

c. **Reagent Preparation**

(1) Sodium carbonate buffer, pH 9.6
1.0 mol/L sodium bicarbonate, NaHCO3 (8.4 g/100mL deionized water ($dH_20$)), 1.0 mol/L sodium carbonate, $Na_2CO_3$ (10.6 g/100 mL $dH_20$). Mix 34 mL 1.0 mol/L sodium bicarbonate solution with 16 mL 1.0 mol/L sodium carbonate solution and 150 mL $dH_20$. The pH should be between 9.59 and 9.69.

(2) Phosphate-buffered saline (PBS)
Place 8.0 g of NaCl , 0.2 g of KCl, 1.2 g of $Na_2HP0_4$ and 0.2 g of $KH_2P0_4$ in a 1-L flask and fill to volume with $dH_2O$. Adjust to pH 7.4 with 10N NaOH.

(3) Wash solution
Prepare PBS with 0.05% (w/v) Tween 20 (Sigma no. P-1379). For example, for 3 L of wash solution, add 1.5 mL of Tween 20 to 3 liters PBS. Adjust the pH to 7.8 with 10 N NaOH.

(4) Blocking solution
Prepare PBS containing 5 g/dL (w/v) bovine serum albumin (BSA). For one plate, mix 1.5 g with 30 mL PBS.

(5) Sample diluent
Prepare PBS containing 1 g/dL (w/v) bovine serum albumin (BSA). For one plate, mix 0.75 g BSA in 75 mLPBS.

(6) Substrate diluent (0.0455 mol/L citric acid buffer, pH 4.0)
Add 4.8 g citric acid monohydrate in 500 mL distilled $H_20$. Adjust the pH with 10 N HCl.

(7) Substrate
Combine 9 mg ABTS, 30 mL citric acid buffer (pH 4.0), and 3 µL 30% $H_2O_2$.

(8) Stop solution (0.1 mol/L citric acid)
Prepare 2.1 g citric acid monohydrate in 100 mL distilled $H_20$.

(9) Antigen to coat on microtiter plates
Dilute Tetanus toxoid refined concentrate 1:250 in carbonate/bicarbonate buffer.

(10) Preparation of second antibody
Dilute peroxidase-conjugated rabbit anti-human IgG 1:4000 in 1 g/dL BSA in PBS. Dilute purchased stock 1:10 initially with 2 mL of 1 g/dL BSA in PBS, added carefully to each vial. (Generally, two vials are prepared at once.) Replace the caps and agitate the vials until a homogeneous solution is obtained. Add the 4 mL thus obtained to 36 mL of 1 g/dL BSA in PBS for a total volume of 40 mL (a 1:10 dilution). Aliquot this solution into smaller vials and freeze at ≤-20 °C. Thaw one aliquot each day and prepare a further dilution of 1:400 using 1 g/dL BSA in PBS. The amount prepared is dependent upon the volume required for the day's plates (e.g., 5 plates require 80 mL total). Therefore, measure 80 mL of 1 g/dL BSA in PBS, remove 200 µL of this solution, and add 200 µL of the 1:10 thawed conjugate. Mix the new solution thoroughly and add it to the plates at a volume of 150 µL/well. The total dilution is 1:4000, created by the initial 1:10 dilution and the subsequent 1:400 dilution of that solution.

d. **Standards Preparation**

(1) 250 U/ml stock tetanus standard
Tetanus immune globulin (human), USP (or "Hyper-Tet") is standardized against the U.S. standard antitoxin and U.S. control tetanus toxin by the manufacturer, and the stock is reported as 250 U/ml of antibody.

(2) 25 U/mL intermediate tetanus standard
Before use, dilute 1:10 in PBS with 1 g/dL BSA, aliquot, and freeze.

(3) Working tetanus standards
For daily working standards, thaw an aliquot of 25 U/mL stock. Prepare PBS with 1 g/dL BSA as described in Table 1.

Table 1
Preparation of Working Standards

| Tube number | BSA (mL) | Tetanus (mL) | Total Volume (mL) |
|---|---|---|---|
| 1 | 1.485 | 0.015 of 25 U/mL stock | 1.5 |
| 2 | 1.66 | 1.0 of tube 1 | 2.66 |
| 3 | 1.0 | 1.0 of tube 2 | 2.0 |
| 4 | 1.0 | 1.0 of tube 3 | 2.0 |
| 5 | 1.0 | 1.0 of tube 4 | 2.0 |
| 6 | 1.0 | 1.0 of tube 5 | 2.0 |
| 7 | 1.0 | 1.0 of tube 6 | 2.0 |
| 8 | 1.0 | 1.0 of tube 7 | 2.0 |
| 9 | 1.0 | 1.0 of tube 8 | 2.0 |

Results from tubes 4 through 9 will be used to generate the standard curve.

(4) Calibration standards

(a) Place the tetanus standard dilutions in column 2 of each plate run. Discard the first dilution, but place each of the other eight in wells A-H in descending order of concentration so that A will have the smallest dilution while H has the greatest dilution. The first two wells are used only as a visual cue in developing the substrate and are masked during measurement to prevent saturated values from skewing the curve.

(b) The QC range standards are formed by pooling serum samples previously reported in the desired ranges. Thaw an aliquot each of high, medium, and, low QC range standards. Dilute each with 1 g/dL BSA. Dilute the high pool 1:250 (12 µL of the high pool + 2988 µL of 1 g/dL BSA). Dilute the medium pool 1 :100 (20 µL of the medium pool + 1980 µL of 1 g/dL BSA). Dilute the low pool 1:10 (200 µl of the low pool + 1800 µL of 1 g/dL BSA). Place the standards in column 3 of each plate run. Place the high pool in wells 3A-3C, the low pool in wells 3D and 3E, and the medium pool in wells 3F-3H.

e. **Preparation of Quality Control Materials**

Each control comprises of a pool of human serum samples already tested and found to have values in selected ranges. The samples are divided into pools with high, low, and medium tetanus concentrations and aliquotted. The pools are tested to ensure that their tetanus antibody concentrations are within the desired ranges for the new pools and then frozen at ≤-70 °C. One aliquot of each pool is thawed for use each day that an analysis is performed.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

Using the "Softmax" program for an IBM-compatible computer, construct a plot using the concentration of the calibration standards versus the respective OD readings from the Vmax using a quadratic curve. If the $R^2$ is lower than 0.98, repeat the assay.

b. **Verification**

(1) Using the Vmax as instructed, begin the plate reading by pressing "F5." The instrument automatically self calibrates for 5 sec prior to analysis. While the Vmax is calibrating, the CALIBRATING status indicator (LED) will be on. After the self calibration is completed, the READING status indicator will light and the values of the plate will be compared with the results of the automatic calibration and stored as the final reading for that plate. The manufacturer claims that "due to the excellent drift management of the electronics on board Vmax, it is possible to override the calibration cycle after the inital time in any setting by using the Speed Read command. Speed Read uses the calibration data from a first reading on air for all sequential endpoint readings until a reading parameter is changed (e.g., wavelength) (6).

(2) The tetanus stock standard is calibrated against the U.S. standard antitoxin and the U.S. control tetanus.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Unpack the samples immediately after arrival, check the vials for possible damage during transport, and check the NCHS numbers of each sample against the packing list for accuracy. Perform the inventory quickly to prevent thawing, and store the samples at ≤-20 °C. Thaw batches of 40 samples for assay.

(2) Thaw samples once at the time of testing. Store samples randomly chosen for reproducibility assays in the refrigerator, and test them within 72 hours of the initial assay. Store residual samples left after the analysis for 1 year at ≤-70 °C.

b. **Sample Preparation**

Thaw serum samples at 20-25 °C and mix the contents of each specimen well.

c. **Instrument Setup for the Vmax Kinetic Reader**

(1) The Vmax reader comes with a plate reading software package called "Softmax." Turn on the power to the Vmax, computer, monitor, and printer (which are connected to the Vmax). After the C:\ prompt appears, change the directory to MDC. Enter the file name "Softmax." A template matching the run plates is stored under the Protocols option (F8).

To set up the template, use an 8-row, 12-column plate. Assign all the wells in column 1 as blanks. Assign column 2 to the calibration curve, S01-S08. Assign wells A3-C3 to the high control, wells D3 and E3 to the low control, and wells F3-H3 to the medium control. NHANES samples will go in the remaining columns. Each column will hold one sample's serial dilutions: 1:100, 1:200, 1:400, etc., until the eighth well contains the 1:12,800 dilution. For the samples requiring the greater dilution range starting at 1:500, the 1:500 dilution will be in row A, 1:1,000 in row B, etc., until the 1:64,000 dilution is in row H. For the samples with a low titer, the dilution range is from 1:10, 1:50, 1:100 and 1:200. For the four dilutions, split the columns. Put one sample's dilutions in rows A-D with another sample's dilutions in rows E-H.

(2) After following the menu option F3 and typing "Y" to recall plate parameters, check the template visually for concordance. Any necessary corrections can be made by using the F2 menu. Once the template is approved, F2 returns the operator to the main menu. F3 sets up the plate for correct conditions and dates. First, assign a file name specific to this plate and then assign the date. "P#" indicates plate number: enter 1-6, according to plate number being read.

(3) Plate description: Enter the assay name, plate number, and date (e.g., Tetanus, Plate 2, 4-29-92). The program prompts the mode applying to the plate. Choose ENDPOINT L1. Assign the reading wavelength. For assaying ABTS substrate, the correct wavelength is "414" (nm). For type of data, choose "Raw." Turn "Automix," "Autocal," and "Autodisk" ON while keeping "Autoprint" OFF. Use the simple toggle switch process accessed by hitting the corresponding function key.

d. **Operation of Assay Procedure**

(1) Prepare a 1:250 dilution of tetanus toxoid with carbonate-bicarbonate buffer. For 10 plates, mix 425 µL of tetanus toxoid in 105 mL buffer.

(2) Add 100 µL of the dilution per well to Immulon I flat bottom 96-well plates.

(3) Incubate the plates overnight at 4-8 °C.

(4) Aspirate and discard the contents of the wells.

(5) Add 200 µL 5 g/dL BSA in PBS pH 7.4 to each well.

(6) Incubate the solution at 37 °C for 30 min.

(7) Wash the plate three times with PBS 0.05% Tween 20, pH 7.8. Blot the plate on paper towel after the last wash.

(8) Using the microtiter plates to make the sequential dilutions, prepare dilutions of samples using 1 g/dL BSA in PBS, pH 7.4. Test each sample from a subject younger than 50 years old at the following dilutions: 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800. Test each sample from a subject ≥50 years is tested at the following dilutions: 1:10, 1:50, 1:100, and 1:200. If any specimen has a high titer, repeat the determination using the following dilutions: 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, and 1:64,000.

(9) To the plates, add 100 µL of 1 g/dL BSA in PBS to the first column (blanks). Transfer 100 µL of each calibration standard in descending order to the wells of the second column. Next, add the high, low, and medium quality control materials to the third column, placing 100 µL of the high control in rows A-C, 100 µL of the low control in rows D and E, and 100 µL of the medium control in rows F-H. The following 9 columns are for the sample dilutions; transfer them from the dilution plates onto the coated plates.

(10) Incubate the coated plates for 2 hours at 37 °C. Wash the plate three times after incubation.

(11) Prepare the conjugate, using PBS-l g/dL BSA as a diluent.

(12) Add 150 µL of diluted conjugate to each well.

(13) Incubate the plate for 1 hour at room temp.

(14) When the incubation period has elapsed, wash the plate three times.

(15) Prepare the substrate immediately before its use.

(16) Add 100 µL of substrate to each well and start a timer.

(17) At the end of 2 min ± 30 sec, stop the reaction with 100 µL of 0.1 mol/L citric acid per well. Add the stop solution in the same order as the substrate was added.

(18) To read the first plate, place the plate in the bracket on the Vmax's sliding drawer. Return to the main menu (F2) and press read plate (F5). After the plate is read, it will re-emerge from the Vmax. Remove it and proceed with the next plate. For the same template, press F3, instrument set up. Alter the plate name and description to indicate the new plate number, return to main menu, and read the new plate. Repeat as necessary. If the new plate requires a different template, then choose option F8, protocols. Recall the new plate protocol and repeat the appropriate steps before reading the plate.

e. **Recording of Data**

After all the plates have been read, press F6 to review the data files. Press F3 to recall printing. F1 shows the template. Use the arrows to move to the well corresponding to the first standard on the calibration curve (S01) and mask this well (F6). Repeat for the second point (S02). (Masking is required because the top two points are used to determine the length of development for the rest of the plate. They are allowed to develop past the linear stage of the curve and therefore should not be included for analysis.) Return to the main menu and display the raw data by following menu options (F2). For the first plate of the day, subtract the blanks (F6). All other plates will have blanks automatically subtracted. F4 prints the plate data. Return to main menu (F2) and recall analyzed data (F4). F1 will plot the quadratic curve fitted to the calibration standard points. The correlation coefficient should be $\geq$0.98. If not, check the curve for an outlying point. If a single outlier exists, mask the point and reread the curve. If all points are read and the correlation coefficient is not $\geq$0.98, this plate is unacceptable and must be reanalyzed. For any plates having acceptable curves, print the curve (F4). Copy the data to another program by pressing F5 to view the report, then press F10 to export the data. Change the export destination to C:\ELISA. A custom-written program receives the analyzed data, multiplies them by the assigned dilution factors, and prints the results by patient ID number, well number, the original value (interpretation of the OD versus the calibration curve), and actual value ( original value multiplied by the dilution factor). After the data have been shipped to the ELISA directory, print the report (F4). Return to the main menu and repeat the procedure for the next plate. When all plates, curves, and reports have been printed, exit Softmax (F10, "Y").

Once back in MS-DOS, change the directory to "C:\elisa" and type "ELISA" at the prompt for the file name to be printed. Type in the first plate name from Softmax and add the suffix ".001." (Every time Softmax reads a plate with the same plate name, it gives it a new suffix, .001, .002, etc.) Enter carriage return when the plate name is typed correctly. The custom program will prompt for the type of template. Type "1" for plates holding samples with eight dilutions each. If the entire plate had samples with four dilutions each, type "2." For mixed plates, type "1" and make the necessary alterations to the dilution factors and actual values on the printout. After the file has been printed, the program will prompt for the next file name. Repeat until all files are printed. Exit the program.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Keep a supply of printer ribbon and paper on hand; reorder as needed.

(2) Keep a supply of DS/DD 5 1/4" floppy diskettes available for archival material; reorder as needed.

(3) Keep 5-cc syringes with Luer-lock tips available for replacing the barrel of the Labindustries repetitive

dispenser when appropriate.

(4) Keep graduated calibration tips available for use in the laboratory. The pipettor is set at a single set point and the calibration tip is attached. Use only room temperature deionized water. The water should align with the calibration mark on the tip. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Keep calibration records for each pipettor by serial number.

g. **Calculations**

(1) Each of the quality control ranges has an assumed dilution factor of 1. Manually correct the ranges: multiply the high values by 250 and average the results. Multiply the medium values by 100 and average the results. Multiply the low values by 10 and average. Check each of the averages against its expected range. If at least two of the three quality control averages are within the 95% limits, the assay data are acceptable. If two or more of the averages are out of the expected range, reanalyze the plate. Record the quality control average values for each plate in the log book.

(2) Turn to the last two pages for the print-outs from the "ELISA" program. Using the assay's template, fill the blanks with the samples' ID numbers. Run each sample as a series of four or eight dilutions, but enter only those values reading between 0.8 and 0.1 OD as unknowns when calculating antibody concentrations. Average the actual values (optical density interpreted values times the dilution factor). Discard values not linear on the calibration curve. Discard any false readings on the well. Ideally, at least three or more values should be averaged for that sample. If the range of values begins with *, the sample was too concentrated and needs to be repeated at a greater dilution. If only one value is reported (.3, .2, .1 or 0), the sample needs to be repeated at lower dilutions. If the 1:10 dilution value is 0, run this sample again at the same concentration. If the dilution value still 0, report as such. If only two values have a high degree of similarity (e.g., .4 and .5), accept their average. If they vary by more than 10%, rerun the sample. If the values are not tightly clustered, rerun the sample.

(3) Manually enter the reported average values on the report sheet, transfer the values to the computer file, and copy them onto disk. Print them out using the provided "NCHS2" program and mail the printout back to the National Center for Health Statistics (NCHS) along with the disk and copies of the original raw data.

## 9. REPORTABLE RANGE OF RESULTS

There is no established normal range for tetanus antibody concentrations. Most values fall between 0 and 30 U/mL, but higher values are occasionally found.

There is some controversy about the minimal protective level. When antibodies are detected by bioassay, the accepted limit is 0.001 U/ml. When measured by EIA, 0.16 U/ml has been recommended as a safe cut-off, reflecting the fact that all antibodies are equally detected by EIA, even those with limited neutralizing ability.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the Medical University of South Carolina. The method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-the-art. The primary standard used is "Hyper-Tet," tetanus human immune globulin. Estimates of imprecision can be generated from long-term results from quality control pools.

Bench quality control specimens are inserted in duplicate or triplicate by the analyst in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

All samples will be recorded by analytical batch number. The bench quality control pools comprise three levels of concentration ranges: low, medium, and high. Three controls will be run in each analytical session, and if the mean of two controls falls outside if the established 95% limits, the run will be repeated. The run will also be repeated if the values in 10 consecutive runs for a given control are consistently 1 SD in the same direction of the assigned value, or if the calibration standards fail to fit a polynomial least squares curve (i.e., $r^2 < 0.98$).

The high, medium, and low controls consist of previously tested serum that has been aliquoted, stored at ≤-70 °C, and thawed once prior to each assay. Aliquots for each control are expected to last for at least 1 year of analysis. When new controls are needed, they are run with the current controls for at least 10 runs to establish the mean value and coefficients of variation for each new control for quality control comparisons. A record is kept of samples that have been rerun and the reason for rerunning them.

A laboratory log will be kept listing the arrival date of samples, the condition of the samples on arrival, and any discrepancies between the information on the vial label and packing sheets. A log will be kept to record when samples are tested, thawed, and refrozen.

A list is kept of all reagents, suppliers, dates of arrival, lot numbers and expiration dates, and, where applicable, the date the reagent was reconstituted and aliquoted.

All equipment is calibrated as recommended by the specific manufacturer or as needed. Pipettes will be checked periodically for accuracy and recalibrated.

A high and a low standard are included in each run. The high standard contains between 2.8 and 5.6 U/mL of antibody and the low between 0.01 and 0.05 U/mL. Five of every 100 samples received will be randomly selected at arrival for reproducibility studies.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. If a run is rejected, recheck the lot numbers and aliquot identification codes of the reagents and controls.

b. Recheck pipettes and the Vmax reader.

c. Include fresh controls in the repeat run.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

High concentrations of lipids, particularly VLDL and chylomicrons, may interfere with the assay by affecting the accuracy of pipetting (because of abnormally high viscosity). Marked hemolysis may cause falsely elevated values because of non-specific absorption of red cell peroxidase to the microtiter plate.

**13. REFERENCE RANGES (NORMAL VALUES)**

There are no established normal ranges for this assay.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach and maintain 20-25 °C during analysis.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no alternative methods of analysis. If the analytical system fails, refrigerate specimens until the analytical system is restored. If long-term interruption (greater than 4 weeks) is anticipated, store the serum at ≤-70 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (e.g., computer data files and hard copies) should be used to track specimens. It is recommended that records, including related QA/QC data, be retained for 6 years, and that duplicate records be kept.

Only numerical identifiers should be used (e.g., NCHS ID numbers) -- all personal identifiers are masked and available only to the project coordinator to safeguard confidentiality.

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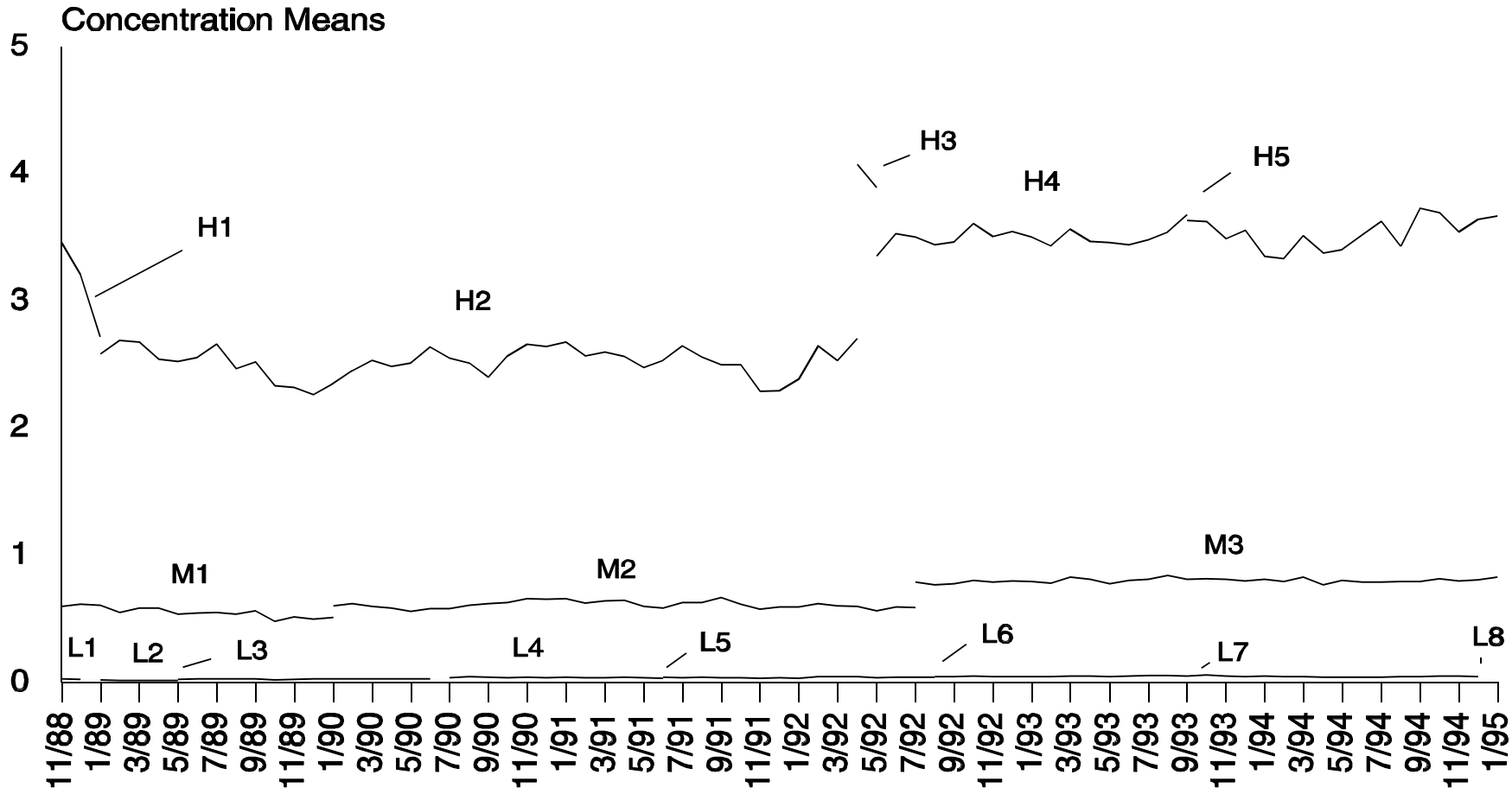
SUMMARY STATISTICS FOR
TETANUS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| H1 | 11/01/88 - 01/26/89 | 3.17364 | 0.60371 | 19.0225 | 132 |
| H2 | 01/31/89 - 04/07/92 | 2.51983 | 0.24242 | 9.6204 | 2149 |
| H3 | 04/08/92 - 05/05/92 | 4.05274 | 0.37138 | 9.1636 | 62 |
| H4 | 05/06/92 - 09/14/93 | 3.50038 | 0.28427 | 8.1212 | 758 |
| H5 | 09/21/93 - 01/12/95 | 3.56750 | 0.29153 | 8.1718 | 743 |
| M1 | 11/01/88 - 01/05/90 | 0.55174 | 0.07261 | 13.1602 | 679 |
| M2 | 01/08/90 - 07/08/92 | 0.61484 | 0.06559 | 10.6682 | 1778 |
| M3 | 07/09/92 - 01/12/95 | 0.80312 | 0.05467 | 6.8069 | 1360 |
| L1 | 11/01/88 - 12/29/88 | 0.02915 | 0.00999 | 34.2615 | 100 |
| L2 | 01/04/89 - 05/25/89 | 0.01930 | 0.00504 | 26.1186 | 222 |
| L3 | 05/31/89 - 06/28/90 | 0.03081 | 0.00595 | 19.2952 | 682 |
| L4 | 07/03/90 - 06/12/91 | 0.04206 | 0.00634 | 15.0657 | 718 |
| L5 | 06/13/91 - 08/04/92 | 0.04269 | 0.00680 | 15.9296 | 743 |
| L6 | 08/05/92 - 09/01/93 | 0.05162 | 0.00717 | 13.8830 | 619 |
| L7 | 09/02/93 - 12/27/94 | 0.05051 | 0.01027 | 20.3328 | 780 |
| L8 | 01/03/95 - 01/12/95 | 0.03143 | 0.00882 | 28.0753 | 21 |

# Tetanus Monthly Means




The downward trend noted for pool H1 is due to deterioration of the pool.
This trend is not noted in other pools used during the same period.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A qualitative determination of total antibody to hepatitis A virus (anti-HAV) contained in human serum or plasma is measured by using solid-phase competitive enzyme immunoassay (EIA) (1-3). A test sample is mixed with detection-phase reagent in a reaction well. The detection-phase reagent consists of anti-HAV conjugated with peroxidase (anti-HAV/PO). The sample-conjugate mixture is incubated with a bead coated with HAV antigen. Any anti-HAV in the test sample competes with the conjugate for HAV epitopes present on the bead. Thus, at the end of the incubation period, the amount of conjugate immunochemically bound to the bead will be inversely proportional to the concentration of anti-HAV in the sample. The beads are washed to remove any unbound material. The beads are then incubated with a hydrogen peroxide/o-phenylenediamine ($H_2O_2$/OPD) chromogenic substrate solution. The reaction of substrate solution with peroxidase yields a yellow-orange color. The reaction is stopped by the addition of 1-N sulfuric acid. The intensity of the color generated is measured spectrophotometrically at 492 nm. The instruments used to measure the test results are equipped with software that calculates a cutoff value. The cutoff calculation is based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HAV.

This is an FDA-licensed method commercially obtained in kit form. The literature and instructions with each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in the determination of whether an individual has been infected with hepatitis A virus, for which anti-HAV is a marker. It can also be used to assess the efficacy of HAV vaccines.

The presence of anti-HAV in human serum is indicative of past or present infection with hepatitis A virus. Virus specific anti-HAV IgM is the most reliable marker for determining the acute stage of disease. Total anti-HAV may persist for years after recovery as anti-HAV IgG. The test for total anti-HAV is primarily used to determine previous exposure to Hepatitis A virus. The results of these tests may be used to assess immune status (5,6) for epidemiological studies (7-12).

## 2. SAFETY PRECAUTIONS

EIA test kits for anti-HAV contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, treat components of test kits as though they are capable of transmitting disease.

Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eye wear, and lab coat during all steps of this method because of infectious contamination hazards. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. Keep these bags in appropriate containers until sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished. Biosafety Level 2 containment and practice as described in CDC/NIH publication #88-8395 are recommended for handling test specimens and kit reagents (13).

Material safety data sheets (MSDSs) for sulfuric acid, hydrochloric acid o-phenylenediamine, and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) computer network. Risk is minimal because of the small quantity of chemicals, the packaging of the chemicals, and limited handling required by the operators using the test kits.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Raw data are transcribed manually from instrument readout sheets into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results. It functions within PARADOX software (Borland Co., Scott's Valley, CA). Test values are compared with a cutoff value calculated from controls. Results are expressed as "positive" or "negative" for anti-HAV. Other information in the database may typically include the HRL identification number, the specimen number, the date collected, the date tested, and results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer.

b. Finished data are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete) the supervisors will be transmit the results to the NCHS mainframe computer along with the other NHANES III data.

d. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.

e. Documentation for data system maintenance is contained in hard copies of data records for 2 years.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (S. Lambert, 5/92).

b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

c. Specimens may be serum, recalcified plasma, or plasma. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers.

d. Required sample volume is 10 µL for the assay, and 1.0 mL will permit repeat analysis.

e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Serum or plasma samples are collected aseptically to minimize hemolysis and bacterial contamination.

g. Samples are stored in labeled 2-mL Nalge cryovials or an equivalent vial.

h. Serum is best stored frozen. However, freeze/thaw cycles should be minimized. Specimens may be stored at 4-8 °C for up to 5 days.

i. For storage >5 days, samples are held at ≤-20 °C. Samples held in long-term storage at ≤-20 °C are indexed in the database for easy retrieval.

j. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.

k. Do not use heat-inactivated specimens.

l. Specimens containing sodium azide may give false-positive reactions and should not be tested.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) Abbott QWIKWASH, model 6258-27 (Abbott Laboratories, North Chicago, IL).

(2) Abbott COMMANDER dynamic incubator, model 6210-01, set at 40 °C for incubations (Abbott Laboratories).

(3) Abbott QUANTAMATIC spectrophotometer, model 7553, set for reading at 492 nm (Abbott Laboratories).

(4) Gilson Pipetman micropipettors, 10- and 200-µL sizes (Rainin Instrument Co., Woburn MA).

b. **Materials**

(1) HAVAB-EIA solid phase EIA kit, 100- or 1000-test sizes, cat. no. 789524 (Abbott Laboratories).

(2) Reaction trays, Costar cat. no. 4870 (VWR Scientific, Bridgeport, CT).

(3) Cover seals provided as part of the anti-HAV test kit (Abbott Laboratories).

(4) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(5) 1.0 N sulfuric acid, cat. no. 7212 (Baxter).

(6) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).

(7) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).

(8) 2-mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(9) Cryovial boxes, cat. no. 5026-0909 (Nalge).

(10) 1.5-mL microtubes (Marsh Biomedical Products, Rochester, NY).

(11) 50-mL polypropylene tubes (Corning Glass Works, Corning, NY).

(12) 5.25% sodium hypochlorite, household bleach (any vendor).

c. **Reagent Preparation**

(1) Reagents for these procedures are prepared by the manufacturer of the test kits. Each kit contains the following:

(a) Hepatitis A virus (human) coated beads
1 bottle (100 beads).

(b) Antibody to hepatitis A virus (human)
1 vial (20 mL). Peroxidase (horseradish) conjugate. Minimum concentration: 0.05 µg/mL in buffer. Antimicrobial agents.

(c) Negative control
1 vial (0.45 mL). Recalcified human plasma nonreactive for anti-HAV, HBsAg, and anti-HIV-1. Antimicrobial agents.

(d) Positive control
1 vial (0.30 mL). Recalcified human plasma reactive for anti-HAV, nonreactive for HBsAg and anti-HIV-1. Titer 1:200 ± 2 $\log_2$ dilutions. Antimicrobial agents.

(e) Diluent for o-phenylenediamine•2HCL (OPD)
1 bottle(55 mL). Citrate-phosphate buffer containing 0.02% (v/v) hydrogen peroxide.

(f) o-phenylenediamine•2HCL (OPD)
1 bottle (10 tablets), each 12.8 mg.

(2) OPD substrate solution

Bring OPD reagents to 20-25 °C. Five to 10 min prior to color development, prepare the OPD substrate solution by dissolving the OPD tablets in diluent for OPD. Using clean pipettes and metal-free containers, transfer the solution into a suitable container 5 mL of diluent for OPD for each tablet to be dissolved. Transfer an appropriate number of OPD tablets into a measured amount of diluent using nonmetallic forceps or equivalent. Allow the tablets to dissolve. *The OPD substrate solution must be used within 60 min of preparation and must not be exposed to strong light*. Just prior to dispensing the solution for color development, swirl the container gently to obtain a homogeneous solution. Remove air bubbles from the dispenser tubing and prime dispenser prior to use.

d. **Standards Preparation**

This method does not involve the use of conventional calibrators or standards. Calibration is based on the results of defined "positive" and "negative" controls.

Positive and negative control reagents are supplied with each test kit. The assay cutoff value is automatically calculated from values obtained from these controls by the instrument.

(7) An "in-house control" (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled "In-House Control: T anti-HAV" (S. Lambert, 8/92). (See Section 6.e.).

e. **Preparation of Quality Control Material**

(1) Kit positive and negative controls are prepared and quality controlled by the manufacturer.

(2) In-house controls are prepared according to HRL specifications.

A pool of anti-HAV positive serum is calibrated by serial dilution in a controlled-process serum-based diluent. *When tested by the anti-HAV EIA, the final anti-HAV IHC reagent must consistently generate a signal-to-cutoff ratio of 0.5 to 1.0*.

Dilute the previously characterized anti-HAV serum pool 1:25, 1:50, 1:100. 1:400 and 1:800 in a defibrinated, dialyzed-filtered, plasma pool diluent that is nonreactive for hepatitis A, B, and C. Test each dilution plus diluent at least in duplicate in HAVAB-EIA. Pick a dilution that yields a signal-to-cutoff value between 0.6 and 0.9. Prepare 8-9 mL of the chosen dilution. Test at least in duplicate in HAVAB-EIA to confirm that the signal-to-cutoff value is between 0.6 and 0.9. Aliquot the control material into labeled vials with 40-50 µL per vial. Store at ≤-20 °C. Thaw each in-house control as needed, use once, and discard.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibration curve is generated by the user as part of these assay methods. Calibration of instruments is either automatic or performed periodically by contracted service personnel.

b. **Verification**

(1) The instrument used to read assay results (Section 6.e.4) is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

(2) Test results that fall within ±10% of the calculated cutoff value are reanalyzed.

(3) If two negative control values fall outside 0.7-1.3 times mean, repeat the test.

(4) Calculate the difference between the negative control and positive control mean absorbance values (N-P value) as a measure of the validity of the test. For the run to be valid, the N-P value should be at least 0.400. If it is not, repeat the run. If the N-P value is consistently less than 0.400, deterioration of reagents may be the cause.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Reagents are used per kit of 100 or 1000 tests. Kit components are occasionally interchanged within a manufacturer's lot, but never interchanged between lots.

(2) Remove the test kit from 4-8 °C storage. Allow 30-40 min for the reagents to warm to 20-25 °C. Swirl gently before use. Adjust the incubator to 40 ±1 °C.

(3) Assay the negative, positive, and in-house controls in triplicate with each run of specimens.

(4) Ensure that all reaction trays are subjected to the same process and incubation times.

(5) Once the assay has been started, complete all subsequent steps without interruption and within the

recommended time limits.

(6) After each step, visually verify the presence of solution and bead in each well.

b. **Sample Preparation**

(1) Bring serum specimens to 20-25 °C. Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix them gently before testing.

(2) Identify the reaction tray wells for each specimen or control.

c. **Instrument Setup**

(1) Operation of the Abbott QWIKWASH

The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

(a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the "Power" indicator on the instrument.

(b) Ensure that the "Low Pressure" and "Low Water Level" indicators are not illuminated before washing beads. See note below.

(c) Place the bead tray on the QWIKWASH with the first row of beads aligned with the washing heads.

(d) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.

(e) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.

(f) Repeat until all of the beads have been washed. Then proceed directly to the next step of the assay procedure.

*NOTE: The wash water is held in a stainless-steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on, fill the tank with deionized water and empty the waste tank. Never fill the water tank without also emptying the waste container. Add approximately 200 mL of bleach to the waste container prior to reconnecting it to the system so that waste water can be discarded down the sink as "decontaminated liquid waste." Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless-steel pressure tank.*

(2) COMMANDER incubators: Set at 40 °C.

(3) Operation of the QUANTIMATIC Plate Reader

*NOTE: These Abbott instruments were approved for use with these test kits as part of FDA licensure of the kits.*

(a) After the final reaction has been stopped, place the tube rack(s) into the appropriate QUANTAMATIC

carrier tray(s).

(b) Place the carrier tray(s) onto the QUANTAMATIC to be automatically fed into the tube pick-up area.

(c) On the instrument keypad choose RUN ASSAYS. Answer the prompts as shown in Table 1.

Table 1
QUANTAMATIC Parameters

| Prompt | Response |
|---|---|
| Run which assay? | Assay # from TABLE 1 |
| Lot #/Tech | Kit lot # & initials |
| Positive ID? | NO |
| Number of patients? | Number of patients |
| Tray 1 size - 20? | YES if tray size is 20<br>NO if tray size is 60 |
| Is tray in back track? | YES if tray is in the back track<br>NO if tray is in the front track |
| How many tubes in tray? | Total number of tubes |
| Enter pat no. ID | NO |
| List operator entries? | NO |
| Are trays ready? | YES if trays are ready to be read |

d. **Operation of Assay Procedure**

(1) Pipette 10 µL of controls and serum samples into designated wells of a reaction tray. Run order is three negative controls, followed by two positive controls and samples.

(2) Pipette 200 µL peroxidase-conjugated anti-HAV antibody into each well containing a control or sample.

(3) Add one HAV antigen-coated bead to each well.

(4) Apply the cover seal. Gently tap the tray to cover the beads and remove any trapped air.

(5) Incubate at 20-25 °C for 18-24 hours.

(6) Wash the beads in the tray using the QWIKWASH bead washer.

(7) Transfer the beads to 10- x 75-mm tubes. Pipette 300 µL of substrate solution into two blank tubes and then into each tube containing a bead. Following a 30-min incubation, stop the chromogenic reaction by adding sulfuric acid. Measure the intensity of the color generated using the QUANTAMATIC spectrophotometer.

(9) Measure the absorbance of controls and specimens at 492 nm within 2 hours after adding the acid.

e. **Recording of Data**

(1) Quality Control Data

Multiple positive and negative controls are averaged by the reading instrument and are determined to be valid or invalid.

Raw data are transcribed manually from instrument readout sheet into a computerized database. Quality

control of individual control values is maintained by the QUANTAMATIC, which will reject the test run if control values do not conform to specifications.

(2) Analytical Results

Raw data are expressed as absorbance values and are transcribed manually from the instrument readout sheet into a computerized database.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Instruments are on service contract and except for the most basic daily maintenance are serviced by an Abbott technical representative.

Monitor and document incubator temperature, quality of water used in the QWIKWASH, refrigerator temperature, freezer temperature, and room temperature on a weekly basis

(2) All micropipettors used in testing clinical specimens are checked for calibration every 6 months. Pipettors that do not conform to specifications are autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records are kept for each pipettor by serial number.

g. **Calculations**

Cutoff calculation is done by the reading instrument and by the data management software.

(1) Calculate the negative control mean absorbance ($NC\bar{x}$) by dividing the total absorbance by the total number of negative controls.

*All negative control values should fall within 0.7-1.3 times the mean*. If one value is outside this range discard this value and recalculate $NC\bar{x}$. If two values are outside the range, repeat the test. If more than an occasional value falls outside this range, technique problems should be suspected.

(2) Calculate the positive control mean absorbance ($PC\bar{x}$) by dividing the total absorbance by the total number of positive controls.

(3) Determine the cutoff value by dividing the sum of $NC\bar{x}$ and $PC\bar{x}$ by 2.

(4) Specimens with values that are within ±10% of the cutoff value are reanalyzed.

(5) Calculate the N-P value by subtracting $PC\bar{x}$ from $NC\bar{x}$. The N-P value should be 0.400 or greater. If not, technique problems or reagent deterioration should be suspected and the run repeated.

(6) An in-house control (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to a SOP entitled "In-House Control: T anti-HAV" (S. Lambert, 8/92). A pool of anti-HAV positive serum is calibrated by serial dilution in a controlled process serum-based diluent. *When tested using the anti-HAV EIA the final anti-HAV IHC reagent must consistently generate a signal-to-cutoff ratio of 0.5 to 1.0.*

(7) Specimens with absorbance less than or equal to the cutoff value are considered reactive for anti-HAV. Specimens that are reactive are considered positive by the criteria of the test kit.

h. **Special Procedure Notes**

(1) When dispensing beads, remove the cap from the bead bottle, attach the bead dispenser and dispense beads into wells of the reaction tray.

(2) Do not splash liquid while tapping trays.

(3) When washing beads, follow the directions provided with the washing apparatus.

(4) When transferring beads from wells to assay tubes, align an inverted carton of tubes over their respective wells in the reaction tray. Press the tubes tightly over the wells and invert the tray and tubes together so that

the beads fall into the corresponding tubes. Blot excess water from top.

(5) Avoid strong light during color development.

(6) Dispense acid in the same sequence as the OPD substrate solution.

(7) Do not allow acid or OPD substrate solution to contact metal.

(8) Remove air bubbles prior to reading absorbance.

(9) Visually inspect blank tubes and discard them if they have a yellow-orange color. If both blank tubes are contaminated repeat the entire run.

## 9. REPORTABLE RANGE OF RESULTS

Final results are expressed qualitatively as positive or negative for the presence of anti-HAV antibody in the sample. No quantitative results are determined.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the HRL for epidemiological health studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-the-art.

This quality control system uses bench quality control samples. Positive and negative controls are included with kits. One in-house control is a pool of anti-HAV positive serum calibrated by serial dilution in a controlled-process serum-based diluent. When tested by the anti-HAV EIA, the final anti-HAV IHC reagent must consistently generate a signal-to-cutoff ratio of 0.5 to 1.0.

Three negative controls, two positive controls, and one in-house control are included in each analytical run (a set of consecutive assays performed without interruption). The presence or absence of anti-HAV is determined by comparing the absorbance value of the sample to the cutoff value. This cutoff value is calculated from the negative and positive control absorbance values as explained in the Calculations Section. Specimens with absorbances less than or equal to the cutoff value are considered reactive for anti-HAV. Specimens that are reactive are considered positive by the criteria of the test kit.

The difference between the negative control and positive control mean absorbance values (N-P) is calculated as a measure of the validity of the test. For the run to be valid, the N-P value should be at least 0.400 or greater. Samples with test results that fall within ±10% of the calculated cutoff value are reanalyzed.

The precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA. This method generates coefficients of variation of 5-10% within runs and 8-15% between runs in the HRL.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. If controls do not conform to specifications, reject the results and reanalyze all samples. Do not use data from nonqualifying test runs.

b. Samples with test results that fall within ±10% of the calculated cutoff value are reanalyzed.

c. If two negative control values fall outside 0.7-1.3 times the mean, repeat the test.

d. Calculate the difference between the negative control and positive control mean absorbance values (N-P) as a measure of the validity of the test. For the run to be valid, the N-P value should be at least 0.400. If it is not, repeat the run. If the N-P value is consistently less than 0.400, deterioration of reagents might be the cause.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. The sample is restricted to human serum or plasma.

b. No interfering substances have been identified.

c. Multiple/freeze thaw procedures should be avoided.

d. The HAV EIA test is limited to the qualitative detection of anti-HAV in human serum or plasma.

**13. REFERENCE RANGES (NORMAL VALUES)**

A normal human serum should be negative for hepatitis A antibodies.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may remain at 20-25 °C during preparation and testing for 4 hours.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Other FDA-licensed tests for total anti-HAV may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Test methods may not be substituted without approval from NCHS.

Alternative methods of storage are not recommended. In case of system failure, samples should be refrigerated at 4-8 °C for no more than 5 days. For longer periods, the specimens should be stored at ≤-20 °C until the system is functioning properly.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Test results are documented through the lab management database (Section 3). Generally, studies conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

Specimens in long-term storage are arranged by study group. The storage location of each sample is listed with the test data.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

**REFERENCES**

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We gratefully acknowledge the contributions of Miriam Alter, Joni Kaluba, Stephen Lambert, Brian Mahy, Ph.D., Harold Margolis, M.D., Linda Moyer and Mar Yi Than who assisted in developing the methodology, performing the analysis for the antibody to hepatitis A in the NHANES III study, and preparing the manuscript for this chapter.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Semiquantitative determination of hepatitis B surface antigen (HBsAg) contained in human serum or plasma is measured by using sandwich radioimmunoassay (1-3). A control or test sample is pipetted into wells of a reaction tray. A plastic bead coated with (guinea pig) anti-HBsAg is added to each well. During an overnight incubation, HBsAg, if present, will bind to the anti-HBsAg on the bead. After the beads are washed, $^{125}$I-conjugated (human) anti-HBsAg is added. During incubation, if HBsAg is present and immobilized on the bead, the conjugate will in turn bind to it, forming a sandwich of HBsAg between bead-bound and conjugate anti-HBsAg. Following another wash step, the beads are transferred to test tubes and analyzed for the presence of $^{125}$I by using a gamma counter. The software program automatically divides each sample signal by the average of the negative controls expressed as sample ratio units (SRUs) or P/N value.

This is an FDA-licensed method used in a commercially available kit. The literature and instructions included with each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in the determination of whether an individual is infected with hepatitis B virus.

## 2. SAFETY PRECAUTIONS

This assay employs $^{125}$I as a tracer, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the *CDC Radiation Safety Manual*. In addition, all personnel must successfully complete the CDC training course *Radiation Safety in the Laboratory* or demonstrate equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eye wear, and lab coat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines.

Any laboratory using radioimmunoassay (RIA) kits must hold a current NRC Certificate of Registration and conform to all of the storage, handling, and disposal requirements.

Test kits contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, they should be treated as capable of transmitting disease. Serum and plasma specimens accepted for testing may contain any number of pathogenic microorganisms. We recommend biosafety Level 2 containment and practice as described in CDC/NIH publication #88-8395 for handling test specimens and kit reagents (4).

Material Safety Data Sheets (MSDS) for sodium azide and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) computer network. Risk is minimal because the chemicals supplied in the test kit are in small quantities, are well packaged, and require limited handling by the operators.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Currently, raw data are transcribed manually from instrument readout sheets into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results. It functions within the PARADOX (Borland Co., Scott's Valley, CA) software program (v. 3.5). For HBsAg, test values are divided by the average of the negative controls and reported as SRUs. Other information in the database may typically include the HRL identification number, the specimen number, the date collected, the date tested and the results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer.

b. Finished data sets are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the values to the NCHS mainframe computer along with the other NHANES III data.

c. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.

d. Documentation for data system maintenance is contained in printed copies of data records for 2 years.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (S. Lambert, 5/92).

b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

c. Specimens may be human serum or plasma. Serum specimens may be collected by using regular red-top or serum separator Vacutainers.

d. The required sample volume is 200 µL for the assay, and 1.0 mL of serum will permit repeat analysis.

e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Serum or plasma samples are collected by using techniques that minimize hemolysis and bacterial contamination.

g. Samples are stored in labeled 2-mL Nalge cryovials or an equivalent vial.

h. Samples can be stored at 4-8 °C for a maximum of 5 days.

i. For storage of more than 5 days, samples are held at ≤-20 °C. Samples held in long-term storage at ≤-20 °C are indexed in the database for easy retrieval.

j. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.

h. Multiple freeze/thaw cycles should be avoided.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Abbott QWIKWASH, model 6258-27 (Abbott Laboratories, North Chicago, IL).

(2) ANSR gamma counter, model 7157 (Abbott Laboratories).

(5) Abbott COMMANDER dynamic incubator, model 6210-01 (Abbott Laboratories).

> *NOTE: The Abbott instruments cited above were approved for use with these test kits as part of FDA licensure of the kits.*

(6) Gilson Pipetman micropipettors, 200 µL size (Rainin Instrument Co., Woburn, MA).

b. **Materials**

(1) Antibody to hepatitis B Surface Antigen (Human) AUSRIA II-$^{125}$I Diagnostic Kit, cat. no. 780224, available in 100- and 1000-test sizes, (Abbott Laboratories, North Chicago IL).

(2) Reagent trays, Costar cat. no. 4870 (VWR Scientific, Bridgeport, CT).

(3) Cover seals provided as part of the test kit (Abbott Laboratories).

(4) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(5) Pipet tips, Rainin cat. nos. RT20 & RT200 (Rainin Instrument Co.).

(6) Protective gloves, Tronex or Flexam, small/medium/large (any vendor).

(7) 2-mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(8) Cryovial boxes, cat. no. 5026-0909 (Nalge).

(9) 1.5-mL microtubes, (Marsh Biomedical Products, Rochester, NY).

(10) 50 mL-polypropylene tubes, (Corning Glass Works, Corning, NY).

(11) 5.25% sodium hypochlorite, household bleach. (Any vendor) .

(12) Virotrol-I, cat. no. 00100 (Blackhawk Biosystems, Inc, San Ramon, CA).

(13) Defibrinated, dialyzed, filtered plasma pool, nonreactive for hepatitis A, B, and C.

c. **Reagent Preparation**

Reagents for these procedures are prepared by the manufacturer of the test kits. Volumes are for 100-/1000-test kit sizes.

(1) Beads coated with antibody to hepatitis B surface antigen (guinea pig)
1 bottle (100 beads)/2 bottles (500 beads). This is supplied as a single reagent, ready to use. Store at 2-8 °C.

(2) Antibody to hepatitis B surface antigen $^{125}$I (human)
2 vials (10 mL each), potency adjusted with Tris buffer containing protein stabilizers. Radioactivity maximum: 0.74 µCi/mL (28 kBq/mL) preservative: 0.1 g/dl sodium azide.

*Note: This material contains $^{125}$I and should be properly handled with gloves and disposed of according to radiation safety guidelines.*

d. **Standards Preparation**

No calibration curve is generated by the user as part of this assay method. Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls.

e. **Preparation of Quality Control Materials**

This method does not employ conventional calibrators or standards. Calibration is based on the results of defined "positive" and "negative" controls.

(1) Kit positive and negative controls
Supplied ready to use by the manufacturer of the test kits, Abbott Laboratories.

(a) Negative control
1 vial (5 mL)/1 vial (35 mL), recalcified human plasma, nonreactive for HBsAg and anti-HBs.

(b) Positive control (human)
1 vial (3 mL)/1 vial (16 mL), human plasma reactive for HBsAg. Potency: 20±5 ng/mL adjusted with Tris buffer containing protein stabilizers. Preservative: 0.1 g/dL sodium azide.

(2) In-house control (IHC)
Included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled "In-

House Control: HBsAg (S. Lambert, 8/92) in which a pool of HBsAg positive serum is calibrated to give a signal/cutoff value of 0.8-1.2 by using the Abbott AUSRIA-II.

(a) Dilute Virotrol-I in plasma diluent in polypropylene tubes as shown in Table 1.

Table 1
Preparation of Dilutions for Quality Control Materials

| Dilution | Diluent (µL) | Virotol I (µL) |
|---|---|---|
| 1:2.5 | 900 | 600 |
| 1:5 | 750 | 750 of 1:2.5 dilution |
| 1:10 | 750 | 750 of 1:5 dilution |
| 1:20 | 750 | 750 of 1:10 dilution |
| 1:40 | 750 | 750 of 1:20 dilution |
| 1:80 | 750 | 750 of 1:40 dilution |

(b) Select a dilution that has signal to cutoff ratio between 1.2 and 1.9 and dilute sufficient volume to yield approximately 150 vials containing 225-250 µL per vial.

(c) Confirm the values by testing in duplicate in corresponding assays. If the values are within the appropriate range, aliquot the QC material into labeled tubes. Store at ≤-20 °C.

(d) Thaw each in-house control as needed, use once, and discard.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

No calibration curve is generated by the user as part of these assay methods except in preparation of the in-house control reagent. Calibration of instruments is either automatic or performed periodically by contracted service personnel.

### b. Verification

(1) The instrument used to read assay results (Section 6.e.3.) is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

(2) Retest all samples with values between 2.1 and 5.0 SRU.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Reagents are used per kit of 100 or 1000 tests. Kit components are occasionally interchanged within a manufacturer's lot, but never interchanged between lots.

(2) Remove the test kit from 4-8 °C storage. Allow 30-40 min for the reagents to warm to 20-25 °C. Swirl gently before use.

(3) Assay seven negative controls, three positive controls, and the in-house control with each run.

(4) Ensure that all reaction trays are subjected to the process and incubation times that are specified in the manufacturer's instructional literature.

(5) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

b. **Sample Preparation**

(1) Bring the serum specimens to 20-25 °C. Serum and plasma samples may settle into layers when frozen or stored at 4-8 °C for extended periods. Mix them gently before testing.

(2) Label the reaction tray wells for each specimen or control.

c. **Instrument Setup**

(1) Operation of the Abbott QWIKWASH

The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

(a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the "Power" indicator on the instrument.

(b) Ensure that the "Low Pressure" and "Low Water Level" indicators are NOT illuminated before washing beads. See note below.

(c) Place the bead tray on the QWIKWASH with the first row of beads aligned with the washing heads.

(d) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.

(e) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.

(f) Repeat until all of the beads have been washed. Then proceed directly to the next step of the assay procedure.

*NOTE: The wash water is held in a stainless-steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on, fill the tank with deionized water AND empty the waste tank. Never fill the water tank without also emptying the waste container! Add approximately 200 mL of bleach to the waste container prior to reconnecting it to the system so that waste water can be discarded down the sink as "decontaminated liquid waste." Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless steel pressure tank.*

(2) Operation of COMMANDER incubator

Set the temperature to 45 °C.

(3) Operation of the ANSR

The ANSR gamma counter is programmed by the manufacturer to read the results of these assay methods. The user needs only to answer a simple set of menu-driven questions regarding the number and placement of samples to be read.

(a) After the beads have been transferred to tube rack(s), place the rack(s) into the appropriate ANSR

carrier tray(s).

(b) Place the carrier tray(s) onto the ANSR to be automatically fed into the tube pick-up area.

(c) On the instrument keypad choose RUN ASSAYS. Answer the prompts as shown in Table 2.

Table 2
ANSR Parameters

| Prompt | Response |
|---|---|
| Run which assay? | Assay # from TABLE 2 |
| Lot #/Tech | Kit lot # & initials |
| Auto data entry? | YES |
| How many patients? | Number of patients |
| Save QC data? | NO |
| Tray 1 size - 20? | YES if tray size is 20<br>NO if tray size is 60 |
| Is tray in back track? | YES if tray is in the back track<br>NO if tray is in the front track |
| How many tubes in tray? | Total number of tubes |
| Are trays ready? | YES if trays are ready to be read |

d. **Operation of Assay Procedure**

(1) Pipette 200 µL of each control or test specimen into the wells of a reaction tray.

(2) Carefully add one bead to each well containing a specimen or control.

(3) Apply the cover seal. Gently tap the tray to cover the beads and remove any trapped air.

(4) Incubate at 20-25 °C overnight (20 ±2 hours).

(5) Remove and discard the cover seal. Wash the beads in the tray using the QWIKWASH bead washer.

(6) Pipette 200 µL of $^{125}$I anti-HBs into each well containing a bead.

(7) Apply a new cover seal. Gently tap the tray to cover the beads and remove any trapped air.

(8) Incubate at 45 °C for 1 hour.

(9) Remove and discard the cover seal. Wash the beads in the tray using the QWIKWASH bead washer.

(10) Transfer the beads to 10- X 75-mm tubes. Measure $^{125}$I activity using the ANSR gamma counter, counting each bead for 1 min.

e. **Recording of Data**

(1) Quality Control Data

Raw data are transcribed manually from the instrument readout sheet into a computerized database. Quality control of individual control values is maintained by the ANSR, which will reject the test run if control values do

not conform to specifications.

(2) Analytical Results

Raw data are transcribed manually from the instrument readout sheets into a computerized database. Raw data are expressed as counts per minute (CPM). Data reduction is done by the laboratory management software that is part of the computerized database.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Abbott ANSR is self-monitoring and requires only routine service and is maintained by an Abbott technical representative.

(2) Once a week, check the quality of water used in QWIKWASH, refrigerator temperature, freezer temperature, and room temperature.

(3) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) Cutoff calculation is done by the reading instrument and by the data management software which use the following formula:

$$\text{Cutoff} = NC\bar{x} \times 2.1,$$

where $NC\bar{x}$ is the mean absorbance value of the negative control.

(2) Data are expressed in sample ratio units (SRU) which are calculated by dividing each sample signal by the mean of the negative controls.

h. **Procedure Notes**

(1) When dispensing beads, remove the cap from the bead bottle, attach the bead dispenser and dispense beads into wells of the reaction tray.

(2) Do not splash liquid while tapping trays.

(3) When washing beads, follow the directions provided with the washing apparatus.

## 9. REPORTABLE RANGE OF RESULTS

Final results are expressed SRUs. SRUs are determined by dividing each sample signal by the mean of the negative controls. Retest all samples with SRU values between 2.1 and 5.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used extensively in the HRL for epidemiological studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art.

This quality control system uses bench quality control samples. Seven negative controls, three positive controls, and one in-house control (IHC) are included in each analytical run (a set of consecutive assays performed without interruption). The IHC reagent is produced by the HRL according to an SOP entitled In-House Control: HBsAg (S. Lambert, 8/92), in which a pool of HBsAg positive serum is calibrated to give a signal/cutoff value of 0.8-1.2 in Abbott AUSRIA-II.

Specimens with CPM $\geq$ cutoff value are considered reactive for HBsAg. For the run to be valid, the mean value for the positive controls specimens should be 20 times the $NC\bar{x}$. All negative control values should fall within 0.5-1.5 times $NC\bar{x}$. The $NC\bar{x}$:$PC\bar{x}$ ratio should be $\geq$20.

The precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA. The HRL protocol generates a coefficient of variation of 5-10% within runs and 8-15% between runs.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

By definition, if controls do not conform to specifications, the testing is rejected. All samples are tested again. Data from nonqualifying test runs are not used.

Repeat the test for any violations of the following rules:

- The mean value for the positive control specimens should be 20 times $NC\bar{x}$.
- All negative control values should fall within 0.5-1.5 times $NC\bar{x}$.
- The $NC\bar{x}/PC\bar{x}$ ratio should be $\geq 20$.

Some specimens that are reactive in the screening procedure may not be reactive on repeat testing. This phenomenon is highly dependent on technique used in running the test. Common causes of nonrepeatedly reactive specimens are improper bead washing, cross-contamination of nonreactive specimens caused by transfer of residual droplets of high-titer antigen containing serum, and contamination of specimen holders in the gamma counter.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

(1) The sample is restricted to human serum or plasma.

(2) No interfering substances have been identified.

(3) Although the association of infectivity and the presence of HBsAg is strong, presently available methods for HBsAg detection may not be sensitive enough to detect all potentially infectious units of blood or possible cases of hepatitis.

(4) Kit positive and negative controls must conform to the manufacturer's specifications in the kit insert literature.

(5) In-house controls must have a signal-to-cutoff ratio between 0.8 and 1.2.

**13. REFERENCE RANGES (NORMAL VALUES)**

A normal value for HBsAg should be negative. All samples with SRU values between 2.1 and 5 ("indeterminate") should be repeated.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may remain at 20-25 °C during preparation and testing for 4 hours.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Other FDA-licensed tests for total HBsAg may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Test methods may not be substituted without approval from NCHS.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Test results are documented through the lab management database (Section 3). Generally, studies conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

Specimens in long-term storage specimens are arranged by study group. The storage location of each sample is listed with the test data.

## 19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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1. Abbott Laboratories, Diagnostics Division. AUSRIA II $^{125}$I RIA directional literature included with each assay kit. Document number 83-3661/R13. North Chicago (IL): Abbott Laboratories, 1989.

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## ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Miriam Alter, Joni Kaluba, Stephen Lambert, Brian Mahy, Ph.D., Harold Margolis, M.D., Linda Moyer and Mar Yi Than who assisted in developing the methodology, performing the analysis for the hepatitis B surface antigen in the NHANES III study, and preparing the manuscript for this chapter.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Qualitative determination of total antibody to hepatitis B virus core antigen (anti-HBc) contained in human serum or plasma is measured using solid-phase competitive immunoassay (RIA). A test sample is mixed in a reaction well with detection-phase reagent. The detection-phase reagent consists of $^{125}$I-conjugated antibody directed against HBc. The sample-conjugate mixture is incubated in the presence of a polystyrene bead permanently bound with a specific amount of HBc antigen. Any anti-HBc present in the sample will compete with the conjugate for HBc epitopes present on the bead (1). At the end of the incubation, the amount of conjugate immunochemically bound to the bead will be inversely proportional to the concentration of anti-HBc in the sample. Beads are washed to remove unbound material. A gamma counter is then used to analyze the beads for the presence of $^{125}$I. The instrument used to measure the test results is equipped with software that calculates a cutoff value. The cutoff calculation is based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HBc.

This FDA-licensed method is commercially obtained in kit form. The literature and instructions with each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in its capacity to determine whether an individual has been infected with hepatitis B virus, for which anti-HBc is a marker.

Anti-HBc determinations can be used to monitor the progress of hepatitis B viral infection. Anti-HBc is found in serum shortly after the appearance of hepatitis B surface antigen (HBsAg) associated with acute hepatitis B, and it will persist after the disappearance of HBsAg and before the appearance of detectable antibody to HBsAg (anti-HBs) (2-5). Therefore, in the absence of HBsAg and anti-HBs, anti-HBc may be the only serological marker of recent hepatitis B infection and potentially infectious blood (6-8).

## 2. SPECIAL SAFETY PRECAUTIONS:

This assay employs $^{125}$I as a tracer, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the CDC *Radiation Safety Manual*. In addition, all personnel must successfully complete the CDC training course "Radiation Safety in the Laboratory" or demonstrate equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe Universal Precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines.

Any laboratory using radioimmunoassay (RIA) kits must hold a current Nuclear Regulatory Commission (NRC) Certificate of Registration and conform to all of the storage, handling, and disposal requirements ascribed thereto.

Test kits for anti-HBc contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, they should be treated as capable of transmitting disease. Serum and plasma specimens accepted for testing may contain any number of pathogenic microorganisms. We recommend Biosafety Level 2 containment and practice as described in CDC/NIH publication #88-8395 for handling test specimens and kit reagents (9).

Material Safety Data Sheets (MSDSs) for sodium azide and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) Local Area Network (LAN). Because the chemicals are supplied in the test kit, which contain only a small quantity of chemicals, and because the chemicals are well packaged and undergo limited handling by the operators, risk from exposure to the chemicals is minimal.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Raw data are transcribed manually from instrument readout sheets into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results. It functions within the PARADOX software program (v. 3.5 Borland, Scott Valley, CA). Test values are compared with a cutoff value, which is calculated from the assay controls. Results are expressed as "positive" or "negative" for anti-HBc. Other information in the database may typically include a specimen's HRL identification number and specimen number, the date it was collected, the date it was tested and the results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer. Electronically stored data are

automatically backed up daily.

b. Finished data sets are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the study sponsor will be transmit the results to the National Center for Health Statistics (NCHS) mainframe computer along with the other NHANES III data.

c. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.

d. Documentation for data system maintenance is contained in hard copies of data records for 2 years.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (S. Lambert, 5/92).

b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

c. Specimens should be human serum or plasma. Serum specimens may be collected in regular red-top or serum separator Vacutainers.

d. The required sample volume is 100 µL for the assay, and 1.0 mL will permit repeat analysis.

e. Specimens may be stored in glass or plastic vials as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Serum or plasma samples are collected by using techniques to minimize hemolysis and bacterial contamination.

g. Samples are stored in labeled 2-mL Nalge cryovials or their equivalent.

h. Specimens may be stored at 4-8 °C for a maximum of 5 days.

i. For storage >5 days, samples are held at ≤-20 °C. Samples held in long-term storage at ≤-20 °C are indexed in the database for easy retrieval.

j. Specimens are rejected if contaminated, hemolyzed or stored improperly. However, rejection is done only after consultation NCHS.

k. Multiple freeze/thaw cycles should be avoided.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) Abbott QWIKWASH, model 6258-27, for washing solid-phase beads between incubation steps (Abbott Laboratories, North Chicago, IL) .

(2) Abbott ANSR gamma counter, model 7157, for reading RIA results (Abbott Laboratories).

(3) Gilson Pipetman adjustable micropipettors (Rainin Instrument Co., Woburn, MA).

b. **Other Materials**

(1) Abbott CORAB solid-phase radioimmunoassay kit, cat. no. 1A1B-24 (Abbott Laboratories). Each kit includes the following:

(a) Hepatitis B virus core antigen (E.coli, recombinant) coated beads-1 bottle (100 beads). Supplied as a single reagent, ready to use. Store at 2-8 °C.

(b) Antibody to hepatitis B virus core antigen $^{125}$I (Human) -- 2 vials (5 mL each. Radioactivity maximum: 1.5 µCi/mL (55.5 kBq/mL). Preservative: 0.1% sodium azide. Dye: red no. 33. *This material contains $^{125}$I and should be handled properly with gloves and disposed of according to CDC radiation safety guidelines*.

(c) Negative control (Human) -- 1 vial (3 mL). Recalcified plasma in phosphate buffered saline, nonreactive for anti-HBc, HBsAg, anti-HBs and anti-HIV-1. Preservative: 0.01% gentamicin.

(d) Positive control (Human) -- 1 vial (3 mL). Recalcified plasma in phosphate buffered saline, positive for anti-HBc and anti-HBs. Nonreactive for HBsAg and anti-HIV-1. Human anti-HBc concentration: 125 ± 100 PEI* units/mL. Preservative: 0.01% gentamicin.

*Concentration standardized against the reference standard of the Paul-Ehrlich-Institut, Langen FRG.

(2) Costar reaction trays, cat. no. 4870 (VWR Scientific, Bridgeport, CT).

(3) Cover seals provided as part of the CORAB RIA test kit (Abbott Laboratories).

(4) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(5) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).

(6) Latex protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).

(7) Nalge cryovials, 2-mL, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(8) Nalge cryovial boxes, cat. no.5026-0909 (Nalge).

(9) Microtubes, 1.5-mL (Marsh Biomedical Products, Rochester, NY).

(10) Polypropylene tubes, 50-mL (Corning Glass Works, Corning, NY).

(11) Virotrol-I, cat. #00100 (Blackhawk Biosystems, Inc, San Ramon, CA). Defibrinated, dialyzed, filtered plasma pool, nonreactive for Hepatitis A, B, and C.

c. **Reagent Preparation**

Reagents for these procedures are prepared by the manufacturer of the test kits (Abbott Laboratories).

d. **Standards Preparation**

This method does not employ conventional calibrators or standards. Calibration is based on the results of defined "positive" and "negative" controls.

(1) Positive and negative control reagents are supplied with each test kit.

(2) Calculation of negative control mean ($NC\bar{x}$):

Average the individual net CPM of the three negative controls.

All negative control values should fall within 0.66-1.34 times $NC\bar{x}$. If one value is outside this range, discard

this value and recalculate $\bar{NC}$ . If two values are outside this range, repeat the test. If more than an occasional value falls outside the range, contact Customer Support Center at Abbott Laboratories for assistance.

(3) Calculation of positive control mean ($\bar{PC}$):

Average the individual net CPM of the two positive controls.

(4) Calculation of N/P ratio:

Divide $\bar{NC}$ by $\bar{PC}$.

This ratio should be greater than five to ensure the validity of each run. While the actual CPM for negative and positive controls will vary according to the age of the reagents and the efficiency of the gamma counter used, the N/P ratio is less variable.

(5) Calculation of the cutoff value:

The cutoff value is $\bar{NC}$ + $\bar{PC}$ divided by 2. Specimens with CPM greater than the cutoff value are considered negative for anti-HBc.

(6) An in-house control (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to a SOP entitled In-House Control: T anti-HBc (S. Lambert, 8/92).

e. **Preparation of Quality Control Materials**

(1) Kit positive and negative controls are prepared and quality monitored by the manufacturer.

(2) In-house controls are prepared according to HRL specifications.

A pool of anti-HBc positive serum is calibrated by serial dilution in a controlled process serum-based diluent. When tested with the anti-HBc RIA, the final anti-HBc IHC reagent must consistently generate a signal-to-cutoff ratio of 1.0 ± 0.5.

(a) Using only polypropylene tubes, prepare dilutions as shown in Table 1.

Table 1
Preparation of Dilutions for Quality Control Materials

| Dilution | Diluent (µL) | Virotol I (µL) |
|---|---|---|
| 1:2.5 | 900 | 600 |
| 1:5 | 750 | 750 of 1:2.5 dilution |
| 1:10 | 750 | 750 of 1:5 dilution |
| 1:20 | 750 | 750 of 1:10 dilution |
| 1:40 | 750 | 750 of 1:20 dilution |
| 1:80 | 750 | 750 of 1:40 dilution |

(b) Pick a dilution that results in a signal-to-cutoff ratio between 0.5 and 0.9.

(c) Dilute sufficient volume for approximately 150 vials with 125-150 µL per vial.

(d) Confirm values by testing in duplicate in corresponding assays.

(e) If the dilution is within the appropriate range, aliquot the solution into labeled tubes. Store at ≤-20 °C.

(f) Thaw each in-house control as needed, use once, and discard.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

No calibration curve is generated by the user as part of these assay methods. Calibration of instruments is either automatic or carried out periodically by contracted service personnel.

### b. Verification

(1) The instrument used to read assay results is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

(2) Verify test results that fall within 10% of the calculated cutoff value by repeat testing.

(3) Consider specimens with CPM equal to or lower than the cutoff value to be initially reactive. For the purposes of this study, anti-HBc testing was repeated if the sample was initially anti-HBc positive but nonreactive for both HBsAg and anti-HBs.

(4) Consider specimens that do not react in both tests to be negative for anti-HBc.

(5) No confirmatory test is available for the presence of anti-HBc in the specimen.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Reagents are used per kit of 100 tests. Kit components are occasionally interchanged within a manufacturer's lot, but never interchanged between lots.

(2) Remove the test kit from 4-8 °C storage. Allow 30-40 min for the reagents to warm to 20-25 °C.

(3) Assay three negative controls, two positive controls, and one in-house control with each run.

(4) Ensure that all reaction trays containing controls or reagents are subjected to the process and incubation times that are specified in the manufacturer's instructional literature.

(5) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

### b. Sample Preparation

(1) Allow the serum specimens to come to 20-25 °C. Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix the specimens gently before testing.

(2) Identify the reaction tray wells for each specimen or control.

(3) Record the position of each control or specimen in the reaction tray.

### c. Instrument Setup

(1) Operation of the Abbott QWIKWASH
The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

(a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the "Power" indicator on the instrument.

(b) Ensure that the "Low Pressure" and "Low Water Level" indicators are not illuminated before washing the beads. See note below.

(3) Place the bead tray on the QWIKWASH, aligning the first row of beads with the washing heads.

(4) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.

(5) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.

(6) Repeat until all of the beads have been washed, then proceed directly to the next step of the assay procedure.

*NOTE: The wash water is held in a stainless steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on, fill the tank with deionized water and empty the waste tank. Never fill the water tank without also emptying the waste container! Add approximately 200 mL of bleach to the waste container prior to reconnecting it to the system so that waste water can be discarded down the sink as "decontaminated liquid waste." Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless steel pressure tank.*

(2) Operation of the ANSR

(a) After the beads have been transferred to tube rack(s), place the rack(s) into the appropriate ANSR carrier trays(s).

(b) Place the carrier tray(s) onto the ANSR so as to be automatically fed into the tube pick-up area.

(c) On the instrument keypad, choose RUN ASSAYS. Answer the prompts as shown in Table 2.

Table 2
ANSR Run Settings

| Prompt | Response |
|---|---|
| Run which assay? | Assay # from TABLE 2 |
| Lot #/Tech | Kit lot # & initials |
| Auto data entry? | YES |
| How many patients? | Number of patients |
| Save QC data? | NO |
| Tray 1 size - 20? | YES if tray size is 20, NO if tray size is 60 |
| Is tray in back track? | YES if tray is in the back track, NO if tray is in the front track |
| How many tubes in tray? | Total number of tubes |
| Are trays ready? | YES if trays are ready to be read |

d. **Operation of the Assay Procedure**

(1) Pipette 100 µL of $^{125}$I anti-HBc conjugate into the wells of a reaction tray.

(2) Pipette 100 µL of each control or specimen into appropriate wells containing conjugate. Mix thoroughly by gently tapping the tray. Do not splash liquid outside of the well or on the well rim.

(3) Add a bead to each well.

(4) Apply the cover seal. Gently tap the tray to cover the beads and remove any trapped air.

(5) Incubate trays at 20-25 °C overnight (20 ± 2 hours).

(6) Remove and discard the cover seal. Wash the beads in the tray using the QWIKWASH bead washer.

(7) Transfer the beads to 10- x 75-mm tubes. Place the tubes in the ANSR gamma counter and count each one for 1 min.

e. **Recording of Data**

(1) Quality Control Data
Multiple positive and negative controls are averaged by the reading instrument and are determined to be valid or invalid.

Raw data are transcribed manually from the instrument readout sheet into a computerized database.

(2) Analytical Results
Raw data are expressed as CPM. Raw data are transcribed from the instrument readout sheets into a computerized database.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Instruments are on service contract and, except for the most basic daily maintenance, are serviced by an Abbott technical representative.

(2) Quality of water used in the QWIKWASH, refrigerator temperature, freezer temperature, and room temperature (20-25 °C) are monitored and documented on a weekly basis:

(3) All micropipettors used in testing clinical specimens are checked for calibration every 6 months. Pipettors that do not conform to specifications are autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records are kept for each pipettor by serial number.

g. **Calculations**

The cutoff calculation is done by the reading instrument and by the data management software using the following formula:

$$\text{Cutoff} = \frac{NC\bar{x} + PC\bar{x}}{2}$$

h. **Special Procedure Notes**

(1) When dispensing beads, remove the cap from the bead bottle, attach the bead dispenser, and dispense the beads into the wells of the reaction tray.

(2) Do not splash liquid while tapping the trays.

(3) When washing beads, follow the directions provided with the washing apparatus.

(4) Do not splash specimen or $^{125}$I labeled antibody outside of wells, on well rims, or on the cover seal because it will not be removed in subsequent washings and may be transferred to the tubes, causing test interference.

**9. REPORTABLE RANGE OF RESULTS**

Final results are expressed qualitatively as positive or negative for the presence of anti-HBc antibody in the sample. No quantitative results are determined.

**10. QUALITY CONTROL (QC) PROCEDURES**

Positive and negative controls are included with the kits. Three negative controls, two positive controls, and one in-house control are assayed with each run of specimens.

An in-house control (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled In-House Control: T anti-HBc (S. Lambert, 8/92). A pool of anti-HBc positive serum is calibrated by serial dilution in a controlled process serum-based diluent. When tested by the anti-HBc RIA, the final anti-HBc IHC reagent must consistently generate a signal-to-cutoff ratio of 1.0 ± 0.5.

The precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA. The HRL method generates coefficients of variation of 5-10% within runs and 8-15% between runs.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

By definition, if controls do not conform to specifications, the testing is rejected, and all samples are tested again. Data from nonqualifying test runs are not used.

Consider specimens with CPM equal to or lower than the cutoff value to be initially reactive. For the purposes of this study, anti-HBc testing was repeated if the sample was initially anti-HBc positive but nonreactive for both HBsAg and anti-HBs.

Consider initially-reactive specimens that do not react in both of the duplicate repeat tests to be negative for anti-HBc.

All negative control values should fall within 0.66-1.34 times $NC\bar{x}$. If one value is outside this range, discard this value and recalculate $NC\bar{x}$. If two values are outside this range, repeat the test. If more than an occasional value falls outside the range, contact the Customer Support Center at Abbott Laboratories for assistance.

The in-house control must generate a signal-to-cutoff ratio of 0.5-1.5.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

(a) The sample must be human serum or plasma.

(b) No interfering substances are identified.

(c) The test procedure is limited to the detection of anti-HBc in serum or plasma. Currently available methods for anti-HBc detection may not detect all potentially infectious units of blood or possible cases of hepatitis B. As with any diagnostic test, false reactive results may be obtained.

**13. REFERENCE RANGES (NORMAL VALUES)**

Normal human serum is negative for hepatitis B core antigen.

Samples generating values ±10% of the cutoff are repeated.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may remain at 20-25 °C for 4 hours during preparation and testing. Multiple freeze/thaw cycles are not recommended.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Other FDA-licensed tests for total anti-HBc may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Substitution of test methods may not be done without approval from NCHS. The use of alternate storage procedures is not recommended.

In case of system failure, refrigerate specimens at 4-8 °C for 5 days. For longer periods, store them at ≤-20 °C until the system is functioning.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Test results are documented through the lab management database (See Section 3.) Generally, studies conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be in electronic or printed form. All electronically held data are backed up routinely.

In long-term storage, specimens are arranged by study group. The storage location of each sample is listed with the test data.

## 19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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We gratefully acknowledge the contributions of Miriam Alter, Joni Kaluba, Stephen Lambert, Brian Mahy, Ph.D., Harold Margolis, M.D., Linda Moyer and Mar Yi Than who assisted in developing the methodology, performing the analysis for the total antibody to hepatitis B core antigen in the NHANES III study, and preparing the manuscript for this chapter.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Semiquantitative determination of antibody to hepatitis B surface antigen (anti-HBsAg) contained in human serum or plasma is performed by sandwich enzyme-immunoassay (EIA) (1,2). Control reagent or test sample is pipetted into appropriate wells of a reaction tray. A bead permanently bound with purified hepatitis surface antigen (HBsAg) is added to each well. During an overnight incubation, any anti-HBs antibody in a sample will bind to the HBsAg on the bead. The beads are washed to remove unbound material. Peroxidase-conjugated HBsAg is added to each bead. The beads are incubated at 40 °C for 2 hours. The conjugate will bind to anti-HBs, forming a sandwich of antibody between bead-bound and conjugated HBsAg. Following another wash step, the beads are transferred to test tubes. A substrate solution made up of o-phenylenediamine (OPD) and hydrogen peroxide is added. After 30 minutes the colorimetric reaction is stopped with the addition of sulfuric acid. The reaction is read using an Abbott Quantamatic spectrophotometer. Raw data are expressed as absorbance units at 492 nm. Sample values are compared with the average value derived from three 10-mIU controls and reported as >10 mIU or <10 mIU.

This is an FDA-licensed method commercially obtained in kit form (3,4). The literature and instructions with each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in the determination of whether an individual has been infected with hepatitis B virus (HBV), for which anti-HBs is a marker. This test has value in determining the efficacy of HBV vaccines as well as demonstrating previous exposure to HBV (5-8).

The reporting of results for this assay was modified in the spring of 1993. Prior to that time, final results were reported as reactive or nonreactive for anti-HBsAg. Final reports now express results as ">10" or "<10" milli-international units (mIU).

## 2. SPECIAL SAFETY PRECAUTIONS

Test kits for anti-HBs contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance these reagents are entirely non-infectious. Therefore, treat them as though they are capable of transmitting disease. Serum and plasma specimens accepted for testing may contain any number of pathogenic microorganisms (9,10). We recommend Biosafety Level 2 containment and practice as described in Centers for Disease Control/National Institutes of Health publication #88-8395 handling test specimens and kit reagents (11,12).

Material Safety Data Sheets (MSDS) for sodium azide and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) local area network (LAN). Risk is minimal because of the limited quantity of chemicals, packaging, and limited handling by the operators using the test kits.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. After the data are collected, they are transcribed manually from instrument read-out sheets into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results. It functions within the PARADOX software program (v. 3.5 Borland, Scott Valley, CA). Test values are compared with a control value that is an average of three in-house controls, which each contain 10 milli-international units (mIU) of anti-HBs activity. Samples are reported as containing greater than or less than 10 mIU of anti-HBs. Other information in the database may typically include the HRL identification number, the specimen number, the date collected, the date tested and the results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer.

b. Completed data are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor transmits the values to the National Center for Health Statistics (NCHS) mainframe computer along with the other NHANES III data.

c. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.

d. Documentation for data system maintenance is contained in printed copies of data records for 2 years.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Specimen handling conditions are outlined according to the HRL SOP entitled "Sample Handling" (S. Lambert,

5/92).

b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

c. Specimens should be serum, plasma, or defibrinated plasma. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers.

d. Required sample volume is 200 µL for the assay; 1.0 mL will permit repeat analyses.

e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Serum or plasma samples are collected to minimize hemolysis and bacterial contamination.

g. Samples are stored in labeled 2-mL Nalge cryovials or equivalent.

h. Samples may be stored at 4-8 °C for up to 5 days.

i. For storage >5 days, samples are held at ≤-20 °C. Samples held in long-term storage at ≤-20 °C are indexed in the database for easy retrieval.

j. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Abbott QWIKWASH, model 6258-27, for washing solid-phase beads between incubation steps (Abbott Laboratories, North Chicago, IL).

(2) ANSR gamma counter, model 7157, for reading RIA results (Abbott Laboratories).

(3) Gilson Pipetman micropipettors, 200 µL size, for pipetting samples and reagents (Rainin Instrument Co., Woburn, MA).

b. **Other Materials**

(1) Abbott AUSAB Enzyme Immunoassay kit, cat. no. 755424 (Abbott Laboratories). Each kit contains the following.

(a) Antibody to Hepatitis B surface antigen (guinea pig)-1 bottle (100 beads) or 2 bottles (500 beads each).

(b) Antibody to hepatitis B surface antigen (human), peroxidase-conjugated -- 2 vials (10 mL each) or 5 vials (40 mL each). Potency adjusted by the manufacturer with Tris buffer containing protein stabilizers.

(c) Negative control -- 1 vial (5 mL) or 1 vial (35 mL). Recalcified human plasma nonreactive for HBsAg and anti-HBs. Preservative: 0.1% sodium azide.

(d) Positive control -- 1 vial (3 mL) or 1 vial (16 mL). Human plasma positive for HBsAg. Potency: 20±5 ng/mL adjusted with Tris Buffer containing protein stabilizers. Preservative: 0.1% sodium azide.

(2) Virotrol-I, cat. no. 00100 (Blackhawk Biosystems, Inc, San Ramon, CA).

(3) Defibrinated, dialyzed, filtered plasma pool, nonreactive for hepatitis A, B, and C (CDC in-house preparation).

(4) Costar reagent trays, cat. no. 4870 (VWR Scientific, Bridgeport, CT).

(5) Cover seals, provided as part of the AUSAB test kit (Abbott Laboratories).

(6) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(7) 1 N sulfuric acid, cat. no. 7212 (Abbott Laboratories).

(8) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).

(9) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).

(10) Nalge cryovials, 2-mL cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(11) Nalge cryovial boxes, cat. no. 5026-0909 (Nalge).

(12) Microtubes, 1.5-mL (Marsh Biomedical Products, Rochester, NY).

(13) Polypropylene tubes, 50-mL (Corning Glass Works, Corning, NY).

(14) 10% sodium hypochlorite, household bleach (any vendor) .

c. **Reagent Preparation**

Reagents for these procedures are prepared by the manufacturer of the test kits.

d. **Standards Preparation**

This method does not employ conventional calibrators or standards. Calibration is based on the results of defined "positive" and "negative" controls.

(1) Positive and negative control reagents are supplied with each test kit. The 10 mIU in-house control standard is formulated according to an HRL standard procedure entitled "Calibration of anti-HBs serum to 10 mIU" (S. Lambert, 1992).

(2) Calculation of negative control mean ($NC\bar{x}$):

Average the individual net absorbance value at 492 nm of the three negative controls, where $NC\bar{x}$ = the sum of absorbance values at 492 nm divided by the total number of negative controls.

All negative control values should fall within 0.5-1.5 times $NC\bar{x}$. Discard one negative value outside this range and recalculate $NC\bar{x}$. If a single negative value is consistently outside this range, or if there are more than two aberrant values in a single run, check for technical problems and investigate the assay technique.

(3) Calculation of positive control mean ($PC\bar{x}$):

Average the individual net absorbance readings at 492 nm of the 2 positive controls.

(4) Calculation of P-N ratio:

Subtract $\bar{x}$NC from the $\bar{x}$PC. For the run to be valid, the P-N value must be ).3 or greater.

(5) Calculation of the cutoff value:

A traditional cutoff to denote reactive and nonreactive samples was used in this method from 1988 through the spring of 1993. Since then, the three 10 mIU controls have been averaged and results expressed as greater than or less than 10 mIU.

(6) Three in-house controls (IHCs) are included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled In-House Control: T anti-HBs (S. Lambert, 8/92) (section 6.e.).

e. **Preparation of Quality Control Material**

(1) Kit positive and negative controls are prepared and quality controlled by the manufacturer.

(2) In-house controls are prepared according to HRL specifications. A pool of anti-HBs positive serum is calibrated to 10 mIU/mL by serial dilution in a controlled-process serum-based diluent by using the quantitative australia antibody enzyme immunoassay (AUSAB-EIA). When tested with the anti-HBs EIA, the final anti-HBs IHC reagent must consistently generate a value of 9-11 mIU. The in-house control is included in triplicate in each testing run and is the basis for determining whether an individual is immunologically protected against HBV infection (See also section 14.)

Using only polypropylene tubes, dilute Virotrol-I in plasma diluent as shown in Table 1.

Table 1
Preparation of Dilutions for Quality Control Materials

| Dilution | Diluent (µL) | Virotol-I (µL) |
|---|---|---|
| 1:2.5 | 900 | 600 |
| 1:5 | 750 | 750 of 1:2.5 dilution |
| 1:10 | 750 | 750 of 1:5 dilution |
| 1:20 | 750 | 750 of 1:10 dilution |
| 1:40 | 750 | 750 of 1:20 dilution |
| 1:80 | 750 | 750 of 1:40 dilution |

(a) Select a dilution that has in a result of 10.0 mIU. Plot and extrapolate the dilution curve if necessary.

(b) Dilute sufficient volume for approximately 150 vials with 225-250 µL per vial.

(c) Confirm values by testing in duplicate in corresponding assays.

(d) If results are within the appropriate range, aliquot into labeled tubes. Store at $\leq$-20 °C.

(e) Thaw each in-house control as needed, use once, and discard.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibration curve is generated by the user as part of these assay methods except in preparation of the in-house control reagent. Calibration of instruments is either automatic or is carried out periodically by contracted service personnel.

b. **Verification**

The instrument used to read assay results (see section 6.e.3.) is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Remove the test kit from 4-8 °C storage. Allow 30-40 min for the reagents to warm to 20-25 °C. Swirl gently before use.

(2) Reagents are used per kit of 100 tests. Kit components are occasionally interchanged within a manufacturer's lot but never interchanged between lots.

(3) Three negative controls, two positive controls, and one in-house control in triplicate are assayed with each run of specimens.

(4) Ensure that all reaction trays are subjected to the process and incubation times that are specified in the manufacturer's instructional literature.

(5) Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.

b. **Sample preparation**

(1) Bring the serum specimens to 20-25 °C. Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix gently before testing.

(2) Identify the reaction tray wells for each specimen or control.

c. **Instrument setup**

(1) Operation of the Abbott QWIKWASH
The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

(a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the "Power" indicator on the instrument.

(b) Ensure that the "Low Pressure" and "Low Water Level" indicators are NOT illuminated before washing beads. See note below.

(c) Place the bead tray on the QWIKWASH with the first row of beads aligned with the washing heads.

(d) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.

(e) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.

(f) Repeat until all of the beads have been washed, and then proceed directly to the next step of the assay procedure.

*NOTE: The wash water is held in a stainless steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on the tank should be filled with deionized water and the waste tank should be emptied. Never fill the water tank without also emptying the waste container! Add approximately 200 mL of bleach to the waste container before reconnecting it to the system so that waste water can be discarded down the sink as "decontaminated liquid waste." Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless steel pressure tank.*

(2) Operation of the QUANTAMATIC plate reader

(a) After the beads have been transferred to tube rack(s), place the rack(s) into the appropriate QUANTAMATIC carrier tray(s).

(b) Place the carrier tray(s) onto the QUANTAMATIC to be automatically fed into the tube pick-up area.

(c) On the instrument keypad choose RUN ASSAYS. Answer the prompts as shown in Table 2.

Table 2
QUANTAMATIC Run Settings

| Prompt | Response |
|---|---|
| Run which assay? | Assay # from TABLE 1 |
| Lot #/Tech | Kit lot # & initials |
| Positive ID? | NO |
| Number of patients? | Number of patients |
| Tray 1 size - 20? | YES if tray size is 20; NO if tray size is 60 |
| Is tray in back track? | YES if tray is in the back track; NO if tray is in the front track |
| How many tubes in tray? | Total number of tubes |
| Enter pat no. ID | NO |
| List operator entries? | NO |
| Are trays ready? | YES if trays are ready to be read |

d. **Operation of Assay Procedure**

(1) Pipette 200 µL of each control or test specimen into the wells of a reaction tray.

(2) Carefully add one bead to each well containing a specimen or control.

(3) Apply cover seal. Gently tap the tray to cover the beads and remove any trapped air.

(4) Incubate at 20-25 °C overnight (20 ± 2 hours).

(5) Remove and discard the cover seal. Wash the beads in the tray using the QWIKWASH bead washer.

(6) Add 200 µL of peroxidase-HBsAg conjugate to each well containing a bead.

(7) Apply the cover seal. Gently tap the tray to cover the beads and remove any trapped air.

(8) Incubate the specimens at 20-25 °C for 4 hours.

(9) Remove and discard the cover seal. Wash the beads again as in step 5).

(10) Transfer the beads to 10- x 75-mm tubes.

(11) Add 300 uL of prepared substrate solution to each tube containing a bead.

(12) Cover and incubate the tubes for 30 min at 20-25 °C.

(13) Stop the reaction by adding 1 mL of 1 N sulfuric acid per tube.

(14) Read the reaction using the QUANTAMATIC.

e. **Recording of Data**

(1) Quality Control Data
Raw data are transcribed manually from the instrument readout sheets into a computerized database. Quality control of individual control values is maintained by the QUANTAMATIC, which will reject the test run if control values do not conform to specifications.

(2) Analytical Results
Raw data are transcribed manually from the instrument readout sheets into a computerized database, and are expressed as absorbance units at 492 nm. Data reduction is done by the laboratory management software that is part of the computerized database.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Instruments are on service contract and, except for the most basic daily maintenance, are serviced by an Abbott technical representative.

(a) Abbott QUANTAMATIC is self-monitoring and requires only routine service per contract.

(b) Quality of water used in QWIKWASH, refrigerator temperature, freezer temperature, and room temperature are monitored and documented on a weekly basis.

(c) All micropipettors used in testing clinical specimens are checked for calibration every six months. Pipettors that do not conform to specifications are autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

From 1988-1993, the normal AUSAB-EIA cutoff calculation was performed by the reading instrument and by the data management software which used the following formula:

$$\text{Cutoff} = NC\bar{x} + 0.05$$

where $NC\bar{x}$ is the average Abs492 value of the three negative controls that are included in each testing run. However, as of April, 1993, the data for this method are reported as greater than or less than 10 mIU. The 10 mIU value is determined by including three 10 mIU standards with each test run. These are averaged and compared with each sample value.

h. **Special Procedure Notes**

(1) When dispensing beads, remove the cap from the bead bottle, attach the bead dispenser and dispense the beads into the wells of the reaction tray.

(2) Do not splash liquid while tapping trays.

(3) When washing the beads, follow the directions provided with the washing apparatus.

**9. REPORTABLE RANGE OF RESULTS**

Final results are expressed as ">10" or "<10" mIU. Results are determined by comparing each specimen value with the average value of the in-house control, which contains 10 mIU and is included in triplicate in each testing run.

**10. QUALITY CONTROL (QC) PROCEDURES**

The method described in this protocol has been used extensively in the HRL for epidemiological health studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art.

This quality control system uses bench quality control samples. Positive and negative controls are included with kits. The in-house control is a pool of anti-HBs positive serum calibrated to 10 mIU/mL by serial dilution in a controlled-process serum-based diluent by using the quantitative AUSAB-EIA. When tested with the anti-HBs EIA, the final anti-HBs IHC reagent must consistently generate a value of 9-11 mIU.

Three negative, two positive, and three in-house controls are included in each analytical run (a set of consecutive assays performed without interruption). The presence or absence of anti-HBs is determined by comparing the absorbance value of each unknown at 492 nm with the average absorbance value of the 10 mIU standards at 492 nm. Specimens with mIU values equal to or greater than 10 mIU are considered protective against HBV infection.

The 10 mIU anti-HBs standards included in triplicate with each testing series are produced by the HRL according to an SOP entitled "In-House Control: Total anti-HBs" (S. Lambert, 8/92). A pool of anti-HBs positive serum is calibrated to 10 mIU/mL by serial dilution in a controlled process serum-based diluent by using the quantitative AUSAB-EIA. When tested with the anti-HBs EIA, the final anti-HBs IHC reagent must consistently generate a value of 9-11 mIU. When tested using the anti-HBs RIA the final anti-HBs IHC reagent must consistently generate a value of 8-12 SRU. The in-house control is included in triplicate in each testing run and is the basis for determining whether an individual is immunologically protected against HBV infection. (See also section 14.)

The precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA. The HRL protocol generates a coefficient of variation of 5-10% within runs and 8-15% between runs.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

By definition, if controls do not conform to specifications, the test results are rejected. All samples are tested again. Data from nonqualifying test runs are not used.

Discard negative control values outside the range 0.5-1.5 times NC$\bar{x}$. Typically, all negative control values fall within 0.5-1.5 times NC$\bar{x}$. If a single negative control value is consistently outside this range or if there are more than two aberrant negative control values in a single run, check for technical problems and investigate assay technique. If three or more negative controls are outside the acceptable range, repeat the test.

The P/N ratio should be at least 20. If not, check for problems with the technique or for deterioration of reagents and repeat the run.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

(a) The sample is restricted to human serum or plasma.

(b) No interfering substances have been identified.

(c) Although the association of infectivity and the presence of HBsAg is strong, presently available methods for HBsAg detection may not be sensitive enough to detect all potentially infectious units of blood or possible cases of hepatitis.

**13. REFERENCE RANGES (NORMAL VALUES)**

Since 1993, final results have been expressed as ">10" or "<10" milli-International units (mIU). Results are determined by comparing each specimen value with the average value of the in-house control, which contains 10 mIU and is included in triplicate in each testing run. From 1988-1993, final results were expressed as "reactive" or "nonreactive" for HBsAg.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

A value greater than 10 mIU of anti-HBs is considered protective against HBV infection (13-15). However, this is a generic guideline since there is a considerable degree of error associated with this determination for reasons that may be technical or biologic in nature (16,17).

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may remain at 20-25 °C during preparation and testing for 4 hours.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Other FDA-licensed tests for total anti-HBs may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Substitution of test methods may not be done without approval from NCHS.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Test results are documented through the lab management database (see Section 3). Generally, studies conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

Specimens in long-term storage are arranged by study group. The storage location of each sample is listed with the test data.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Qualitative determination of the human antibody directed against hepatitis C virus (anti-HCV) in human serum or plasma is measured using direct solid-phase enzyme immunoassay. Human serum or plasma is diluted in specimen diluent and incubated with a polystyrene bead coated with recombinant HCV antigen. Following a 1-hour incubation, the beads are washed to remove unbound material. A peroxidase-conjugated antibody directed against human IgG is added to each bead. Following a 30-min incubation, the beads are washed again to remove unbound material. The beads are then transferred to 10- x 75-mm polystyrene tubes, and a peroxidase-specific chromogenic substrate solution is added to each tube. The substrate solution consists of hydrogen peroxide and o-phenylenediamine (OPD) in a citrate buffer. Following a 30-min incubation at 20-25 °C, 1 N sulfuric acid is added to stop the enzyme-substrate reaction.

Anti-HCV antibody will bind to the HCV antigen on the bead. Subsequently, the conjugate binds to that antibody. The reaction of the conjugate with the substrate solution results in the generation of an orange color. Absence of color indicates the absence of anti-HCV in the sample. The intensity of the color generated is measured spectrophotometrically at 492 nm. The instrument used to measure the test results is equipped with software that calculates a cutoff value. The cutoff calculation is based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HCV. Positive specimens are repeated in duplicate according to the same procedure. Repeatedly positive specimens are supplementally tested using the MATRIX instrument (Abbott Laboratories). Matrix is an unlicensed technology that is in use by the HRL under an Investigational New Drug (IND) agreement.

This is an FDA-licensed method commercially obtained in kit form (1-3). The literature and instructions in each kit constitute the standard operating procedure (SOP) for the method. Its diagnostic utility lies in its capacity to determine whether an individual has been infected with hepatitis C. The presence of these antibodies indicates that the individual has been infected with HCV.

## 2. SAFETY PRECAUTIONS

Test kits for anti-HCV contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, treat kit components as though they are capable of transmitting disease. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eyewear, and lab coat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware contaminated with serum in an plastic autoclave bag for disposal. We recommend Biosafety Level 2 containment procedures as described in CDC/NIH publication #88-8395 be used by those handling test specimens and kit reagents (4).

Material safety data sheets (MSDSs) for sodium azide, sulfuric acid, hydrochloric acid, o-phenylenediamine, and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) computer network. Risk is minimal due to the small quantity of chemicals, the safe packaging, and the limited handling by the operators using the test kits.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Raw data are transcribed manually from an instrument readout sheet into a computerized database. This database was custom-designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results, and functions within PARADOX software (Borland Co., Scott Valley, CA). Test values are compared with a cutoff value calculated from the controls. Results are expressed as "positive" or "negative" for anti-HCV. Other information in the database may typically include the HRL identification number, the specimen number, the date collected, the date tested and results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer. Electronically stored data are backed up routinely.

b. Finished data are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the supervisor will transmit the results to the NCHS mainframe computer along with the other NHANES III data.

c. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.

d. Documentation for data system maintenance is contained in printed copies of data records for 2 years.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Specimens submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (S. Lambert, 5/92).

b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

c. Specimens may be serum, recalcified plasma, or plasma. Serum specimens may be collected using regular red-top or serum-separator Vacutainers.

d. Required sample volume is 50 µL for the assay; 1.0 mL will permit repeat analyses as well.

e. Specimens may be stored in glass or plastic vials, as long as vials are tightly sealed to prevent desiccation of the sample.

f. Serum or plasma samples are collected aseptically to minimize hemolysis and bacterial contamination.

g. Samples are stored in labeled 2-mL Nalge cryovials or equivalent.

h. Serum is best stored frozen, and freeze/thaw cycles should be kept to a minimum. Store samples at 4-8 °C for no more than 5 days.

i. For storage >5 days, samples are held at ≤-20 °C. Samples held in long-term storage at ≤-20 °C are indexed in the database for easy retrieval.

j. Specimens are rejected if contaminated, hemolyzed, or stored improperly. However, rejection is done only after consultation with NCHS.

k. Avoid multiple freeze/thaw cycles.

l. Do not use heat-activated specimens.

m. Performance has not been established for cadaver specimens or body fluids other than serum or plasma (such as urine, saliva or pleural fluid.)

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION**

a. **Instrumentation**

(1) Abbott QWIKWASH, model 6258-27 (Abbott Laboratories, North Chicago, IL).

(2) Abbott COMMANDER dynamic incubator, model 6210-01, set at 40 °C (Abbott Laboratories).

(3) Abbott QUANTAMATIC spectrophotometer, model 7553 (Abbott Laboratories).

*Note: The Abbott instruments cited above are approved for use with these test kits as part of FDA licensure of the kits.*

(5) Gilson Pipetman micropipettors, 10- and 200-µL sizes (Rainin Instrument Co., Woburn, MA).

b. **Materials**

(1) Reaction trays, Costar cat.no. 4870 (VWR Scientific, Bridgeport, CT).

(2) Cover seals provided as part of the test kit (Abbott Laboratories).

(3) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(4) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).

(5) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).

(6) 2-mL cryovials, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(7) Cryovial boxes, cat. no. 5026-0909 (Nalge Company, Inc.).

(8) 1.5-mL microtubes (Marsh Biomedical Products, Rochester, NY).

(9) 50 mL-polypropylene tubes (Corning Glass Works, Corning, NY).

(10) 5.25% sodium hypochlorite (household bleach) (any vendor).

(11) Virotrol-I, cat. no. 00100 (Blackhawk Biosystems, Inc., San Ramon, CA)

(12) Abbott HCV EIA 2.0 kit, Hepatis C Virus Encoded Antigen (Recombinant C100-3), HC-31, cat. no. 4A1424 (Abbott Laboratories Diagnostics Division, Abbott Park IL).

c. **Reagents**

Abbott HCV EIA 2.0 kit contains the following reagents; prepared by the manufacturer. Volumes listed are for 100-test / 1000-test kits.

(1) Hepatitis C virus encoded antigen
recombinant c100-3, HC-31, and HC-34) coated beads -- 1 bottle (100 beads).

(2) Conjugate concentrate
3 vials (1-mL) / 3 vials (5-mL). Goat antibody to human IgG (H + L): Peroxidase (horseradish). Minimum concentration: 0.02 µg/mL in Tris buffer with calf serum and red dye no. 33. Preservatives: 0.01 g/dL gentamicin, 0.01 g/dL thimerosal.

(3) Conjugate diluent
3 vials (19 mL) / 3 vials (95 mL), containing 20 g/dL mixed goat and calf serum in Tris buffer. Preservatives: 0.01 g/dL gentamicin, 0.01 g/dL thimerosal.

(4) Negative control
1 vial (2 mL) / 2 vials (2 mL). Human plasma, nonreactive for antibodies to HCV, HBsAg, and HIV. Preservative: 0.1 g/dL sodium azide.

(5) Positive control
1 vial (2 mL) / 2 vials (2 mL). Inactivated human plasma reactive for anti-HCV, nonreactive for HBsAg and anti-HIV-1. Minimum titer 1:2. Preservative: 0.1 g/dL sodium azide.

(6) Specimen diluent
2 vial (20 mL) / 4 vials (100 mL) containing Tris buffer, 0.2 g/dL Triton X-100, protein lysates, and animal sera (goat, calf). Preservative: 0.01 g/dL sodium azide.

(7) o-Phenylenediamine -- 2HCL
(OPD) diluent, 1 bottle (55 mL) / 2 bottles (220 mL). Citrate-phosphate buffer containing 0.02% (v/v) hydrogen peroxide.

(8) o-Phenylenediamine -- 2HCL
(OPD) tablets, 1 bottle (10 tablets) / 2 bottles (40 tablets, each 12.8 mg).

d. **Reagent Preparation**

(1) Bring OPD reagents to 20-25 °C. Five to 10 min prior to color development, prepare the OPD substrate solution by dissolving the OPD tablets in diluent for OPD.

Using clean pipettes, transfer 5 mL of diluent for OPD into a suitable metal-free container for each tablet to be dissolved. Transfer the appropriate number of OPD tablets into a measured amount of diluent using nonmetallic forceps or equivalent. Allow the tablets to dissolve. *The OPD substrate solution must be used within 60 min of preparation and must not be exposed to strong light*. Just prior to dispensing for color development, swirl the container gently to obtain a homogeneous solution. Remove air bubbles from the dispenser tubing and prime dispenser prior to use.

(2) Bring the conjugate concentrate and conjugate diluent to 20-25 °C before mixing. Carefully empty the contents of a conjugate concentrate vial (with red dye) into a vial of conjugate diluent. This can be done most efficiently by slowly squeezing the small vial 2 to 3 times while maintaining the nozzle within the opening of the large vial. Avoid foaming. One vial of diluted conjugate is sufficient for 100 tests (100 test kit)/500 tests (1000 test kit). Reseal the large vial. Mix thoroughly by slowly inverting the vial several times. Do not vortex. Write the date of dilution and the expiration date in the space provided on the conjugate diluent label. The conjugate is stable for 14 days (not to exceed kit expiration date) after dilution when stored at 4-8 °C. Allow the diluted conjugate to equilibrate at 20-25 °C for approximately 60 min prior to use. If storing the diluted conjugate, store it at 4-8 °C. Bring it to 20-25 °C before use. Do not mix vials of diluted conjugate. Separate positive and negative controls must be run with each vial of diluted conjugate.

e. **Standards Preparation**

There are no standards or calibrators in the conventional sense used with these kits. Calibration is based on the results of defined "positive" and "negative" controls.

(1) Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls. The instrument automatically subtracts the blank value from each individual or sample value.

(2) Calculation of negative control mean absorbance ($NC\bar{x}$)

Determine the mean of the negative controls.

Individual negative control values must meet the following criteria:

(a) Individual control values must be less than or equal to 0.150 and greater than or equal to 0.010.

(b) Individual control values should be 0.54-1.46 times the negative control mean.

If one value doesn't meet either of the criteria, exclude it and recalculate $NC\bar{x}$ . All remaining individual control values must meet the above criteria, or the run should be repeated.

(3) Calculation of positive control mean absorbance ($PC\bar{x}$)

Determine the mean of the positive controls.

Individual positive control values must meet the following criteria:

(a) Individual control values must be less than or equal to 1.999 and greater than or equal to 0.400.

(b) Individual control values should be 0.66-1.34 times the positive control mean.

If one value doesn't meet either of the criteria, exclude it and recalculate $PC\bar{x}$. All remaining individual control values must meet the above criteria, or the run must be repeated.

(4) Assay run validity criteria

For the run to be valid, the difference between the mean absorbances of the positive and negative (P-N) must be 0.400 or greater. If not, poor technique or reagent deterioration should be suspected and the run repeated.

(5) An "in-house control" (IHC) is included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled "In-House Control: anti-HCV" (S. Lambert, 8/92). A pool of anti-HCV-positive serum is calibrated by serial dilution via controlled process in a serum-based diluent. *When tested by the anti-HCV EIA, the final anti-HCV IHC reagent must consistently generate a signal-to-cutoff ratio of 1.5 ± 0.5*.

f. **Preparation of Quality Control Materials**

(1) Kit positive and negative controls are prepared and quality-controlled by the manufacturer.

(2) In-house controls are prepared according to HRL specifications.

(a) Prepare serial dilutions of Virotrol-I in defibrinated, dialyzed, filtered plasma pool that is nonreactive for hepatitis C (Table 1). Use only polypropylene tubes for dilution series.

Table 1
Serial Dilutions for Quality Control Materials

| Dilution | Plasma Diluent (µL) | Diluted Virotrol (µL) |
|---|---|---|
| 1:2.5 | 900 | 600 |
| 1:5 | 750 | 750 of 1.25 dilution |
| 1:10 | 750 | 750 of 1:5 dilution |
| 1:20 | 750 | 750 of 1:10 dilution |
| 1:40 | 750 | 750 of 1:20 dilution |
| 1:80 | 750 | 750 of 1:40 dilution |

(b) Select a dilution that yields a signal-to-cutoff ratio between 1.2 and 1.9.

(c) Prepare sufficient volume for approximately 150 vials each containing 225-250 µL. Confirm values by testing in corresponding hepatitis C assays.

(d) If the values are confirmed to be within the appropriate range, dispense 225 -250 µL aliquots into labeled tubes. Store the sample at ≤-20 °C. Use only once as an in-house control and discard any residual sample.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibration curve is generated by the user as part of these assay methods. The calibration of instruments is either automatic or performed periodically by contracted service personnel.

b. **Verification**

(1) The instruments used to read assay results (Section 6.a.4.) are equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

(2) Repeat positive specimens in duplicate. Repeatedly positive specimens are confirmed with MATRIX.

(3) Retest specimens having absorbance values below 0.005 using the same product and test method in order to verify the initial test result because poor technique may have caused the low values. If the specimen has an absorbance value less than the cutoff when retested, the specimen may be considered negative for antibodies by the criteria of the kit. Further testing is not required.

(4) Specimens having absorbance values greater than or equal to cutoff are considered initially reactive by the criteria of the test kit. Before interpretation, however, retest the original sample in duplicate using the same product and test method. If either duplicate is reactive, the specimen may be interpreted to be repeatedly reactive for antibodies by the criteria of the test kit.

(5) Retest initially reactive specimens must be repeated in duplicate using the same product and test method. If both of the duplicate repeat tests are negative, the test is considered negative by the criteria of the test kit.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Reagents are used per kit of 100 or 1000 tests. Kit components are occasionally interchanged within a manufacturer's lot, but never interchanged between lots.

(2) Remove the test kit from 4-8 °C storage. Allow 30-40 min for reagents to warm to 20-25 °C. Swirl gently before use. Adjust the incubator to 40 °C ±2 .

(3) Assay the negative, positive, and in-house controls in triplicate adjacent to the specimens using one bottle of diluted conjugate in the assay with each run of specimens. Use one preparation of working reagents per run.

(4) Ensure that all reaction trays containing controls and samples are subjected to the same process and incubation times.

(5) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

(6) After each step, visually verify the presence of solution and bead in each well.

(7) Make certain that sufficient diluted conjugate is available for the test. If necessary, prepare additional conjugate reagent. Do not mix bottles of diluted conjugate.

(8) Run separate positive and negative controls with each vial of diluted conjugate.

(9) Follow the exact order of specimen and reagent addition as described in this test procedure.

### b. Sample Preparation

(1) Bring serum specimens to 20-25 °C. While one box or rack of samples is being pipetted, the other racks should be refrigerated.

(2) Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix specimens gently before testing.

(3) Identify the reaction tray wells for each specimen or control.

### c. Instrument Setup

(1) Operation of the Abbott QWIKWASH

The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

(a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the "Power" indicator on the instrument.

(b) Ensure that the "Low Pressure" and "Low Water Level" indicators are NOT illuminated before washing beads. See note below.

(c) Place bead tray on the QWIKWASH with the first row of beads aligned with the washing heads.

(d) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.

(e) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.

(f) Repeat until all of the beads have been washed, then proceed directly to the next step of the assay procedure.

> *NOTE: The wash water is held in a stainless steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on, the tank should be filled with deionized water AND the waste tank should be emptied. Never fill the water tank without also emptying the waste container! Add approximately 200 mL of bleach to the waste container prior to reconnecting it to the system so that waste water can be discarded down the sink as "decontaminated liquid waste." Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless steel pressure tank.*

(2) COMMANDER incubators
Set and verify the temperature of the incubator at 40 °C.

(3) Operation of the QUANTAMATIC

(a) After the final reaction has been stopped, place the tube rack(s) into the appropriate QUANTAMATIC carrier trays(s).

(b) Place the carrier tray(s) onto the QUANTAMATIC to be fed automatically into the tube pick-up area.

(c) On the instrument keypad, choose RUN ASSAYS. Answer the prompts as indicated in Table 2.

d. **Operation of Assay Procedure**

(1) Pipet 10 µL of controls or serum samples into designated wells of a reaction tray or into test tubes.

(2) Add 400 µL of specimen diluent (supplied with each kit) to each 10-µL sample and mix by gentle tapping.

(3) Add 200 µL of each diluted sample to the wells of a new reaction tray.

(4) Add one HCV antigen-coated bead to each well containing a diluted sample.

(5) Apply cover seal. Gently tap the tray to cover the beads and remove any trapped air. Incubate at 40 °C for 1 hour.

(6) Wash the beads with deionized water in the tray using a QWIKWASH bead washer.

(7) Add 200 µL of peroxidase-conjugated (goat) anti-human antibody to each well containing a bead.

(8) Cover the tray and incubate at 40 °C for 30 min.

(9) Wash the beads with deionized water in the tray using a QWIKWASH bead washer.

Table 2
QUANTAMATIC Run Parameters

| Prompt | Response |
|---|---|
| Run which assay? | Assay # from TABLE 1 |
| Lot #/Tech | Kit lot # & initials |
| Positive ID? | NO |
| Number of patients? | Number of patients |
| Tray 1 size - 20? | YES if tray size is 20<br>NO if tray size is 60 |
| Is tray in back track? | YES if tray is in the back track<br>NO if tray is in the front track |
| How many tubes in tray? | Total number of tubes |
| Enter pat no. ID | NO |
| List operator entries? | NO |
| Are trays ready? | YES if trays are ready to be read |

(10) Transfer the beads to 12- x 75-mm polystyrene tubes.

(11) Add 300 µL of substrate solution to each tube containing a bead.

(12) Incubate the tubes at 20-25 °C, *protected from direct light*, for 30 min.

(13) Stop the reaction with the addition of 1 mL of 1 N sulfuric acid.

(14) Read the reaction at 492 nm in the Abbott QUANTAMATIC spectrophotometer.

(15) Retest positive samples in duplicate using this procedure.

(16) Confirm repeatedly positive samples with the MATRIX assay.

e. **Recording of Data**

(1) Quality Control Data

Multiple positive and negative controls are averaged by the reading instrument and are determined to be valid or invalid. Raw data are transcribed manually from the instrument readout sheet into a computerized database.

(2) Analytical Results

For EIA, raw data are expressed as absorbance value. Raw data are transcribed manually from the instrument readout sheet into a computerized database.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Instruments are on service contract and, except for the most basic daily maintenance, are serviced by an Abbott technical representative.

(2) The following procedures are monitored and documented on a weekly basis:

- incubator temperature
- quality of water used in QWIKWASH
- refrigerator temperature
- freezer temperature
- room temperature

(3) All micro-pipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for re-calibration in accordance with manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) The cutoff calculation is done by the reading instrument and by the data management software which uses the following formula:

$$\text{Cutoff} = NC\bar{x} + (xPC/4)$$

(2) Calculate the negative control mean absorbance ($NC\bar{x}$) by determining the mean of the negative controls.

(3) Calculate the positive control mean absorbance ($PC\bar{x}$) by determining the mean of the positive controls.

h. **Special Procedure Notes**

(1) When dispensing beads, remove cap the from the bead bottle, attach the bead dispenser and dispense the beads into the wells of the reaction tray.

(2) Do not splash liquid while tapping trays.

(3) When washing beads, follow the directions provided with the washing apparatus.

(4) When transferring the beads from the wells to the assay tubes, align an inverted carton of tubes over their respective wells in the reaction tray. Press the tubes tightly over the wells and invert the tray and tubes together so that the beads fall into the corresponding tubes. Blot excess water from the top of the tubes.

(5) Avoid strong light during color development.

(6) Dispense acid in the same sequence as the OPD substrate solution.

(7) Do not allow acid or OPD substrate solution to contact metal.

(8) Remove air bubbles prior to reading absorbance.

(9) Visually inspect blank tubes and discard them if they have a yellow-orange color. If both tubes are contaminated, the entire run should be repeated.

## 9. REPORTABLE RANGE OF RESULTS

A normal value for HCV should be negative. Final reports express results qualitatively as positive or negative for the presence of anti-HCV antibody in the sample. No quantitative results are determined. All samples testing positive are retested in duplicate.

Specimens that repeatedly test positive are tested by MATRIX for confirmation.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the HRL for epidemiologic studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art.

This quality control system uses bench quality control samples. Positive and negative controls are included with kits. An

in-house control anti-HCV positive serum is calibrated by serial dilution in a controlled process serum-based diluent. When tested by using the anti-HCV EIA, the final anti-HCV in-house control (IHC) reagent must consistently generate a signal-to-cutoff ratio of 1.5 ± 0.5.

Three negative controls, three positive controls, and one in-house control are included in each analytical run (a set of consecutive assays performed without interruption). The presence or absence of anti-HCV is determined by comparing the absorbance value of the sample to the cutoff value. This cutoff value is calculated from the negative and positive control absorbance values (as explained in the calculations section). Specimens with absorbances greater than or equal to the cutoff value are considered reactive for anti-HCV.

For the run to be valid, the difference between the mean absorbances of the positive and negative (P-N) must be 0.400 or greater. If not, poor technique or reagent deteroriation should be suspected, and the run should be repeated.

Individual negative control values must meet the following acceptance criteria:

- Individual control values must be less than or equal to 0.150, and greater than or equal to 0.010.
- Individual control values should be 0.54-1.46 ($NC\bar{x}$) times the negative control mean.

An IHC is included with each testing series. The IHC reagent is produced by the HRL according to an SOP entitled In-House Control: anti-HCV (S. Lambert, 8/92). A pool of anti-HCV positive serum is calibrated by serial dilution in a controlled process of serum-based diluent. When tested using the anti-HCV EIA, the final anti-HCV IHC reagent must consistently generate a signal-to-cutoff ratio of 1.5 ± 0.5.

Precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA.

This method generates coefficients of variation (CVs) of 5-10% within runs and 8-15% between runs in the HRL.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. By definition, if controls do not conform to specifications, the testing is rejected. All samples are tested again. Data from nonqualifying test runs are not used.

b. If one negative or positive value fails to meet either of the acceptance criteria in the quality control procedures, it must be excluded and the $NC\bar{x}$ or $PC\bar{x}$ recalculated. All remaining individual control values must meet the criteria or the run should be repeated.

c. For the run to be valid, the difference between the mean absorbances of the positive and negative (P-N) must be 0.400 or greater. If not, poor technique or reagent deteroriation should be suspected and the run repeated.

d. An assay run is considered valid with respect to substrate blank if the blank has an absorbance value greater than or equal to -0.020 and less than or equal to 0.040. The user must determine assay validity due to substrate blank.

   If the substrate blank falls outside the acceptable range, the preparation of the substrate is in question and the alternate blank may be used. If it is unacceptable, the run must be repeated.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The sample is restricted to human serum or plasma. No interfering substances are identified. Closely monitor this procedure and the interpretation of results when testing serum or plasma specimens for the presence of antibody to HCV. Do not use heat-activated specimens. A negative test does not exclude the possibility of exposure to or infection with HCV. Negative results in this assay in individuals with prior exposure to HCV may be due to antibody levels below the limit of detection of the assay or lack of antibody reactivity to the HCV antigens used in this assay. Specimens may contain antibodies to either vector proteins or fusion proteins associated with the HCV recombinant antigens. Vector and/or fusion protein antibody-containing specimens may demonstrate reactivity that is unrelated to HCV infection. Additional, more specific, tests may be useful in defining the true HCV antibody reactivity.

## 13. REFERENCE RANGES (NORMAL VALUES)

All normal noninfected humans should have negative values for hepatitis C.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may remain at 20-25 °C during preparation and testing for 4 hours.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Other FDA-licensed tests for total anti-HCV may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Substitution of test methods may not be done without approval from the NCHS.

Alternate storage is not recommended.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Test results are documented through the lab management database (Section 3). Generally, studies conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

Specimens in long-term storage are arranged by study group. The storage location of each sample is listed with the test data.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

**REFERENCES**

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Qualitative determination of antibody to hepatitis D virus or delta antigen (anti-HDV) contained in human serum or plasma is measured by using competition enzyme-immunoassay (1-3). Test samples are mixed with a peroxidase-conjugated anti-HDV in the wells of a reaction tray. A bead coated with HDV antigen is added to each well. Following an overnight incubation at 20-25 °C, the beads are washed to remove unbound material. The beads are transferred to 10- X 75-mm polystyrene tubes. A peroxidase-specific chromogenic substrate solution is added to each tube containing a bead. The substrate solution consists of hydrogen peroxide and o-phenylenediamine in a citrate buffer. Following a 30-minute incubation at 20-25 °C, sulfuric acid is added to stop the enzyme-substrate reaction. An orange color is proportional to the amount of conjugated anti-HDV bound to the bead, and thus, inversely proportional to the amount of anti-HDV in the sample. The intensity of the color generated is measured spectrophotometrically at 492 nm.

Anti-HDV antibody competes with the conjugated anti-HDV antibody for binding sites on the bead. A reduction in signal generated by conjugated anti-HDV bound to the HDV antigen on the bead is an indication that anti-HDV is contained in the sample. A cutoff value is calculated from assay controls by the instrument which reads the color intensity. An absorbance value less than the cutoff value is indicative of a sample containing anti-HDV antibody. Results are expressed as "positive" or "negative" for anti-HDV.

The presence of anti-HDV antibody in a serum specimen indicates that the donor has been infected with hepatitis D virus. Because HDV can only replicate in the presence of hepatitis B virus (HBV), anti-HDV is an indirect indicator of HBV infection as well. This is an FDA-licensed method commercially obtained in kit form. The literature and instructions that come with each kit constitute the standard operating procedure (SOP) for the method.

## 2. SPECIAL SAFETY PRECAUTIONS

Test kits for anti-HDV contain components derived from human serum or plasma. Although various treatments in the manufacturing process are sufficient to inactivate most blood-borne pathogens, there is no assurance that these reagents are entirely noninfectious. Therefore, they should be treated as though capable of transmitting disease. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and HBV. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method because of infectious contamination hazards. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. We recommend Biosafety Level 2 containment and practice as described in CDC/NIH publication #88-8395 for handling test specimens and kit reagents (4).

Material Safety Data Sheets (MSDSs) for sodium azide, sulfuric acid, hydrochloric acid, o-phenylenediamine (OPD) and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) Local Area Network (LAN). Risk is minimal because of the small quantity of chemicals, the packaging of the chemicals, and limited handling of chemicals by the operators using the test kits.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Raw data are transcribed manually from the instrument readout sheet into a computerized database. This database was custom designed for the management of CDC Hepatitis Reference Laboratory (HRL) test results. It functions within the PARADOX software program (v. 3.5 Borland, Scott Valley, CA). Test values are compared with a cutoff value that is calculated from controls. Results are expressed as "positive" or "negative" for anti-HDV. Other information in the database may typically include the HRL identification number, specimen number, date collected, date tested, and results of testing for other hepatitis markers. Reporting is done directly from the database in printed form or by electronic transfer. Electronically stored data are backed up routinely.

b. Finished records are reviewed by the supervisor. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the study sponsor will transmit the values to the mainframe computer of the National Center for Health Statistics (NCHS) along with the other NHANES III data.

c. Files stored on the LAN or CDC mainframe are automatically backed up nightly to tape by CDC Data Center staff.

d. Documentation for data system maintenance is contained in printed copies of data records for 2 years.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Specimens that are submitted for testing are handled according to the HRL SOP entitled "Sample Handling" (S. Lambert, 5/92).

b. No special instructions such as fasting or special diets are required. Diurnal variation is not a major consideration.

c. Specimens should be serum, plasma, or recalcified plasma. Serum specimens may be collected by using regular red-top or serum separator Vacutainers.

d. The minimum required sample volume for the anti-HDV assay is 100 µL. A 1-mL sample will permit repeat analyses as well.

e. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

f. Serum or plasma samples are collected aseptically to minimize hemolysis and bacterial contamination.

g. Samples are stored in labeled 2-mL Nalge cryovials or equivalent.

h. Serum is best stored frozen. However, freeze/thaw cycles should be kept to a minimum. Specimens may be stored at 4-8 °C for 5 days.

i. For storage >5 days, samples are held at ≤-20 °C. Samples held in long-term storage at ≤-20 °C are indexed in the database for easy retrieval.

j. Specimens are rejected if contaminated, hemolyzed or stored improperly. However, rejection is done only after consultation with NCHS.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) Abbott QWIKWASH, model 6258-27, for washing solid-phase beads between incubation steps (Abbott Laboratories, North Chicago, IL).

(2) Abbott QUANTAMATIC spectrophotometer, model 7553, set at 492 nm for reading EIA results (Abbott Laboratories).

(3) Gilson Pipetman adjustable micropipettors (Rainin Instrument Co.) or equivalent are used for pipeting samples and reagents. They are calibrated every 6 months.

d. **Other Materials**

(1) Abbott Anti-Delta Enzyme Immunoassay Kit, cat no. 301822 (Abbott Laboratories). Each kit includes the following:

(a) Delta antigen (woodchuck) coated beads -- 1 bottle (50 beads).

(b) Anti-delta conjugate -- 1 vial (5 mL). Antibody to delta antigen (human): Peroxidase (horseradish). Minimum concentration: 0.05 µg/mL in Tris buffer with animal serum (calf) and red dye no. 33.

Preservative: 0.01% gentamicin, 0.01% thimerosal.

(c) Negative control anti-delta -- 1 vial (1.5 mL). Recalcified human plasma, nonreactive for anti-delta and HBsAg. Preservative: 0.01% gentamicin sulfate.

(d) Positive control anti-delta -- 1 vial (1 mL). Recalcified human plasma, positive for anti-delta and HBsAg. Anti-delta minimum titer 1:200 ± 2 $\log_2$ dilutions. Preservative: 0.01% gentamicin sulfate.

(e) OPD ● 2HCL (OPD) tablets -- 1 bottle (10 tablets). OPD/tablet:12.8 mg.

(f) Diluent for OPD -- 1 bottle(55 mL)/2 bottles(220 mL). Citrate-phosphate buffer containing 0.02% hydrogen peroxide.

(g) Positive and negative controls.

(h) Cover seals.

(2) Costar reaction trays, cat. no. 4870 (VWR Scientific, Bridgeport, CT).

(3) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(4) 1 N sulfuric acid, cat. no. 7212 (Abbott Laboratories).

(5) Pipet tips, cat. nos. RT20 & RT200 (Rainin Instrument Co.).

(6) Protective gloves, Tronex or Flexam, small/medium/large (Best Manufacturing, Menlo Park, GA).

(7) Cryovials, 2-mL, cat. no. 5000-0020 (Nalge Company, Inc., Rochester, NY).

(8) Cryovial boxes, cat. no. 5026-0909 (Nalge).

(9) Microtubes, 1.5-mL (Marsh Biomedical Products, Rochester, NY).

(10) Polypropylene tubes, 50-mL (Corning Glass Works, Corning, NY).

(11) 10% sodium hypochlorite solution, household bleach (any vendor) .

c. **Reagent Preparation**

Preparation of OPD substrate solution
Bring OPD reagents to 20-25 °C. Five to 10 min before the end of the incubation period, prepare the OPD substrate solution by dissolving the OPD tablets in diluent for OPD. Using clean pipettes and metal-free containers, transfer 5 mL of diluent for each tablet to be dissolved, into a suitable container. Transfer an appropriate number of OPD tablets into the measured amount of diluent using nonmetallic forceps or their equivalent. Allow the tablets to dissolve. Use the solution within 60 min of preparation. Do not expose it to strong light.

Just prior to dispensing the solution for color development, swirl the container gently to obtain a homogeneous solution. Remove air bubbles from the dispenser tubing and prime the dispenser prior to use.

d. **Standards Preparation**

This method does not employ conventional calibrators or standards. Calibration is based on the results of defined positive and negative controls. Positive and negative control reagents are supplied with each test kit. The assay cutoff value is calculated from values obtained from these controls.

e. **Preparation of Quality Control Materials**

Kit positive and negative controls are prepared and quality monitored by the manufacturer.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

No calibration curve is generated by the user as part of this method. Calibration of instruments is either automatic or is carried out periodically by contracted service personnel.

### b. Verification

The instrument used to read assay results (see section 6.e.3) is equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the product literature, the entire series is invalidated by the instrument.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Remove the test kit from 4-8 °C storage. Allow 30-40 min for the reagents to warm to 20-25 °C.

(2) Reagents are used per kit of 100 tests. Components of kits from the same manufacturer's lot are occasionally interchanged, but components of kits from different lots never are.

(3) Assay three negative and two positive controls with each run of specimens.

(4) Ensure that all reaction trays containing controls and samples are subjected to the same process and incubation times.

(5) Once the assay has been started, complete all subsequent steps without interruption.

### b. Sample Preparation

(1) Bring serum specimens to 20-25 °C.

(2) Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix the samples gently before testing.

(3) Identify the reaction tray wells for each specimen or control.

### c. Instrument Setup

(1) Operation of the Abbott QWIKWASH
The Abbott QWIKWASH is a semi-automated instrument that is used to wash the beads of the Abbott immunoassays between reagent steps. The wash solution is deionized water.

(a) Turn on the QWIKWASH using the toggle switch on the back of the instrument. The vacuum pump will come on, as will the power indicator on the instrument.

(b) Ensure that the "Low Pressure" and "Low Water Level" indicators are not illuminated before the beads are washed. See note below.

(c) Place the bead tray on the QWIKWASH while aligning the first row of beads with the washing heads.

(d) Push down on the handle on the top of the instrument. The beads will automatically go through one wash cycle, which will take about 4 sec.

(e) Raise the handle and slide the tray over until the second row of beads is aligned with the washing heads.

(f) Repeat until all of the beads have been washed, then proceed directly to the next step of the assay procedure.

> *NOTE: The wash water is held in a stainless steel pressure tank near the instrument. Waste water is collected in a plastic container, also nearby. When the "Low Water Level" light on the instrument comes on, the tank should be filled with deionized water and the waste tank should be emptied. Never fill the water tank without also emptying the waste container! Add approximately 200 mL of bleach to the waste container prior to reconnecting it to the system so that waste water can be discarded down the sink as decontaminated liquid waste. Never put any solution other than deionized water into the water tank. If the "Low Pressure" light on the instrument comes on, check the connections and seals on the stainless steel pressure tank.*

(2) Operation of the QUANTAMATIC

(a) After the final reaction has been stopped, place the tube rack(s) into the appropriate QUANTAMATIC carrier trays(s).

(b) Place the carrier tray(s) onto the QUANTAMATIC so that they can be automatically fed into the tube pick-up area.

(c) On the instrument keypad, choose RUN ASSAYS. Answer the prompts as shown in Table 1.

Table 1
QUANTAMATIC Run Settings

| Prompt | Reponse |
|---|---|
| Run which assay? | Assay # from TABLE 1 |
| Lot #/Tech | Kit lot # & initials |
| Positive ID? | NO |
| Number of patients? | Type number of patient samples |
| Tray 1 size - 20? | YES if tray size is 20, NO if tray size is 60 |
| Is tray in back track? | YES if tray is in the back track, NO if tray is in the front track |
| How many tubes in tray? | Total number of tubes |
| Enter pat no. ID | NO |
| List operator entries? | NO |
| Are trays ready? | YES if trays are ready to be read |

The QUANTAMATIC is programmed by the manufacturer to read the results of these assays methods. The user follows a simple set of menu-driven questions regarding the number and placement of samples to be read.

d. **Operation of Assay Procedure**

(1) Record the position of each control or specimen in the tray.

(2) Pipette 100 µL of antibody conjugate solution into the appropriate wells of a reaction tray.

(3) Pipette 100 µL of each control or specimen into the wells containing antibody conjugate solution. Mix thoroughly by vigorously tapping the tray. Do not splash.

(4) Add an HDV antigen-coated bead to each well.

(5) Apply the cover seal. Gently tap the tray. Incubate the tray at 20-25 °C overnight (20 ± 2 hours).

(6) Remove the cover seal. Wash the beads with deionized water in the tray using the QWIKWASH bead washer.

(7) Transfer the beads to 12- x 75-mm polystyrene tubes.

(8) Add 300 µL of substrate solution into two empty tubes (substrate blanks) and into each tube containing a bead.

(9) Cover and incubate the solution at 20-25 °C, away from direct light, for 30 min.

(10) Stop the reaction by adding 1 mL of 1 N sulfuric acid.

(11) Visually inspect blank tubes and discard any with yellow-orange color. If both blank tubes are contaminated, the entire run should be repeated.

(12) Measure the absorbance at of each tube at 492 nm using the Abbott QUANTAMATIC spectrophotometer.

e. **Recording of Data**

(1) Quality control data
Multiple positive and negative controls are averaged by the reading instrument and are determined to be valid or invalid. Raw data are transcribed manually from the instrument readout sheets into a computerized database.

(2) Analytical results
For EIA, raw data are expressed as absorbance values. Raw data are transcribed manually from the instrument readout sheet into a computerized database.

(a) Specimens having absorbance values greater than the cutoff value are considered nonreactive and therefore negative by the criteria of the kit.

(b) Specimens having absorbance values lower than or equal to the cutoff value are considered reactive by the criteria of the test kit.

(c) Specimens having absorbance values within 10% of the cutoff value are retested.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Instruments are self-monitoring and maintained under service contract and except for the most basic daily maintenance, they are serviced by an Abbott technical representative.

Incubator temperature, quality of water used in QWIKWASH, refrigerator temperature, freezer temperature, and room temperature are monitored and documented on a weekly basis.

(2) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) Cutoff calculation is done by the reading instrument and by the data management software, which uses the following formula:

Cutoff = (0.4 X NC)+(0.6 X PC).

(2) To calculate the negative control mean absorbance ($NC\bar{x}$), average the individual absorbance values of the three negative controls.

(3) To calculate the positive control mean absorbance ($PC\bar{x}$), determine the mean of the two positive controls.

(4) To determine the cutoff value, use the following formula:

Cutoff = $0.4(NC\bar{x}) + 0.6(PC\bar{x})$.

(5) To determine N-P, use the following formula:

N-P = $PC\bar{x} - NC\bar{x}$.

For the run to be valid, the difference between the mean absorbencies of N-P must be 0.300 or greater. If not, errors in technique or reagent deterioration should be suspected and the run repeated.

h. **Special Procedure Notes**

(1) When dispensing the beads, remove the cap from the bead bottle, attach the bead dispenser, and dispense the beads into wells of the reaction tray.

(2) Do not splash liquid while tapping the trays.

(3) When washing beads, follow the directions provided with the washing apparatus.

(4) When transferring beads from wells to assay tubes, align an inverted carton of tubes over their respective wells in the reaction tray. Press the tubes tightly over the wells and invert the tray and tubes together so that beads fall into their corresponding tubes. Blot excess water from the top of the tubes using the tube carton.

(5) Avoid strong light during color development.

(6) Dispense acid in the same sequence as the OPD substrate solution.

(7) Do not allow acid or OPD substrate solution to contact metal.

(8) Remove air bubbles prior to reading absorbance.

(9) Visually inspect blank tubes and discard any that have a yellow-orange color. If both blank tubes are contaminated, repeat the entire run.

## 9. REPORTABLE RANGE OF RESULTS

Final results are expressed qualitatively as positive or negative for the presence of anti-HDV antibody in the sample. No quantitative results are determined.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the Hepatitis Reference Laboratory for epidemiologic studies. This method has proven to be accurate, precise, and reliable. The instrumentation used is state-of-art.

This quality control system uses bench quality control samples. Positive and negative controls are included with kits. Three negative and two positive controls are included in each analytical run (a set of consecutive assays performed without interruption). The presence or absence of anti-HDV is determined by comparing the absorbance value of the sample to the cutoff value. This cutoff value is calculated from the negative and positive control absorbance values as explained in the calculations section. Specimens having absorbance values lower than or equal to the cutoff value are considered reactive by the criteria of the test kit.

Individual negative control values should be 0.5-1.5 times $NC\bar{x}$. If one value falls outside the range, discard that value and recalculate $NC\bar{x}$. If two values are outside this range, repeat the run. If more than one value is consistently outside the range, look at possible technique problems.

For the run to be valid, the difference between the mean absorbances of N-P must be 0.300 or greater. If not, errors in technique or reagent deterioration should be suspected and the run repeated.

The precision of these procedures is as claimed for licensure and is maintained by the manufacturer under the authority of the FDA.

The experience of the HRL is that this kit generates coefficients of variation (CVs) of 5-10% within runs and 8-15% between runs.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

(a) By definition, if controls do not conform to specifications, reject the test results and retest all samples. Do not use data from nonqualifying test runs.

(b) An assay run is considered valid with respect to the substrate blank if the blank has an absorbance value from -0.020 to 0.040 in order for the assay to be valid. The determination of assay validity due to the substrate blank must be done by the user.

If the substrate blank falls outside the expected range, consider the preparation of the substrate to be in question and use the alternate blank. If the alternate blank is unacceptable, repeat the run.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

No interfering substances have been identified. The Abbott anti-delta EIA is limited to the detection of anti-delta in human serum or plasma.

Specimens containing sodium azide may give false-positive reactions and should not be tested.

**13. REFERENCE RANGES (NORMAL VALUES)**

Normal human serum should be negative for hepatitis anti-delta antibodies.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may remain at 20-25 °C for 4 hours during preparation and testing.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Other FDA-licensed tests for total anti-HDV may be substituted but must be accompanied by validation data to show substantial equivalence with these assays. Substitution of test methods may not be done without approval from NCHS. In case of system failure, specimens may be refrigerated at 4-8 °C for 5 days. For longer periods, specimens should be stored at $\leq$-20 °C until the system is functioning.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Test results are documented through the lab management database (Section 3). Generally, studies that are conducted in the HRL are sponsored by a CDC epidemiologist who communicates the findings to other participants in the study. Final reports may be electronic or in printed form. All electronically held data are backed up routinely.

In long-term storage, specimens are arranged by study group. The storage location of each sample is listed with the test data. Standard record-keeping means (e.g., electronic, mainframe data files, laboratory notebook, floppy disks) should be used to track specimens. We recommend that records, including QA/QC data and duplicate records, be maintained for 10 years.

Only numerical identifiers (e.g., case ID numbers) should be used.

## 19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

## REFERENCES

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## ACKNOWLEDGMENTS

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Although extensive antigenic cross-reactivity exists between the two viral types of herpes, a viral glycoprotein specific for herpes simplex virus type 2 (HSV-2) (designated gG-2), and a glycoprotein specific for herpes simplex virus type 1 (HSV-1) (designated gG-1) have been identified. Monoclonal antibodies and affinity chromatography have been used to purify these glycoproteins and thus provide antigens for type-specific herpes serologic assays. Solid-phase enzymatic immunodot assays are used to detect antibodies reactive to these antigens. The purified glycoprotein, gG-1 or gG-2, is adsorbed to the center of a nitrocellulose disk. The rest of the disk surface is coated with bovine serum albumin (BSA) to prevent further nonspecific protein adsorption. Incubation of test serum with the disk allows specific antibodies, if present, to bind to the immobilized antigen. After extensive washing to remove nonreactive antibodies, the bound antibodies are detected by sequential treatment with peroxidase-conjugated goat-anti-human IgG and the enzyme substrate ($H_2O_2$ with chromogen 4-chloro-1-naphthol). A positive reaction is demonstrated by the appearance of a blue dot at the center of the disk. Serum reactive to an immunodot charged with gG-1 indicates previous and probable latent HSV-1 infection. Serum reactive with gG-2 indicates previous and probable latent HSV-2 infection.

HSV-1 is typically associated with infection of the upper body. HSV-2 commonly infects the genitalia and is primarily transmitted sexually. Perinatal transmission, usually of HSV-2, is comparatively infrequent but results in a severe, often fatal disease in newborns. Assays that can detect and distinguish antibodies to these viruses are of clinical and epidemiological importance.

## 2. SPECIAL SAFETY PRECAUTIONS

All human serum specimens are pretreated with 0.5% (v/v) Triton X-100 to inactivate enveloped viruses, including the human immunodeficiency virus, which may be present. However, observe universal precautions. Wear gloves, a lab coat, and safety glasses when handling all human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container. Discard all disposable glassware into a sharps waste container. Place all liquid hazardous waste materials in closed containers labelled as hazardous waste and stating the composition of waste being contained. These materials are decontaminated by autoclaving at 250 $^{o}$F, 19 pounds pressure, for 1 hour.

Protect all work surfaces by absorbent benchtop paper. Discard the benchtop paper into the biohazard waste container daily or whenever blood contamination occurs. Wipe down all work surfaces with 10% (v/v) sodium hypochlorite weekly. Material Safety Data Sheets (MSDSs) for sodium hypochlorite, Triton X-100, 4-chloro-1-napthol, methanol, and hydrogen peroxide are maintained in the Emory School of Medicine.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (OUTPUT.TXT) on a 5¼" high-density (HD) floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, or plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. After the data are calculated and the final values are approved by the reviewing supervisor for release, the data entry clerk transcribes the results into the NHANES III data base using Rbase software; data entry is proofed by the supervisor and clerk. Periodically, data from the laboratory database are downloaded onto floppy diskettes and delivered to the data manager of the Centers for Disease Control and Prevention.

   After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete) the NHANES laboratory supervisor transmits the values to the NCHS mainframe computer along with the other NHANES III data.

c. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff or CDC Data Center staff, respectively.

d. Documentation for data system maintenance is contained in hard copies of data records.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are necessary. Blood is collected in a red-top Vacutainer tube by standard venipuncture procedures.

b. Specimens for HSV-1 and HSV-2 analysis should be fresh or frozen serum.

c. A 0.5-mL sample of serum is preferable. The minimum sample volume required for analysis is 50 µL. Specimens are rejected if insufficient quantity is available for analysis.

d. The appropriate amount of serum is dispensed into a Nalgene cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, specimens are stored at ≤-20 °C until analyzed. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at ≤-20 °C. Samples thawed and refrozen several times are not compromised, but multiple brief freeze/thaw cycles should be avoided.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable to this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Multiple-head 96-hole puncher (Emory University, Atlanta, GA).

(2) TekPro rotating platform (American Hospital Supply Corp., Evanston, IL).

(3) Manual ELISA washer (Corning Glassworks, Corning, N.Y.)

b. **Other Materials**

(1) Nitrocellulose membrane sheets (Schleicher & Schuell, Inc., Keene, NH).

(2) Polyvinyl chloride plates, 96-well (Dynatech Laboratories, Inc., Alexandria, VA).

(3) Microsyringe fitted with a repeating dispenser (Hamilton Co., Reno, NV).

(4) Bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO).

(5) Hydrogen peroxide, $H_2O_2$, 30% (Sigma Chemical Co.).

(6) 4-chloro-1-napthol, $C_{10}H_7ClO$ (Sigma Chemical Co.).

(7) Methanol, $CH_3OH$ (Sigma Chemical Co.).

(8) Horseradish peroxidase-conjugated goat anti-human IgG (Jackson Immunological Laboratory, West Grove, PA).

(9) 0.55% Triton X-100 in Tris-buffered saline.

(10) Antigens, gG-1 and gG-2 (Dr. Lenore Periera, University of California, San Francisco, CA).

(11) Tris-HCl (Sigma Chemical Co., St. Louis, MO).

(12) Trizma base (Sigma Chemical Co.).

(13) Sodium chloride (NaCl) (Sigma Chemical Co.).

(14) Distilled water (American Sterilizer Co., Erie, PA).

(15) In-house HSV-1 and HSV-2 positive and negative control serum (Emory University, Atlanta, GA).

c. **Reagent Preparation**

(1) gG-1 and gG-2 Antigens
The gG-1 and gG-2 antigens have been prepared by affinity chromatography using specific monoclonal antibodies (H1379-2 and H1206), respectively. The purified materials are diluted 1:64 in Tris-buffered saline (pH 7.2) before they are used.

(2) Conjugate solution
Horseradish peroxidase-conjugated goat anti-human IgG. Dilute 1:1000 in phosphate-buffered saline (pH 7.2) containing 3% bovine serum albumin and 1% goat serum.

(3) Buffer solution
Tris-buffered saline (pH 7.2) containing 3 g/dL bovine serum albumin.

(4) Substrate solution
6 mg 4-chloro-1-napthol ($C_{10}H_7ClO$) dissolved in 2 mL methanol mixed with 10 mL TBS and 5 µL of 30% (v/v) hydrogen peroxide($H_2O_2$).

(5) Tris-buffered saline (TBS), pH 7.2
Dissolve 6.6 g Tris-HCl, 1.0 g Trizma base, and 11.6 g NaCl and bring to volume with 1000 mL distilled water in a 1-L flask.

d. **Standards Preparation**

There are no standards used in this assay, since no calibration curve is generated as part of this method.

e. **Preparation of Quality Control Materials**

In-house HSV-1, HSV-2, and negative control serum pools were prepared at Emory University. High-titered serum samples from patients with primary HSV-1 infection were pooled and then diluted to be used as HSV-1 positive controls. Serum samples from convalescent patients with primary HSV-2 infections were pooled, diluted and used as HSV-2 positive controls. Both positive pools are monospecific, i.e. they do not cross-react with the other virus type. Serum samples from healthy donors, nonreactive to both HSV types in the screening ELISA, were pooled, diluted, and used as negative controls. The dilution scheme for controls is shown in Table 1.

Table 1
Dilution for Controls

| Control | Dilution(s) |
|---|---|
| HSV-1 positive pool 1 | 1:800, 1:3200 |
| HSV-1 positive pool 2 | 1:50, 1:200 |
| HSV-2 positive pool 1 | 1:400, 1:1600 |
| HSV-2 positive pool 2 | 1:50. 1:200 |
| HSV negative pool | 1:50 |

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibration curve is generated by the user as part of these assay methods.

b. **Verification**

Verification for this assay is not possible in the conventional manner. The investigators who read assay results are trained to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the protocol, the results for the entire series are invalidated, and the series is retested in duplicate to confirm the initial test result.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Prepare dilutions of controls, conjugate, buffer, substrate, and antigens.

(2) Assay one negative and two positive controls in duplicate for each virus type with each run of specimens.

(3) Ensure that all disks and plates are subjected to the same process and incubation times.

(4) Once the assay has been started, complete all subsequent steps without interruption and within the recommended time limits.

### b. Sample Preparation

(1) Bring serum specimens to 20-25 °C.

(2) Mix serum samples gently before testing to eliminate stratification which may occur when serum is frozen or stored at 4 °C for extended periods.

(3) Identify the reaction tray wells for each specimen or control.

(4) Dilute test serum initially 1:10 in 0.55% Triton X-100 in TBS. After the incubation at room temperature, further dilute with Tris-buffered saline (pH 7.2) containing 3% bovine serum albumin, to a final serum dilution of 1:50.

### c. Instrument Setup

There is no instrument required for this solid-phase enzymatic immunodot assay. Purified HSV-1 or HSV-2 antigens are immobilized on a small disk and incubated with test serum. A positive reaction is demonstrated by the appearance of a bluish-purple dot at the center of the disk.

### d. Operation of Assay Procedure

HSV-1 and HSV-2 assays are run simultaneously in separate wells. Half of each plate is precoated with gG-1 antigen for HSV-1; the other half of the plate is precoated with gG-2 antigen for HSV-2.

(1) Prepare and deposit small disks of nitrocellulose membrane directly in the 96-well polyvinyl chloride plates with a 96-hole punch.

(2) Wash nitrocellulose disks in each well once with distilled water. Dry the discs completely at 20-25 °C.

(3) Onto the center of each disk, deliver 1 µL of appropriatly diluted antigen with a microsyringe fitted with a repeating dispenser.

(4) After drying the disks at 20-25 °C overnight, wash them twice with TBS for 10 min each.

(5) Add 100 µL of buffer containing 3% BSA to each well and incubate at 20-25 °C for 30 min on a rotating platform.

(6) Remove the buffer by suction.

(7) Add 100 µL of diluted serum or control to duplicate wells and incubate at 20-25 °C overnight on a rotating platform.

(8) Remove the serum from each well by suction using the manual washer. Add 100 µL of TBS and incubate at 20-25 °C for 10 min on rotator. Remove the TBS by suction. Repeat this procedure two times. Add 100 µL of buffer (3% BSA) to each well and incubate for 30 min.

(9) Remove the buffer by suction. Add 100 µL diluted conjugate solution to each well and incubate at 20-25 °C for 2 hours on a rotating platform.

(10) Remove the conjugate by suction. Add 100 µL TBS to each well and incubate at 20-25 °C for 10 min on rotator. Remove TBS by suction. Repeat this procedure two times.

(11) Remove the TBS by suction. Add 100 µL of freshly prepared substrate solution to each well.

(12) After 15 min, stop the reaction by removing the substrate and washing the plate twice with distilled water.

(13) Dry the plates overnight at room temperature in the dark. Examine the disks for color development. A positive reaction is demonstrated by the appearance of a bluish-purple dot at the center of the disk.

e. **Recording of Data**

(1) Quality Control Data
Positive and negative controls are determined to be valid or invalid. Results of each dilution of assay controls are recorded in standard forms as the test results are read by the investigators. The sample data are then entered into the computer database.

(2) Analytical Results
Results of each assay sample are recorded in standard forms as the test results are read by the investigators. The sample data are then entered into the computer database.

**f. Replacement and Periodic Maintenance of Key Components**

(1) Monitor and document the refrigerator temperature, freezer temperature, and room temperature on a weekly basis.

(2) Pipettors

All micropipettors that are used in testing clinical specimens should be checked for calibration every six months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

h. **Special Procedure Notes - Emory University**

(1) With the availability of mouse monoclonal antibodies, it has become possible to purify HSV-2 proteins that fail to express type-common antigenic determinants detectable in serological assays.

(2) The use of gG-2, purified from extracts of HSV-2-infected cells, led to the development of an assay of high sensitivity, specificity, and reproducibility.

(3) This immunodot assay is suitable for large numbers of serum samples because it requires a small amount of purified glycoprotein.

(4) Purified gG-2 retains antigenicity at ≤-70 °C for over 5 years if stored in glass (but not plastic) ampules.

(5) BSA from different sources could cause significant reductions in the sensitivity of the gG-2 assay. This problem is overcome by testing different batches of BSA from several sources and choosing a large stock of the best batch.

**9. REPORTABLE RANGE OF RESULTS**

Final reports express results as positive or negative for the presence of anti-HSV-1 or HSV-2 antibody in the sample.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Although extensive antigenic cross-reactivity exists between the two viral types of herpes, a viral glycoprotein specific for herpes simplex virus type 2 (HSV-2) (designated gG-2), and a glycoprotein specific for herpes simplex virus type 1 (HSV-1) (designated gG-1) have been identified. Monoclonal antibodies and affinity chromatography have been used to purify these glycoproteins and thus provide antigens for type-specific herpes serologic assays. Solid-phase enzymatic immunodot assays are used to detect antibodies reactive to these antigens. The purified glycoprotein, gG-1 or gG-2, is adsorbed to the center of a nitrocellulose disk. The rest of the disk surface is coated with bovine serum albumin (BSA) to prevent further nonspecific protein adsorption. Incubation of test serum with the disk allows specific antibodies, if present, to bind to the immobilized antigen. After extensive washing to remove nonreactive antibodies, the bound antibodies are detected by sequential treatment with peroxidase-conjugated goat-anti-human IgG and the enzyme substrate ($H_2O_2$ with chromogen 4-chloro-1-naphthol). A positive reaction is demonstrated by the appearance of a blue dot at the center of the disk. Serum reactive to an immunodot charged with gG-1 indicates previous and probable latent HSV-1 infection. Serum reactive with gG-2 indicates previous and probable latent HSV-2 infection.

HSV-1 is typically associated with infection of the upper body. HSV-2 commonly infects the genitalia and is primarily transmitted sexually. Perinatal transmission, usually of HSV-2, is comparatively infrequent but results in a severe, often fatal disease in newborns. Assays that can detect and distinguish antibodies to these viruses are of clinical and epidemiological importance.

## 2. SPECIAL SAFETY PRECAUTIONS

All human serum specimens are pretreated with 0.5% (v/v) Triton X-100 to inactivate enveloped viruses, including the human immunodeficiency virus, which may be present. However, observe universal precautions. Wear gloves, a lab coat, and safety glasses when handling all human blood specimens. Place all plastic tips, sample cups, and gloves that contact blood in a biohazard waste container. Discard all disposable glassware into a sharps waste container. Place all liquid hazardous waste materials in closed containers labelled as hazardous waste and stating the composition of waste being contained. These materials are decontaminated by autoclaving at 250 $^{o}$F, 19 pounds pressure, for 1 hour.

Protect all work surfaces by absorbent benchtop paper. Discard the benchtop paper into the biohazard waste container daily or whenever blood contamination occurs. Wipe down all work surfaces with 10% (v/v) sodium hypochlorite weekly. Material Safety Data Sheets (MSDSs) for sodium hypochlorite, Triton X-100, 4-chloro-1-napthol, methanol, and hydrogen peroxide are maintained in the Emory School of Medicine.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (OUTPUT.TXT) on a 5¼" high-density (HD) floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, or plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. After the data are calculated and the final values are approved by the reviewing supervisor for release, the data entry clerk transcribes the results into the NHANES III data base using Rbase software; data entry is proofed by the supervisor and clerk. Periodically, data from the laboratory database are downloaded onto floppy diskettes and delivered to the data manager of the Centers for Disease Control and Prevention.

   After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete) the NHANES laboratory supervisor transmits the values to the NCHS mainframe computer along with the other NHANES III data.

c. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by EHLS LAN support staff or CDC Data Center staff, respectively.

d. Documentation for data system maintenance is contained in hard copies of data records.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are necessary. Blood is collected in a red-top Vacutainer tube by standard venipuncture procedures.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no alternate methods for performing analysis for HSV-1 or HSV-2 antibodies. If the analytical system fails, specimens may be stored at ≤-20 °C until the system is returned to functionality.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this assay method.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard recording keeping involves using the mainframe computer, floppy disks, and the hard copy results themselves to track specimens. Records are maintained indefinitely. Only numerical identifiers (e.g., case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality.

For the NHANES study, residual serum is retained at ≤-70 °C for 1 year and then returned to NCHS serum bank.

## 19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

### REFERENCES

1. Lee FK, Coleman RM, Pereira L, et al. Detection of herpes simplex virus type 2-specific antibody with glycoprotein G. J Clin Microbiol 1985; 22:641-4.

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## ACKNOWLEDGMENTS

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Antibody to human immunodeficiency virus type 1 (HIV-1) is measured by Food and Drug Administration (FDA) licensed kits. For enzyme immunoassay (EIA), purified, inactivated HIV is adsorbed onto wells of a microwell plate. Diluted samples are added to each well, incubated and washed. If HIV-1 antibodies are present, they bind to the antigen and are not removed by washing. Conjugate and peroxidase-labeled goat anti-human immunoglobulin is added to the wells. The conjugate will bind to the antibody-antigen complex, if present. Unbound material is removed by washing. The substrate is added and incubated to allow color development. Color develops in proportion to the amount of antibody bound to the antigen-coated plate. The optical absorbance of controls and specimens is measured with a spectrophotometer at 450 nm.

For Western blot analysis, specific HIV-1 proteins are fractionated according to molecular weight by electrophoresis on a polyacrylamide slab gel in the presence of sodium dodecylsulfate (SDS). The separated proteins are transferred by blotting to a nitrocellulose membrane, which is then washed, blocked and packaged. Specimens determined to be repeatedly reactive by EIA are tested by Western blot analysis. Individual nitrocellulose strips are incubated with serum or plasma. If HIV-1 antibodies are present during incubation they bind to the viral antigens bound to the nitrocellulose strip. The strips are washed to remove unbound material. Any HIV-1 proteins are visualized by using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with horseradish peroxidase, and the substrate 4-chloro-1-naphthol. Antibodies to any of the major HIV-1 proteins can be visually determined by the appearance of blue-black bands corresponding to molecular weights of 17, 24, 31, 41, 51, 55, 66, 120, and 160 kD. The presence of any two of the 24, 41, or 120/160 kD bands is the interpretive criterion that the Centers for Disease Control and Prevention (CDC) uses to identify a positive result.

The acquired immunodeficiency syndrome (AIDS) is caused by a virus transmitted by sexual contact, exposure to blood (including exposure caused by sharing contaminated needles and syringes) or certain blood products, or transmission from an infected mother to her fetus or child during the perinatal period (1). HIV-1 has been isolated from people with AIDS and AIDS-related complex (ARC) and from healthy people at high risk for AIDS (2,3). The incidence of antibodies specific for HIV-1 among AIDS and ARC people and among people at increased risk for AIDS is high. The prevalence of infection with HIV-1 among people at low risk is not known. The HIV-1 antibody enzyme-linked immunoassay (EIA) was developed to detect antibodies to HIV-1 and to identify potentially infectious units of donated blood and plasma. It has been established that repeatedly reactive units of blood and plasma should be eliminated from the blood supply (4,5).

For clinical and public health applications of the EIA, both the degree of risk for HIV-1 infection of the person studied and the degree of reactivity of their serum may be of value in interpreting the test. In most settings it is appropriate to investigate reactive specimens by additional, more specific, or supplemental tests. For this survey, the Western blot method is used as an additional, more specific test on human serum specimens found to be repeatedly reactive with the EIA screening procedure.

People demonstrated to have antibodies to HIV-1 should be referred for medical evaluation, which may include testing by other techniques. A clinical diagnosis of AIDS can be made only if a person meets the case definition of AIDS established by CDC (6).

## 2. SAFETY PRECAUTIONS

Consider all serum samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Perform HIV serology under Biosafety Level Two conditions. Wear lab coats, gloves, and safety glasses at all times when working in the laboratory. Use a biosafety cabinet if any possibility of aerosolization exists. Place disposable products in either sharps containers or autoclave bags and autoclave the containers or bags before their disposal. Clean all work surfaces with 10% sodium hypochlorite if any spills occur and at the end of each work day.

Dispose of all specimens and materials used in the Western blot kit as biohazard waste. Do not permit substrate, especially 4-chloro-1-naphthol, to contact skin. If contact occurs, flush skin thoroughly with water.

Material Safety Data Sheets (MSDSs) for sulfuric acid, thimerosal, citric acid, trimethylbenzidine, dimethylsulfoxide (DMSO), 4-chloro-1-naphthol, sodium azide, Tween 20, glycerol, tris, sodium chloride, and sodium hypochlorite are available through the National Center for Infectious Diseases (NCID) computer network. Risk is minimal because of the small quantity of chemicals, the packaging of the chemicals, and limited handling of chemicals by the operators using the test kits.

**3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT**

a. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data and by storing the data in multiple computer systems. Data files provided to the Laboratory Investigations Branch by the field stands consist of 5 1/4" diskettes with demographic data for specimens collected. HIV-1 antibody status is added via Wordstar; data is saved to the hard drive as well. The completed data files are returned by mail to NCHS in Hyattsville, MD.

b. As part of routine backup, the hard copies of all serology printouts and NCHS transmittals are stored permantly.

c. Documentation for system maintenance is contained in hard copies of data records, as well as in the "system log" files on the local hard drive used to archive data.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. No special instructions such as fasting or special diets are required.

b. Specimen type: whole blood with or without anticoagulant; either serum or plasma may be tested.

c. The optimal amount of specimen is 1.0 mL to allow repeat analysis; the minimum volume is 200 µL (0.2 mL).

d. Acceptable collection tubes range from 2 to 10 mL. Anticoagulants, if used, may be either EDTA or heparin. Sterile collection technique should be used.

e. Specimen stability has been demonstrated for several years at $\leq$-20 °C.

f. The criteria for unacceptable specimens are low volumes, gross contamination or hemolysis, or clotted anticoagulated specimens.

g. Whole blood specimens are transported and stored at 4-8 °C.

h. Serum or plasma is transported on dry ice. Once received, the aliquots should be stored at $\leq$-20 °C. Freezing and thawing of specimens should be kept to a minimum.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) BioTek EL1312 96-well microplate reader (Genetic Systems Corp., Seattle WA)

(2) BioTek EL403H microwell plate washer (Genetic Systems Corp.)

(3) Precision pipets to deliver 10 µL, 100 µL, 1 mL, 5 mL, and 10 mL (Rainin Instrument Co., Woburn, MA).

(4) Rocker platform (Genetic Systems Corp.).

(5) Aspirator with disinfectant trap (Genetic Systems Corp.).

b. **Materials**

(1) Genetic Systems LAV EIA kit, no. 0218 (Genetic Systems Corp.) 100-, 200-, or 500-test sizes. Each kit contains EIA specimen diluent concentrate (10X), conjugate concentrate, conjugate diluent, wash solution concentrate (30X), buffered substrate, EIA chromogen reagent, stopping reagent,

specimen diluent, conjugate reagent, chromogen/Buffered substrate reagent, wash solution, and positive and negative controls.

(2) Cambridge Biotech HIV-1 Western Blot Method (Worcester, MA). Each kit contains wash buffer, blotting buffer, conjugate 1, conjugate 2, substrate A, substrate B, blotting powder, diluted wash buffer, working blotting buffer, working conjugate 1, working conjugate 2, working substrate solution, and tri-level controls.

(3) Pipet tips (Rainin Instrument Co.).

(4) Appropriate containers to prepare diluted specimens (any vendor).

(5) Dry-heat incubator set at 37±1 °C (Genetic Systems Corp.).

(6) Deionized water (Continental Water Systems, Inc., San Antonio, TX).

(7) Sodium hypochlorite (bleach), 10% (any vendor).

(8) Forceps (any vendor).

(9) Plate sealers: 1, 2, or 8 pads of 25 sealers each pad. (Genetic Systems Corp.).

(10) Protective gloves, Tronex or Flexam, small/medium/large (Fisher Scientific, Atlanta, GA).

(11) Nitrocellulose strips for Western blot analysis contains separated, bound antigenic proteins from partially purified inactivated HIV-1 in sufficient quantity to detect human antibodies. Bovine protein is present as a blocking agent (Cambridge Biotech Corp.).

(12) Incubation trays for Western blot analysis, 3 trays of 9 wells/tray, (Cambridge Biotech Corp.).

(13) 2-, 10-, or 50-microwell plates or plate holder with strips (Genetic Systems Corp.)

c. **Reagent Preparation**

Because the EIA and Western blot kits are FDA-licensed assays, the reagent preparation must follow exactly the manufacturer's kit inserts, which are available in the HIV Branch Laboratory in the Standard Operating Procedures (SOP) Manual.

(1) Genetic Systems LAV EIA kit, cat. no. 0218, volumes are listed for 100-, 200-, or 500-test sizes.

(a) EIA specimen diluent concentrate (10X)
1, 2, or 10 bottle(s) of diluent for specimen containing 0.1 g/dL thimerosal ($C_9H_9HgO_2SNa$).

(b) EIA conjugate concentrate
1 or 5 vial(s) (1.5 mL) containing anti-human IgM and IgG (goat) peroxidase (horseradish) conjugated solution and 0.01 g/dL thimerosal. Dilute with conjugate.

(c) EIA conjugate diluent
1 bottle (25 mL or 120 mL) or 5 bottles (120 mL) containing normal goat serum and 0.01 g/dL thimerosal. Ready to use.

(d) EIA wash solution concentrate (30X)
1, 2, or 10 bottle(s) (120 mL) containing sodium chloride and Tween-20 (polyoxyethylene-20 sorbitan monolaurate). Dilute to working solution with deionized water.

(e) EIA buffered substrate
1 or 5 bottle(s) (120 mL) containing hydrogen peroxide, citric acid and phosphate buffer. Ready to use.

(f) EIA chromogen reagent
1 or 5 vial(s) (1.5 mL) containing tetramethylbenzidine, and dimethylsulfoxide (DMSO). Dilute with EIA buffered substrate.

*Handle with care as DMSO is readily absorbed through the skin.*

(g) EIA stopping reagent
1 bottle (20 or 120 mL) or 5 bottles (120 mL) of 1 N $H_2SO_4$. Ready to use.

(h) EIA specimen diluent
This reagent is a 1:10 dilution of the specimen diluent concentrate. Thoroughly mix the specimen diluent concentrate. For example, prepare 1 mL concentrate with 9 mL of deionized water. Note lot number and date of preparation on the bottle. Store in the refrigerator at 2-8 °C for up to 2 weeks.

(i) EIA conjugate reagent
Prepare a 1:101 dilution of the conjugate concentrate in conjugate diluent. For each plate, add 120 µL of the concentrate to 12 mL of the diluent. Note the lot number, time and date of preparation on the bottle. Use within 8 hours.

(j) EIA chromogen/Buffered substrate reagent
Prepare a 1:101 dilution of the chromogen reagent in buffered substrate. For each plate, add 120 µL of the chromogen reagent to 12 mL of the buffered substrate. Note the lot number, time, and date of preparation on the bottle. Keep the bottle in the dark at 20-25 °C and use within 8 hours.

(k) EIA wash solution
Prepare a 1:31 dilution of wash solution concentrate in deionized water. Add 120 mL of wash solution concentrate to 3.6 L of deionized water. Store at 20-25 °C up to 4 weeks. Note the lot number and date prepared on the container.

(2) Cambridge Biotech HIV-1 Western Blot Method

(a) Wash buffer
1 bottle (60 mL) supplied as a 20X concentrate. When diluted, the buffer contains 0.02 mol/L tris ($C_4H_{11}NO_3$), 0.1 mol/L NaCl, 0.3% (v/v) Tween-20, and 0.005 g/dL thimerosal, pH 7.4.

(b) Blotting buffer
1 bottle (18 mL) supplied as a 10X concentrate. When diluted, the buffer contains 0.02 mol/L Tris, 0.1 mol/L NaCl, heat-inactivated normal goat serum, and 0.01 g/dL thimerosal, pH 7.4.

(c) Conjugate 1
1 vial (160 µL) [blue] contains biotinylated goat anti-human IgG (heavy and light chain) antibodies and 0.002 g/dL thimerosal.

(d) Conjugate 2
1 vial (160 µL) [black] of avidin-conjugated horseradish peroxidase containing 0.01 g/dL thimerosal.

(e) Substrate A
1 bottle (30 mL) containing a 7.8 mM solution of 4-chloro-1-naphthol in an ethanol solution.

(f) Substrate B
1 bottle (30 mL) containing an aqueous hydrogen peroxide solution ($H_2O_2$, 0.02%, v/v) in citrate buffer.

(g) Blotting powder
1 package (minimum of 9.0 g) of sterile nonfat dry milk.

(h) Diluted wash buffer
Dilute 1 volume of wash buffer (20X) with 19 volumes of reagent-grade water. For 1 strip, add 1 mL wash buffer to 19 mL reagent-grade water. Mix well. Store at 20-25 °C for 3 months.

When diluted, the solution contains 0.02 mol/L Tris, 0.1 mol/L NaCL, 0.3% (v/v) Tween-20, and 0.005% thimerosal, pH 7.4.

(i) Working blotting buffer
Dilute 1 volume of blotting buffer (10X) with 9 volumes of reagent-grade water. Mix well. Prepared fresh prior to use.

Use 1.0 g of blotting powder per 20 mL of the diluted blotting buffer. If the entire kit is used within 5 days, add 9.0 g to 180 mL of diluted blotting buffer. Store at 2-8 °C. When diluted, the solution contains 0.02 mol/L Tris, 0.1 mol/L NaCl, heat-inactivated normal goat serum, and 0.01 g/dL thimerosal, pH 7.4.

(j) Working conjugate 1
Use the supplemental instructions sheet for the dilution appropriate for the working conjugate 1 solution. Prepare a fresh solution prior to use.

(k) Working conjugate 2
Use the supplemental instructions sheet for the dilution appropriate for the working conjugate 2 solution. Prepare a fresh solution prior to use.

(l) Working substrate solution
Mix equal parts of substrate A and substrate B. Mix well and prepare a fresh solution prior to use.

d. **Standards Preparation**

No calibration curve is generated by the user as part of the EIA method or the Western blot method.

e. **Preparation of Quality Control Materials**

(1) EIA positive control
1 or 5 vial(s), each 0.8 mL, of human serum (containing anti-HIV-1 immunoglobulin) non-reactive for HBsAg, 0.1 g/dL sodium azide ($NaN_3$), and 0.01 g/dL thimerosal are supplied ready to use.

(2) EIA negative control
1 or 5 vial(s), each 0.8 mL, of human serum nonreactive for HBsAg and antibody to HIV-1, 0.1 g/dL $NaN_3$, and 0.01 g/dL thimerosal are supplied ready to use.

(3) WB nonreactive control
1 vial (160 µL) [green] containing normal human serum nonreactive for HIV-1 antibodies and HBsAg, 0.1 g/dL $NaN_3$, and 0.005 g/dL thimerosal.

(4) WB weakly reactive control
1 vial (160 µL) [lavender] of inactivated human serum (nonreactive for HBsAg) containing a low titer of antibodies to HIV-1 antigens, 0.1 g/dL $NaN_3$ and 0.005 g/dL thimerosal.

(5) WB strongly reactive control
1 vial (160 µL) [red] of inactivated human serum (nonreactive for HBsAg) having a high titer of antibodies to HIV-1 antigens, 0.1 g/dL $NaN_3$ and 0.005 g/dL thimerosal.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

No calibration curve is generated by the user as part of these assay methods. Calibration of instruments is either automatic or performed periodically by contracted service personnel.

b. **Verification**

The instruments used to read assay results are equipped to analyze the positive and negative controls for each test series. If, within a testing series, positive or negative controls do not conform to specifications as defined in the

product literature, the entire series is invalidated by the instrument.

**8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS**

a. **Preliminaries**

(1) Bring all reagents except the conjugate concentrate to 20-25 °C before beginning the assay procedure.

(2) For EIA, prepare working concentrations of the wash solution, conjugate reagents, specimen diluent, and substrate reagent; mix well. For WB, prepare working concentrations of the wash buffer, conjugate reagents, blotting buffer, and substrate reagent; mix well.

(3) If sample identity is not maintained by an automatic procedure, identify the individual wells for each specimen or control on a data sheet. Label the tubes used for specimen dilution.

(4) Generally for EIA, two positive controls and three negative controls are assayed with each plate or partial plate of unknowns. All microwell plates containing controls and unknowns must be subjected to the same process and incubation times.

For Western blot analysis, two reactive controls and one nonreactive control are assayed with each run of unknown specimens.

b. **Sample Preparation**

(1) Allow the serum specimens to warm to 20-25 °C.

(2) Serum and plasma samples may stratify when frozen or stored at 4-8 °C for extended periods. Mix them gently before testing.

(3) For EIA, all controls and unknown specimens are diluted 1:401 in specimen diluent and mixed thoroughly.

c. **Instrument Setup**

BioTek EL1312 96-well microplate reader

(1) For users of an FDA-licensed EIA kit, the manufacturer supplies the spectrophotometer and analysis software used with its kit.

(2) Turn on the computer, printer and spectrophotometer.

(3) Set the parameters as shown in Table 1.

Table 1
Instrument Parameters for the BioTek EL1312

| Parameter | Setting |
|---|---|
| Wavelength | 450 nm |
| Bandwidth | 8 nm HBW |
| Absorbance Range | 0 to 2 AU |
| Repeatability | ±0.5% and ±0.005 O.D. units |
| Linearity | 1% from 0 to 2.0 O.D. units |

BioTek EL403H microwell plate washer

(1) The settings of the WASH program parameters determine the fill volume of the wells (F1), the number of wash cycles (F2), the delay between wash cycles (F3), the aspiration depth (F4), and the option to aspirate the last

drop (F5). Beginning with F1 and ending with F5, the instrument will prompt the order for entering the settings as shown in Table 2.

Table 2
Parameters for the BioTek EL403H

| Test Procedure | Program Number | Fill Volume | Wash Cycles | Delay Time | Asp. Width | Aspirate Last Drop (Y/N) |
|---|---|---|---|---|---|---|
| | | F1 | F2 | F3 | F4 | F5 |
| LAV | 3 | 375 | 3 | 0 | N/01 | Y |

(2) Empty the waste bottles.

(3) Check the dispense bottle fluid levels.

(4) Check the stopcocks.

(5) Turn on the washer, vacuum pump, and pressure delivery modules.

(6) Check the pressure on the pressure delivery module. Set the pressure at 1.5 psi if the module is on a counter top and 3.0 psi if the module is on the floor.

(7) Insert a null microplate.

(8) Press RINSE, then press START. When the washer has finished rinsing, press PRIME, then press START.

(9) Press DISPENSE, then press START. Ensure that all wells are filled to an even level.

(10) Press ASPIRATE, then press START. Check for complete aspiration of the wells.

(11) Press WASH. Use the OPTION key to select program desired. Check the wash program settings.

(12) Press START. Ensure that a full wash cycle is completed.

(13) Initial the preventive maintenance checklist located in the Preventive Maintenance Module.

d. **Operation of Assay Procedure**

(1) Genetic Systems LAV EIA method

(a) Add 100 µL of diluted control or unknown specimen to the appropriate well on the microplate.

(b) Cover the microwell plate with a plate sealer and incubate it at 37±1 °C for 60 min.

To begin timing a plate, choose the "T" option from the main menu of BioTek EL312, or press the F5 key from any screen. The run number has automatically been entered by the machine when the prompt "input run number" appears. The instrument displays the "select operator" list, plate information, and incubation times for the assay. Select the operator who diluted or added the specimens or reagents to the plate. Press the ENTER key. The prompt reads "Place plate in incubator and press F1 to continue." Pressing the F1 key starts the incubation timer. This key is used consistently to indicate readiness to continue on to the next step. When F1 is pressed, the "Status of Plates" column displays the incubation status and time remaining for each step.

(c) The system will sound an alarm and prompt "Remove plate ## from incubator and press F1 to continue." Press F1 to silence the alarm.

After incubation, carefully remove the plate sealer and aspirate the fluid from each well into a biohazard container. The prompt reads "Wash the plate ## and add conjugate. Press F1 to continue."

(d) Wash the plate three times with approximately 300 µL of wash solution per well per wash. Do the following to prime and wash the microplates:

i. Close the distilled water stopcock and open the wash buffer stopcock.

ii. Press PRIME, then press START. When complete, press PRIME and START again.

iii. The washer is now primed with wash buffer.

iv. Remove the null microplate and insert the plate to be washed.

v. Press WASH. Select the desired program and check the settings.

vi. Press START. Ensure that a full wash cycle is completed and that the plate is aspirated completely.

vii. Remove the assay plate and blot. Thoroughly blot the plate by sharply striking the inverted plate on a pad of clean absorbent towels placed on the counter top. Continue striking the plate until no droplets remain in the well.

Repeat the cycle two times. After the last wash, blot the inverted plate on absorbent paper towels.

(e) Add 100 µL conjugate reagent to each well.

(f) Cover the microwell plate with a plate sealer and incubate at 37±1 °C for 60 min. The "AUTOMATIC RESTART" timing mode prompts "Put plate ## back in incubator and press the F1 to continue." Press F1 key. Again, the operator list is displayed. Select the operator who added conjugate. Press the ENTER key.

(g) The system will sound an alarm and prompt "Remove plate ## from incubator and press F1 to continue." Press F1 to silence the alarm. The prompt reads "Wash plate ## and add chromogen. Press F1 to continue." After incubation, carefully remove the plate sealer and aspirate the fluid in each well into a biohazard container.

(h) Wash the plate three times with approximately 300 µL of wash solution per well per wash. After the last wash, blot the inverted plate on absorbent paper towels. Follow the wash instructions in section d.

(i) Add 100 µL chromogen/buffered substrate reagent to each well.

(j) Cover the microwell plate with a plate sealer. After F1 is pressed, the "AUTOMATIC RESTART" timing mode prompts "Incubate plates in the dark at 20-25 °C and press F1 to continue." Incubate plates in the dark at 20-25 °C for 30 min. Press the F1 key. Select the operator who added chromogen from the displayed list. Press the ENTER key.

(k) The system will sound an alarm and the prompt will read "Remove plate ## from the dark and press F1 to continue." Press F1 to silence the alarm. The prompt reads "Add stop solution to plate ## and press F1 to continue." Carefully remove the plate sealer and add 100 µL stopping reagent to each well to terminate the reaction.

(l) Again, from the operator list select the operator who added the stop solution. Press the ENTER key. From any menu, the prompt reads "Initializing plate reader, communicating with reader, press 'ESC' to abort." When the reader initialization is complete, the plate carrier slides out of the reader and the prompt reads "Place plate number ## in reader and press F1 to continue." Insert the microwell plate into the plate holder with well A1 on the left hand of the plate. Press the F1 key. Access the software to "Run Plate." The plate carrier will slide into the reader and prompt "Using NAME reader, Reading plate ##-Test Type (Sample filter: xxxnm, Reference filter: xxxnm), Communicating with reader, Press ESC to abort." Read absorbance within 30 min.

(m) The spectrophotometer will analyze the negative and positive controls to see if specified limits are met.

(n) If the controls are within established limits, the machine will continue reading the optical density of the remaining wells.

(o) Turn off the machine when readings are complete and printed.

(2) Cambridge Biotech HIV-1 Western blot method

(a) Add 2.0 mL diluted wash buffer to each well used.

(b) Using forceps, carefully remove a nitrocellulose strip from the vial and place it numbered side up, into a well containing diluted wash buffer.

(c) Place the tray on a rocker or rotary platform for 30 min at 20-25 °C, then remove the buffer by aspiration.

(d) Add 2.0 mL working blotting buffer to each well used.

(e) Add 20 µL of each undiluted control or specimen to each well containing its assigned strip in the working blotting buffer. CAUTION: Use a different pipet tip for each sample.

(f) Cover the tray and incubate the contents on a rocking platform overnight at 20-25 °C.

(g) Carefully uncover the tray to avoid splashing or mixing specimens. Remove condensation or droplets on the incubation tray lid by rinsing the lid with diluted wash buffer or wiping it with absorbent towels.

(h) Aspirate the mixture from the wells into a trap containing disinfectant. To avoid cross contamination, rinse the aspirator tip with diluted wash buffer or deionized water after each sample is added.

(i) To each strip, add 2.0 mL diluted wash buffer and rock by hand several times. Remove buffer by aspiration.

(j) Add 2.0 mL diluted wash buffer to each strip for a minimum of 5 min. Aspirate the wash buffer between washes. Repeat a second time. Perform all wash steps at 20-25 °C on a rocking platform.

(k) Add 2.0 mL working conjugate 1 solution to each well and incubate the solution for 60 min at 20-25 °C on a rocking platform.

(l) Aspirate the conjugate from the wells. Wash each strip three times for five minutes as in step 8.d.1.d.

(m) Add 2.0 mL working conjugate 2 solution to each well and incubate the solution for 60 min at 20-25 °C on a rocking platform.

(n) Aspirate the conjugate from the wells. Wash each strip three times for five minutes as in step 8.d.1.d.

(o) Add 2.0 mL working substrate solution to each well and incubate the solution at 20-25 °C on a rocking platform for 10-15 min (or until a weak positive reaction is shown by the appearance of p24 or gp160 bands).

(p) Aspirate the substrate and stop the reaction by rinsing the strips several times with distilled water.

(q) Air dry the strips between absorbent towels in the dark. For best results and consistency, strips should be scored soon after air drying. The strips can then be mounted and stored between clear plastic sheets. When mounting with tape, do not tape over developed bands. This will cause the bands to fade.

(r) The strips may be photographed with high-resolution film. Developed strips will retain their color if stored in the dark. Exposure to light and air will eventually cause the bands to fade.

(s) Read banding patterns as stated in the kit insert.

e. **Recording of Data**

(1) Quality control data
The EIA positive and negative control data are printed as part of the machine read-out. Means and ranges are calculated automatically. Western blot controls are recorded by visual examination.

(2) Analytical results
The EIA specimen results are calculated automatically from the control data from that particular run. Optical densities are recorded for all specimens. Specimens with optical densities exceeding the calculated positive cut-off are noted as reactive on the printout. For Western blot analysis, all bands are recorded for all specimens on the worksheet.

(3) All forms are given to the supervisor who, using Wordstar, adds the EIA and Western blot results to a floppy disk that accompanied the shipment. The disk is then sent to the NCHS. The supervisor keeps the original copies and also stores the data on a computer hard drive.

f. **Replacement and Periodic Maintenance of Key Components**

(1) BioTek EL1312

(a) Source lamp: a spare lamp should be available.

(b) Printer ribbon: spare printer ribbons should be available.

(c) The Bio-Tek EL312 Microplate Reader basically requires no operator maintenance. The service representative provides scheduled maintenance on the instrument.

(d) Light bulb replacement: The tungsten-halogen light bulb in the reader must be replaced if it is burned out or has degraded to such an extent that reader performance is erratic. Check for bulb burnout by lifting the hinged compartment cover on the rear of the reader while the power is on. If no light is visible, the light bulb needs replacing.

(2) BioTek EL403H

(a) Daily maintenance

i. Press MAINT. Press START three times.

ii. When "WETTED CONDITION" is displayed, turn off the washer, pump, and pressure delivery system.

iii. Initial the preventive maintenance checklist located in the preventive maintenance module.

(b) Shut down

Check the waste bottles. If they are over half full, decontaminate them with 10% bleach solution and empty them before proceeding with the washer shut down.

i. Turn on the washer, pump, and pressure delivery module.

ii. Close the wash buffer stopcock and open the distilled water stopcock.

iii. Press MAINT.

iv. Press OPTION until "OVERNIGHT MAINT M2" is displayed.

v. Press START three times. The system will now rinse, prime, and leave the prongs submerged.

vi. Turn off the washer, pump, and pressure delivery module.

vii. Before discarding the waste, decontaminate it at least 30 min by adding 100 mL of a 10% bleach solution.

viii. Wipe up any spills with disinfectant (70-80% alcohol or Wescodyne).

ix. Initial the preventive maintenance checklist located in the preventive maintenance module.

(c) Perform twice monthly decontamination and quarterly acid/base maintenance as indicated in the instrument manual.

(3) **Pipettors**

All micropipettors that are used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) Genetic Systems LAV EIA method

When FDA-licensed kits are used, the calculations are performed automatically by the software provided by the manufacturer. When the plate has been read, the system reports on the validity of the controls. The system then gives the values of the controls, the mean of negative controls ($NC\bar{x}$), the mean of the positive controls ($PC\bar{x}$), and the value of the cutoff.

Table 3
Example of QC Report for Genetic Systems LAV/EIA Method

| Well | Ctrl ID | Value | Status |
|---|---|---|---|
| A1 | Pos Ctrl | x.xxx | (OK,High,Low) |
| B1 | Pos Ctrl | x.xxx | (OK,High,Low) |
| C1 | Pos Ctrl | x.xxx | (OK,High,Low) |
| D1 | Pos Ctrl | x.xxx | (OK,High,Low) |
| E1 | Pos Ctrl | x.xxx | (OK,High,Low) |

Table 3 shows an example of the QC report that is generated by the software. The report is followed by either "Controls, OK: $NC\bar{x}$ = x.xxx, $PC\bar{x}$ = x.xxx, Cutoff = ($NC\bar{x}$+x.xxx) = x.xxx" or "Invalid controls: Cutoff = ($NC\bar{x}$+x.xxx) = ????." If the controls are acceptable, the next prompts are "Remove plate number## from reader and discard"; "Calculating results....completed"; and Updating Statistics....completed." Press the F1 key to print the complete results.

If the controls are invalid, the system prompts "press F1 to read plate## again or press ESC to abort." Pressing F1 is the last chance to re-read an invalid plate. Pressing ESC returns the user to the menu. Use the "copy platemap" function on the Edit Menu to re-read an invalid plate. Before displaying the next screen, the system displays the prompt "Run number ## has a status of 'inval'"; "Create a copy of plate to repeat assay? Y/N:_"; "Press ESC to abort." Press Y or N.

The system tests for the validity of the positive and negative controls according to the instructions in the package insert. It reports overrange O.D. values as "*x.xxx" where "x.xxx" is the overrange value of the reader used.

The following formulas are used for calculating the means of the negative and positive controls:

(a) Calculation of negative control mean absorbance ($NC\bar{x}$)
Determine the mean of the negative control values, where $NC\bar{x}$ = total absorbance divided by total

number of negative controls. The individual negative control values must be >0.000 but ≤ 0.140 O.D. units. If one value is outside this range discard this value and recalculate $\bar{NC}$.

(b) Calculation of positive control mean absorbance ($\bar{PC}$)
Determine the mean of the positive control values. The mean of the positive controls must be ≥0.700 OD units. The individual positive control values must be within the range of 0.65-1.35 times the mean of the positive controls. No positive control value may be discarded.

(c) Determination of the cutoff value
The cutoff value is the mean of the negative controls + 0.225 OD units. Specimens with absorbance values less than the cutoff are considered negative for HIV antibody. Specimens with absorbance values equal to or greater than the cutoff are considered initially reactive and retested in duplicate. If either duplicate retest is reactive, the specimen is considered repeatably reactive and must get confirmatory testing. Initially-reactive specimens which do not react in either duplicate repeat tests are considered negative for antibodies to HIV-1.

(2) Cambridge Biotech HIV-1 Western blot method

There are no calculations for the Western blot method. Band intensities are read visually and scored. The band patterns are reviewed in order to determine the total result of the analysis. The presence or absence of antibodies to HIV-1 in a specimen and the identity of any antibodies present are determined by comparing each nitrocellulose strip with the strips used for the nonreactive and weakly reactive controls tested with that run and with the strip used for the strongly reactive control tested once with the kit.

The interpretation process requires three steps. First, each band that appears on the test strip must be identified on the basis of the strongly reactive control strip. Second, each band is assigned a reactivity score on the basis of its intensity. Third, the strip is interpreted on the basis of the combination of band pattern and reactivity.

Intensity of bands present on strips used to test specific specimens may be scored as shown in Table 4.

Table 4
Reactivity Scores

| Intensity of Band | Reactivity Score |
|---|---|
| Absent | - |
| < Intensity of p24 on the weakly reactive control strip | ± |
| At least as intense as p24 on the weakly reactive strip but < the intensity of p24 on the strongly-reactive strip | + |
| ≥ To the intensity of p24 on the strongly reactive strip | ++ |

Using the strongly reactive control strip as a reference for position and the p24 band on the weakly reactive control strip as a reference for intensity, assign a reactivity score to each band on a strip. When analyzing test specimens, it is helpful to place the control strips side by side with the unknown strips to facilitate the assignment of molecular weights and intensities of each band. The result of blotting can be interpreted as negative, indeterminate, or positive on the basis of the pattern present, as shown in Table 5.

Table 5
Interpretation of Western Blot

| Pattern | Interpretation |
|---|---|
| No bands are present. | Negative |
| At least one band is present, but the pattern does not meet criteria for positive. | Indeterminate |
| At least two of the following bands are present: p24, gp41, and gp120/160. Each band has a reactivity score of + or greater. | Positive |

Because reactivity of any degree with any of the virus-specific proteins identified on the strip is presumptive evidence of antibodies to HIV-1, any results interpreted as indeterminate must be taken as suspicious and should trigger repeat testing and follow-up testing. *Indeterminate assay results must not be considered positive or negative*.

h. **Special Procedure Notes**

(1) Genetic Systems LAV EIA method

(a) Do not use the kit beyond the expiration date.

(b) Do not mix controls, conjugate concentrate, conjugate diluent, chromogen reagent, or plates from different lots of the kit.

(c) Exercise care in opening and removing aliquots from vials to avoid microbial contamination of the reagents.

(d) Use a clean, dedicated dispenser for the conjugate reagent. Exposure to sodium azide will result in inactivation of the conjugate reagent.

(e) Avoid exposing the chromogen reagent or chromogen/buffered reagent to strong light during storage or incubation. Discard the chromogen reagent if it is a distinct yellow-orange or if the chromogen/buffered substrate reagent is a distinct blue color.

(2) Cambridge Biotech HIV-1 Western blot method

(a) Do not use the kit beyond the expiration date.

(b) Do not exchange reagents or other kit contents from one kit lot with those from another kit lot.

(c) Grossly contaminated specimens or strips may result in the development of dark spots on the strip that should not be interpreted. To prevent this problem, pay careful attention to the storage of reagents and kits.

(d) Shield working substrate solution from sunlight during its preparation and use within 30 min of mixing. If substrate A shows color, do not use it.

(e) Use reagent grade water to dilute reagents.

(f) Do not remove nitrocellulose strips from the storage tube until immediately before use. Open the tube only after the strips have reached
20-25 °C. Close the tube immediately after removing the strips for use.

(g) Use only the controls supplied with the kit.

(h) Do not cut the strips. Narrower strips can lead to misinterpretation because strips may flip over the incubation tray, or artifacts in the reaction zones my be mistaken for possible bands or may prevent recognition of positive bands.

(i) Measure all reagents. Use extreme care and calibrated pipettors with good quality tips when preparing working conjugate solutions.

**9. REPORTABLE RANGE OF RESULTS**

EIA results are reported as either nonreactive or repeatably reactive. Western blot results are reported as either negative, positive, or indeterminate.

**10. QUALITY CONTROL (QC) PROCEDURES**

When FDA-licensed EIA kits are used, the QC procedures must be performed in order for the kit to be finished and read. The individual negative control values must be >0.000 but $\leq$0.140 O.D. units. The individual positive control values must be 0.65-1.35 times the mean of the positive controls. For the run to be valid, the positive and negative controls must be within the acceptable range. If not, technique or reagents should be suspected and the run repeated.

For EIA, two positive controls and three negative controls are assayed with each plate of unknowns. All microwell plates containing controls and unknowns must be subjected to the same process and incubation times. Only one of three negative controls and no positive control can be discarded when their means are calculated. The controls are supplied ready to use in the kit.

For Western blot analysis, the QC procedures involve the use of nonreactive, weakly reactive and strongly reactive controls that must have the correct number of bands as specified in the manufacturer's kit insert. If the correct numbers of bands are not apparent or the negative control has visible bands, then the run must be repeated.

The nonreactive and weakly reactive controls must be included with each run, regardless of the number of specimens tested or nitrocellulose strips used. The strongly reactive control is used to establish criteria for the reactivity of bands and is included with the first run of specimens for each kit. The strongly reactive control need not be included in subsequent runs unless the strip is misplaced or faded.

In order for the results obtained from any run of specimens to be considered valid, the following conditions must be met:

a. Nonreactive control: No bands should be visible on the nitrocellulose strip used to test the nonreactive control.

b. Strongly reactive control: All relevant molecular weight bands must be visible on the nitrocellulose strip used to test the strongly reactive control. These bands are p17, p24, p31, gp41, p51, p55, p66, and gp160. A gp120 band may also be seen but is not a requirement for acceptable performance.

c. Weakly reactive control: The nitrocellulose strip used to test the weakly reactive control provides a measure of the sensitivity of the Western blot kit and must exhibit bands at p24 and gp160. Additional weak bands may appear but are not required in order for acceptable performance to be demonstrated.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. Repeat the entire EIA or Western blot run using new dilutions.

b. If controls continue to fail, consult the supervisor for other appropriate actions.

c. Do not report results from runs in which the controls did not meet acceptable limits.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

EIAs and Western blot assays were validated by using human serum or plasma specimens. Insufficient data exist to interpret results performed on other body specimens, and testing of specimens other than serum or plasma is not recommended.

Grossly contaminated or hemolyzed specimens have a greater propensity to give false reactions in EIA and unreadable reactions in Western blot analysis. In the Western blot method, sodium azide interferes with horseradish peroxidase activity.

Although a positive result for antibodies to HIV-1 on the EIA or Western blot analysis indicates infection with the virus, a

clinical diagnosis of AIDS can be made only if a person meets the case definition of AIDS established by CDC. People who show evidence of having antibodies to HIV-1 should be referred for medical evaluation, which may include testing by other techniques.

A negative test result does not exclude the possibility of infection with HIV-1.

**13. REFERENCE RANGES (NORMAL VALUES)**

A normal value for HIV-1 should be negative.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Testing for antibodies to HIV-1 in this survey is anonymous. Any link with the original personal identifier was removed before the specimens were shipped to CDC.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach 20-25 °C during testing. Excess specimen is refrozen at ≤-20 °C.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no alternate methods for performing HIV antibody analysis. If the analytical system fails, specimens can be stored at ≤-20 °C until the system is returned to functionality. For Western blot analysis, specimens can be stored up to 2 weeks at 2-8 °C. For longer intervals, specimens should be frozen at ≤-20 °C prior to testing.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Testing for antibodies to HIV-1 is completely anonymous. No reporting is possible. These data are to be used for national prevalence estimates only.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Records are stored on the CDC mainframe computer, on floppy diskettes, and in hard-copy form. Records are maintained indefinitely. Testing for antibodies to HIV-1 in this survey is anonymous. Excess serum is archived at CDC and is not returned to the NCHS specimen repository.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The thrombin clotting time of dilute plasma is inversely proportional to the fibrinogen concentration of the plasma. This principle provides the basis for a simple quantitative assay for fibrinogen in which the clotting time of a specimen is compared with the clotting time of a standardized fibrinogen preparation. The enzyme thrombin converts the soluble plasma protein, fibrinogen, into its insoluble polymer, fibrin. At relatively high thrombin concentrations (>30 NIH units/mL) and low fibrinogen concentrations (2.0-30.0 mg/dL), the clotting time is dependent on the fibrinogen level. A semi-log plot of the thrombin clotting time versus the fibrinogen concentration is linear.

If vascular trauma or injury occurs, circulating fibrinogen must be at sufficient levels to arrest bleeding and repair tissue. Thus, the determination of fibrinogen in plasma is important in assessing thrombolytic disorders. Fibrinogen is an acute-phase reactant; its concentration in plasma increases as a result of inflammation, pregnancy, and oral contraceptive use. Decreased levels are associated with certain pathological states including liver disease and disseminated intravascular coagulation. Congenital deficiencies include afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia (1-3).

## 2. SAFETY PRECAUTIONS

Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe Universal Precautions: wear gloves, scrubs, laboratory coats, and face shields while handling all human blood products. Place disposable plastic, glass, and paper (pipet tips, tubes, gloves, etc.) that contact blood in the biohazard container located at the work sites. Seal and label containers when they are 3/4 full, and transport them to the biohazard storage facility until they are removed by a commercial contractor. Wipe down all work surfaces with 10% sodium hypochlorite solution daily.

> *Dispose of all biological samples and diluted specimens in a biohazard container at the end of the analysis.*

Material Safety Data Sheets (MSDSs) for veronal buffer and sodium hypochlorite are located adjacent to the instruments.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transferred data from a printed copy of the output file and storing data in multiple computer systems. Data files containing the date, analytical run ID, specimen analytical results by specimen ID, and method code are stored in archive files in an ASCII format. An ASCII file of the data is copied to a 5¼" high-density (HD) diskette to be sent to the National Center for Health Statistics (NCHS). The file is copied onto the main laboratory computer system in the laboratory administration area.

b. Routine backup procedures include 1) weekly backup of the hard drive and 2) archive on 3½" HD floppy diskettes.

c. Documentation for system maintenance is contained in hard copies of recorded data and in files on the local tape drives used for archival of data.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Do not use hemolyzed specimens.

b. Specimen type: plasma, from a blue-top Vacutainer tube containing sodium citrate. This is the only acceptable anticoagulant for this analysis. Separate plasma from cells within 1 hour of collection.

c. The optimal amount of specimen is 4.5 mL whole blood in a 5.0-mL Vacutainer; the minimum amount is 1.8 mL in a 2.0-mL Vacutainer.

d. Acceptable containers include 2.0- or 5.0-mL blue-top Vacutainer tubes for specimens sent directly to the laboratory, and a 2.0-mL Nalge vial containing plasma for NHANES III specimens.

e. Specimens should be refrigerated if not used immediately. Specimens stored longer than 72 hours should be

frozen at ≤-20 °C. Specimen stability has been demonstrated for at least 6 months at ≤-20 °C.

f. Specimens that contain inadequate blood volume, that have undergone hemolysis, that are labelled improperly, or that have had prolonged contact with cells are inadequate for fibrinogen analysis.

g. Serum specimens from NCHS collection sites are transported on dry ice and stored at ≤-70 °C until analysis. Residual samples are refrozen at ≤-70 °C. Multiple freeze/thaw cycles do not compromise this analyte.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Coagamate XC Plus automated coagulation analyzer, including four reagent pumps, two reagent wells, and a storage compartment. Thermal printer with paper tape to record the first test, last test, plate temperature, test mode, pump volumes, blank time, activation time, maximum time, test station number, test results, date and time, and 37 °C temperature (Organon Teknika, Durham, NC).

(2) Sealpette variable-volume micropipets: 2-20, 20-200, and 200-1000 μL volumes (Cole Scientific, Moorpark, CA).

(3) Pipet-aid (Drummond Scientific Co., Broomall, PA).

(4) Selectapette variable micropipettor (Baxter, Miami, FL).

b. **Materials**

(1) Thrombin reagent (Organon Teknika).

(2) Owren's veronal buffer (Organon Teknika).

(3) Fibrinogen calibrator plasma (Organon Teknika).

(4) Human-based QC plasma (Organon Teknika).

(5) 1.0- and 5.0-mL volumetric pipets (any vendor).

(6) 5.0-mL serological pipets (any vendor).

(7) Ultrapure water from the Barnstead E-pure water purification system with a resistivity of >16 megOhm-cm (Culligan Water Systems, Alamogordo, NM)

(8) 3.5-mL Beral polypropylene transfer pipettes (Sarstedt, Newton, NC)

(9) Coagamate XC Plus reaction cuvette disks (Organon Teknika, Miami, FL).

(10) Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).

(11) Clean room vinyl gloves (Baxter Healthcare, Valencia, NC).

(12) Biohazard autoclave bags (Curtin-Matheson Scientific, Inc., Atlanta, GA).

(13) 10% sodium hypochlorite (bleach) solution (any vendor).

c. **Reagent Preparation**

(1) Thrombin reagent
Contains a lyophilized preparation of 100 NIH units/mL of bovine thrombin per vial with added stabilizers. Reconstitute a vial of thrombin reagent with 5.0 mL distilled deionized water. Invert the vial gently to mix and allow it to stand until the reagent is dissolved. The reconstituted thrombin is stable for 8 hours at 20-25 °C or 1 week at 4-8 °C.

(2) Owren's veronal buffer
Contains 0.0284-M sodium barbital, 0.125-M sodium chloride and 0.01 g/dL thimerosal, pH 7.35. The buffer is ready for use as packaged and is stable until the expiration date indicated on the label when stored at 4-8 °C.

d. **Standards Preparation**

(1) Fibrinogen calibrator plasma
Lyophilized citrated normal human plasma assayed for fibrinogen. Constituent concentrations are specific for each lot used. Store the unopened calibrator at 4-8 °C until the expiration date on the vial.

(a) Bring ultrapure water to 20-25 °C before use.

(b) Remove the calibrator vial from 4-8 °C storage. Tap the calibrator bottle lightly to dislodge the lyophilized material.

(c) Using a volumetric pipette, transfer exactly 1.0 mL of ultrapure water into the bottle of calibrator.

(d) Cover and then invert calibrator vial several times to cover all inside surfaces of the bottle. Allow the vial to stand 30 min until dissolved. Do not shake. Mix gently.

(e) Store the reconstituted calibrator at 4-8 °C until use. This preparation is stable for 24 hours.

(2) Calibration standards

(a) The calibration curve is prepared from a dilution of the fibrinogen calibration reference plasma in Owren's veronal buffer as shown in Table 1.

Table 1
Dilution of Fibrinogen Reference Plasma

| Dil.# | Ref. Plasma (mL) | Buffer (mL) | Dilution | Dilution Factor | Fib. (mg/dL)* |
|---|---|---|---|---|---|
| 1 | 0.5 ref. | 2.0 | 1:5 | 2.0 | ---- |
| 2 | 1.0 dil #1 | 1.0 | 1:10 | 1.0 | ---- |
| 3 | 1.0 dil #2 | 1.0 | 1:20 | 0.5 | ---- |
| 4 | 1.0 dil #3 | 1.0 | 1:40 | 0.25 | ---- |

* The concentration of fibrinogen (mg/dL) for the dilution is determined by multiplying the assigned value designated on the vial label of the Fibrinogen calibration reference by the dilution factor.

e. **Preparation of Quality Control Materials**

"Verify" normal and low fibrinogen QC plasma is a human-based material that has been assayed by the manufacturer.

The QC materials are commercial preparations of human citrated plasma with added human and animal tissue extracts and preservatives.

Verify that normal and abnormal QC serum samples are reconstituted as follows:

(1) Bring all vials of control plasma to 20-25 °C before reconstitution.

(2) Tap the control bottles lightly to dislodge the lyophilized material.

(3) Using a volumetric pipette, transfer exactly 1.0 mL of deionized water into each bottle of the control.

(4) Let the vials stand for 30 min at 20-25 °C and then mix the contents by gently swirling.

(5) Store reconstituted control vials at 4-8 °C between each use. Invert them gently before each use to ensure total homogeneity.

(6) The preparation is stable 24 hours after reconstitution at 4-8 °C.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

(1) Prior to calibration, run the four standard curve dilutions in the fibrinogen mode. Run all samples in duplicate and average the appropriate values.

(2) Press the FIBRINOGEN key, STD CURVE key, and CALIB key. Press the appropriate keys on the numeric keypad to enter the number of standards. Press ENTER.

(3) The display will prompt: "1 mg/dL=____." Enter the concentration of the calibrator at the 1:5 dilution and press ENTER. The display will indicate "1 mg/dL=XXX.X TIME=____." Enter the corresponding mean time value for that standard via the numeric keypad and press ENTER.

(4) The display will continue to prompt for both the concentration (mg/dL) and time for the remaining standard dilutions. After each prompt, enter the concentration and the time for each dilution and press ENTER.

(5) After all values have been entered, the display will prompt "Calibration Printout (Y/N)?"

(a) If "Y" is pressed, the printer will print mg/dL, clotting times, and the coefficient of determination. The coefficient of determination is an indication of the line of best fit drawn through a series of data points. If the coefficient of determination for the calibration curve is $\leq$0.985, the curve should be carefully evaluated.

(b) If "N" is pressed, the display will prompt, "New Calibration (Y/N)?"
If "N" is selected, the display returns to the "Fibrinogen/Std" screen CURVE configuration. If "Y" is pressed, the display will prompt the user through steps 1-6 of the calibration procedure.

### b. Verification

Each time a new lot of calibrator is received, linear dilutions are prepared and analyzed. The resulting standard curve is compared with the previous curve stored in the instrument's memory. If the curve parameters are acceptable, the new calibration lot number may be put into use.

An in-house comparison of this method on the Coagamate XC Plus, using the fibrometer method as reference, resulted in the following linear regression statistics (3):

*Coagamate = 1.044(fibrometer) - 0.289, r = 0.985*

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Allow frozen specimens, QC serum samples, and calibration serum samples to reach 20-25 °C and mix by inversion for 10 sec.

(2) Prepare a sufficient number of cuvettes for the samples being tested.

b. **Sample Preparation**

Frozen specimens must be thawed at 37 °C prior to analysis and kept refrigerated until analysis.

c. **Instrument Setup for the Coagamate XC Plus Coagulation Analyzer**

(1) Set the parameters for the Coagamate XC Plus as described in Table 2.

Table 2
Coagamate XC Plus Parameters

| Parameter | Setting | Display |
|---|---|---|
| Pump 1 volume | 0.10 mL | 100 |
| Pump 2 volume | 0.000 mL | 0.0 |
| Blank time | 1.0 sec | 1.0 |
| Maximum time | 60.0 sec | 60.0 |
| Activation time | 0.0 sec | 0.0 |

(1) Press the rocker switch to the on position. The instrument will automatically proceed through a power-up the self diagnostic procedure. The display and printer will indicate "System Self Check."

(2) After the self-check is complete, the display will indicate "Coagamate xc unit warming, proceed with calibrations" and will automatically enter the PT mode. The printer will indicate:

COAGAMATE XC
REV. X VERSION XXXXXX
Z PUMP BCT
where Z = 4

(3) Press the FIBRINOGEN key on the front panel. The LED on the key will light. Allow the instrument to reach the operating temperature of 37.5±0.6 °C. The LED on the "NOT READY/NO" key will remain illuminated during the instrument warm-up period. The "READY/YES" LED will illuminate when the instrument is at operating temperature, and the display and printer will indicate "Warm-up Complete."

(4) START cannot be activated until the unit has concluded the system self-test with the analog channel test and returns to the PT mode. Press the FIBRINOGEN and STD CURVE key to activate the fibrinogen mode, in which the clotting time and mg/dL are printed on the header of each run.

d. **Operation of Assay Procedure**

(1) Prepare the thrombin reagent according to instructions. Place the vial of reagent in the left-hand reagent well. Do not add a stir bar. Cap the vial with a 3-hole rubber stopper. Insert ends of reagent tubing until the pick-up tip touches the bottom of the vial.

(2) Dilute 50 $\mu$L plasma sample with 450 $\mu$L Owren's buffer. Use immediately.

(3) Transfer 0.2 mL of plasma dilution to each cuvette of the test tray. Participant samples must be analyzed in duplicate.

(4) Standard instrument parameters set the "FIRST TEST" to 1 and the "LAST TEST" to 12. To modify the first and last test, press the appropriate key (FIRST or LAST TEST), the station number required, and ENTER.

(5) Prime the reagent tubing with reagent by pressing the PRIME 1 key. Deliver excess reagent into a suitable receptacle held one-half inch under the delivery tip of the reagent incubation arm. Inspect the tubing for bubbles and re-prime if bubbles are visible.

(6) Position and seat the test tray on the incubation test plate, matching the notch in the tray with the notch in the hub. Lower the reagent incubation arm. Do not lift the reagent incubation arm during a test cycle.

(7) Press START to initiate a test cycle. The printer will advance and display first test, last test, plate temperature, test mode, and instrument parameters. In the fibrinogen mode, samples are incubated for 60 sec. The display will count incubation time, indicating "Cuvette warming...seconds left = XXX." After the warm-up period, the display will indicate "Test in progress." During the test cycle, the only active controls are CANCEL and ON/OFF.

(8) The instrument operates automatically until the last test has been completed; results are then displayed and printed. Duplicate determinations must be within 10%. Values outside this range must be repeated for verification.

(9) Raise the reagent incubation arm. Remove and discard the test tray containing the clotted samples.

(10) When a test cycle has been completed, press RECALL for an additional tape printout.

(11) Flush the reagent tubing thoroughly with purified water by pressing, YES, PRIME 1 followed by YES, PRIME 2. Do not allow any reagent to remain in the reagent tubing for more than 30 min after a run is completed.

(12) Remove the reagent tubing from the reagent dispenser pump. It is not necessary to remove the tubing from the reagent incubation arm. Turn power off.

e. **Recording of Data**

(1) Quality Control Data
The QC data are recorded daily. The data are accumulated over a 1-month period and entered into the computer. At the end of each month, print out Levey-Jennings charts, the mean, the 2-SD range, and the percent coefficient of variation (% CV). Repeat these steps for each of the controls. This report must be kept in the *Coagulation QC Manual*.

(2) Analytical Results
Results are collated on the NHANES report sheet and include 1) participant demographics, 2) names of tests performed, 3) units for each parameter, 4) results, and 5) any footnotes. The operator must retest specimens whose results are deemed unacceptable by the supervisor. After being approved by the supervisor, the form is given to the computer analyst to verify results against the ASCII file printed from the host computer system. After verification, the computer analyst will transfer the ASCII file to a 5¼" HD diskette and mail the diskette to the NCHS coordinator. A printout of the ASCII file will be filed in the study notebook.

(3) The QC data and Levey-Jennings charts are stored in the QC manual and reviewed monthly.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Reagent tubing must be rinsed daily with distilled water: Remove the reagent pickup tip from the reagent vial and place it in a container of distilled water. Lift the reagent incubation arm and hold a waste container under the dispensing tip. Press PRIME for approximately 20 sec. Remove the reagent pickup tip from water and press PRIME for 20 sec. Alternate air and water cycles and end with an air cycle to remove all water from the tubing. Clean any splatter or residual reagent from the underside of the reagent incubation arm.

(2) Lightly lubricate the area between tubing collars on a daily basis. Each time the tubing is lubricated, the pump must be calibrated.

(3) Wash tubing weekly or on alternate days if the instrument is used across all shifts. Rinse the reagent tubing and remove it from instrument. Hold the tip of the tubing upwards until you reach a sink. Roll the tubing with your palms to loosen any particles or debris adhering to the inner tubing wall. Reinstall the tubing in the same arm and pump position and rinse. Recalibrate the pump.

(4) Tubing verification should be performed weekly or when a calibration error is detected during the pump calibration. Remove the tubing from the reagent incubation arm but not from the pump. Place the pickup tip in a container of distilled water. Connect the dispensing tip of the tubing to a 1.0-mL serological pipet using a

section of tubing for the connection. Holding the pipet in an inverted position, press PRIME to pump water to the 0.5-mL division on the pipet. Turn the stator volume-adjustment screw toward the back of the instrument until the water level begins to descend. Press PRIME and determine if the water volume increases by an increment of 0.1 mL. If the volume inccrease is >0.1 mL, perform a pump calibration. If the volume increase is ≤0.1 mL, discard the tubing.

(5) Replace the reagent tubing monthly or more often if the instrument usage increases. Remove the reagent pickup tip from the reagent vial; grasp the tubing on either side of the pump stator and gently lift to remove. Remove the tubing from the groove and gently lift the dispensing tip from the hole in the reagent incubation arm.

(6) Clean the pump rotor monthly. Loosen the screw in the center of the pump rotor and remove the rotor. Immerse it in isopropanol for several min. Rollers should rotate freely when dust and old lubricant are removed. Moisten an absorbent tissue with isopropanol to wipe dust and excess lubricant from the pump stator. Replace the rotor and tighten the screw. The rotor should be flush with the top of the instrument. Perform tubing verification and pump calibration using new tubing.

(7) The temperature must be verified semi-annually. Press YES when turning the instrument ON. When the display prompts "Select menu number (0-16)____," press 7 and ENTER. The display prompts "Select A/D type (0-3) = ___." Press 2 and ENTER to monitor the incubation-arm temperature. Press CANCEL, 3, and ENTER to monitor the incubation-plate temperature. If the temperature is out of the operating range of 37.5± 0.6 °C, contact the service representative. Turn the instrument OFF to exit this mode.

g. **Calculations**

(1) The instrument automatically plots and stores a standard curve. The participants' samples are assayed and reported in sec and expressed in mg/dL when the "Standard Curve Option" is used.

(2) Concentration values can be calculated for manually entered clotting times.

(a) Press the ASSAY MODE key of the desired standard curve calculation. The fibrinogen concentration is calculated by using a log-log best fit line determined by least squares analysis.

(b) Press STD CURVE and MODIFY CALIB.

(c) For an existing curve, the display prompts, "Standard curve printout (Y/N)?" Press YES for a printout of the existing standard curve.

(d) The display prompts: "New calibration (Y/N)?" Press NO and enter the concentration and clotting times for each point.

(e) If an existing curve is used or after a new curve is entered the display prompts: "Manual calculation (Y/N)?" Press YES.

(f) The display prompts: "Time____.__." Enter the clotting time. The calculated concentration is displayed and printed.

(g) The display prompts: "Manual calculation (Y/N)?" Press YES if additional calculations are desired or press NO to return to fibrinogen mode.

(3) Repeat a specimen analysis when results are outside the ±2-SD range. Duplicates must agree within 10%. When reanalyzing any specimen with a concentration >800 mg/dL, prepare a 1:20 dilution (0.1 mL plasma + 1.9 mL buffer) of the plasma with buffer. Read the value from the curve and multiply by the dilution factor of 2.

(4) For fibrinogen values <50 mg/dL: if a prolonged clotting time is obtained by using the 1:10 dilution of the participant's plasma, test a 1:5 (0.2 mL plasma + 0.8 mL buffer solution) or a 1:2 (0.2 mL plasma + 0.2 mL buffer solution) dilution. Divide the value obtained by the dilution factor (2 for a 1:5 dilution, 5 for a 1:2 dilution).

(5) Lack of clotting with a 1:2 dilution suggests a fibrinogen concentration <15 mg/dL or a fibrinogen abnormality.

## 9. REPORTABLE RANGE OF RESULTS

Normal ranges on the Coagamate XC Plus system are 200 to 400 mg/dL. If the fibrinogen value is >800 mg/dL, the specimen should be diluted and reanalyzed.

## 10. QUALITY CONTROL (QC) PROCEDURES

This method is performed in accordance with the Clinical Laboratory Improvement Act (CLIA) and College of American Pathologists (CAP) guidelines. The method has proven to be accurate, precise, and reliable. Imprecision studies can be generated from use of a long-term lot number of QC material.

QC material with normal and abnormal levels of fibrinogen is used to establish our QC program. These assayed controls have a moving 2-SD range of fibrinogen levels determined by assay on our instrument and through our participation in CAP proficiency surveys. We also compare our 2-SD range with those of laboratories nationwide. All levels of this parameter are assessed by taking these control samples through the complete analytical process. The data from these materials are used to estimate methodological imprecision, shifts, and trends.

The "Verify" assayed QC material manufactured by Organon Teknika, comprises material with normal and abnormal fibrinogen levels as determined by each coagulation test performed on the Coagamate XC Plus. The QC samples are lyophilized preparations of pooled human citrated plasma from pooled human sources. The constituent concentrations are specific for each lot and are assayed by Organon Teknika. One set of assayed controls is analyzed at the beginning and end of the analyzer run to verify calibration and as an accuracy check. If the amount of these materials in one lot becomes low, another lot is ordered in time to analyze concurrently with the lot currently in use.

The CAP proficiency survey program evaluates the accuracy and performance not only of individual laboratories but the state of the art of laboratory science. Unknown samples are sent to the laboratory on a quarterly basis. These unknowns are treated as routine samples and receive no special consideration. After analysis, the results and information concerning the method of analysis are recorded on a report form and the form is sent to CAP . For the evaluation, results for each sample are grouped by analyte and methodology. The performance of the group is determined by calculating a mean and SD for each specimen by majority opinion of referees. The performance of the laboratory within that group is evaluated by comparison with the group performance. The consensus result for the survey specimen is considered the accurate value for that sample. The CAP proficiency survey program accomplishes the following goals in our QC program:

- It assesses the current state of the art of laboratory medicine.
- It provides information needed to select the best methods and reagents.
- It satisfies the CLIA-88 regulatory requirements.
- It provides an education peer-comparison program with which to compare our performance with those of our peers.

Table 3
Precision and Accuracy of Representative Plasma Fibrinogen QC Pools Used for NHANES III

| Pool | Mean (mg/dL) | 95% limits (mg/dL) | 99% limits (mg/dL) | Runs | Total CV (%) |
|---|---|---|---|---|---|
| Within Run Normal | 252 | 236-268 | 220-284 | 20 | 2.3 |
| Within Run Abnormal | 95 | 91-98 | 88-102 | 20 | 1.7 |
| Total Normal | 252 | 232-271 | 222-281 | 20 | 3.9 |
| Total Abnormal | 95 | 89-101 | 86-101 | 20 | 3.2 |

After the standards and assayed QC materials are analyzed, the monthly Levey-Jennings control charts, stored in the host computer system, are consulted to determine if the analyte is "in control".

(a) The data are acceptable if all controls are within 2 SD of the mean. Report the participant results.

(b) When at least one control observation is more than 2 SD outside the mean, hold the participant's results and inspect the control data further, using additional control rules. Inspect control data within the run.

(1) Reject the run when one control is more than 3 SD outside the mean. Do not report the participant's results.

(2) Reject the run when two control observations are more than 2 SD outside the mean. Do not report participant's results.

(3) Reject the run when one control observation is more than 2SD above the mean and another is more than 2 SD below the mean. Do not report the participant's results.

(c) Inspect control data across runs.

(1) Reject when the previous observation on the same control material was more than 2SD outside the mean. Do not report the participant's results.

(2) Reject when the last four consecutive control observations were more than 1 SD outside the mean. Do not report the participant's results.

(3) Reject when the previous three control observations on the same control material were more than 1 SD outside the mean. Do not report the participant's results.

(4) Reject when the last 10 consecutive observations fall on the same side of the mean. Do not report the participant's results.

(5) Test with the $10_x$ rule within control materials. Reject when nine previous observations on the same control material fall on the same side of the mean. Do not report the participant's results.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more QC samples fall outside the ±2 SD range or a within-run control sample shifts 2 SD or more from its previous value, take the following steps:

a. Determine the type of errors occurring (random, systematic, or both) on the basis of the control rules being violated.

b. Refer to the Coagamate XC Plus troubleshooting guide to inspect the components of the method or instrument that can cause the type of error observed.

c. Correct the problem and reanalyze the donor's samples and control samples, testing for statistical control by the same procedure.

d. Consult the Hematology technical supervisor for any decision to report data when there is a lack of statistical control.

e. The Hematology technical supervisor may make a decision to report data when there is a lack of statistical control in the following situations:

(1) The control problem is due to the control materials themselves.

(2) The control problem resulted from an isolated event that would not have affected the rest of the run.

(3) The control problem occurs in a concentration range that is different from the concentration range of the donor's samples. The method is in-control in the range of the donors's samples.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Immunochemical methods measure total fibrinogen, and levels of total fibrinogen may not always parallel levels of physiologically active fibrinogen.

## 13. REFERENCE RANGES (NORMAL VALUES)

The normal range for fibrinogen by this method, as determined by Organon Teknika for the Coagamate XC Plus, is 150-

400 mg/dL.

Depending upon method of analysis, the validity of results may be limited if the donor is receiving anticoagulant therapy. In cases of dysfibrinogenemia, results of fibrinogen determination will vary widely. Results of individual methods may also vary widely, and the usefulness of some methods may be limited under specific circumstances.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

a. Medical intervention is indicated for donors with plasma fibrinogen <100 mg/dL.

b. The NCHS coordinator should be notified of laboratory results.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens must be maintained at 4-8 °C prior to analysis, and only 1 aliquot should be removed and heated to 37 °C during analysis. After a run is accepted, store samples at ≤-70 °C for long-term storage.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

The fibrinogen analysis can be performed on the BBL fibrometer or the Helena Cascade 480 in the event of a malfunction of the Coagamate XC Plus. The reagents, calibrators, and controls from the Coagamate XC Plus are used to perform the analysis on the alternate instruments. Either of the two alternate instruments may be used to analyze NHANES III samples until functionality of the Coagamate can be restored.

Normal ranges of 150-400 mg/dL, established by Helena Laboratories, Inc., are used with the Helena Cascade 480 instrument. Normal ranges of 200-400 mg/dL, established by Dade, Inc., are used with fibrometer instrument.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Notify the NCHS coordinator by telephone or FAX. The NCHS physician will then notify the participant's medical provider.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (electronic and log accession books) are used to track specimens. All records, including related QC data, are maintained for a minimum of 7 years in electronic and hard copy format. Excess serum is returned to the serum repository in Rockville, MD.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

Because these were assayed controls no statistics were accumulated to generate monthly summary statistics or graphs.

**REFERENCES**

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This highly sensitive, direct radioimmunoassay system is based on the use of a highly purified, stable preparation of $^{125}$I-labeled thyroglobulin (1). The assay is standardized against the MRC (Medical Research Council) thyroglobulin auto-antibody First International Reference Preparation (coded 65/93). Specimens containing known amounts of thyroglobulin antibody and specimens containing unknowns are incubated for 1 hour with the purified $^{125}$I-thyroglobulin. During this incubation, the thyroglobulin antibodies present bind with labeled thyroglobulin. Protein A is added and the tubes are incubated for another hour. During this incubation, the Fc portion of thyroglobulin antibodies present is bound by the protein A. Assay buffer is added and the tubes are centrifuged. After centrifuging, the supernatants are decanted or aspirated. The amount of radioactivity in the pellets is directly proportional to the amount of thyroglobulin antibody contained in the calibrators and unknowns. Calibrator concentrations are plotted on semilog graph paper, and the concentration of antibody in the unknowns is interpolated from the curve.

The measurement of thyroglobulin auto-antibodies can be of considerable value in the diagnosis of thyroid diseases, such as autoimmune thyroiditis, Graves' disease, endemic goiter, and subacute thyroiditis (2). These measurements, as part of a thyroid autoimmune diagnostic profile that would include antithyroid peroxidase antibody and thyrotropin receptor antibody, can be very useful in the differential diagnosis and management of thyroid disease (3-5).

## 2. SPECIAL SAFETY PRECAUTIONS

The assay employs $^{125}$I as a tracer and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the *U.S.C. Radiation Safety Manual*. In addition, all personnel must successfully complete the training course *Radiation Safety in the Laboratory* or demonstrate having received equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective eyewear, gloves, and lab coat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines. Wipe countertops with a 10% sodium hypochlorite (bleach) solution after assay completion.

Material safety data sheets (MSDSs) for sodium chloride, TRIS-hydrochloric acid, $^{125}$I- thyroglobulin tracer, sodium hypochlorite, and sodium azide are available through the laboratory office.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III Mobile Examination Center (MEC) contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" high-density (HD) diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e. whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data and by storing data in multiple computer systems.

c. A cumulative computerized laboratory log is generated for all specimens received. The log includes batch receipt dates and updated details concerning the handling, manipulation, and analysis of specimens as well as any problems that occur.

d. Documentation for system maintenance is contained in hard copies of data records.

e. The results from the assay, which are transcribed from the dedicated instrument printouts to the assay sheets and computer record, include ancillary details such as the analysis date for each test and the identity of the technician operator(s). All entries are proofread. Batch data will be transmitted by both IBM DOS floppy diskettes and on report form printout to the National Center for Health Statistics (NCHS). The final test values for each specimen will be identified along with the dates of analysis, the identity of the technical operators involved, whether repeat determinations were required, and if so, for what reason.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Serum is the preferred sample. Do not use plasma. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers.

c. Minimum sample volume is 0.5 mL.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, specimens should be stored at ≤-20 °C until analyzed. Residual specimens are refrozen at ≤-20 °C.

f. Specimens may be stored at 4-8 °C for up to 7 days, or stored frozen at ≤-20 °C.

g. Subsequent freezing and thawing must be avoided as it may lead to a loss of activity.

h. Slightly and moderately lipemic, hemolyzed, or icteric samples are acceptable. Samples that are grossly lipemic, icteric, or hemolyzed are to be brought to the attention of the laboratory manager or technical supervisor.

i. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Micromedic Systems, Inc., model 24000 autodilutor (Polymedco, Inc., Yorktown Heights, NY).

(2) Multi-tube vortexer, cat. no. 58816-115 (VWR Scientific, San Francisco, CA).

(3) Genesys 5000 series, 10-well gamma counter, model 5010R-B (Laboratories Technologies, Inc, Schaumburg, IL).

(4) Beckman GPR refrigerated centrifuge with swing-out rotor (Beckman Instruments, Fullerton, CA).

### b. Other Materials

(1) Kronus Thyroglobulin Antibody RIA kit (Kronus, Dana Point, CA).

(2) 3.5-mL, 55- x 12-mm polypropylene tubes, cat. no. 55.484 (Sarstedt Laboratories, Newton, NC).

(3) 5-mL, 75- x 12-mm polypropylene tubes, cat. no. 55.476 (Sarstedt Laboratories).

(4) Push-in stoppers for 12- x 55-mm and 12- x 75-mm polypropylene tubes, white, cat. no. 65.809.505, (Sarstedt Laboratories).

(5) Eppendorf pipet, 100- to 1000-µL, cat. no. 022 44 020 9 (Brinkman Instruments, Inc, Westbury, NY).

(6) Eppendorf pipet, 10- to 100-µL, cat. no. 022 44 010 1 (Brinkman Instruments, Inc.).

(7) Eppendorf pipet, 100-µL, cat. no. 022 44 180 9 (Brinkman Instruments, Inc.).

(8) Eppendorf pipet, 2- to 20-µL, cat. no. 022 44 005 5 (Brinkman Instruments, Inc.).

(9) Rubber gloves, 100% nitrile (Best Manufacturing, Menlo, GA).

(10) Latex gloves, style 312 (Perry X-AM, Smith & Nephew Perry, Massilon, OH).

(11) Biohazard disposable bag, cat. no. 13166 (VWR Scientific).

(12) Bleach, 10% sodium hypochlorite (NaOCl) solution (any vendor).

(13) Kim-Wipe lintless tissues (Kimberly Clark Corp., Green Bay WI).

(14) Singlefold towels, 227-04 (Fort Howard Corp., Green Bay WI).

(15) Alconox detergent (any vendor).

c. **Reagent Preparation**

Materials are supplied ready-to-use by the manufacturer, Kronus, San Clemente, CA.

(1) $^{125}$I - Thyroglobulin tracer (lyophilized)
Reconstitute each vial with 5.0 mL assay diluent at least 30 min before use. Write the analyst's initials and the date reconstituted on the vial. Stable for 4 weeks after reconstitution. Store at 4-8 °C.

(2) Assay diluent
Supplied ready to use. Contains 0.15 mol/L NaCl, 0.01 mol/L Tris-HCl ($(HOCH_2)_3CNH_2$ HCl), pH 7.5, 5 mg/mL bovine serum albumin, and 0.002 mol/L sodium azide ($NaN_3$) as a preservative. Stable for 1 year; store at 4-8 °C.

(3) Protein A reagent (lyophilized)
Reconstitute with 5.0 mL assay diluent. Contains microbiological protein. Write new expiration date on vial. Stable for 8 weeks after reconstitution; store at 4-8 °C.

d. **Standards Preparation**

Thyroglobulin antibody calibration standards
Supplied in ready-to-use 1-mL vials in concentrations of 0.0, 0.3, 1.0, 10.0, and 30.0 U/mL. Calibrators contain 0.15 mol/L NaCl, 0.02 mol/L Tris-HCl (pH 7.5), 5 mg/mL bovine serum albumin and 0.002 mol/L sodium azide ($NaN_3$) as a preservative.

e. **Preparation of Quality Control Materials**

In-house quality control (QC) pools are prepared in the low, medium, and high concentration ranges of the curve by the USC Endocrine Services Laboratory. These QC pools are made by selective pooling of previously analyzed, excess patient serum, and aliquoting it into appropriately small quantities to minimize any influence of repeat freezing and thawing. Pools are coded and identified by the pooling date. These serum pools, stored at $\leq$-20 °C, are considered to have a shelf life of 2 years.

Sufficient material is always pooled and aliquoted to ensure a supply of QC material sufficient to last more than 1 year under normal work volumes. New pools are constructed and their normal ranges established from a minimum of 10 individual assay runs, not less than three months before the current pool is exhausted. The ranges for each control are documented in the Quality Control Book. When more than 10 unknowns are run in a single assay, the low, medium, and high controls are placed after the calibrators and after the last unknown sample. When fewer than 10 unknowns are run in a single assay, the low, medium, and high controls are placed after the last unknown sample.

This laboratory has previously determined that fresh serum pools are a better QC material than commercially available, modified, lyophilized, serum-based material. Pooled serum is a matrix identical to the test serum sample and better reflects when a run is "out of control."

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration

The Genesys model 5010R-B has full data-reduction capabilities. It produces a standard curve showing a direct relationship between levels of radioactivity measured in corrected counts per min (CPM) and the concentration of ATA in the serum sample. Serum results are expressed as U/mL.

Thyroglobulin antibody calibrators are supplied in ready-to-use 1-mL vials in concentrations of 0.0, 0.3, 1.0, 10.0, and 30.0 U/mL.

### b. Verification

The kit calibration standards are prepared by Kronus and standardized against the MRC thyroglobulin autoantibody First International Reference Preparation coded 65/93, available from the National Institute for Biological Standards and Control (NIBSC), Hertfordshire, England. The NIBSC material is the only available internationally recognized source of human ATA.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Unpack samples immediately after their arrival and check the vials for possible damage during transport. Verify the NCHS numbers of each sample against the packing list for accuracy. Perform the inventory quickly to prevent thawing. Store the samples at ≤-20 °C. Thaw testing batches of 50 samples for assay.

(2) Identify each batch with the box number that will be used throughout all analyses to identify the batch.

(3) Analyze batches of 50 serum specimens or fewer in a single assay. Analyze batches containing more than 50 specimens in two separate assays to avoid assay drift.

### b. Sample Preparation

(1) Allow specimens to thaw at 20-25 °C. (Thawing occurs within 1-2 hours.)

(2) Mix each thawed specimen thoroughly by vortexing.

(3) For the first dilution use only unknowns and controls; do not use calibrators. Program the Abbott dilutor by pressing 2, RECALL to make the first dilution of each serum specimen and low, medium, and high controls (if needed) for ATA analysis. Make the 1:20 dilutions in 12- x 55-mm tubes, using 50 µL serum and 950 µL of assay diluent. Gently vortex the tubes.

Using antibody-free diluent, dilute specimens with concentrations >20 U/mL 1:20, 1:50, or more if necessary, and rerun the assay.

(4) After the dilutions are prepared, transfer the specimens from the Micromedic racks to 12- x 75-mm assay racks.

### c. Instrument Setup of the Genesys 5000

(1) Assay programming for the Genesys 5000 series:

- Response Variable: CPM
- Standard Curve Axes: RESPONSE VS LIN(DOSE)
- Standard Curve Fit: POINT-TO-POINT

(2) Label and load the tubes as shown in Table 1.

Table 1
Tube Loading Order

| Tube | Replications | Dose |
|---|---|---|
| TC (total counts) | 2 | -- |
| NS (non-specific binding) | 2 | -- |
| $B_0$ (0.0 standard) | 2 | -- |
| STD 1 | 2 | 0.3 |
| STD 2 | 2 | 1.0 |
| STD 3 | 2 | 10.0 |
| STD 4 | 2 | 30.0 |
| UNK (unknown) | 2 | -- |

(3) Ensure that enough printer paper is available for the entire run. If necessary, replace paper.

(4) Place the tubes to be counted into the tray holder; insert the tray into the wells.

(5) At the Genesys screen, select EXIT until the SYSTEM MENU is displayed.

(6) Select ASSAY LIBRARY.

(7) Select the assay being analyzed (e.g. ATA).

(8) Select VIEW/EDIT ASSAY SUMMARY.

(9) Select RUN ASSAY.

(10) Select START COUNT. While the tubes are being counted, the user will see the counting time (60 sec), tray number, and time left to count on the topmost line, followed by a display of the actual counts in each well. Adjacent to this line is displayed the assay ID, below which is displayed the protocol ID (AMA/ATA/T4).

(11) After the standards have been counted, press PROCESS UNKNOWNS on the screen displaying the curve. The Genesys 5000 will continue counting the samples and generate a printout.

(12) When the last tray is counted, touch the well number to show the last well used.

(13) When the count screen reappears, touch COUNTING COMPLETE two times.

**d. Instrument Setup of the Beckman Refrigerated Centrifuge**

(1) Temperature range: 6-8 (±2) °C.

(2) Speed control: 3000 (±10) rpm.

(3) Timer: 20 min.

e. **Operation of Assay Procedure**

(1) Ensure that the pumps on the Micromedic (MM) are set to pipet 50 µL of the standards, controls, and first serum dilutions, and dispense these solutions with 450 µL of assay diluent (e.g., ensure that the 200-µL sampler is set at 25% and the 1-mL dispenser is set at 45%). Be sure the toggle switch is set to duplicate on the MM.

(2) Set up the MM racks with tubes in the appropriate places according to the ATA work sheet.

(3) Prime the MM with assay diluent. Discard 3-5 complete cycles of assay diluent. Set the MM ready for the assay from the prime position.

(4) Start running the assay; check the tip depth and wiper pads. Aspirate the specimen and dispense the diluent manually into the first two tubes before switching to automatic. As the racks are completed, check the liquid level and look for samples with inadequate volume. Place those samples in the incubation rack.

(5) Using an Eppendorf Repeater set at 1, and a 2.5-mL combitip, fill the combitip with the tracer by priming it several times. After equilibrating the combitip, discard the first volume back into the vial, and add 50 µL of thyroglobulin tracer to each tube. Be sure to pipette the tracer into two additional TC tubes. Set the TC tubes aside; no further reagents will be added to them.

(6) Gently vortex the tubes.

(7) Incubate all tubes for 60 (±3) min at 20-25 °C.

(8) Mix the protein A reagent immediately prior to use. Using an Eppendorf Repeater set at 1, and a 2.5-mL combitip, equilibrate with the protein A by priming the combitip several times. After equilibrating the combitip, discard the first volume back into the vial and add 50 µL of protein to all tubes *except* the TC tubes. Every time the combitip is refilled, prime it at least twice to ensure a homogeneous mixture of protein A. Avoid air bubbles.

(9) Vortex the tubes, and incubate them for 60 (±3) min at 20-25 °C.

(10) To all tubes except the "total counts" tubes, add 1 mL assay diluent and centrifuge at 3000 RPM for 20 min at 6-8 °C.

(11) Aspirate and discard the supernatants. Count the precipitate in the tubes for 1 min in the counter using the preprogrammed data-reduction program for ATA.

f. **Recording of Data**

(1) Quality Control Data
The USC Thyroid Clinical Laboratory uses the Bio-Rad QC program. The program is set up to allow real-time data entry, providing the user with immediate validation of actual participant results. Data are evaluated and screened according to the multirule system formulated by Westgard. The control data is recorded in the Quality Control Log for NHANES and interpreted for acceptability of assay. When entering the QC results, the analyst indicates any problems with QC runs or any corrective action taken on the "Corrective Action Log" and in the computer. The laboratory technical director and supervisor check the individual test results worksheet and compare the original to the computer printout to detect clerical errors before releasing participant reports.

(2) Analytical Results
Prepare worksheets from the transmittal that was sent from the NHANES MEC. Record each of the duplicate results and the mean result on this worksheet. For results below the detection limit of the method, record "less than 1.0/mL." For results greater than the highest standard, dilute the specimens appropriately (e.g., 1:2) and re-assay them.

g. **Replacement and Periodic Maintenance of Key Components**

(1) Genesys 5000

(a) Perform a daily background check.

(b) Perform a daily detector efficiency check using $^{125}$I multi-calibrators. This reference source is Na($^{125}$I) embedded homogeneously in a cationic resin. The dried point-sources are sealed in 12- x 75-mm polypropylene tubes.

(3) Replace the print head cartridge as needed.

(4) The following items are checked and preventative maintenance is performed by service representative:

- Average efficiency
- High-low efficiency
- Percent spread
- Background
- Background high-low ratio
- Normalization factors of iodine-125, cobalt-57, and dual label
- Check fan, printer, printout, keyboard, display, and software

(2) Pipettors

The calibration of all micropipettors used in testing clinical specimens should be checked every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

h. **Calculations**

(a) Automated: The Genesys model 5010R-B has full data reduction capabilities. It produces a linearized standard curve showing the direct relationship of radioactivity levels in corrected counts per min (CPM) to the concentration of ATA in the serum sample. Serum results are expressed as U/mL.

(2) Manual:

(a) Average the counts in each set of duplicate tubes.

(b) Calculate the percent bound for each assay tube as follows:

$$\frac{\text{average of duplicate counts}}{\text{average of total counts}} \times 100 = \%\ \text{bound}$$

(c) Plot the percent bound versus the concentration of calibrators on semilog graph paper.

(d) Interpolate unknown results from the calibration curve.

(3) The binding in the zero calibrator should be $\leq$5% of the total counts and is calculated as:

$$\frac{STD_0}{TC} \times 100 = \%\ \text{bound in the zero calibrator}$$

(4) The binding in the 30 calibrator should be $\geq$45% of the total counts and is calculated as:

$$\frac{STD_{30}}{TC} \times 100 = \%\ \text{bound in the 30 calibrator}$$

i. **Special Procedure Notes -- USC Modifications**

(1) Allow all reagents to come to 20-25 °C before use.

(2) After making the first dilution, freeze control aliquots until the next assay.

**9. REPORTABLE RANGE OF RESULTS**

The normal range for antithyroglobulin in human serum is <1.0 U/mL.

The routine working range of the assay is 1.0-20.0 U/mL.

Results <1.0 U/mL are reported as <1.0 U/mL.

Results >20.0 U/mL are diluted 1:20 or 1:50 with antibody-free diluent for serum and rerun. The resulting value is multiplied by the dilution factor used. The value is reported as a whole number with a decimal.

For serum samples with concentrations >3000 U/mL after appropriate dilutions, the final results are reported as >3000 U/mL.

NOTE: CAP Survey results are reported as international units (IU). (1 IU = 10 U/mL).

**10. QUALITY CONTROL (QC) PROCEDURES**

In addition to the Bio-Rad QC program (see section 8.f.1). The USC Thyroid Clinical Laboratory applies the floating mean principle. If a trend or shift occurs, the problem is investigated and corrected. A new mean is calculated from the last 2 months' data by using a months' fixed mean until a new floating mean is established (6,7).

Two types of QC systems are used in this analytical method, the sample QC system and the bench QC system. With the sample QC system, 5% of the specimens are randomly selected and analyzed either within an assay or between assays for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is reanalyzed. Bench QC specimens are placed before and after all specimens to be analyzed.

An internal QC program monitors the accuracy and precision of laboratory performance on a daily basis. The analyst inserts bench QC specimens two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. Three separate serum pools having low, medium, and high range values for the analyte are inserted in each assay. Each of three pools is run twice in each assay -- once at the beginning and once at the end of the run. That is, specimens with unknown values are "sandwiched" between the QC pools to ensure that the assay run has not been subjected to methodologic drift.

If the stock of these controls becomes low, another batch is prepared in time to analyze it concurrently with the current QC materials. The new controls are used only after their means and ranges are established following 10 characterization runs. Sometimes, more runs are used to update control means and ranges if 10 runs are deemed inadequate to represent the overall characteristic of the method over time. All updates of control means and ranges are performed only after approval from NCHS.

In addition, the assay mean (the mean of all the values falling within the normal limits for that analyte) will be used as an additional medium-range QC.

The USC Thyroid Clinical Laboratory, a state-licensed facility, is externally monitored and certified by the College of American Pathologists, an interlaboratory comparison system that performs proficiency testing designed to compare performance with those of other laboratories.

a. Determining the acceptability of control results:

(1) Analyze samples of three different control levels. Make replicate measurements on each control material at the beginning and end of each run when testing for statistical control. Record these observations as the daily average.

(2) Accept the run when two or more mean control observations are within 2 standard deviations (SD) of the mean. Report participant's results. When at least one control observation is more than 3 SDs from the mean, hold the participant results and inspect the control data further using additional control rules.

(3) Inspect control data within the run:

- Reject the run when one control observation falls outside the mean ±3 SD limits. Do not report participant results.
- Reject the run when two or more control observations fall more than 2 SDs outside the same mean. Do not report participant results.
- Accept the run if one control falls outside the mean by more than 2 SDs but less than 3 SD, if other controls are within 2 SDs.

(4) Inspect control data across run:

- Reject the run when previous observations on the same control level fell more than 2 SDs outside the same mean. Do not report participant results.
- Reject the run when the last 10 consecutive observations of a pool fall on the same side of the mean. Do not report participant results.

(5) Accept the run when none of the above rules indicate a lack of statistical control. Report participant results.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. When the analytical run is out of control take the following actions:

(1) Determine the type of errors occurring (random, systematic, or both), on the basis of the control rules being violated. A review of control data on all control levels will help detect errors occurring throughout the concentration range tested by these controls. A review of control data on a single control level (within control materials) will help detect errors in a particular concentration range.

(2) Refer to a troubleshooting guide to inspect the components of the method or instrument that contributes to the type of error observed.

(3) Correct the problem, then reanalyze the participant and control samples, testing for statistical control by the same procedures. Do not include data from previous out-of-control situations in the cumulative data. If results were more than 3 SDs outside the mean, indicate corrective action taken on the "Corrective Action Log" and in the computer when entering the QC result.

(4) Consult the laboratory manager or technical supervisor before reporting data when there is a lack of statistical control.

b. In the following situations, the laboratory manager or technical supervisor may decide to report the data even when there is lack of statistical control.

(1) The control problem is shown to be due to the control materials themselves.

(2) The control problem is shown to have resulted from an isolated event not affecting the rest of the run (e.g., an interchange of two samples or a clerical transcription error).

(3) The control problem occurs in a concentration range different from the concentrations of the participant samples. The method is in-control in the range of the participant samples.

(4) The size of the analytical error is judged to be small relative to the medical usefulness requirements. For example, small shifts of the analytical mean relative to medical needs may be ignored when associated with reagent changes.

(5) Participant data can be released even if QC results fall outside pre-established statistical limits, provided participant care is not compromised.

c. Check the clinical appropriateness of results before reporting:

Each day the laboratory technical director and supervisor will check the cumulative summary of the results for each participant specimen to determine the clinical appropriateness of the results. They will check the individual test results worksheet and compare the original to the computer printout to detect clerical errors before releasing participant reports.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

This test should only be run after one has become familiar with the information provided by Kronus in the product insert. This laboratory has determined that neither repeated freezing and thawing, storing serum specimens for 6 to 8 hours at 20-25 °C, nor storing them for 24-48 hours at 4 °C will affect the stability of these thyroid tests.

No interfering substances have been identified.

**13. REFERENCE RANGES (NORMAL VALUES)**

Expected normal values for antithyroglobulin in human serum are less than 1.0 U/mL.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable to this procedure.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should be allowed to reach 20-25 °C during testing. Specimens can be refrigerated at -8 °C overnight for the TSH analysis. After analysis, specimens are immediately refrozen at ≤-20 °C and thawed only for repeat testing.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Currently, there is no alternative method for performing this test but a backup instrument is maintained. If the analytical system fails, specimens may be stored at 4-8 °C for up to 7 days, or they may be stored frozen at ≤-20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Results are recorded electronically on floppy discs provided by the NHANES MEC. Records of QC data, specimen results, dilutions, and random and repeat results are maintained in the laboratory.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard records such as computer data files and printed copies should be used to track specimens. We recommend that records, including related QA/QC data, be maintained for 6 years, and that duplicate records be kept. Only numerical identifiers (e.g., NCHS ID numbers) should be used; all personal identifiers are kept masked and available only to the project coordinator to safeguard confidentiality.

Residual specimens are stored at ≤70 °C for 1 year and are returned to the NCHS serum bank.

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1. Kronus 1991 Product Insert: P/N 114J, dated 3/91, Thyroglobulin Antibody (TgAb) RIA Kit.

2. Ericsson UB, Christensen SB, Thorell JI. A high prevalence of thyroglobulin auto-antibodies in adults with and without thyroid diseases as measured with a sensitive solid-phase immunosorbent radioassay. Clin Immunol & Immunopathol 1985;2:154-62.

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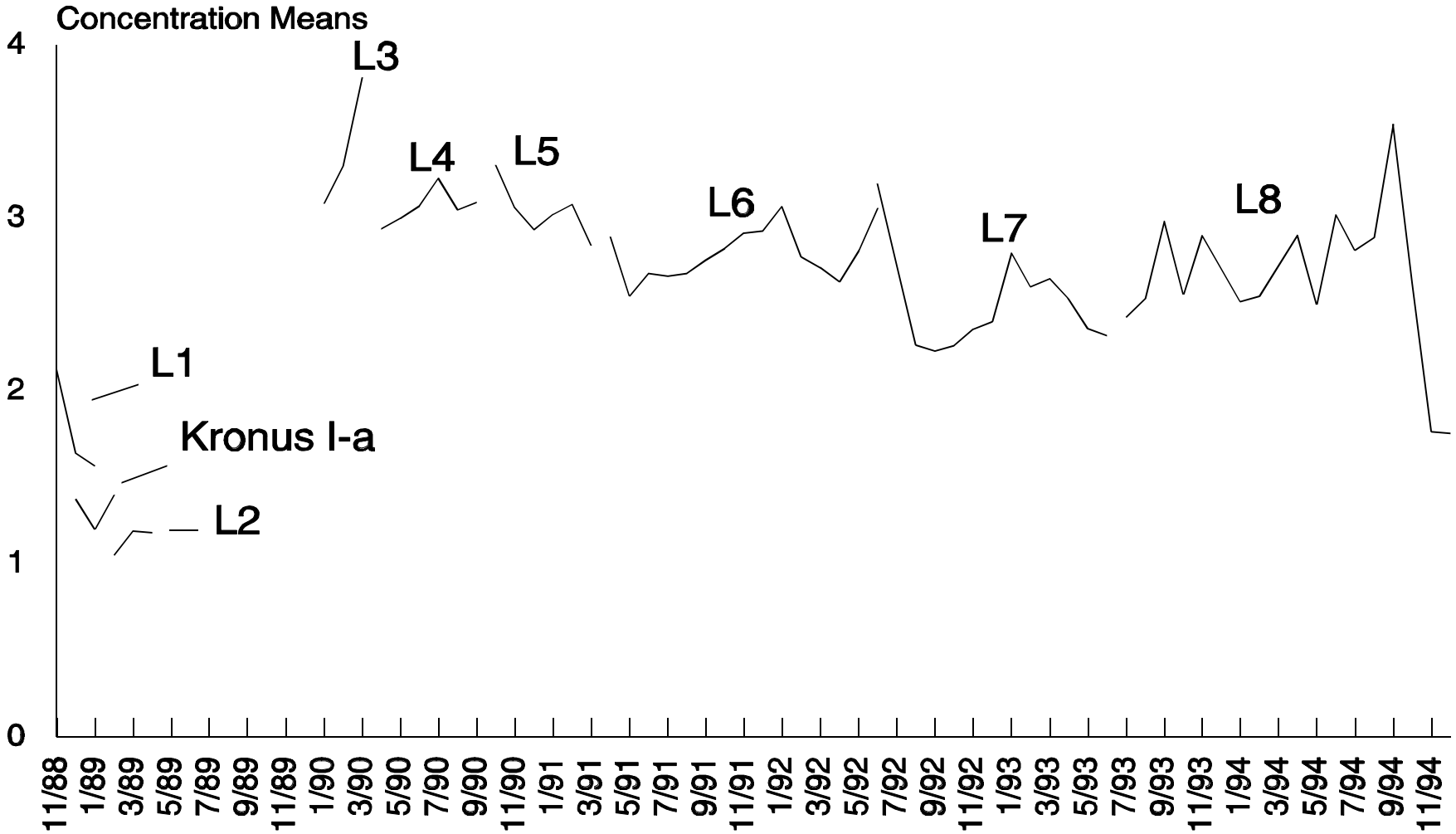
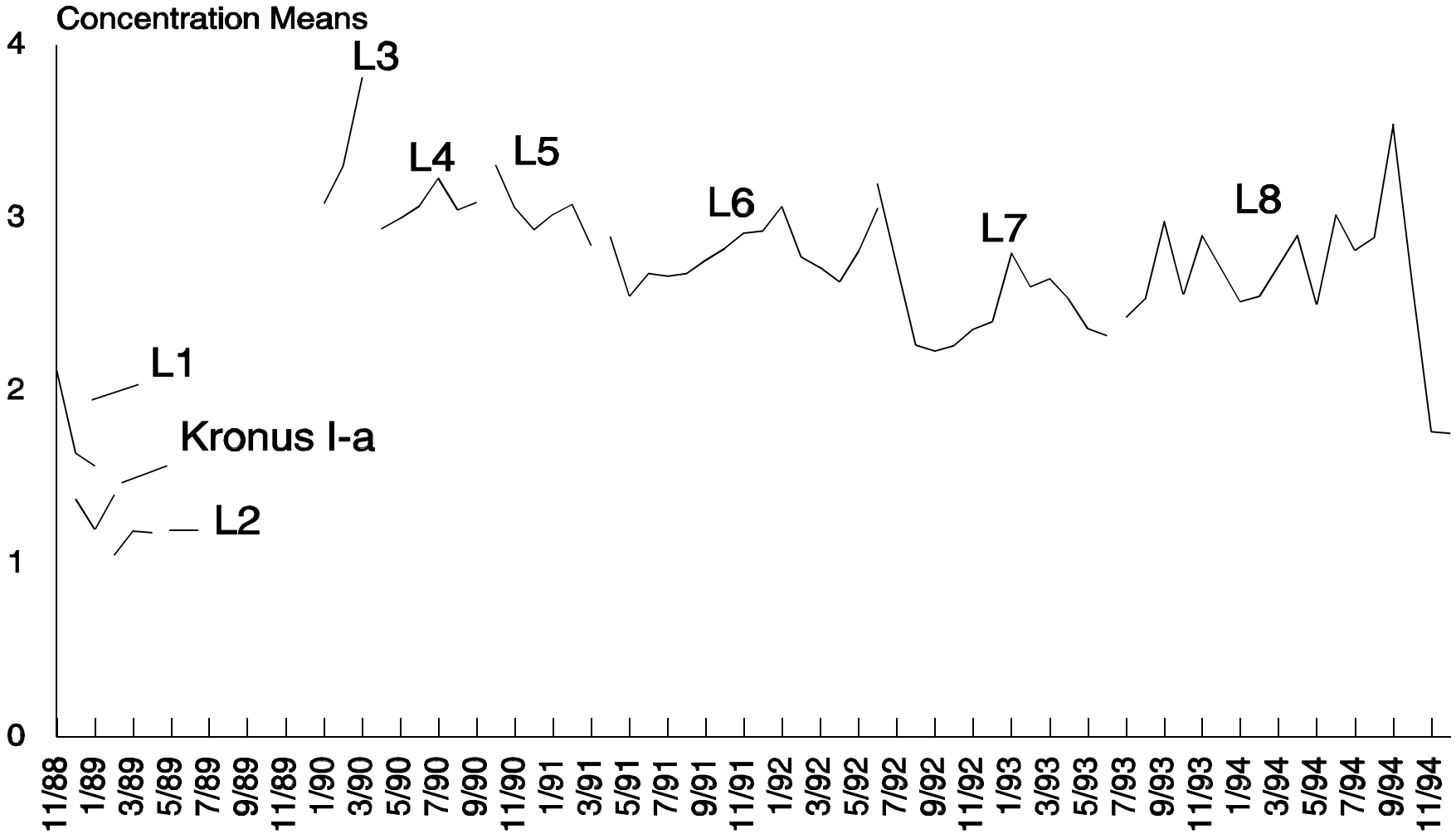
SUMMARY STATISTICS FOR
ANTITHYROGLOBULIN ANTIBODY (ATA) - LOW POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1 | 11/09/88 - 01/24/89 | 1.78333 | 0.53865 | 30.2047 | 24 |
| L2 | 02/14/89 - 04/11/89 | 1.15769 | 0.46232 | 39.9345 | 26 |
| L3 | 01/03/90 - 03/14/90 | 3.30625 | 0.37840 | 11.4449 | 32 |
| L4 | 04/04/90 - 09/30/90 | 3.06000 | 0.16782 | 5.4843 | 50 |
| L5 | 10/04/90 - 03/31/91 | 3.02817 | 0.42095 | 13.9010 | 71 |
| L6 | 04/07/91 - 06/20/92 | 2.79716 | 0.28813 | 10.3009 | 176 |
| L7 | 06/28/92 - 06/28/93 | 2.50574 | 0.37731 | 15.0579 | 122 |
| L8 | 07/19/93 - 12/22/94 | 2.70735 | 0.51231 | 18.9228 | 204 |
| KR1A | 12/01/88 - 02/02/89 | 1.28182 | 0.24421 | 19.0515 | 11 |

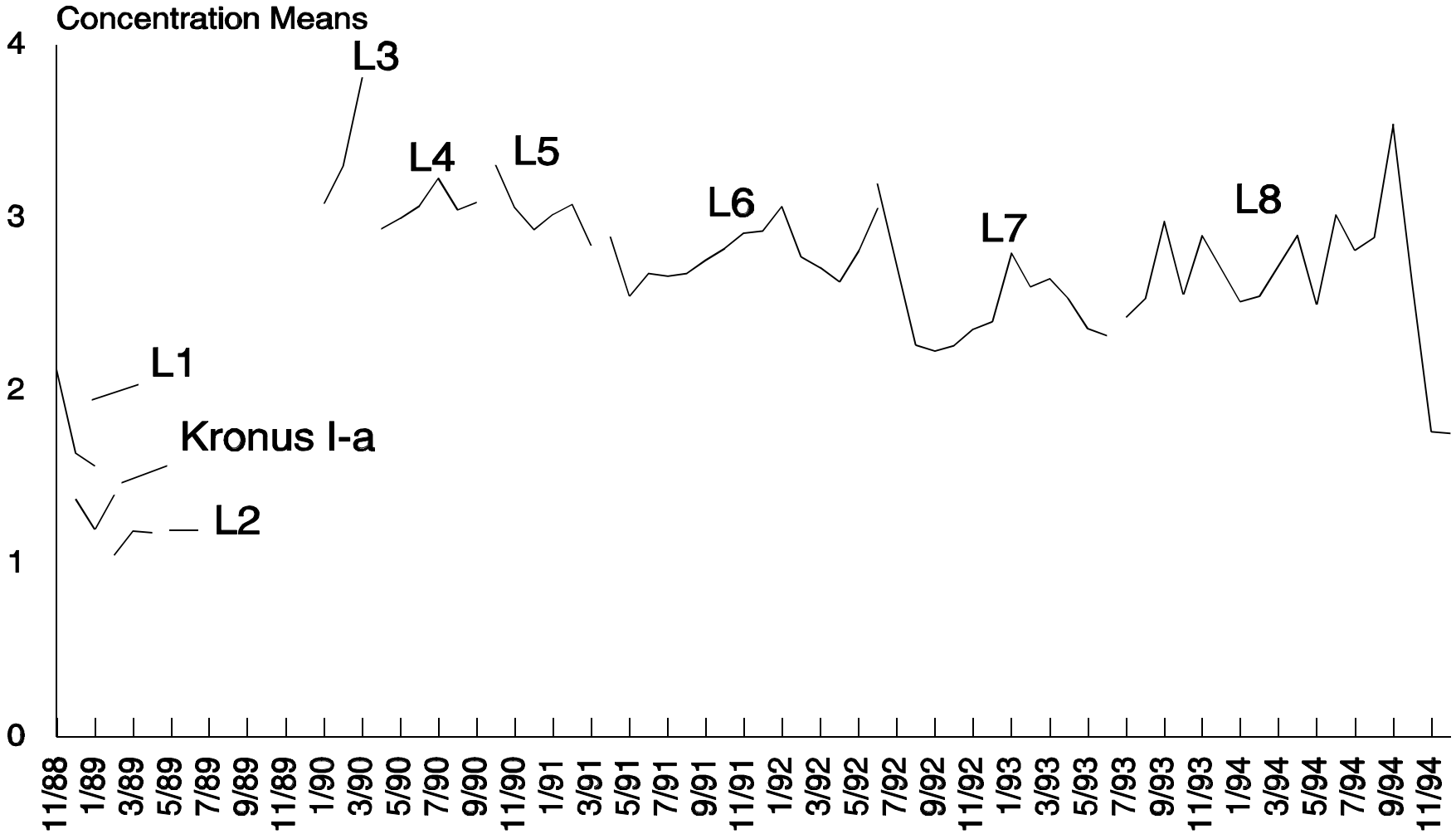
# Antithyroglobulin Antibody (ATA) Monthly Means - Low



# Antithyroglobulin Antibody (ATA) Monthly Means - Low



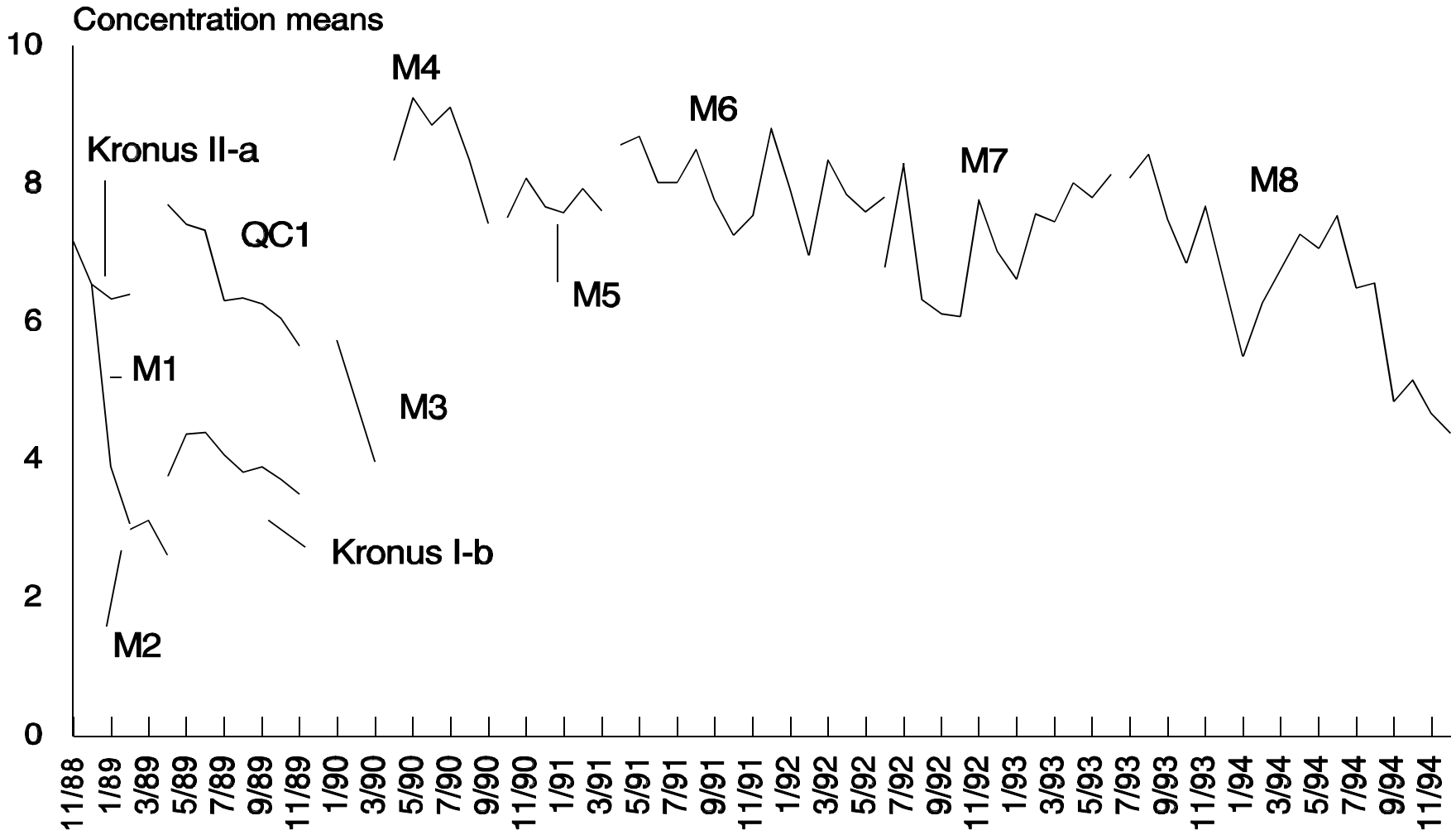
# Antithyroglobulin Antibody (ATA) Monthly Means - Low



SUMMARY STATISTICS FOR
ANTITHYROGLOBULIN ANTIBODY (ATA) - MEDIUM POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| M1 | 11/22/88 - 02/10/89 | 5.23571 | 1.99392 | 38.0830 | 28 |
| M2 | 02/14/89 - 04/11/89 | 2.98077 | 0.99157 | 33.2656 | 26 |
| M3 | 01/03/90 - 03/14/90 | 5.12813 | 0.95756 | 18.6728 | 32 |
| M4 | 04/04/90 - 09/09/90 | 8.83409 | 0.60615 | 6.8615 | 44 |
| M5 | 09/15/90 - 03/31/91 | 7.60263 | 0.63979 | 8.4153 | 76 |
| M6 | 04/07/91 - 06/20/92 | 7.95575 | 0.73070 | 9.1846 | 174 |
| M7 | 06/28/92 - 06/26/93 | 7.36379 | 0.93534 | 12.7018 | 116 |
| M8 | 07/19/93 - 12/22/94 | 6.72921 | 1.34922 | 20.0502 | 202 |
| KR1B | 04/17/89 - 11/02/89 | 4.01892 | 0.52643 | 13.0989 | 37 |
| KR2A | 12/01/88 - 02/02/89 | 6.42500 | 0.31079 | 4.8372 | 12 |
| QC1 | 04/17/89 - 11/02/89 | 6.74384 | 0.77656 | 11.5152 | 73 |

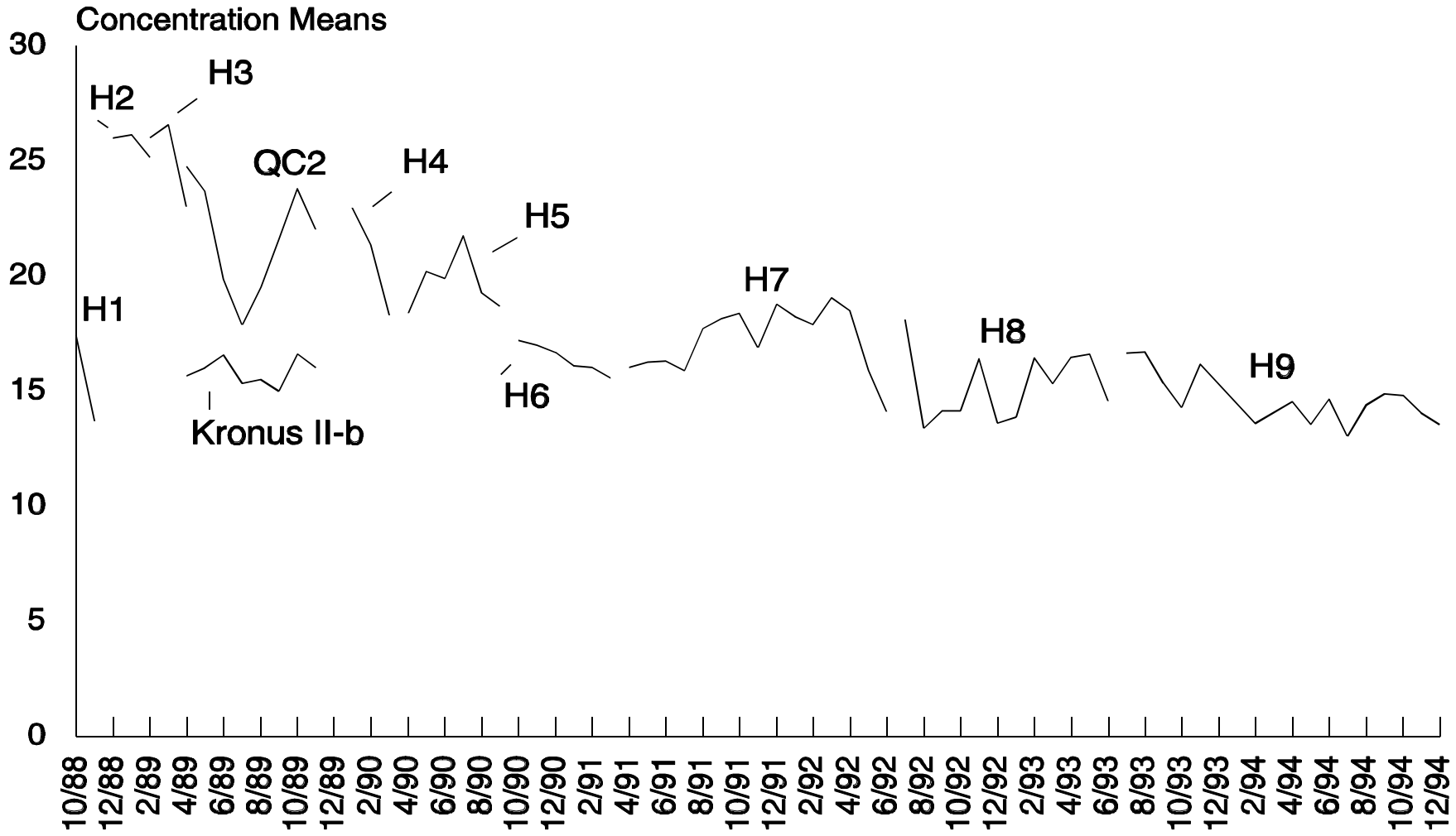
# Antithyroglobulin Antibody (ATA) Monthly Means - Medium



SUMMARY STATISTICS FOR
ANTITHYROGLOBULIN ANTIBODY (ATA) - HIGH POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| H1 | 10/31/88 - 11/09/88 | 15.5375 | 2.42425 | 15.6025 | 8 |
| H2 | 12/22/88 - 02/10/89 | 25.8688 | 1.40320 | 5.4243 | 16 |
| H3 | 02/14/89 - 04/11/89 | 25.7200 | 1.88237 | 7.3187 | 25 |
| H4 | 01/03/90 - 03/14/90 | 21.5806 | 2.46000 | 11.3991 | 31 |
| H5 | 04/04/90 - 09/09/90 | 19.8043 | 1.34362 | 6.7845 | 46 |
| H6 | 09/15/90 - 03/31/91 | 16.2260 | 1.33795 | 8.2457 | 77 |
| H7 | 04/07/91 - 06/20/92 | 17.2188 | 1.73783 | 10.0926 | 176 |
| H8 | 07/12/92 - 06/28/93 | 15.4416 | 1.65663 | 10.7283 | 113 |
| H9 | 07/19/93 - 12/22/94 | 14.7964 | 1.69193 | 11.4347 | 195 |
| KR2B | 04/17/89 - 11/02/89 | 15.8108 | 1.24360 | 7.8655 | 37 |
| QC2 | 04/17/89 - 11/02/89 | 21.6707 | 2.49471 | 11.5119 | 82 |

# Antithyroglobulin Antibody (ATA) Monthly Means - High



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This highly sensitive, direct radioimmunoassay system is based on the use of a highly purified, stable preparation of $^{125}$I-labeled thyroid peroxidase enzyme (TPO). The assay is standardized against the MRC (Medical Research Council) Microsomal Antigen Auto-Antibody First International Reference Preparation (coded 66/387) (National Institute for Biological Standards and Control (NIBSC), Hertfordshire, England).

Specimens containing known amounts of TPO antibody and those containing unknown amounts are incubated for 1 hour with the purified $^{125}$I-TPO. During this incubation, the TPO antibodies present bind with labeled TPO. Protein A (microbiological protein) is added and the tubes are incubated for another hour. During this incubation, the Fc portion of TPO antibodies present are bound by the protein A. Assay buffer is added and the tubes are centrifuged. After centrifuging, the supernatants are decanted or aspirated. The amount of radioactivity in the pellets is directly proportional to the amount of TPO antibody contained in the calibrators and unknowns. Calibrator concentrations are plotted on semilog graph paper, and the concentration of antibody in the unknowns is interpolated from the curve (1).

Measurement of thyroid microsomal autoantibodies can be of considerable value in the diagnosis of thyroid diseases, such as autoimmune thyroiditis, Graves' disease, endemic goiter, and subacute thyroiditis (2). These measurements, as part of a thyroid autoimmune diagnostic profile that would include thyroglobulin antibody and thyrotropin receptor antibody, can be very useful in the differential diagnosis and management of thyroid disease (3-6).

## 2. SPECIAL SAFETY PRECAUTIONS:

The assay employs $^{125}$I as a tracer and all necessary radiation safety considerations for isotope management and disposal must be observed according to the guidelines of the *U.S.C. Radiation Safety Manual*. In addition, all personnel must successfully complete the training course *Radiation Safety in the Laboratory* or demonstrate having received equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective eyewear, gloves, and lab coat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines. Wipe countertops with a 10% sodium hypochlorite (bleach) solution after assay completion.

Material safety data sheets (MSDSs) for sodium chloride, TRIS-hydrochloric acid, $^{125}$I- TPO tracer, sodium azide, and sodium hypochlorite are available through the laboratory office.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III Mobile Examination Center (MEC) contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" high-density (HD) diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data and by storing data in multiple computer systems.

c. A cumulative computerized laboratory log, which is generated with all specimens received, contains batch receipt dates and updates on all details and problems associated with specimen handling, manipulation, and analysis.

d. Documentation for system maintenance is contained in hard copies of data records.

e. The results from the assay are transcribed from the dedicated instrument printouts to the assay sheets and computer record. All entries are proofread. Batch data will be transmitted to NCHS via both IBM DOS floppy diskettes via a report form printout. The final results for each specimen will be identified along with the dates of analysis, technical operators involved, whether repeat determinations were required, and if so, for what reason.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Serum is the preferred sample. Do not use plasma. Serum specimens may be collected using regular red-top or serum separator Vacutainers.

c. Minimum sample volume is 0.5 mL.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, specimens should be stored at ≤-20 °C until analyzed. Residual aliquots after testing are refrozen at ≤-20 °C.

f. Specimens may be stored at 4-8 °C for up to 7 days or may be stored frozen at ≤-20 °C.

g. Avoid subsequent freezing and thawing as it may lead to a loss of activity.

h. Slightly and moderately lipemic, hemolyzed, or icteric samples are acceptable. Samples that are grossly lipemic, icteric, or hemolyzed are to be brought to the attention of the laboratory manager or technical supervisor.

i. Specimens may be stored in glass or plastic vials, as long as vials are tightly sealed to prevent desiccation of the sample.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Micromedic Systems, Inc. Model 24000 autodilutor (Polymedco, Inc., Yorktown Heights, NY).

(2) Multi-tube vortexer, cat. no. 58816-115 (VWR Scientific, San Francisco, CA).

(3) Genesys 5000 series, 10-well gamma counter, model 5010R-B (Laboratories Technologies, Inc., Schaumburg, IL).

(4) Eppendorf pipet, 100- to 1000-µL, cat. no. 022 44 020 9 (Brinkman Instruments, Inc., Westbury, NY).

(5) Eppendorf pipet, 10- to 100-µL, cat. no. 022 44 010 1 (Brinkman Instruments, Inc.).

(6) Eppendorf pipet, 100-µL, cat. no. 022 44 180 9 (Brinkman Instruments, Inc.).

(7) Eppendorf pipet, 2- to 20-µL, cat. no. 022 44 005 5 (Brinkman Instruments, Inc.).

b. **Other Materials**

(1) Kronus TPO Antibody RIA Kit (Kronus, Dana Point, CA).

(2) 3-5 mL, 55- x 12-mm polypropylene tubes, cat. no. 55.484 (Sarstedt Laboratories, Newton, NC).

(3) 5-mL, 75- x 12-mm polypropylene tubes, cat. no. 55.476 (Sarstedt Laboratories).

(4) Push-in stoppers for 12- x 55-mm and 12- x 75-mm polypropylene tubes, white, cat. no. 65.809.505 (Sarstedt Laboratories).

(5) Rubber gloves, 100% nitrile (Best Manufacturing, Menlo, GA).

(6) Latex gloves, style 312 (Perry X-AM, Smith & Nephew Perry, Massilon, OH).

(7) Biohazard disposable bags, cat. no. 13166 (VWR Scientific).

(8) Bleach, 10% sodium hypochlorite solution (NaOCl) (any vendor).

(9) Kim-Wipe lintless tissues (Kimberly Clark Corp., Green Bay, WI).

(10) Singlefold towels, 227-04 (Fort Howard Corp., Green Bay, WI).

c. **Reagent Preparation**

Materials are supplied ready-to-use by Kronus.

(1) $^{125}$I - TPO Tracer (lyophilized)
Reconstitute each vial with 5.0 mL assay diluent at least 30 min before use. Write tech initials and date reconstituted on the vial. Stable for 4 weeks after reconstitution; store at 4-8 °C.

(2) Assay diluent
Diluent is supplied ready to use. Contains 0.15 mol/L NaCl, 0.02 mol/L Tris-HCl ($(HOCH_2)_3CNH_2$ HCl), pH 7.5, bovine serum albumin, and 0.002 mol/L sodium azide ($NaN_3$) as preservative. Stable for 1 year; store at 4-8 °C.

(3) Protein A reagent (lyophilized)
Reconstitute with 5.0 mL assay diluent. Contains microbiological protein. Write new expiration date on vial. Stable for 8 weeks after reconstitution; store at 4-8 °C.

(4) Antibody-free diluent for serum
Diluent is supplied ready to use. Contains 0.1% sodium azide ($NaN_3$), tris buffer, and animal protein. For use in diluting neat serum of specimens having values greater than the highest calibrator. Stable for 1 year; store at 4-8 °C.

d. **Standards Preparation**

AMA calibration standards are supplied in 1-mL vials, ready to use in concentrations of 0.0, 0.3, 1.0, 10.0 and 30.0 U/mL. Calibrators contain 0.15 mol/L NaCl, 0.02 mol/L Tris-HCl ($(HOCH_2)_3CNH_2$ HCl) (pH 7.5), 5 mg/mL bovine serum albumin and 0.002 mol/L sodium azide ($NaN_3$) as a preservative.

Make a 2:3 dilution of the 30.0 U/mL standard by adding 0.3 mL of the 0.0 U/mL standard and 0.7 mL of the 30.0 U/mL standard to obtain a 21.0 U/mL standard; store at 4-8 °C.

e. **Preparation of Quality Control Materials**

In-house controls consist of specimens with low, medium, and high concentrations of AMA (USC Endocrine Services Laboratory, Los Angeles, CA).

In-house QC pools are prepared in the low, medium, and high concentration ranges of the curve. These QC pools are made by selective pooling of previously analyzed, excess serum, and aliquoting it into appropriately small quantities to minimize any influence of repeat freezing and thawing. Pools are coded and identified by the pooling date. These serum pools, stored at ≤-20 °C, are considered to have a shelf life of 2 years.

Sufficient material is always pooled and aliquoted to ensure a supply of QC material sufficient to last more than 1 year under normal work volumes. New pools are constructed and their normal ranges established from a minimum of 10 individual assay runs, not less than 3 months the current pool is exhausted. The ranges for each control are documented in the Quality Control Book. When more than 10 unknowns are run in a single assay, the low, medium, and high controls are placed after the calibrators and after the last unknown sample. When fewer than 10 unknowns are run in a single assay, the low, medium and high controls are placed after the last unknown sample.

This laboratory has previously determined that serum pools are a better QC material than commercially available, modified, lyophilized, serum-based material. Pooled serum is a matrix identical to the test serum sample and better reflects when a run is "out of control."

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

The Genesys model 5010R-B has full data-reduction capabilities. It produces a standard curve showing a direct relationship between levels of radioactivity measured in corrected counts per min (CPM) to concentration of AMA in the serum sample. Serum results are expressed as U/mL.

a. **Calibration**

TPO antibody calibrators are supplied in ready-to-use, 1-mL vials, in concentrations of 0.0, 0.3, 1.0, 10.0 and 30.0 U/mL. These calibrators are assayed with each run.

b. **Verification**

The AMA kit calibration standards are prepared by Kronus and standardized against the MRC microsomal antigen autoantibody First International Reference Preparation, coded 66/387, available from the National Institute for Biological Standards and Controls (NIBSC) Hertfordshire, England. The NIBSC material is the only available internationally recognized source of human AMA.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Unpack samples immediately after arrival, and check the vials for possible damage during transport. Verify the NCHS numbers of each sample against the packing list. Complete the inventory quickly to prevent thawing, and store samples at ≤-20 °C. Thaw testing batches of 50 samples for assay.

(2) Identify each batch with the box number which will be used throughout all analyses to identify the batch.

(3) Analyze batches of 50 serum specimens or fewer in a single assay. Analyze batches containing more than 50 specimens in two separate assays to avoid assay drift.

b. **Sample Preparation**

(1) Allow specimens to thaw at 20-25 °C. (Thawing occurs within 1-2 hours.)

(2) Mix each thawed specimen thoroughly by vortexing.

(3) For the first dilution use only unknowns and controls; do not use calibrators here. Program the Abbott dilutor by pressing 2, RECALL to make the first dilution of each serum specimen and low, medium, and high controls (if needed) for AMA analysis. Make the 1:20 dilutions in 12- x 55-mm tubes, using 50 µl serum and 950 µl of assay diluent. Gently vortex the tubes.

Using antibody-free diluent for serum, dilute specimens with results >20, 1:20, 1:50, or more if necessary and rerun the assay.

**Instrument Setup of the Genesys 5000**

(1) Assay programming for the Genesys 5000 series:

- Response Variable: CPM
- Standard Curve Axes: RESPONSE VS LIN(DOSE)
- Standard Curve Fit: POINT-TO-POINT

(2) Label and load the tubes as shown in Table 1.

(3) Ensure that enough printer paper is available for the entire run. If necessary, replace paper.

(4) Place the tubes to be counted into the tray holder; insert the tray into the wells.

Table 1
Tube Loading Order

| Tube | Replications | Dose |
|---|---|---|
| TC (total counts) | 2 | -- |
| NS (non-specific binding) | 2 | -- |
| $B_0$ (0 standard) | 2 | -- |
| STD 1 | 2 | 0.3 |
| STD 2 | 2 | 1.0 |
| STD 3 | 2 | 10.0 |
| STD 4 | 2 | 20.0 |
| UNK (unknowns) | 2 | ? |

(5) At the Genesys screen, select EXIT until the SYSTEM MENU is displayed.

(6) Select ASSAY LIBRARY.

(7) Select the assay being analyzed (e.g., AMA).

(8) Select VIEW/EDIT ASSAY SUMMARY.

(9) Select RUN ASSAY.

(10) Select START COUNT. Count the tubes for one min on the Genesys gamma counter using the preprogrammed data reduction program entitled ANTI-TPO. While the tubes are being counted, the user will see the counting time, tray number, and time left to count on the topmost line, followed by a display of the actual counts in each well. Adjacent to this line is the assay ID, below which is displayed the protocol ID (AMA/ATA/T4).

(11) After the standards have been counted, select PROCESS UNKNOWNS from the screen displaying the standard curve. The instrument will continue counting the samples and generate a printout.

(12) When the last tray is counted, touch the well number to show the last well used; the other wells are covered.

(13) When the count screen reappears, touch COUNTING COMPLETE two times.

**d. Instrument Setup of the Beckman Refrigerated Centrifuge**

(1) Temperature setting: 20 (±2) °C.

(2) Speed control: 3000 (±10) rpm.

(3) Timer: 20 min.

e. **Operation of Assay Procedure**

(1) Ensure that the pumps on the Micromedic (MM) are set to sample 50 µL of the standards, controls, and first serum dilutions, and dispense each of these samples along with 450 µL of assay diluent (e.g., ensure that the 200-µL sampler is set at 25% and the 1-mL dispenser is set at 45%). Be sure the toggle switch is set to duplicate on the MM.

(2) Set up the MM racks with tubes in the appropriate places according to the AMA work sheet.

(3) Prime the MM with assay diluent. Discard 3-5 complete cycles of assay diluent then set the MM ready for the assay.

(4) Begin the assay; check the tip depth, wiper pads, specimen aspirating, and diluent dispensing manually for the first two tubes before switching to automatic. As the racks are completed check the liquid level and look for samples with inadequate volume. Place the samples in the incubation rack.

(5) Using an Eppendorf Repeater set at 1 and a 2.5-mL combitip, fill the tip with the tracer by priming the combitip several times. After equilibrating the combitip, discard the first volume back into the vial, and add 50 µL of TPO tracer to each tube. Be sure to pipette the tracer into two additional TC tubes. Set the TC tubes aside. No further reagents will be added to them.

(6) Gently vortex the tubes.

(7) Incubate all tubes for 60 (±3) min at 20-25 °C.

(8) Mix the protein A reagent immediately prior to use. Using an Eppendorf Repeater set at 1 and a 2.5-mL combitip, fill the tip with the protein A by priming the combitip several times. After equilibrating the combitip, discard the first volume back into the vial and add 50 µL of protein A to all tubes except the TC tubes. Every time the combitip is refilled, prime it at least twice to ensure a homogeneous mixture of protein A. Avoid air bubbles.

(9) Vortex the tubes, and incubate all tubes for 60 (±3) min at 20-25 °C.

(10) To all tubes *except* "total counts", add 1 mL assay diluent and centrifuge at 3000 rpm for 20 min at 6-8 °C.

(11) Aspirate and discard the supernatants and count the precipitate in the tubes for 1 min in counter using the preprogrammed data-reduction program, ANTI-TPO.

f. **Recording of Data**

(1) Quality Control Data

The USC Thyroid Clinical Laboratory uses the Bio-Rad QC program. The program is set up to allow real-time data entry, providing the user with immediate validation of actual participant results. Data is evaluated and screened according to the multirule system formulated by Westgard. The control data is recorded in the Quality Control Log for NHANES and interpreted for acceptability of assay. Any problem with QC runs and corrective action taken is indicated on the "Corrective Action Log" and in the computer when QC result is entered. The laboratory technical director and supervisor check the individual test results worksheet and compare the original to the computer printout to detect clerical errors before releasing participant reports.

(2) Analytical Results

Prepare worksheets from the transmittal from the NHANES MEC. Record each of the duplicate results and the mean result on this worksheet. For results below the detection limit of the method, record "less than 0.5 U/mL." For results greater than the highest standard, dilute the specimens appropriately (e.g., 1:2) and re-assay them.

g. **Replacement and periodic maintenance of key components**

(1) Genesys model 5010R-B

(a) Perform a daily background check.

(b) Perform a daily detector efficiency check using $^{125}$I multi-calibrators. This reference source is: Na($^{125}$I) embedded homogeneously in a cationic resin. The dried point-sources are sealed in 12- x 75-mm polypropylene tube.

(c) The following items are checked and preventative maintenance is performed by service representative:

- Average efficiency
- High-low efficiency
- Percent spread
- Background
- Background high-low ratio
- Normalization factors of iodine-125, cobalt-57 and dual-label
- Check fan, printer, printout, keyboard, display and software

(2) Pipettors

Every 6 months check the calibration of all micropipettors used in testing clinical specimens. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

h. **Calculations**

(1) Automated

The Genesys model 5010R-B has full data reduction capabilities. It produces a linearized standard curve showing the direct relationship of levels of radioactivity measured in counter per min (CPM) to the concentration of AMA in the serum sample. Serum results are expressed as U/mL.

(2) Manual

(a) Average the counts in each set of duplicate tubes.

(b) Calculate the percent bound for each assay tube as follows:

$$\frac{\text{average of duplicate counts}}{\text{average of total counts}} \times 100 = \%\ \text{bound}$$

(c) Plot the percent bound versus the concentration of calibrators on semilog graph paper.

(d) Interpolate unknown results from the calibration curve.

(3) The binding in the zero calibrator should be $\leq$5% of the total counts and is calculated as:

$$\frac{STD_0}{TC} \times 100$$

(4) The binding in the 30 calibrator should be $\geq$30% of the total counts and is calculated as:

$$\frac{STD_{30}}{TC} \times 100$$

i. **Special Procedure Notes - USC Modifications**

(1) Allow all reagents to come to 20-25 °C before use.

(2) After making the first dilution, freeze the control aliquots until the next assay.

**9. REPORTABLE RANGE OF RESULTS**

The normal range for AMA in human serum is <0.5 U/mL.

The routine assay working range is 0.5-20.0 U/mL.

Results <0.5 are reported as <0.5 U/mL.

Results >20.0 U/mL are diluted 1:20 or 1:50 with antibody-free diluent for serum and rerun. The resulting value is

multiplied by the dilution factor used. The value is reported as a whole number (e,g., 160).

For serum samples with AMA concentrations >3000 after appropriate dilutions, the final results are reported as >3000 U/mL.

NOTE: The CAP Survey results are reported in international units (IU) (1 IU = 10 U/mL).

**10. QUALITY CONTROL (QC) PROCEDURES**

The USC Thyroid Clinical Laboratory uses the Bio-Rad QC program. The program is designed to allow real-time data entry, providing the user with immediate validation of actual participant results. Data is evaluated and screened according to the multi-rule system formulated by Westgard. This system helps maximize laboratory resources by decreasing unnecessary assay rejection and simultaneously increases sensitivity to error. The lab QC manager generates QC reports on a monthly basis for individual laboratory statistics (e.g., Levy-Jennings).

The USC Thyroid Clinical Laboratory applies the floating mean principle. If a trend or shift occurs, the problem is investigated and corrected. A new mean is calculated from the last 2 months' data by using a fixed mean until a new floating mean is established (6,7).

Two types of QC systems are used in this analytical method, the sample QC system and the bench QC system. With the sample QC system, 5% of the specimens are randomly selected and analyzed either within an assay or between assays for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is reanalyzed. Bench QC specimens are placed before and after all specimens to be analyzed.

An internal QC program monitors the accuracy and precision of laboratory performance on a daily basis. The analyst inserts bench QC specimens two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. Three separate serum pools having low, medium, and high range values for the analyte are inserted in each assay. Each of three pools is run twice in each assay -- once at the beginning and once at the end of the run. That is, specimens with unknown values are "sandwiched" between the QC pools to ensure that the assay run has not been subjected to methodologic drift.

If the stock of these controls becomes low, another batch is prepared in time to analyze it concurrently with the current QC materials. The new controls are used only after their means and ranges are established following 10 characterization runs. Sometimes, more runs are used to update control means and ranges if 10 runs are deemed inadequate to represent the overall characteristic of the method over time. All updates of control means and ranges are performed only after approval from NCHS.

In addition, the assay mean (the mean of all the values falling within the normal limits for that analyte) will be used as an additional medium-range QC.

The USC Thyroid Clinical Laboratory, a state-licensed facility, is externally monitored by the College of American Pathologists, an interlaboratory comparison system that performs proficiency testing designed to compare performance with those of other laboratories.

a. Determining the acceptability of control results:

   (1) Analyze samples of three different control levels. Make replicate measurements on each control material at the beginning and end of each run when testing for statistical control. Record these observations as the daily average.

   (2) Accept the run when two or more mean control observations are within 2 standard deviations (SD) of the mean. Report participant's results. When at least one control observation is more than 3 SDs from the mean, hold the participant results and inspect the control data further using additional control rules.

   (3) Inspect control data within the run:

   - Reject the run when one control observation falls outside the mean ±3 SD limits. Do not report participant results.
   - Reject the run when two or more control observations fall more than 2 SDs outside the same mean. Do not report participant results.

- Accept the run if one control falls outside the mean by more than 2 SDs but less than 3 SD, if other controls are within 2 SDs.

(4) Inspect control data across runs:

- Reject the run when previous observations on the same control level fell more than 2 SDs outside the same mean. Do not report participant results.
- Reject the run when the last 10 consecutive observations of a pool fall on the same side of the mean. Do not report participant results.

(5) Accept the run when none of the above rules indicate a lack of statistical control. Report participant results.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. When the analytical run is out of control take the following actions:

(1) Determine the type of errors occurring (random, systematic, or both), on the basis of the control rules being violated. A review of control data on all control levels will help detect errors occurring throughout the concentration range tested by these controls. A review of control data on a single control level (within control materials) will help detect errors in a particular concentration range.

(2) Refer to a troubleshooting guide to inspect the components of the method or instrument that contributes to the type of error observed.

(3) Correct the problem, then reanalyze the participant and control samples, testing for statistical control by the same procedures. Do not include data from previous out-of-control situations in the cumulative data. If results were more than 3 SDs outside the mean, indicate corrective action taken on the "Corrective Action Log" and in the computer when entering the QC result.

(4) Consult the laboratory manager or technical supervisor before reporting data when there is a lack of statistical control.

b. In the following situations, the laboratory manager or technical supervisor may decide to report the data even when there is lack of statistical control.

(1) The control problem is shown to be due to the control materials themselves.

(2) The control problem is shown to have resulted from an isolated event not affecting the rest of the run (e.g., an interchange of two samples or a clerical transcription error).

(3) The control problem occurs in a concentration range different from the concentrations of the participant samples. The method is in-control in the range of the participant samples.

(4) The size of the analytical error is judged to be small relative to the medical usefulness requirements. For example, small shifts of the analytical mean relative to medical needs may be ignored when associated with reagent changes.

(5) Participant data can be released even if QC results fall outside pre-established statistical limits, provided participant care is not compromised.

c. Check the clinical appropriateness of results before reporting:

Each day the laboratory technical director and supervisor will check the cumulative summary of the results for each participant specimen to determine the clinical appropriateness of the results. They will check the individual test results worksheet and compare the original to the computer printout to detect clerical errors before releasing participant reports.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

This test should only be run after one has become familiar with the information provided by Kronus in the product insert.

This laboratory has determined that neither repeated freezing and thawing, storing serum specimens for 6 to 8 hours at 20-25 °C, nor storing them for 24-48 hours at 4 °C will affect the stability of these thyroid tests.

No interfering substances have been identified.

**13. REFERENCE RANGES (NORMAL VALUES)**

Normal values for anti-microsomal antibody in serum are less than 0.5 U/mL.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable for this procedure.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should be allowed to reach 20-25 °C during testing. Specimens can be refrigerated at 4-8 °C overnight for the TSH analysis. After analysis, specimens are immediately refrozen at ≤-20 °C and not thawed again unless it needs repeating.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Currently, the laboratory does not have an alternative method for performing this test, but maintains a backup instrument. Specimens are stored at 4-8 °C for up to seven days, or are stored frozen at ≤-20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Results are recorded on floppy disc provided by the NHANES MEC. Records of QC data, specimen results, dilutions, random and repeats are maintained in the laboratory.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard records such as computer data files and printed copies should be used to track specimens. We recommend that records, including related QA/QC data, be maintained for 6 years, and that duplicate records be kept. Only numerical identifiers (e.g., NCHS ID numbers) should be used.

Residual specimens are stored at ≤70 °C for 1 year and are returned to the NCHS serum bank.

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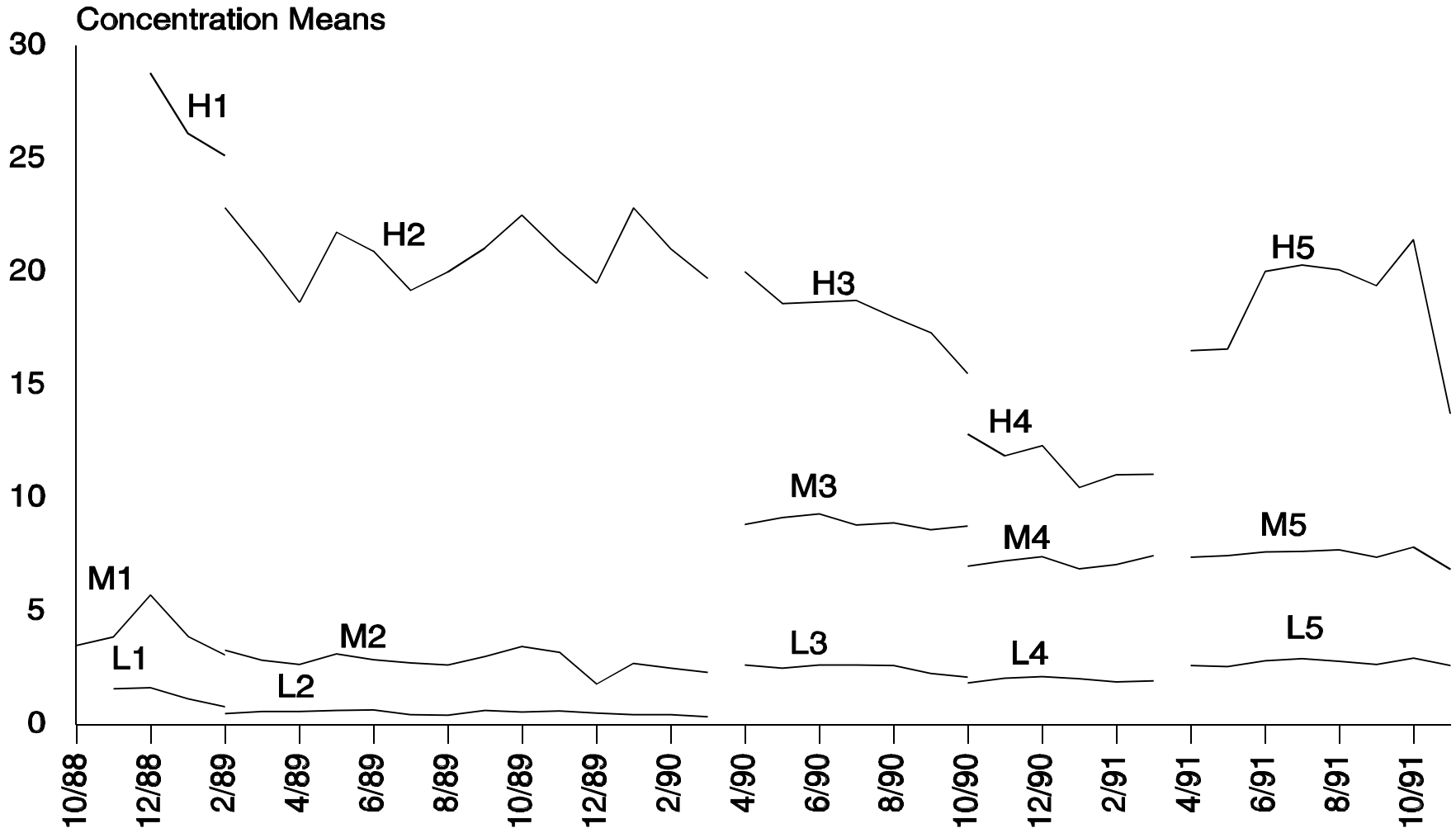
SUMMARY STATISTICS FOR
ANTIMICROSOMAL ANTIBODY (AMA) - PHASE 1
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| H1 | 12/20/88 - 02/10/89 | 26.5000 | 2.29526 | 8.6614 | 18 |
| H2 | 02/14/89 - 03/19/90 | 20.9612 | 2.29989 | 10.9721 | 147 |
| H3 | 04/03/90 - 10/20/90 | 18.1452 | 1.86290 | 10.2666 | 62 |
| H4 | 10/21/90 - 03/31/91 | 11.5475 | 1.39127 | 12.0483 | 59 |
| H5 | 04/07/91 - 11/17/91 | 18.9922 | 2.92120 | 15.3811 | 102 |
| M1 | 11/09/88 - 02/10/89 | 4.2972 | 1.67238 | 38.9177 | 36 |
| M2 | 02/14/89 - 03/19/90 | 2.8581 | 0.40692 | 14.2372 | 148 |
| M3 | 04/03/90 - 10/20/90 | 8.9371 | 0.54359 | 6.0824 | 62 |
| M4 | 10/21/90 - 03/31/91 | 7.2258 | 0.35401 | 4.8993 | 66 |
| M5 | 04/07/91 - 11/17/91 | 7.5412 | 0.36271 | 4.8097 | 102 |
| L1 | 11/22/88 - 02/10/89 | 1.3519 | 0.56116 | 41.5104 | 27 |
| L2 | 02/14/89 - 03/19/90 | 0.5358 | 0.13603 | 25.3881 | 148 |
| L3 | 04/03/90 - 10/20/90 | 2.4625 | 0.29032 | 11.7896 | 64 |
| L4 | 10/21/90 - 03/31/91 | 1.9864 | 0.18388 | 9.2570 | 66 |
| L5 | 04/07/91 - 11/17/91 | 2.7618 | 0.26253 | 9.5057 | 102 |

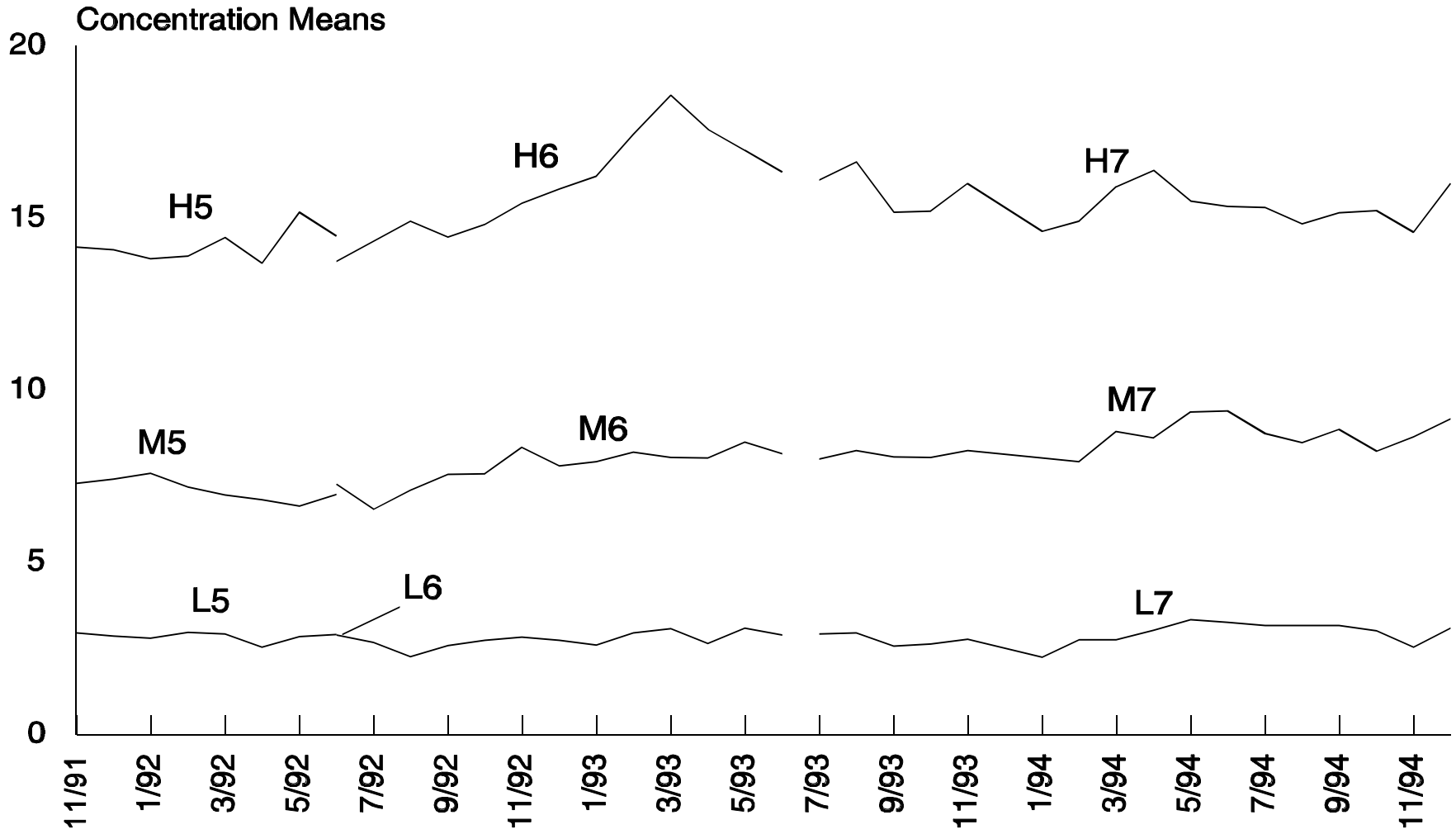
# Antimicrosomal Antibody (AMA) Monthly Means - Phase 1



SUMMARY STATISTICS FOR
ANTIMICROSOMAL ANTIBODY (AMA) - PHASE 2
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| H5 | 11/24/91 - 06/20/92 | 14.2183 | 1.01338 | 7.1273 | 82 |
| H6 | 06/28/92 - 06/26/93 | 16.2600 | 1.67760 | 10.3174 | 130 |
| H7 | 07/10/93 - 12/20/94 | 15.5437 | 1.05062 | 6.7591 | 213 |
| M5 | 11/24/91 - 06/20/92 | 7.0878 | 0.40258 | 5.6799 | 82 |
| M6 | 06/28/92 - 06/26/93 | 7.9131 | 0.51960 | 6.5663 | 130 |
| M7 | 07/10/93 - 12/20/94 | 8.4958 | 0.63703 | 7.4983 | 212 |
| L5 | 11/24/91 - 06/20/92 | 2.8488 | 0.27988 | 9.8245 | 82 |
| L6 | 06/28/92 - 06/26/93 | 2.8085 | 0.31550 | 11.2339 | 130 |
| L7 | 07/10/93 - 12/20/94 | 2.9696 | 0.41118 | 13.8464 | 214 |

# Antimicrosomal Antibody (AMA) Monthly Means - Phase 2



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This method is used for the quantitative measurement of total circulating thyroxine (T4). T4 is present in two forms, free and protein-bound. The test system is designed to measure total thyroxine (e.g., the sum of the free and protein-bound forms).

Radioimmunoassay has been applied in various forms to the measurement of total serum T4. The major differences are in the method of extracting T4 from serum-binding proteins and in techniques of separating the free or unbound T4 from the T4 bound to the antibody during an incubation period. This test system uses 8-anilino-1-napthalene sulfonic acid (ANS) to extract T4 from its binding protein in the presence of buffer to inhibit further binding of T4 by the proteins. The T4 is precipitated by a second antibody reagent added simultaneously with the other assay constituents (1-5).

T4, whether radio-labeled or not, will bind specifically with a specific antibody or antiserum in a mixture containing a limited amount of antiserum in proportion to T4.

When T4 from the serum is equilibrated with $^{125}$I-T4 and T4 antiserum, the amount of $^{125}$I-T4 bound to the antiserum will be inversely proportional to the amount of nonradioactive T4 present in the sample or standard solution. By separating the antiserum complex from the unbound T4 and measuring the radioactivity of the $^{125}$I-T4 bound in the complex, one can plot concentrations of added T4 versus percentages of the $^{125}$I-T4 bound. The standard curve can be used to determine the amount of T4 in the participant serum.

## 2. SAFETY PRECAUTIONS

The assay employs $^{125}$I as a tracer. Observe all necessary radiation safety considerations for isotope management and disposal according to the guidelines of the U.S.C. *Radiation Safety Manual*. In addition, all personnel must successfully complete the training course *Radiation Safety in the Laboratory* or demonstrate having received equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eyewear, and lab coat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that contaminated by the radioactive tracer in an plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines. Wash countertops with a 10% sodium hypochlorite (bleach) solution after assay completion.

Material safety data sheets (MSDSs) for 8-anilino-1-napthalene sulfonic acid sodium salt, $^{125}$I, sodium hypochlorite and sodium azide are available through the laboratory office.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III Mobile Examination Center (MEC) contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" high density (HD) floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data and by storing of data in multiple computer systems.

c. A cumulative computerized laboratory log is generated for all specimens received. The log contains batch receipt dates and updated details concerning the handling, manipulation, and analysis of specimens, as well as any problems as they occur.

d. Documentation for system maintenance is contained in hard copies of data records.

e. The results from the assay are transcribed from the dedicated instrument printouts to the assay sheets and computer record. All entries are proofread. Batch data will be transmitted by both IBM DOS floppy diskettes with their report form printout to the National Center for Health Statistics (NCHS). The final test values for each specimen will be identified along with the dates of analysis, the technical operators involved, whether repeat determinations were required, and if so, for what reason.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. The preferred sample type for T4 analysis is serum. Do not use plasma. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers.

c. Minimum sample size is 0.5 mL.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field should be frozen and then shipped on dry ice by overnight mail. Once received, specimens are stored at ≤-20 °C until analyzed. Residual specimen after analytical aliquots are withdrawn are refrozen at ≤-20 °C.

f. Specimens may be stored at 4-8 °C for up to 24 hours.

g. For longer storage, freeze samples at ≤-20 °C.

h. Avoid repeated thawing and freezing of samples.

i. Completely mix each thawed sample before analyzing it.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Micromedic Systems, Inc. model 24000 autodilutor (Polymedco, Inc, Yorktown Heights, NY).

(2) Multi-tube vortexer, cat no. 58816-115 (VWR Scientific, San Francisco, CA).

(3) GENESYS 5000 series, 10-well gamma counter, model 5010R-B (Laboratories Technologies, Inc, Schaumburg, IL).

(4) Beckman GPR refrigerated centrifuge with swing-out rotor (Beckman Instruments, Fullerton, CA).

(5) Eppendorf pipet, 100- to 1000-µL, cat no. 022-44-020-9 (Brinkman Instruments, Inc., Westbury, NY).

(6) Eppendorf pipet, 10- to 100-µL, cat no. 022-44-010-1 (Brinkman Instruments, Inc.).

(7) Eppendorf pipet, 100 µL, cat no. 022-44-180-9 (Brinkman Instruments, Inc.).

(8) Eppendorf pipet, 2- to 20-µL, cat no. 022-44-005-5 (Brinkman Instruments, Inc.).

b. **Other Materials**

(1) 3.5-mL, 55- x 12-mm polypropylene tubes, cat no. 55.484 (Sarstedt Laboratories, Newton, NC).

(2) 5-mL, 75- x 12-mm polypropylene tubes, cat no. 55.476 (Sarstedt Laboratories).

(3) 5-mL, 75- x 12-mm borosilicate glass tubes, cat no. 60825-550 (VWR Scientific).

(4) Push-in stoppers for 12- x 55-mm and 12- x 75-mm polypropylene tubes, white, cat no. 65.809.505 (Sarstedt

Laboratories).

(5) Rubber gloves, 100% nitrile (Best Manufacturing, Menlo, GA)

(6) Latex gloves, style 312 (Perry X-AM, Smith & Nephew Perry, Massilon, OH).

(7) Biohazard disposable bag, cat no. 13166 (VWR Scientific).

(8) Bleach, 10% sodium hypochlorite solution (NaOCl) (Any vendor).

(9) Kim-Wipe lintless tissues (Kimberly Clark Corp., Green Bay, WI).

(10) Singlefold towels, 227-04 (Fort Howard Corp., Green Bay, WI).

(11) Laboratory detergent (various vendors).

(12) $^{125}$I-T4 (New England Nuclear Products, Claremont, CA) cat no. NEX-111X. $^{125}$I-T4L carrier-free (Dupont Co/NEN Products, Boston, MA). One shipment per month of fresh tracer. High specific activity = 5500 CPM.

(13) Rabbit anti-T4 antibody, lot no. 62617 (Abbott Laboratories). Stored in 0.5-mL aliquots.

(14) Donkey anti-rabbit (DAR) second antibody, code B-5. Neat DAR, gift of Dr. Dee Warren. Stored frozen in 400-µL aliquots.

(15) 8-anilino-1-napthalene sulfonic acid (ANS), no. A-3125 (Sigma Chemical Company, St. Louis, MO).

(16) Sodium azide (Sigma Chemical Company, St. Louis, MO).

(17) Boric acid (Sigma).

(18) Albumin, bovine (RIA grade) (Sigma).

(19) T4 stock standard, (1 µg/mL or 100 µg/dL) purchased from Endocrine Sciences. (Abbott Research Laboratory, Chicago, IL). Stored in 0.5-mL aliquots.

c. **Reagent Preparation**

0.2 mol/L borate buffer (BBSA)
0.5 g/dL BSA, 0.1 g/dL sodium azide ($NaN_3$), pH 8.6. Using 123.5 g boric acid ($H_3BO_3$) + 10 g sodium azide + 50 g albumin bovine (RIA grade), bring volume to to 10 L with distilled water. Adjust pH to 8.6 using 10 N NaOH.

d. **Standards Preparation**

Procedure for preparing standards:

(1) Prepare enough resin-stripped serum to make standards for at least 2 years.

(2) Reconstitute the T4 stock standard (1 µg/mL or 100 µg/dL) in accordance with the directions on the vial.

(3) Prepare a 1:10 dilution using 100 µL of T4 stock standard and 900 µL of resin-stripped serum, to yield 10 µg/dL solution.

(4) Assay the solution prepared in step 3 at the end of a T4 RIA assay to confirm its value.

(5) Prepare the dilutions shown in Table 1 after confirming that the value of the resin-stripped serum = 0.0 µg/dL.

(6) Aliquot 0.5 mL of each standard into four 12- x 75-mm tubes. Freeze three of the aliquots and the remaining solution for each standard at -10 to -30 °C.

(7) Use the aliquoted tubes to run the new standards at the end of the T4 assay for 20 runs.

Table 1
Preparation of Standards

| Final Concentration (µg/dL) | Vol. 10 µg/dL T4 Stock Std. (mL) | Vol. Resin Stripped Serum (mL) |
|---|---|---|
| 20.0 | 20.0 | 80.0 |
| 15.0 | 15.0 | 85.0 |
| 10.0 | 10.0 | 90.0 |
| 7.5 | 7.5 | 92.5 |
| 5.0 | 5.0 | 95.0 |
| 2.5 | 2.5 | 97.5 |
| 1.0 | 1.0 | 99.0 |

(8) Average the values of each standard separately, check for outliers, and establish values for the standards. Consult with the laboratory manager or technical supervisor at this time.

(9) Run the new standards with the current controls being used and confirm values. Consult the laboratory manager or technical supervisor again at this time.

(10) When final approval is obtained from the laboratory manager or technical supervisor, thaw and mix the frozen standards and proceed to make 0.5-mL aliquots of each standard. Store the aliquots at -10 to -30 °C.

e. **Preparation of Quality Control Materials**

In-house quality control (QC) pools are prepared in the low, medium and high concentration ranges of the curve. These QC pools are made by selective pooling of previously analyzed, excess patient serum, and aliquoted into appropriately small quantities to minimize any influence of repeat freezing and thawing. Pools are coded and identified by the pooling date. These serum pools, stored at ≤-20 °C, are considered to have a shelf life of 2 years.

Sufficient material is always pooled and aliquoted to ensure a supply of QC material to last more than one year under normal work volumes. New pools are prepared and their normal ranges established from 10 individual assay runs, not less than three months prior to exhaustion of the current pool. The ranges for each control are documented in the Quality Control Book. When more than 10 unknowns are to be run in a single assay, the low, medium and high controls are analyzed after the calibrators and after the last unknown sample. When fewer than 10 unknowns are to be run in a single assay, the low, medium, and high controls are analyzed after the last unknown sample.

This laboratory has previously determined that fresh serum pools are a better QC material than commercially available, modified, lyophilized, serum-based material. Pooled serum is a matrix identical to the test serum sample and, as such, better reflects when a run is "out of control."

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

The Genesys model 5010R-B has full data reduction capabilities using L0GIT($B/B_0$) VS L0G(DOSE). This program calculates a linearized calibration curve and specimen concentrations.

b. **Verification**

A purified T4 stock standard is prepared, and serial dilutions are made from the stock. These dilutions are assayed twice yearly as unknowns to verify method linearity, accuracy and precision.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Unpack samples immediately after they arrive and check the vials for possible damage during transport. Verify the NCHS numbers of each sample against the packing list for accuracy. Perform the inventory quickly to prevent thawing. Store samples at ≤-20 °C. Thaw batches of 50 samples for analysis.

(2) Label each batch with the box number used throughout all analyses to identify the batch.

(3) Analyze batches of 50 serum specimens or fewer in a single assay. Analyze batches containing more than 50 specimens in two separate assays to avoid assay drift.

(4) Assay all specimens, standards and QC materials in duplicate.

(5) Check the volume of all standards and QC materials, thaw new standards and QC aliquots, if necessary.

b. **Sample preparation**

(1) Allow specimens to thaw at 20-25 °C for 1-2 hours.

(2) Mix thawed specimens by vortexing.

c. **Instrument setup of Genesys instrument**

(1) Assay programming for the GENESYS 5000 series.

- Response variable: $\%B/B_0$
- Standard curve axes: L0GIT($B/B_0$) VS L0G(D0SE)
- Standard curve fit: POINT-TO-POINT

(2) The tube loading order is shown in Table 2.

Table 2
Tube Loading Order

| Tube | Replicates | Dose |
|---|---|---|
| TC (total counts) | 2 | -- |
| NS (non-specific binding) | 2 | -- |
| $B_0$ (0.0 standard) | 2 | -- |
| STD 1 | 2 | 0.6 |
| STD 2 | 2 | 1.2 |
| STD 3 | 2 | 2.4 |
| STD 4 | 2 | 5.3 |
| STD 5 | 2 | 12.5 |
| STD 6 | 2 | 15.9 |
| STD 7 | 2 | 21.0 |
| UNK | 2 | -- |

(3) Ensure that enough printer paper is available the entire run. If necessary, replace paper.

(4) Place the tubes to be counted into the tray holder; insert the tray into the wells.

(5) At the GENESYS screen, select EXIT until the SYSTEM MENU is displayed.

(6) Select ASSAY LIBRARY.

(7) Select the assay being analyzed, T4.

(8) Select VIEW/EDIT ASSAY SUMMARY.

(9) Select RUN ASSAY.

(10) Select START COUNT. While the tubes are being counted, the user will see the COUNTING TIME, TRAY NUMBER, and TIME LEFT TO COUNT on the topmost line, followed by a display of the actual counts in each well. Adjacent is displayed the ASSAY ID, below which is displayed the PROTOCOL ID (AMA/ATA/T4).

(11) After the standards have been counted, a screen displaying the curve has five options:

(a) Process Unknowns
(b) View/Edit Standards
(c) Term Assay
(d) View/Edit QC
(e) Manual Update

(12) Touch PROCESS UNKNOWNS in the lower left corner to generate a printout, and continue counting until the last tray is reached.

(13) When the last tray to be counted is reached, touch the well number to show the last well used; the other wells will be covered.

(14) When the count screen reappears, touch COUNTING COMPLETE two times.

d. **Instrument Setup of the Beckman Refrigerated Centrifuge**

(1) Temperature range: 4-8 °C (±2).

(2) Speed control: 3000 rpm (±10).

(3) Timer: 30 min.

e. **Operation of Assay Procedure**

(1) Process samples, assigning Micromedic (MM) numbers on the basis of the computer-generated worksheet. If there are research samples, add them at the end of the routine clinical samples.

(2) Check the volume of all standards and QCs, and thaw new aliquots if necessary. If the total number of samples is fewer than 10, run only one set of QC materials at the end of the samples.

(3) Decide whether a half or full batch of mix is needed: A half batch of mix = 21 mg of ANS + 40 mL BBSA + 1 T4 Ab aliquot (0.5 mL) + 1 DAR aliquot (0.5 mL). This is enough for 70 tubes (20 samples). A full batch of twice the preceding amounts will be enough for 40 samples. Weigh out ANS and dissolve in BBSA. Cover with parafilm and invert graduated cylinder to mix. Remove sufficient T4 Ab and DAR from the freezer to run a half or full batch.

(4) Check the pumps on the MM. Set the 1-mL dispenser at 50%; set the 20-µL sampler at 50%, or if a 50-µL sampler is used, set it at 20%. Set the high-speed-unit (HSU) 200-µL dispenser at 50%.

(5) Make up the T4 tracer. Use a premeasured scintillation vial containing 20 mL BBSA + 20 µL concentrated T4 tracer to obtain approximately 18,000 to 24,000 CPM/100 µL/tube.

(6) Using a beaker and stirring bar, prepare the T4 mix for a half or full batch as needed. Once the T4 Ab and DAR are added, mix for 15 min.

(7) Prime the MM and HSU with BBSA for two min. Set up the MM racks with the tubes in appropriate places according to the T4 worksheet. Vortex the standards and controls vials, then uncap them.

(8) When the T4 mix has finished equilibrating (15 min), begin priming the MM with the T4 mix. Introduce an air bubble and prime the T4 mix until it is dispensed at the tip. Recirculate the T4 mix for 2 min. Place the T4 mix back on the stirring base.

(9) Prime the HSU with T4 tracer prepared in step 5-6 for two min.

(10) Prime both the mix and the tracer simultaneously and discard approximately 2-3 complete cycles. Synchronize the MM and the HSU from the prime position.

(11) Begin the assay, checking the tip depth and the aspirating and dispensing functions with the first two tubes before switching to automatic. Be sure to have two total count tubes at the end of the assay.

(12) As the racks are completed, reverse them to check the liquid level and look for bubbles that might indicate samples with inadequate volume. Vortex the tubes and place them in the incubation rack. Cover the tubes with parafilm and incubate them at 20-25 °C for 18 - 24 hours (overnight).

(13) Remove the T4 mix and tracer and prime the MM and HSU with detergent solution for 2 min, followed by distilled water for 2 min, followed by air. The MM and HSU are now ready for the next assay.

(14) After the overnight incubation, centrifuge all tubes (except TC) at 3000 RPM for 30 min at 4-8 °C. Aspirate the supernatant and count each tube for 1 min on the counter using the preprogrammed data-reduction program, T4.

f. **Recording of Data**

(1) Quality Control Data

Enter QC data into the Bio-Rad Laboratories Quality Control Program, as well as in the Quality Control log for NHANES. These data are then interpreted to determine the acceptability of the assay. Any problem with QC runs and corrective action taken is indicated on the "Corrective Action Log" and in the computer when the QC result is entered. The laboratory technical director and supervisor check individual test results worksheet and compare the original to the computer printout to detect clerical errors before releasing participant reports.

(2) Analytical Results

Prepare worksheets from the transmittal from the NHANES MEC. Record each of the duplicate results and the mean result on this worksheet. For results below the detection limit of the method, record "less than 1.0 µg/dL." For results greater than the highest standard, dilute the specimens appropriately (e.g., 1:2) and reassay them.

g. **Replacement and Periodic Maintenance of Key Components**

(1) On the Genesys gamma counter, perform a daily background count. Perform a daily efficiency check using Multi-Calibrators. This reference source is Na ($^{125}$I) embedded homogenously in a cationic resin. The dried point-source is sealed in a 12- x 75-mm polypropylene tube.

(2) Replace the print-head cartridge as needed.

(3) The following items are checked and preventative maintenance performed as necessary by a contract service technician.

- Average efficiency
- High-low efficiency
- Percent spread

- Background
- Background high-low ratio
- Normalization factors of Iodine-125, Cobalt-57, and dual-label
- Check fan, printer, printout, keyboard, display and software

(4) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

h. **Calculations**

(1) Automated: The Genesys model 5010R-B has full data-reduction capabilities. The curve is obtained with a linear point to point function. The curve plots logit ($B/B_0$) vs log concentration.

(2) Manual:

(a) Average the counts in each set of duplicate tubes.

(b) Calculate percent bound for each assay tube as follows:

$$\frac{\text{average of duplicate counts}}{\text{average of total counts}} \times 100 = \%\ \text{bound}$$

(c) Plot the percent bound versus the concentration of calibrators on semilog paper.

(d) Interpolate unknown results from the calibration curve.

(e) Record the T4 $^{125}$I lot number and the initials of the technical person running the assay on the worksheet.

i. **Special Procedure Notes - USC Modifications**

(1) Allow all reagents to come to 20-25 °C before use.

(2) Return standards and controls to the cold room after use in accordance with the manufacturer's instructions.

## 9. REPORTABLE RANGE OF RESULTS

a. The normal range is 4.5 - 13.2 µg/dL.

b. The working range of the assay is 1.0 - 20 µg/dL.

c. All results <1.0 µg/dL are reported as <1.0 µg/dL.

d. All specimens with concentrations >20 µg/dL are diluted 1:2 with the zero standard or stripped serum. The resulting value is then multiplied by the dilution factor used.

## 10. QUALITY CONTROL (QC) PROCEDURES

The USC Thyroid Clinical Laboratory uses the Bio-Rad QC program. The program is designed to allow real-time data entry, providing the user with immediate validation of actual participant results. Data are evaluated and screened according to the multirule system formulated by Westgard (6). This system helps maximize laboratory resources by decreasing unnecessary assay rejection and simultaneously increases sensitivity to error. The lab QC manager generates QC reports on a monthly basis for individual laboratory statistics and Levy-Jennings plots.

The USC Thyroid Clinical Laboratory applies the floating mean principle, and if a trend or shift in the mean occurs, the problem is investigated and corrected. A new mean is calculated from the last two months' data by using a fixed mean until a new floating mean is established (6,7).

Two types of QC systems are used in this analytical method: the sample QC system and the bench QC system. With the sample QC system, 5% of the specimens are randomly selected and analyzed either within an assay or between assays for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is repeated. Bench QC specimens are placed before and after all specimens to be analyzed.

An internal QC program monitors the accuracy and precision of laboratory performance on a daily basis. The analyst inserts bench QC specimens two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. Three separate serum pools having low, medium, and high range values for the analyte are inserted in each assay. Each of three pools is run twice in each assay -- once at the beginning and once at the end of the run. That is, specimens with unknown values are "sandwiched" between the QC pools to ensure that the assay run has not been subjected to methodologic drift.

If the stock of these controls becomes low, another batch is prepared in time to analyze it concurrently with the current QC materials. The new controls are used only after their means and ranges are established following a minimum of 10 characterization runs. Sometimes, more runs are used to update control means and ranges if 10 runs are deemed to be inadequate to represent the overall characteristic of the method over time. All updates of control means and ranges are performed after approval from NCHS.

In addition, the assay mean (the mean of all the values falling within the normal limits for that analyte) will be used as an additional medium range QC.

The USC Thyroid Clinical Laboratory is a state-licensed facility and as such is externally monitored by the College of American Pathologists, an interlaboratory comparison system (proficiency testing) designed to compare laboratory performance with that of other laboratories.

a. Interpretation of acceptability of control results:

(1) Analyze samples of three different control levels. Make one measurement on each control material at the beginning and end of each run when testing for statistical control. Record these observations as the daily average.

(2) Accept the run when both control observations are within mean ±2 SD limits. Report participant results. When at least one control observation exceeds the mean ±3 SD limits, hold the participant results and inspect the control data further using additional control rules.

(3) Inspect control data within the run:

- Reject the run when one control observation exceeds the mean by 3 SDs. Do not report participant results.
- Reject the run when 2 or more control observations exceed the same mean by 2 SD. Do not report participant results.
- Accept the run if one control exceeds the mean by more than 2 SD, but less than 3 SD, if other controls are within 2 SD.

(4) Inspect control data across run:

- Reject the run when previous observation on the same control level exceeded the same mean by more than 2 SD. Do not report participant results.
- Reject the run when the last 8 consecutive observations fall on the same side of the mean. Do not report participant results.

(5) Accept the run when none of the above rules indicates a lack of statistical control. Report participant results.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. When the analytical run is out of control:

(1) Determine the type of errors occuring (random, systematic or both), based on the control rules being violated. A review of control data on all control levels will help detect errors that occur throughout the concentration range tested by these controls. A review of control data on a single control level (within control materials), will

help detect errors in a particular concentration range.

(2) Refer to a troubleshooting guide to inspect the components of the method or instrument that contributes to the type of error observed.

(3) Correct the problem, then reanalyze the participant samples and control samples, testing for statistical control by the same procedures. Do not include data from previous out-of-control situations in the cumulative data. If results were more than 3 SD from the mean, indicate corrective action taken on the "Corrective Action Log" and in the computer when entering the QC result.

(4) Consult the laboratory manager or technical supervisor for any decision to report data when there is a lack of statistical control.

b. The laboratory manager or technical supervisor may decide to report the data when there is lack of statistical control in the following situations:

(1) The control problem can be shown to be due to the control materials themselves.

(2) The control problem can be shown to have resulted from an isolated event that would not have affected the rest of the run (e.g., an interchange of two samples or a clerical transcription error).

(3) The control problem occurs in a concentration range that is different from the concentrations of the participant samples. The method is in-control in the range of the participant samples.

(4) The size of the analytical error is judged to be small relative to the medical usefulness requirements. For example, small shifts of the analytical mean relative to medical needs may be ignored when associated with reagent changes.

(5) Participant data can be released even if QC results fall outside pre-established statistical limits, provided that participant care is not compromised.

c. Check the clinical appropriateness of results before reporting:

The laboratory technical director and supervisor will check daily the cumulative summary of the results for each participant specimen to determine clinical appropriateness of the results. They will also check individual test results worksheets and compare the original to the computer printout ones to detect clerical errors before releasing participant reports.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Recovery studies have indicated that icteric, lipemic, or hemolyzed serum does not cause any variation in assay results. Serum containing antithyroxine antibodies cannot be measured accurately without interference.

## 13. REFERENCE RANGES (NORMAL VALUES)

The expected range is 4.5 - 13.2 µg/dL (1).

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

T4 values <4.5 µg/dL and T4 values >13.2 µg/dL are reported by FAX to NCHS.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should be allowed to maintain a temperature of 20-25 °C during testing. Specimens can be refrigerated at 4-8 °C overnight for the T4 analysis. After analysis specimens are immediately refrozen and stored at ≤-20 °C and thawed only for repeat testing.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Currently, the laboratory does not have an alternative method for performing this test, but maintains a backup instrument.

If the analytical system fails, specimens may be stored at 4-8 °C for up to seven days, or may be stored frozen at ≤-20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Results are recorded electronically on a floppy disk provided by the NHANES. Records of QC data, specimen results, dilutions, random and repeat results are maintained in the laboratory.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record keeping means such as computer data files and hard copies should be used to track specimens. It is recommended that records, including related QA/QC data, be maintained for 6 years, and that duplicate records be kept. Only numerical identifiers (e.g., Case ID numbers) should be used.

Residual specimens are stored at ≤-70 °C for 1 year and returned to NCHS serum repository.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

The qc summary statistics and graphs won't be released until the actual thyroxine data is released.

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A solid-phase fluorescent immunoassay for the measurement of human urinary albumin is described by Chavers et al. (1). The fluorescent immunoassay is a non-competitive, double-antibody method for the determination of human albumin in urine. Antibody to human albumin is covalently attached to derivatized polyacrylamide beads. The solid-phase antibody is reacted with a urine specimen. The urine albumin antigen complexes with the solid-phase antibody which reacts with fluorescein-labeled antibody. Unattached fluorescent antibody is removed by washing during centrifugation. The fluorescence of the stable solid-phase antibody complex is determined with a fluorometer and is directly proportional to the amount of urine albumin present. The standard curve is 0.5 to 20 µg/mL albumin.

Increased microalbuminuria is a sign of renal disease and may be predictive of nephropathy risk in patients with insulin-dependent diabetes. Results of the fluorescent immunoassay (FIA) are reproducible, and the test is accurate and sensitive for the detection of human urinary albumin excretion. It is especially useful for measurement of low levels of urinary albumin not detectable by dipstick methods. The FIA assay resembles the radio-immunoassay (RIA) in technique and sensitivity without the potential health hazards associated with the handling of isotopes in the laboratory (1).

## 2. SPECIAL SAFETY PRECAUTIONS

a. Follow the Laboratory Safety and General Laboratory Practice regulations from the College of American Pathologists (CAP), the Clinical Laboratory Improvement Act (CLIA), and Occupational Safety and Health Administration (OSHA).

b. Treat all specimens for analysis as if they are known to be infectious. Observe Universal Precautions. Wear blue hospital laboratory coats and gloves when handling urine specimens.

c. Cover the work surface with disposable, absorbent paper toweling.

d. Place contaminated tubes, pipet tips, gloves, toweling, etc., and residual urine specimens into biohazard bags. Discard the bags after autoclaving.

e. Clean the work surfaces with 3% phenolic disinfectant detergent, "O-Syl", or 10% sodium hypochlorite.

f. On the label for all reagents, indicate the date of preparation, the expiration date, the formula, any hazards posed by the reagent, and the initials of the technician.

g. We recommend that laboratory personnel performing the assay are vaccinated against hepatitis B.

Material Data Safety Sheets for O-Syl, hydrochloric acid, potassium hydroxide, potassium phosphate monobasic, sodium azide, phosphate buffered saline, physiological phosphate buffered saline, sodium chloride, and sodium hypochlorite are in section #5 of the notebook "Safety Procedures for the UMHC Clinical Chemistry Laboratory, Minnesota, Employee Right to Know."

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Format a template document using Excel software on the MacIntosh computer. The document should include sample identification information, the specimen Albustix value, the dilution factor and the duplicate percent relative fluorescence (RF%) values, the linear regression calculation for the standard line, the specimen values converted to µg/mL, and the calculated total µg/ml albumin concentration. Follow the Excel operator's manual for information regarding formatting. Prepare an Excel spreadsheet from the template to use for each assay.

The integrity of the specimen data is established with routine verification of the transcribed information against the specimen identification on the specimen tube. The linear regression data generated by the MacIntosh computer (slope, y-intercept, correlation coefficient, and the calculated values of the three control urine samples) is verified by comparison with data obtained on the Hewlett-Packard (HP) calculator.

The data is stored on the Macintosh computer hard drive, back-up diskettes and hard copies organized in notebooks.

NHANES provides IBM computer diskettes with each shipment of specimens. The MacIntosh computer-generated data are entered onto this format on the IBM computer. The IBM diskettes formatted by NCHS contain the donor's ID, age, and sex, and the date of collection for each specimen. The program calculates albumin (µg/mL) X 1.04 and the index

value formula:

*10 (µg albumin) ÷ (mmol creatinine)*

The IBM diskettes are returned to NCHS with hard copies of the data. The NHANES data on worksheets, MacIntosh, and IBM are reviewed by the laboratory supervisor.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Random urine specimens from sample persons are collected at the survey mobile examination centers in accordance with the NHANES protocol.

b. No special instructions such as fasting or special diets are requested.

c. The urine specimens are shipped on dry ice via an overnight courier and stored at ≤-20 °C upon arrival.

d. Specimens are thawed for analysis at refrigeration temperature and remain at refrigeration temperature for up to 3 weeks until analyses are complete. Specimen stability at ≤-20 °C for at least 1 year has been documented.

e. The optimum sample volume is 3 mL, and the minimum acceptable volume is 1 mL.

f. The specimen tube is a 4.5-mL Vangard polypropylene cryogenic vial.

g. Visibly hematuric specimens are unacceptable for analysis.

h. Handle all urine specimens as if they are capable of transmitting any infectious agent.

i. Discard specimens after the supervisor verifies the integrity of the data.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Sequoia-Turner Fluorometer, model 450, digital (cat. no. 450-000). Excitation energy provided by a quartz halogen lamp (cat. no. 450-201). Wavelength selection is accomplished with a filter application set for fluorescein, SC 515 and NB 490 (Sharp cut emission (cat no. 450-151) transmits >515 nm and narrow band excitation 490 nm peak, respectively). Adjust the filter at 450-151 for excitation=485 nm, emmision=525 nm. (Sequoia-Turner Corp., Mountain View, CA).

(2) Micromedic model 25004 automatic pipette, with 50-µL sampling and 200-µL dispensing pumps (Micromedic Systems, Division of Rohm and Haas, Horsham, PA).

(3) Water bath, model 185 (Precision Scientific, Inc., Chicago, IL).

(4) Continuous automatic dispenser 0.5- to 20-mL adjustable volume dispenser, cat. no.8885-047004 (Oxford, Division of Sherwood Medical, St. Louis, MO).

(5) Vortex-Genie, model K-550-G (Scientific Industries, Inc., Bohemia, NY).

(6) Multi-tube vortex mixer, model 2600 (Scientific Manufacturing Ind., Emeryville, CA).

(7) Top-loader balance, model H15, 1000g/10mg (Mettler Instrument Corp., Hightstown, NJ).

(8) Top-loader balance, model XE-100A, 100g/0.1mg (Fisher Scientific, Chicago, IL).

(9) Accumet pH meter, model 15 (Fisher).

(10) Magnestir magnetic stir plate, cat. no. 214-924 (Curtin Matheson Scientific Inc., Eden Prairie, MN) and stir bars (general storehouse, Univ. of Minn.).

(11) Micro-centrifuge, model 235C (Fisher).

(12) Centrifuge, model UV, with swinging buckets to hold 12- X 75-mm tubes (International Equipment Co., Boston, MA).

(13) Centrifuge, model J2-21, with JA-10 fixed-angle rotor (Beckman Instruments, Palo Alto, CA).

(14) Autoclave, isothermal, Eagle series 2022 (AMSCO Scientific, Apex, NC).

(15) Calculator, model 11C, and operator's manual (Hewlett-Packard, Corvallis, OR).

(16) MacIntosh computer and laser printer (Apple, Inc., Cupertino, CA) and EXCEL software (Microsoft Corp., Redmond, WA) and manufacturer's operating manuals.

(17) IBM PC-compatible platform computer and printer and manufacturer's operating manuals (EPS Technologies, Jefferson, SD).

(18) Gilson Pipetman, adjustable, 101- to 1000-µL and Rainin 101- to 1000-µL nonsterile, blue tips (Rainin Instrument Co., Inc., Woburn, MA).

(19) Oxford micro-pipet, 2-10 µL (Oxford) and ultramicro tips, cat. no. 21-197-2F (Fisher).

(20) Oxford micro-pipet P-7000 series, 50-µL (Oxford, Division of Sherwood Medical, St. Louis, MO) and Fisherbrand Oxford-style tips, cat no. 21-244-1 (Fisher).

(21) Eppendorf 20-µL pipet (Brinkman Instruments Inc., Westbury, NY) and Fisherbrand Eppendorf-style yellow tips, cat no. 21-375-D (Fisher).

(22) Eppendorf repeater pipettors, 50- to 250-µL sizes and Eppendorf disposable pipette Combitips (Brinkman).

(23) Excalibur 99 micro pipette, 50- to 250-µL sizes (Excalibur Labs, Melbourne, Australia) and Fisherbrand reference tips, 1-250 µL, cat.no. 21-381-10A (Fisher).

b. **Other Materials**

(1) Kandiyohi distilled water, Type II (Kandiyohi Bottled Water, Willmar, MN).

(2) Hydrochloric acid, HCl, F.W. 36.46, A.C.S. grade (EM Science, Gibbstown, NJ). Store in acid cabinet for up to 2 years at 20-25 °C. *HAZARD: Caustic and poisonous*.

(3) Potassium hydroxide, KOH, F.W. 56.11, A.C.S. grade (EM Science, Gibbstown, NJ). Store at 20-25 °C for up to 5 years. *HAZARD: Caustic and poisonous.*

(4) Potassium phosphate monobasic, $KH_2PO_4$, F.W. 136.09, A.C.S. grade (Mallinckrodt, Paris, KY). Store at 20-25 °C for up to 5 years.

(5) Sodium azide, $NaN_3$, F.W. 65.02 (Sigma Chemical Co., St. Louis, MO). Store at 20-25 °C for up to 5 years. *CAUTION: Toxic. Wear safety goggles, mask, and gloves when handling the dry powder.*

(6) Sodium chloride, NaCl, A.C.S. grade (Fisher Scientific Co.). Store at 20-25 °C for up to 5 years.

(7) Ovalbumin, chicken egg albumin, grade V, cat. no. A-5503, average F.W. 44,287 (Sigma Chemical Co., St. Louis, MO). Store refrigerated in desiccator according to the package expiration date.

(8) Immunobead active matrix (beads), 20 mg/mL, 1 g of polyacrylamide beads in 50 mL of 0.005 mol/L phosphate buffer, pH 6.3, cat. no. 170-5911 (Bio-Rad Laboratories, Richmond, CA). Store at 4 °C for up to 5 years.

(9) Goat anti-human albumin, IgG fraction (GAHA), 5-mL vial of 40-50 mg lyophilized antibody protein per vial, cat. no. 55028 (Organon Teknika Corp., West Chester, PA). Store at ≤-70 °C for up to 5 years.

(10) EDAC, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl, M.W. 191.7, cat. no. E-6383, protein sequencing grade (Sigma). Allow for complete return to 20-25 °C before opening to avoid condensation. Store at ≤-70 °C with desiccant for up to 5 years. *CAUTION: Toxic. Wear safety goggles, mask and gloves when handling the dry powder.*

(11) Fluorescein-conjugated goat anti-human albumin, IgG fraction, (Fl GAHA) cat. no. 55162 (Organon Teknika). Store at ≤-70 °C protected from light for up to 5 years.

(12) Albustix test strips, cat. no. AM-2870 (Ames Division of Miles Laboratories, Elkhart, IN). Follow the package insert for explicit directions for use. Store at 20-25 °C according to package instructions and expiration date.

(13) Human serum albumin, cat. no. A-8763, approximately 99% pure by agarose electrophoresis, essentially globulin-free, derived from Sigma cat. no. A-1653 Cohn Fraction V (Sigma). Store in a desiccator at 4-8 °C according to the product expiration date. *CAUTION: Handle as if capable of transmitting any infectious agent.*

(14) CAP Urine Protein Reference Material, human albumin Cohn Fraction V (College of American Pathologists, Skokie, IL). *CAUTION: Handle as if capable of transmitting any infectious agent.*

(15) 400-mL centrifuge bottles for immunobead preparation (Nalge Co., Rochester, NY).

(16) 0.65-mL micro-centrifuge conical-bottom tube, RNAse-free, presiliconized, different colors, for freezer storage of aliquots of human serum albumin and quality control urine (Intermountain Scientific, Bountiful, UT).

(17) 1.5-mL micro-centrifuge conical-bottom tube, cat. no. 72-690, for the assay procedure (Sarstedt, Inc., Princeton, NJ).

(18) 12- X 75-mm tubes, borosilicate, disposable (Kimble Co, Toledo, OH).

(19) 16- X 100-mm tubes, borosilicate, disposable (Kimble).

(20) 4.5-mL cryogenic storage vials, cat. no. MS-4505, for urine specimens (Vangard International, Inc., Neptune, NJ).

(21) 3.5-mL cryogenic storage vials, cat. no. 223-9835, for storage of aliquots of Fl GAHA (Bio-Rad).

(22) Centaur microfilters, 45-µm, cat. no. FT-2002 (Centaur West, Sparks, NV).

(23) 100-mL disposable Nalgene filterware, 0.2-µm, sterile (Nalge).

(24) Spectra-por 4 dialysis tubing. Store at 4-8 °C. (Spectrum Medical Industries, Los Angeles, CA).

(25) 99% isopropyl alcohol (Univ. of Minn. chemical storehouse). Store in flammable safety cabinet for up to 2 years. *HAZARD: Flammable.*

(26) O-Syl phenolic disinfectant detergent (General Medical Corp., Minneapolis, MN). Store the concentrate at 20-25 °C for up to 5 years. Prepare a 3% v/v working solution with distilled water and store at 20-25 °C for up to 2 years.

(27) General laboratory supplies: gloves, bio-hazard bags, safety glasses, bleach, disinfectant, lab coats, disposable absorbent bench-top toweling, weighing boats, 50-mL volumetric flasks (Univ. of Minn. chemical storehouse).

c. **Reagent Preparation**

(1) Sterile distilled water
Filter the Kandiyohi distilled water through sterile, 100-mL, 0.2-µm, disposable Nalgene Filterware under vacuum. Do not retain unused water.

(2) 1 mol/L HCl pH solution
Dilute 8.3 mL in 100 mL distilled water and mix well. Store at 20-25 °C for up to 1 year. *CAUTION: Prepare in safety hood. Wear safety glasses and gloves. Add acid to water.*

(3) 5 mol/L KOH pH solution
Dissolve 70.1 gm KOH in 250 mL distilled water. Store at 20-25 °C for up to 1 year. *CAUTION: Prepare in safety hood.*

(4) 0.3 mol/L $KH_2PO_4$ stock solution
Dissolve 40.8 g of $KH_2PO_4$ in 1 L of distilled water and mix well. Store at 20-25 °C for up to 1 year.

(5) 0.003 mol/L $KH_2PO_4$ (bead coupling dialysis buffer)
Dilute 20 mL of 0.3 mol/L $KH_2PO_4$ to 2 L with distilled water and mix well. Adjust the pH to 6.3 with 5 mol/L KOH. Prepare the day before use and refrigerate overnight. Use cold; discard after 24 hours.

(6) Phosphate buffered saline stock solution concentrated (20X PBS)
Dissolve 175.4 g NaCL and 2 g $NaN_3$ in 666 mL of 0.3 mol//L $KH_2PO_4$. Dilute to l L with distilled water and mix well. Store at 20-25 °C for up to 1 year.

> *WARNING: $NaN_3$ is toxic and may react explosively with lead and copper plumbing.*

(7) Physiological phosphate buffered saline (PBS)
Dilute 200 mL of 20X PBS to 4 L with distilled water and mix well. Adjust pH to 7.2 with 5 mol/L KOH. The final concentration is 0.01 mol/L $KH_2PO_4$, 0.15 mol/L NaCl, 0.015 mmol/L $NaN_3$. Prepare 4 L weekly for assay performance and prepare 12 L for the immunobead coupling procedure. Use cold. Store refrigerated for up to 1 week.

(8) 1.4 mol/L NaCl in PBS
Dissolve 262 g NaCl in 3200 mL PBS for bead coupling preparation. Prepare 3.2 L the day before use and refrigerate overnight. Use cold; discard after 24 hours.

(9) Ovalbumin in physiological phosphate buffered saline (oval/PBS)
Dissolve 0.5 g ovalbumin in 100 mL PBS and filter through 0.2 µm sterile Nalge Filterware. Store in this filter unit in a refrigerator for a maximum of 1 week.

(10) Beads coupled to goat anti-human albumin (immunobeads)
Prepare 400 mL. Do not prepare more than a 3-month supply. The final concentration of the immunobeads is 10 mg/mL in oval/PBS with 0.15 mmol/L $NaN_3$. (This procedure is adapted from the Bio-Rad package insert (6).)

Reconstitute each of four vials of GAHA with 5 mL of sterile distilled water and refrigerate the vials at 4-8 °C for 1 hour. Combine the vials into approximately 25 cm of dialysis tubing and use an additional 2 mL of sterile distilled water to rinse out the vials. Dialyze against 2 L of 0.003 mol/L $KH_2PO_4$, pH 6.3, for 4 hours at 4-8 °C in the cold room.

Combine the beads from each of the four 50-mL (1-g each) bottles. Adjust the phosphate buffer concentration from 0.005 mol/L to 0.003 mol/L by diluting the buffer to 350 mL with distilled water. Adjust the pH to 6.3 with 1 mol/L HCl or 5 mol/L KOH. Cover and refrigerate at 4-8 °C for 1 hour.

Add dialyzed GAHA to the beads, adjust the pH to 6.3 with 1 mol/L HCl or 5 mol/L KOH and refrigerate for 1 hour at 4-8 °C. The final concentration is 40-50 mg of antibody protein per 1 g of beads. Quickly add 800 mg

of EDAC while stirring. The pH will oscillate rapidly. Use 1 mol/L HCL and 5 mol/L KOH as needed to maintain the pH at 6.3 for 30 min during this equilibration process. After 30 min, the pH will stabilize.

Divide the immunobeads equally into each of four 400-mL Nalgene centrifuge bottles. Store refrigerated overnight.

Prepare the Beckman J2-21 centrifuge. Insert the JA-10 rotor, set the temperature at 4-8 °C, and allow the centrifuge to cool overnight. Prepare the immunobead washing buffers (12 L of PBS and 3.2 L of 1.4 mol/L NaCl-PBS) and refrigerate at 4-8 °C overnight.

Stop and continue the procedure the following day.

Perform six washing procedures with cold buffers and a refrigerated centrifuge. For each wash, fill the bottle with buffer, centrifuge at 3000 rpm (2000 x g) for 10 min in the Beckman J2-21 centrifuge with the JA-10 rotor and the brake on high, decant and blot, and resuspend the beads in a small volume of the next wash solution with a gentle rolling motion. Wash twice with PBS and then wash twice with 1.4 mol/L NaCl-PBS. Wash twice more with PBS. Resuspend the contents of each bottle with 100 mL of PBS and refrigerate for at least 3 hours. Do a final wash in PBS.

Resuspend and combine the contents of each bottle in a total volume of 400 mL with oval/PBS and add 0.4 g of sodium azide. Divide four 100-mL portions into each of the original Bio-Rad bottles and relabel the bottles. Store refrigerated at 4-8 °C for 3 months.

(11) Fl-GAHA stock solution
Reconstitute each vial of lyophilized product with 2 mL of sterile distilled water. Combine all vials together and place them at 4-8 °C, protected from light, for 1 hour. Aliquot 0.4 mL into 3.5-mL Bio-Rad cryogenic storage vials. Store the vials at ≤-70 °C protected from light for up to 2 years.

(12) Fl-GAHA working solution
Prepare a 1:10 dilution. Add 3.6 mL of oval/PBS to the cryogenic vial containing the 0.4-mL aliquot of FL-GAHA stock solution (or other proportions as determined by the limiting curve data, see below). Centrifuge at 2100 rpm (1000 x g) for 5 min to remove precipitates. Transfer the supernatant to a 16- X 100-mm tube and use at 20-25 °C. Return the excess to a light-protected container and store refrigerated for up to 1 mo. Centrifuge to remove cryo-precipitates before use on each assay day. Plot a limiting curve. Test new lots of FL-GAHA to determine the optimum concentration to use when there is a lot variation of the fluorescein/protein ratio (F/P). Assay the 20 µg/mL albumin standard line preparation against serial dilutions of Fl GAHA and graph the data. Select a dilution that is 2 times the concentration at the point (or limit) at which the graph detours from a straight line and begins to plateau.

d. **Standards Preparation**

(1) Human serum albumin (HSA) working standard
Prepare a 1 mg/mL solution. Weigh 50 mg of lyophilized HSA using a balance with a 0.1 mg sensitivity and dissolve in 50 mL of sterile distilled water using a 50-mL volumetric flask. Prepare 0.5-mL aliquots in 0.65-mL conical-bottom tubes and label each aliquot "HSA." Store at ≤-70 °C for 1 year. *CAUTION: Handle as if capable of transmitting infectious agents.*

(2) Calibration standards
The concentrations of the calibration material used are 0, 0.5, 1, 2, 5, 10, and 20 µg/mL.

Thaw and vortex the HSA working standard. Dilute the HSA with oval/PBS in 12-X 75-mm tubes using the Oxford micropipet as directed in the Table 1.

e. **Preparation of Quality Control Materials**

Screen urine from a number of volunteers to obtain albumin concentrations of approximately 1.0, 7.0, and 15.0 µg/mL. Filter each urine collection through sterile 0.2-µm Nalge Filterware under vacuum. Aliquot 0.4 mL into 0.65-mL micro-centrifuge tubes using a different tube or cap color for each concentration level. Label each aliquot. Prepare 400 tubes for an estimated 2-year supply for each of the three albumin control concentrations. Store at ≤-70 °C for up to 2 years. Thaw one tube of each control pool on the day of an assay. *CAUTION: Handle the*

*pools as if they are capable of transmitting any infectious agent.*

Table 1
Preparation of Standards

| HSA Standard (µg/mL) | Volume of HSA (µL) | Volume of oval/PBS (mL) |
|---|---|---|
| 20 | 20 | 1 |
| 10 | 10 | 1 |
| 5 | 5 | 1 |
| 2 | 4 | 2 |
| 1 | 2 | 2 |
| 0.5 | 1 | 2 |
| 0 | 0 | 1 |

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

For each assay, a calibration curve is constructed by using dilutions of the 1 mg/mL HSA standard material prepared as described in sections 6d. and 8d. The concentrations of the calibration material used are 0, 0.5, 1, 2, 5, 10, and 20 µg/mL. To establish a known relationship between the measurement response and the true value of the albumin analyte, adjust the fluorometer to read 0% relative fluorescence (RF) and 100% RF for the calibration limits 0 to 20 µg/mL, respectively. The calibration graph is linear with a y-intercept near zero.

### b. Verification

For each assay, the calibration curve correlation coefficient (r) must be > 0.9900. Also, results for at least two of the three quality control urine specimens must be within the expected 95% limits as described in section 10.

Albumin reference value: The 1 mg/mL HSA used for the standard line is checked against a reference material of known value (CAP Urine Protein Reference Material, human albumin Cohn Fraction V). Reconstitute the material as described in the package insert. Devise a dilution schematic that correlates with the concentrations of the HSA of the standard line for comparison. Store at $\leq$-70 °C according to the product expiration date.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

Prior to the assay day:

(1) Attach the Centaur microfilters to the pipet tips for the Excalibur pipet.

(2) Number a 1.5-mL micro-centrifuge tube corresponding to each urine specimen.

(3) Using a waterproof marker, number duplicate 12- X 75-mm tubes for each of the three quality control urine samples, the standard line (labeled from 0 to 20 µg/mL), and the urine specimens.

(4) Prepare a worksheet to compile the information corresponding with the information on the Excel spreadsheet described in section 3. On the worksheet place one of the 3 quality control urine samples in the 0 position before specimen number 1 and the other two after the specimens. Rotate these positions in subsequent assays.

(5) On the day of the assay: Thaw one aliquot of each of the three urine controls, one aliquot of HSA, and the appropriate quantity of Fl-GAHA stock solution needed for the number of assays. Prepare Fl-GAHA working

solution, protecting it against exposure to light, and keeping it at 20-25 °C on the assay day until use.

b. **Sample preparation**

(1) Number the specimen tubes so that their numbers correspond with the specimen identification numbers and the worksheet.

(2) Mix the thawed urine specimens by inverting the tubes eight times.

(3) Measure and record the Albustix protein value as directed on the package insert. Albustix results will be used to determine the specimen dilution factor.

(4) Transfer 250 µL urine to the corresponding conical-bottom tube with the Excaliber pipet and the attached pipet tip with the Centaur microfilter. Centrifuge the conical-bottom tubes for 2 min in the microfuge.

(5) Prepare sample dilutions for samples reading trace, +, ++, or +++ on the Albustix test according to Table 2. Specimens with a reading of "non-detect," "negative," or "negative-trace" need not be prediluted. Dilutions for these samples may be prepared directly on the micromedic as described in Table 3.

(6) Combine samples or diluted samples with 75 µL of immunobeads in 12- x 75-mL borosilicate glass tubes using the Micromedic set as described in Table 3.

(7) Vortex the diluted specimens.

(8) Vortex the dilutions of HSA standards. Note: Centrifugation is not required. The dilution factor is 1X. Use the Micromedic with the sampling syringe set at 40% (20 µl).

(9) Vortex the three urine controls. Using the Excaliber pipet and the attached pipet tip with the Centaur microfilter, transfer each to a 12- X 75-mm tube then to the correspondingly labeled conical-bottom tube. Centrifuge for 2 min in the microfuge. The dilution factor is 1X. Use the Micromedic set at 40% on the sampling syringe.

Table 2
Initial Sample Dilutions With PBS

| Albustix Result | Dilution | Urine (µL) | PBS Buffer (µL) |
|---|---|---|---|
| Non-detect* | -- | 40 | -- |
| Negative* | -- | 20 | -- |
| Negative/Trace* | 1:2 | 10 | 10 |
| Trace | 1:10 | 50 | 450 |
| + | 1:50 | 20 | 980 |
| ++ | 1:100 | 10 | 990 |
| +++ | 1:1000 | 2 | 2000 |

* Dilutions can be made on Micromedic during the addition of immunobeads (Table 3).

c. **Instrument Setup for the Instruments**

(1) Waterbath
Preset and maintain at 37 °C. Fill the chamber with tap distilled water. Check and record the temperature on the assay day prior to the incubation.

Table 3
Final Dilution of Urine with 75 µL of Immunobeads
(37% setting on a 200-µL Micromedic dispensing syringe)

| Albustix value | Concentration of 1st Dilution | Vol of 1st Dilution | 50-µL Sampling Syringe Setting (%) | Sample Vol. (µL) | Final Vol. with Addition of 75 µL Immunobeads (µL) |
|---|---|---|---|---|---|
| N-D | Undiluted | 40 | 80 | 40 | 115 |
| Neg | Undiluted | 20 | 40 | 20 | 95 |
| Neg Tr | 1:2 | 20 | 20 | 10 | 85 |
| Tr | 1:10 | 500 | 40 | 20 | 95 |
| + | 1:50 | 1000 | 40 | 20 | 95 |
| ++ | 1:100 | 1000 | 40 | 20 | 95 |
| +++ | 1:1000 | 1000 | 40 | 20 | 95 |

(The final volume is in essence the same as after the first dilution because amount of immunobeads is the same for all specimens.)

(2) IEC Centrifuge
Set at 2100 rpm (1000 x g) and 10 min for each of the washes. Use a swinging bucket and centrifuge at 20-25 °C with the brake set at maximum.

(3) Micromedic
Immerse the tubing in constantly stirring immunobeads and prime the Micromedic by washing through the bead solution with the dials set for maximum volume dispensing, just enough to fill the lines without the presence of bubbles. Take care to prevent the beads from settling in the tubing while the instrument is not in operation.

Set the instrument for automatic sampling and dispensing. The number dialed on each syringe pump equals the percentage of the syringe volume. The maximum volume of the right syringe (dispensing) is 200 µL; the maximum volume of the left (sampling) is 50 µL.

To Prime -- push both buttons down.

To Set -- push the left button down and the right button up to dial in percentages. Set the left (sampling) dial at 20%, 40% or 80% according to dilution factor schematic. Keep the right dial at 37.5%.

To Run -- set the right button in the "down" position and left button in the "up" position. Press the foot pedal to siphon the sample. (Avoid disturbing the pellet.) Press it again to eject the sample combined with the beads into the 12- X 75-mm tubes. The final volume is 20 µL of sample combined with 75 µL of immunobeads.

When finished, flush the Micromedic by running several cycles with 5 mL of isopropyl alcohol followed by 5 mL of distilled water. Store the Micromedic with tubing filled with distilled water.

(4) Oxford continuous automatic dispenser
Prime with PBS to fill the lines.

Adjust settings for dispensing the 2-mL volume and the 4-mL volume when required in the procedure. When finished, flush the PBS out and leave the lines dry.

(5) Fluorometer
Turn the instrument on and allow it to warm up for a minimum of 15 min prior to use. Set the GAIN at "5." The raw data is read as relative fluorescence (RF%). The 12- X 75-mm tubes function as disposable cuvettes.

d. **Operation of Assay Procedure**

(1) Stir the immunobeads gently on a magnetic stir plate in a beaker of crushed ice, replenishing with ice as needed to ensure that the beads are kept cold throughout the procedure.

(2) Use the Micromedic as described in Section 8.c.3. of this method. Adjust the Micromedic settings as directed in the dilution schematic table to obtain the correct dilutions for the Albustix values of the specimens listed on the worksheet and also for the standard line tubes and the quality control urine samples. Transfer the sample combined with the immunobeads into the appropriate duplicate 12- X 75-mm tubes.

(3) Mix the rack of tubes gently, cover them, and incubate them for 1.5 hours in the 37 °C water bath.

(4) Add 50 µL of Fl GAHA working solution to each tube with the repeating Eppendorf set on 5. The pipette will deliver eight 50-µL aliquots.

(5) Mix the rack of tubes gently, cover them and incubate them 2 hours in the 37 °C water bath.

(6) Perform two washing procedures to remove the unbound Fl GAHA from the immunobead-antigen complex. Add 4 mL of cold PBS to each tube with the Oxford continuous automatic dispenser, centrifuge the tubes for 10 min as directed in the Section 8.c.2. of this method. Decant the supernatant and blot. Add 2 mL of PBS, and vortex. Add 2 mL of PBS, centrifuge, decant the supernatant and blot. Add 2 mL of PBS.

(7) Fluorometer Procedure -- Vortex and wipe the outside of each tube until clean and dry. Insert each tube and record the displayed RF% immediately after vortexing while the beads are in suspension. Read the specimens as soon as possible because of possible deterioration of the photosensitivity of the fluorescein.

Calibrate the 0 µg/mL (blank) tube to 0% RF: insert the 0 µg/mL tube and adjust the zero button to "0." Calibrate the 20 µg/mL tube to 100% RF: insert the 20 µg/mL tube and adjust the scan button to 100% RF.

Read the standard line tubes and the three urine quality control tubes. Record the RF% values on the worksheet.

Perform linear regression on the HP calculator to generate the standard line. Record the y-intercept, slope, and correlation coefficient (r) onto the worksheet.

Verify the assay by calculating the three urine control values. Discard the assay if the r is <.9900 or if the results of the QC material are outside limits as described in section 10.

If the assay is verified, read the specimen tubes and record the values on the worksheet.

To substantiate the continued accuracy of the test method periodically verify that the calibrations remain in adjustment by re-checking the 0 µg/mL and the 20 µg/mL samples at 0% RF and 20% RF, respectively.

Because fluorescein is photosensitive, the final solution is not stable.

e. **Recording of Data**

Type the worksheet data for both QC and analytical samples onto the Excel spreadsheet. Record the linear regression values of slope, y-intercept and r. Record the RF% values of the standard line, controls, and specimens. Convert the RF% values of the samples into µg/mL HSA by performing a "fill-down" of equations previously formatted on the template document.

Print the Excel document and verify that every value (i.e., specimen identification number(s), volume, time, dilution factor and RF%) matches those on the worksheet.

Transcribe the MacIntosh Excel data onto the NHANES IBM formatted diskettes. Flag unknown samples with values for albumin >1000 mg/mL or "too low to read."

Record the values for the three quality control urine samples on the Levy-Jennings charts.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Fluorometer

Determine acceptable reagent performance by documenting the performance of reagents when lot numbers are changed for the following: beads, GAHA, HSA, Fl GAHA, and new bead prep. Concurrently assay a standard line and controls using the new reagent to be evaluated. The parameter used to determine acceptable performance is the verification that the results of the three control urine samples are within their acceptable 95% limits.

Perform calibrations and maintenance quarterly or as necessary on instrumentation and pipets. Record accuracy and precision for pipets.

(2) Pipettors

All micro-pipettors used in testing clinical specimens are checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

Apply the equation for a straight line, $y = mx + b$. Use linear regression electronically on the Excel template. The linear regression data generates slope (m), y-intercept (b), r, and fitted line. The RF% value is directly proportional to the µg/mL of the standard line. The dilution factor is multiplied by the derived µg/mL from the RF% to obtain the total µg/mL albumin concentration.

The urine albumin concentration is reported in µg/mL and is significant to one decimal point. For the NHANES project, micrograms of albumin are calculated as albumin µg/mL X 1.04, and an index value describing the relationship of albumin to creatinine is calculated. The formula is:

*10 X (albumin µg/mL ÷ creatinine mmol/L).*

## 9. REPORTABLE RANGE OF RESULTS

(a) The reportable range of values for albumin is >0.5 µg/mL to <20 µg/mL for undiluted specimens as read on the fluorometer. Specimens with values >20 µg/mL are diluted and the results multiplied by their dilution factor. Results up to 20 mg/mL are reported for diluted specimens.

(b) The range of test values for the fluorometer instrument is 0% to 100% RF, which corresponds with the range of linearity for the standard line, 0 to 20 µg/mL. Follow the dilution schematic described in section 8.b. to obtain appropriately diluted specimens.

(c) Specimens below 0.5 µg/mL when assayed at the 2X dilution are reported as "too low to read."

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the University of Minnesota Hospital. The method has proven to be accurate, precise, and reliable. The instrumentation used is "state-of-the-art." The primary standard used is a CAP Urine Protein Reference Material, human albumin Cohn Fraction V (College of American Pathologists, Skokie, IL). Estimates of imprecision can be generated from long-term QC pool results.

Low, medium, and high range bench QC specimens are analyzed in duplicate in each analytical run (a set of assays performed on a given day) so that judgements may be made on the day of analysis.

Tolerance limits for controls are established by using the Westgard Rules as guidelines. Permanent QC limits, the average difference between duplicates, and the range (R) for the assay are established by using 100 pairs for each of the three control urine levels. The control urine samples are the same in all assays. When new control urine samples are initiated into the assay, the QC parameters are established by using 20 pairs analyzed on 20 different days to calculate the range and the mean. The standard deviation overall (SDo) is calculated on one control result from each assay day for a total of 100 days. Tolerance limits are defined by the Westgard Rule as the mean ±3 SDo (or 99% action limit) and the mean ±2 SDo (or 95% warning limit).

Prepare a Levy-Jennings QC plot for each of the 3 control urine samples. Chart the means and tolerance limits on graph paper and plot each control urine value as a dot for all assays. Repeat the assay if two of three control urine samples are outside of the 95% limit. Watch for trends in the dot plot. If the dot plot scatter is out of range, perform function checks on the reagents and instrumentation. Do not provide assay results until the problem is corrected. The NHANES project officer and the hospital clinical chemistry laboratory manager review the Levy-Jennings plots.

QC for interassay variation is monitored by repeating 5% of the specimens from previous assay(s). Interassay variation must be $\leq$10% of the coefficient of variance (CV%).

The system is declared "out-of-control" if any of the following events occur:

On the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two or more pools fall either both above or both below the 95% limit.
- Two successive run means for a single pool fall either both above or both below the 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

On the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

Quality assurance is monitored by recording the following for each assay: date of assay, name of specimen group, the measured and expected QC values, the dates of changes in reagents with the lot numbers, and the reason for repeat analysis of samples.

The quality of the assay is maintained by ensuring that samples are collected and preserved correctly, that samples are rejected if the criteria are not met, and that instructions are followed in handling, shipping and storing specimens. Results from specimen storage, preservation, and freeze/thaw studies have provided guidelines for the laboratory.

External proficiency testing to evaluate the assay performance is conducted through the Centers for Disease Control and Prevention Interlaboratory Quality Assessment Program for urine microalbumin.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Retest specimens with discordant duplicate RF% values. The acceptable duplicate ranges in RF% are as follows:

| | | |
|---|---|---|
| 0-20 | RF% | 4 units |
| 21-50 | RF% | 5 units |
| 51-100 | RF% | 8 units |

Retest specimens at appropriate dilutions to obtain duplicate RF% values within the reportable range of values for the fluorometer.

Repeat the entire assay if QC urine samples are outside of the limits as described in section 10, or if the r value is <0.9900. Replace reagents or recalibrate instrumentation, if necessary, for corrective action. Introduce reagents with new lot numbers into the assay one at a time to facilitate identifying problem reagents.

Do not report results until corrective action is documented and the assay is in statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

The lowest reportable value is <0.5 µg/mL. Any values less than the %RF for the 0.5 µg/mL standard when diluted two-fold are reported as "too low to read." These values are below the limit of detection for the assay.

Female participants who are menstruating may have contaminated urine specimens resulting in falsely elevated albumin levels.

**13. REFERENCE RANGES (NORMAL VALUES)**

Table 4
Urine Albumin in Healthy Subjects

| Number of Subjects | Author | Method | µg/mL | µg/min | mg/24 hr |
|---|---|---|---|---|---|
| 50 adults | Chavers (2) | FIA | 0.7-16.4* | 0.9-14.0 | 1.3-20.5 |
| 20 adults | Morgenson (3) | RIA | | 5-20 | 3.6-23 |
| 11 adults | Howey (4) | RIA | 8.13+ | 7.1 **,+ | 10.2+ |
| 20 children | Fielding (5) | ELISA | | 1.2-15.9** | 1.7-22.9 |

* obtained from subject data in the published manuscript
** calculated from the published units, mg/24 hour
\+ mean values

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Specimens visibly hematuric or testing positive for hemoglobin on a dipstick are unacceptable. Urine albumin is not measured in hematuric specimens and NHANES is notified.

Albumin values >1000 µg/mL or "too low to read" are flagged for attention.

No medical intervention is indicated for unusual albumin values.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach 20-25 °C during the following procedures: pipetting into the conical-bottom tubes and centrifugation in the microfuge and during the Micromedic dilution procedure. The only critical temperature during the testing procedure is the 37 °C incubation, after which incubation ends and cold buffer is introduced for the centrifugation/washing procedure. During the fluorometric procedure, the samples remain relatively cool but may approach 20-25 °C.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

Manual volumetric pipets can be substituted for the automatic diluters (Micromedic and Oxford). If the fluorometer fails, no alternative instrumentation is available until it is returned to service.

Return specimens to ≤-20 °C if the test system is out of operation for >3 weeks.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

The albumin data is reported in µg/mL with one decimal point.

Albumin values >1000 µg/mL, or those designated "too low to read" are flagged for attention.

Mail to NCHS, MacIntosh print-outs of the Excel spreadsheets containing the albumin assay data, QC reports, the IBM print-outs, and the IBM diskettes.

Quarterly progress reports are sent to NCHS regarding QC, assay performance, and miscellaneous information. Included is information regarding specimen results with abnormal values (values >1000 µg/mL albumin or "too low to read") or unacceptable specimens.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

All notebooks, discs, and files containing raw data, final data, QC information, communications, etc. are saved. These are the property of the NHANES project and will be released upon request. Specimens are discarded after the integrity of the specimen data has been verified by the laboratory supervisor.

### REFERENCES

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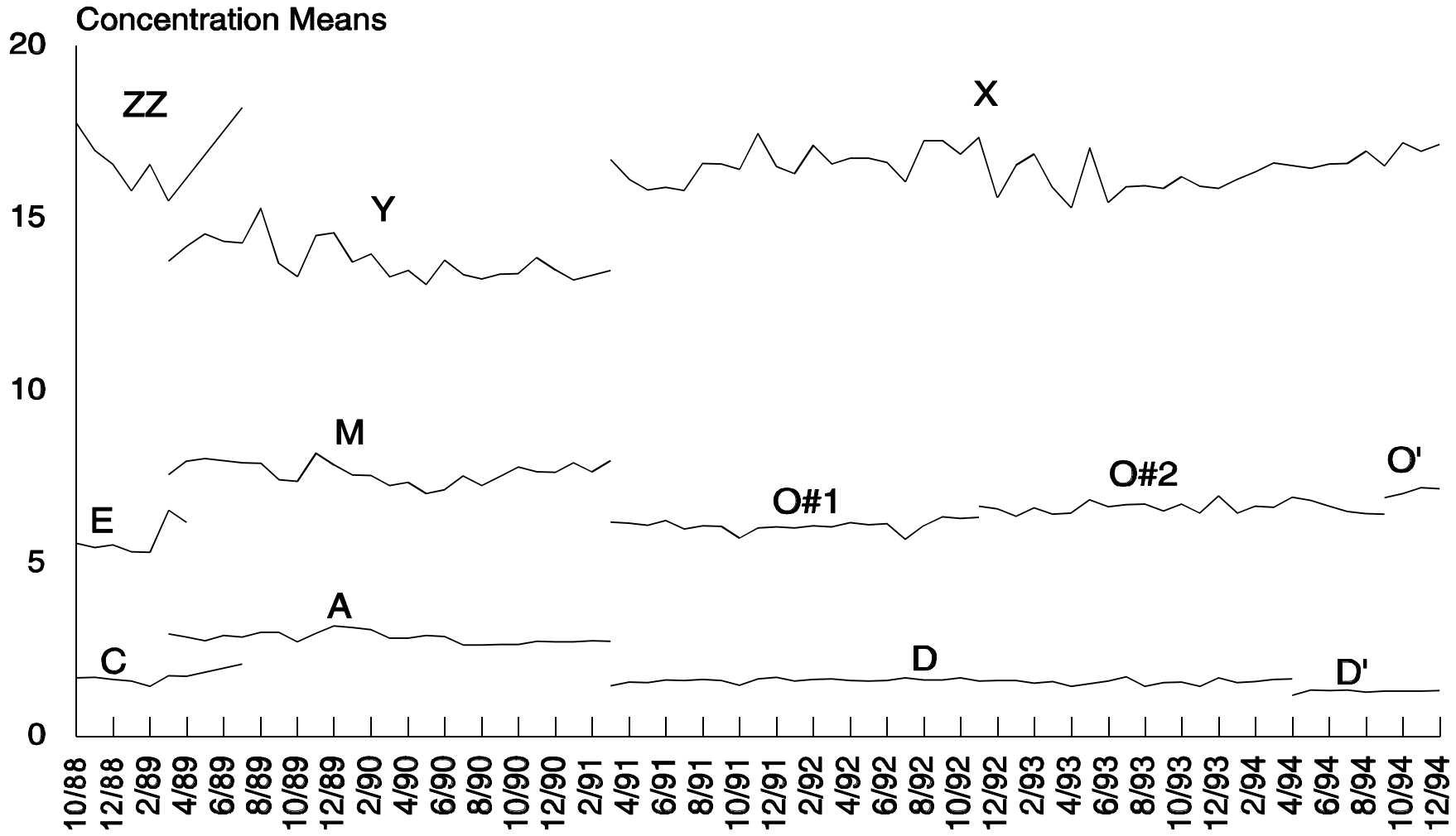
SUMMARY STATISTICS FOR
URINE MICROALBUMIN
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| A | 03/27/89 - 03/21/91 | 2.8557 | 0.27934 | 9.7819 | 397 |
| C | 10/31/88 - 07/07/89 | 1.6650 | 0.26796 | 16.0940 | 60 |
| D | 03/25/91 - 04/26/94 | 1.6163 | 0.18835 | 11.6527 | 588 |
| D' | 04/28/94 - 12/15/94 | 1.3208 | 0.13159 | 9.9635 | 130 |
| E | 10/31/88 - 04/17/89 | 5.5333 | 0.65828 | 11.8966 | 57 |
| M | 03/06/89 - 03/18/91 | 7.6053 | 0.60855 | 8.0017 | 414 |
| O#1 | 03/21/91 - 11/15/92 | 6.1279 | 0.36964 | 6.0321 | 340 |
| O#2 | 11/18/92 - 09/08/94 | 6.6245 | 0.39722 | 5.9962 | 335 |
| O' | 09/13/94 - 12/15/94 | 7.1125 | 0.34247 | 4.8151 | 48 |
| X | 03/27/91 - 12/15/94 | 16.5004 | 1.02609 | 6.2185 | 716 |
| Y | 03/06/89 - 03/25/91 | 13.7106 | 0.92922 | 6.7774 | 417 |
| ZZ | 10/31/88 - 07/07/89 | 16.4914 | 1.75322 | 10.6311 | 58 |

# Urine Microalbumin Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

A Jaffé rate reaction, in which creatinine reacts with picrate in an alkaline solution to form a red creatinine-picrate complex, is used for creatinine analysis and is measured with an ASTRA analyzer. The rate of the color development is measured 25.6 sec after sample injection at 520 nm. The observed rate measurement is proportional to the concentration of creatinine in the reaction cup. The procedures described below are the standard protocols of the University of Minnesota Hospital and Clinic (UMHC) (1-5).

Creatinine is the waste product derived from creatine and is released into the plasma at a relatively constant rate. The amount of creatinine per unit of muscle mass is constant; therefore, creatinine is the best indicator of impaired kidney function. Both blood urea nitrogen (BUN) and creatinine are measured primarily to assess renal function.

## 2. SPECIAL SAFETY PRECAUTIONS

Follow all procedures and policies in the UMHC Laboratory Safety Manual, including the Universal Blood and Body Substance Technique (UBBST). Consider all specimens received for analysis potentially positive for infectious agents. Wear gloves, lab coat, and safety glasses while handling all specimens. Dispose of contaminated supplies in biohazard bags; seal and autoclave the bags. Wipe all work surfaces 3% phenolic disinfectant solution.

> *Special care should be taken when handling picric acid. Avoid contact with skin. If spilled, flush with copious amounts of water.*

The material safety data sheets (MSDSs) for sodium hypochlorite, ethanol, and picric acid are located in UH3-555 CC1 paperwork area.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Tests are ordered and accessioned in the Knowledge Data Systems laboratory information system (LIS). The UMHC Microalbumin Laboratory specimen code is reported for verification of NHANES specification identification.

The integrity of specimen identification is maintained through a two-step verification process. The specimen number is entered in the ASTRA prior to specimen analysis, and results are automatically transmitted to the LIS via an instrument interface. A technologist then reviews result data prior to result verification and release. Results are available to the Microalbumin Laboratory immediately via screen display, or within 24 hours via hard copy printout.

Results are reported from the ASTRA in mg/dL and converted to mmol/L by the LIS according to the formula:

$$mg/dL \times 0.0884 = mmol/L.$$

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Either a random or a timed urine specimen is acceptable; a random urine specimen is used in NHANES III.

b. Acidified urine specimens are acceptable.

c. Angiogram/IVP dyes do not interfere with analysis.

d. Mix and filter cloudy urine specimens prior to analysis.

e. Centrifuge urine specimens containing blood or precipitate prior to analysis.

f. The optimum specimen volume is 1 mL, and the minimum acceptable volume is 30 µL.

g. The UMHC Microalbumin Laboratory sends 250-µL aliquots of urine in 0.5-mL capped vials to the Chemistry

Laboratory for creatinine analysis. Aliquots are stored at 4-8 °C until analysis. Analysis is completed within 36 hours of receipt.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Beckman Synchron AS/ASTRA clinical analyzer (Beckman Instruments, Inc., Brea, CA). Hot Line 1-800-854-3633; Field Service 1-800-345-0424.

(2) Milli-Q water system, (Millipore Corporation, San Antonio, TX).

### b. Other Materials

(1) Wash solution concentrate, cat. no. 668601 (Beckman Instruments, Inc.).

(2) Alkaline buffer, cat. no. 668306 (Beckman Instruments, Inc.).

(3) Picric acid solution, cat. no. 668306 (Beckman Instruments, Inc.)

(4) 5% (v/v) sodium hypochlorite (bleach) solution, cat. no. CX23880 (UMHC Pharmacy, Minneapolis, MN). Stable indefinitely at 20-25 °C. *CAUTION: Corrosive and irritant.*

(5) 95% (v/v) ethyl alcohol ($C_2H_5OH$), 1-L bottle, (UMHC Pharmacy). Store supply in flammable section under UH3-555 hood. Stable indefinitely at 20-25 °C. *CAUTION: Flammable.*

(6) Calibrator I and Calibrator II, cat. no. 668304 (Beckman Instruments, Inc).

(7) ChemTrak chemistry control level 1 and 3, liquid, unassayed, cat. no. CU1-1001 (Medical Analysis Systems, Inc., Camarillo, CA).

(8) Urine I control, human, assayed, product code 9035 (Ciba-Corning Diagnostics Corp., Irvine, CA).

(9) Sample cups, polystyrene, conical, 0.5-mL capacity, 1000 cups/bag, cat. no. 339-788 (Curtin-Matheson Scientific, Inc., Eden Prairie, MN).

(10) Syringe, sterile 5-cc disposable, 100/pkg, cat. no. 301603 (Becton-Dickinson, Inc., Franklin Lakes, NJ).

(11) Luer-tip cap, 10/pkg (Becton-Dickinson, Inc.).

### c. Reagent Preparation

(1) Wash Solution

The wash concentrate is stable at 20-25 °C until the expiration date and contains bacteriostat and other nonreactive ingredients. Dilute 50 mL of wash concentrate to 2000 mL with Milli-Q deionized water. Mix well. The diluted solution is stable 1 month at 20-25 °C.

After using the wash solution bottle, rinse it with 70% ethanol, followed by Milli-Q water. Allow the bottle to dry completely before re-using. Record the new lot number and date of preparation on the bottle.

(2) Alkaline buffer

Contains 0.188 mol/L sodium hydroxide (NaOH) buffered with sodium borate ($Na_2B_4O_7$) and sodium phosphate ($NaH_2PO_4$) and other nonreactive ingredients. Store the kit at 20-25 °C. The solution is stable until the manufacturer's expiration date.

(3) Picric acid solution
Contains 0.05 mol/L picric acid. Store the kit at 20-25 °C. The solution is stable until the manufacturer's expiration date.

> *CAUTION: Avoid contact with skin. If spilled, flush with copious amounts of water.*

(4) Creatinine Reagent
Add 400 mL of picric acid solution to a 1600-mL bottle of alkaline buffer. Cap tightly and mix vigorously. Record the lot number of the alkaline picrate solution and the date on the buffer bottle. This solution is stable 30 days at 20-25 °C after mixing. Do not use combined reagent until 1 hour after preparation to allow bubbles to dissipate.

NOTE: Precipitation of some components may occur at low temperatures. Bring reagent to 20-25 °C and mix it by inversion before use. Do not use the reagent until all particulate matter has dissolved and the reagent is clear.

(5) 2% (v/v) sodium hypochlorite (bleach) solution
Dilute 400 mL of 5% bleach to 1 L with Milli-Q water. The solution is stable indefinitely at 20-25 °C. *CAUTION: Corrosive and irritant.*

(6) 70% (v/v) ethyl alcohol ($C_2H_5OH$)
Dilute 700 mL of 95% ethyl alcohol to 1 L with Milli-Q water. The solution is stable indefinitely at 20-25 °C. *CAUTION: Flammable.*

b. **Standards Preparation**

(1) Aqueous calibration standard
Aqueous calibration I and II standards are supplied ready-to-use by Beckman Instruments and are referenced against NIST SRM 914A creatinine (National Institute of Standards and Technology, Gaithersburg, MD).

Calibrator I contains 5 mg/dL creatinine.

Calibrator II contains 0 mg/dL creatinine.

Calibrators are ready-to-use and are stable until the expiration date on the label when stored at 4-8 °C. Calibrators are stable for 1 month at 20-25 °C after date of opening.

(2) To validate a new lot number of aqueous calibrators:

(a) Calibrate using the current (old) lot of calibrators. Run the current and new lot numbers of aqueous calibrators (i.e., alternating between them) five times each.

(b) Average the result of each lot number.

(c) Divide the average of the new lot number by the average of the old lot number and multiply by 100. Acceptable limit: 100 ± 1%.

If the results of calibrator comparisons are not within the acceptable limit, the new lot of calibrators should be returned to the manufacturer and replaced with a different lot.

c. **Quality Control Preparation**

(1) ChemTrak chemistry control (Levels 1 and 3)
Store unopened vials at ≤-20 °C; stable until the expiration date on the box. Unopened material is stable for 60 days at 2-8 °C.

To use, open 1 vial and aspirate 3-5 mL into a 5-mL disposable syringe. Cap with a luer-syringe cap and

store the material at 2-8 °C. Dispense aliquots as needed for analysis. Once a vial is opened, the contents are stable for 14 days or until depleted.

(2) Urine I control

Store unreconstituted material at 2-8 °C; material is stable until the expiration date on the box. To use, bring vial components to 20-25 °C. Remove the metal seal and rubber stopper from the vial. Add 25 mL Milli-Q water and allow the solution to stand for 5-10 min. Swirl contents gently until the solution is homogeneous. Aliquot entire vial contents into 0.5-mL sample cups, cap the vial and freeze at ≤-20 °C; the solution is stable for 1 month at ≤-20 °C. Thaw aliquots as needed for analysis.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

Creatinine calibration lasts 8 hours. If the reagent is replaced or maintenance/troubleshooting is performed, prime and recalibrate the instrument.

The calibration standards are purchased from Beckman Instruments and are referenced against NIST SRM 914A creatinine. This method results in a linearized 2-point standard curve and is linear from 0-400 mg/dL.

To calibrate creatinine, prepare fresh cups of aqueous calibration standards I and II, and place in positions 39 and 40 on the tray. Press the CALIBRATE button and enter the analytes to be calibrated, the tray number, and the first two digits of the reorder number (04) on the calibrator bottles.

The ASTRA primes each channel twice, samples, and analyzes each calibration solution at least twice. The ASTRA evaluates the calibration by comparing the analog-digital count (ADC) number to the preset ranges.

The instrument evaluates calibration against the preset ranges as shown in Table 1.

Table 1
Acceptable Ranges for Calibrators

| Parameter | ADC Range |
|---|---|
| Calibrator I | 175 to 375 |
| Calibrator II | -45 to 45 |
| Span of Calibrator I and II | ≥165 |
| Difference of duplicates for each calibrator | ≤9 |

If an analyte is imprecise, a third or fourth replicate of the standard will be measured. If any calibration fails, the beeper alarm will sound.

If calibration is interrupted, the beeper alarm will sound. Press the STATUS button for a message explaining the interruption. Press the HOME button and, when the green light quits flashing, proceed with a new calibration. Acceptable range, precision, and sensitivity limits for ADC numbers are found under SPECIAL FUNCTIONS (#1 Calibration, ENTER, ENTER, #7 Calibration Data Display, ENTER).

### b. Verification

New standards are validated with a minimum of five overlapping assays performed with existing standards. When quality control values in all five assays using the new standard lots meet acceptable criteria, the new standards are included in assay runs.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

Record reagent dates and lot numbers. Perform daily start-up maintenance:

(1) Check reagent levels.

(2) Check drain lines. Dispose of waste.

(3) Check the red heater lamp on the creatinine module; the heater lamp lights when the module reaches the correct temperature.

(4) Check the source lamp on the creatinine module and check for bubbles in the creatinine module.

(5) Prime all modules five times. Check the stirrer in the module and inspect the reagent lines to ensure that all lines are pumping.

(6) Wipe the outside of the probes with an alcohol prep. Check the height of the sample probe tips and adjust if necessary. The probe tips should be approximately a credit card width (1mm) above the modules.

(7) Soak the sample probes in 2% (v/v) bleach by doing the following:

(a) Fill a sample cup with 2% bleach and place it in a vacant slot on the sample tray.

(b) Program the instrument to sample the cups for all available tests.

(c) Press RUN/START.

(d) When the sample probes are over the wash cup, press STOP. *Do not allow bleach to be dispensed in any of the modules.* Allow the bleach to sit in the probes approximately 5 min.

(e) Press HOME and prime the sample module 10 times.

b. **Sample Preparation**

Recap sample cups immediately to maintain sample integrity for repeat analysis, if needed.

c. **Instrument Setup of the Beckman Synchron AS/ASTRA Clinical Analyzer.**

Calibrate the instrument for creatinine as described in section 7.

d. **Operation of the Assay Procedure**

(1) Pipet 50 µL of control or test specimens into sample cups. To prevent evaporation, do not load more than five samples in advance. Cups are sampled every 70 sec when all tests are requested.

(2) To program the tray, press PROGRAM to enter the tray number. Cups 1-34 are available for test programming.

(3) To program the analysis of controls, press the key for creatinine. Press CONTROL to enter the control number (level 1 = 1; level 2 = 3; urine = 4). Analyze two levels of controls after each calibration to verify the calibration. Analyze urine controls whenever unknowns are analyzed. Analyze one level of control for at least every 10-15 samples throughout the run.

(4) To program the analysis of specimens, press the key for creatinine, press URINE, and enter the specimen number. If an error is made, press CLEAR to clear the entire line.

(5) When five specimens are programmed, press RUN/START to begin the analysis. To continue programming as specimens are analyzed, repeat steps 2-4.

(6) The instrument will print results as testing is completed. Whenever ASTRA completes all samples programmed on a tray, the message "Program for this tray completed" prints and the instrument returns to standby mode.

(7) The instrument has memory for two trays. To clear a completed tray, press CLEAR, then enter tray number

to be cleared. Trays are identified as 1-99.

e. **Recording of Data**

(1) Urine creatinine is reported in mg/dL to the nearest whole number.

(2) Record all dilution factors or any pertinent specimen information on the protocol pages.

(3) Results of duplicate tests of sample urine specimens should be within 6 mg/dL. Report the original result rather than averaging two results when duplicate resulta are within 6 mg/dL.

(4) The laboratory computer will calculate the urine creatinine in mmol/L from mg/dL and report both values on the worksheet.

f. **Replacement and Periodic Maintenance of Key Components**

Beckman Clinical Analyzer

(1) Weekly Maintenance

(a) Inspect in-line filters in the wash module and reverse flush if they are dirty.

(b) Inspect sample trays and covers; if dirty, clean in soapy water, rinse, and dry.

(2) Two-Week Maintenance

(a) Wipe the outside of module covers and wipe any spills and dust from the inside of the module compartment. Clean the plexiglass window, module covers, and video display with anti-static spray.

(b) Wipe the transport bar with a Kim-wipe moistened with 70% ethanol.

(c) Clean wash reagent lines and the reaction cup every 2 weeks with 2% bleach solution and 70% ethanol as follows:

(i) Place the wash reagent pickup line in 2% bleach. Prime 15 times.

(ii) Place wash pickup lines in 70% ethanol and prime 10 times. Allow the solution to stand in the lines and reaction cup for 5-10 min.

(iii) Place the wash pickup lines in the wash solution and prime five times.

(iv) Remove wash pickup lines from the wash solution and prime five times with air.

(v) After wiping the pickup lines, place them in the wash solution.

(d) Prime the creatinine module 30 times with hot Milli-Q water.

(e) Clean the creatinine cup with Milli-Q water:

i. Drain the cup using Special Function 4, Option 2.

ii. Remove the stirrer and clean with a Kim-wipe wet with Milli-Q water.

iii. Swab the reaction cup with a cotton-tipped applicator wet with Milli-Q water.

iv. Replace the stirrer.

(f) Drain the wash cup by manually turning the drain pump. Clean the wash cup and stirrer with Milli-Q water.

(g) Clean the drain trough with 50 mL warm water by disconnecting the drain line on the sample wash module and using a syringe to push the water into the drain.

(h) Clean and regrease the black sample syringe. Do not regrease the blue sample syringes.

(i) Clean the air filter on the back of the disc drive by banging the filter against a waste basket or by using a vacuum cleaner.

(j) Clean the disc drive following the instructions on the kit.

(k) Prime the modules as necessary.

(4) Perform 4-, 8-, and 12-week maintenance as directed in the operators manual.

(5) Refer to the ASTRA Operating and Service instructions for troubleshooting procedures. Further assistance can be obtained from the Beckman Hotline (1-800-854-3633) or Beckman Field Service (1-800-845-0424).

(6) Notes

(a) Never place metal objects in the creatinine analysis cup, and be careful when removing the stirrer, as the optical surfaces facing the cup are easily scratched.

(b) Fishing line may be used to clean the inside of the sample probe.

Pipettors

All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors not conforming to specifications should be autoclaved and mailed for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

This method results in a linearized 2-point standard curve and is linear from 0-400 mg/dL. Results are reported from the ASTRA in mg/dL, and are subsequently translated into mmol/L through the use of the KDS worksheet.

## 9. REPORTABLE RANGE OF RESULTS

The range of linearity for urine creatinine analysis is 10-400 mg/dL.

Dilute specimens that have urine creatinine results >400 mg/dL with Milli-Q water and reanalyze them.

Reanalyze specimens with values <10 mg/dL on the serum channel.

Report values <1.0 mg/dL as <1 mg/dL.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in the University of Minnesota Hospital. The method has proven to be accurate, precise, and reliable. Estimates of imprecision can be generated from long-term QC pool results.

In this analytical method, bench QC specimens are inserted by the analyst two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. Five percent of the specimens from previous assays(s) are also sent for blind retesting. The results of the blind specimens are decoded and reviewed by the supervisor. With both bench and blind QC systems, all levels of concentration are assessed throughout the complete analytical process. The data from these materials are then used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Three Westgard rules (6) are used to determine whether data are acceptable (i.e., in statistical control) and thus to be reported:

- Warning Rule, 1-2s (1 value > 2 SD): If one control observation falls outside the 2 SD limits, additional inspection of control data and application of the rules below are required before the analytical run is accepted or rejected.
- Action Rule, 1-3s (1 value > 3 SD): Consult the charge technologist. If one control observation falls outside the 3 SD limits, spot-check an appropriate number of test samples analyzed since the last acceptable control or since the problem detected occurred.
- Action Rule, 2-2s (2 values > 2 SD): Consult the charge technologist if two consecutive control observations fall outside the same 2 SD limit (mean±2 SD). This rule applies to 2 different controls within a run or 2 consecutive values on the same control in 2 consecutive runs.
- Accept/Reject: Accept the run if the rules indicate that the run is in statistical control.

QC for interassay variation is monitored by the Microalbumin Laboratory. Interassay variation must be ≤10% of the coefficient of variance (CV).

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. Discontinue testing until the problem is resolved and both calibration and QC meet acceptable criteria.

b. Do not report patient results from runs that are not in statistical control.

c. Document all actions and information (i.e., reagent changes, instrument problems, etc.) relating to the "out-of-control" run.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

a. Creatinine Interferences:

Specimens having the following interferences on the ASTRA should be analyzed on the EKTACHEM and results reported.

(1) A hemoglobin level >400 mg/dL
Gross hemolysis falsely lowers the creatinine level.

(2) Acetoacetate levels of 0.25 mmol/L (normal = 0.05-0.15 mmol/L)
This corresponds to a "trace-positive" Ketostix (ketone screen) and will falsely elevate creatinine values by 0.8 mg/dL at a 2.0 mg/dL creatinine level. Creatinines should be analyzed on the EKTACHEM for all samples with a positive ketone screen.

(3) Acetone level of 100 mg/dL
Although this problem would be rarely encountered, acetone falsely elevates creatinine value by 0.4 mg/dL at a 1.1 mg/dL creatinine level.

(4) Cephalosporin levels of 300 µg/mL
This drug concentration will falsely elevate creatinine values by 1.1-1.5 mg/dL at a 1.1 mg/dL creatinine level. Higher levels of cephalosporin will cause a proportionally higher interference.

The current Physicians Desk Reference states that cephalothin and cefoxitin concentrations >100 µg/mL may interfere with test results, and serum samples should not be analyzed for creatinine if drawn within 2 hours of drug administration. It is advisable that patients have their blood drawn for creatinine tests immediately before the next dose of cephalosporin. Because of the potential interference, it is crucial to assess the BUN and creatinine fluctuations and investigate further, as this is the only way to discover the problem.

d. Other substances causing false positive creatinine results are; lithium bromide, acetoacetate, acetone, glucose at levels ≥2000 mg/dL (it falsely elevates creatinine results by 25%), ammonium chloride, M-dopa at levels ≥200 µg/mL (it falsely elevates creatinine results by 0.6 at a level of 2.0 mg/dL), and sodium pyruvate.

e. Factors causing false negative creatinine results are gentistic acid at levels ≥20 mg/dL (it falsely lowers creatinine results by 0.5 at a level of 2.2 mg/dL), bilirubin, lipemia, hemolysis, and L-dopa at levels ≥250 µg/mL (it falsely lowers creatinine results by 0.6 at a level of 2.0 mg/dL).

f. See Synchron advisory (2) a for further list of substances interfering with Beckman Chemistries.

## 13. REFERENCE RANGES (NORMAL VALUES)

Although urine creatinine concentrations are very dependent upon skeletal muscle mass, approximate normal ranges (based on 24-hour urine collecions) are presented in Table 2.

Table 2
Normal Ranges for Urine Creatinine

| Age, years | Range |
|---|---|
| 2-3 | 6-22 mg/kg/d |
| 4-16 | 12-30 mg/kg/d |
| ≥16, male | 1.0-2.0 g/d |
| ≥16, female | 0.8-1.8 g/d |

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

No medical intervention is indicated for unusual urine creatinine results on random urine specimens.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Store specimens at 4-8 °C until analysis; complete testing within 36 hours of receipt in the laboratory.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

The ASTRA is the only instrument used for analysis of NHANES urine creatinines. The Kodak Ektachem is used only for those specimens which are shown to contain substances that may interfere with the ASTRA chemistry. In the event of instrument malfunction or unacceptable calibration or QC, samples are stored at 4-8 °C. If the system is inoperable >36 hours, fresh urine aliquots are requested from the Microalbumin Laboratory. Specimens may be refrozen until analysis is possible.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable for this procedure.

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

All notebooks, disks, and files containing raw data, final data, QC information, communications, etc. are saved. These are the property of the NHANES III project and will be released to NCHS. Specimens are discarded after the integrity of the specimen data has been verified by the laboratory supervisor.

## REFERENCES

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5. Kaplan LA, Pesce AJ, editors, Clinical Chemistry Theory, Analysis and Correlation. St. Louis: CV Mosby Company, 1984:416-8, 1032-5, 1044-5, 1051-6, 1075-8, 1234-9, 1247-53, 1257-61.

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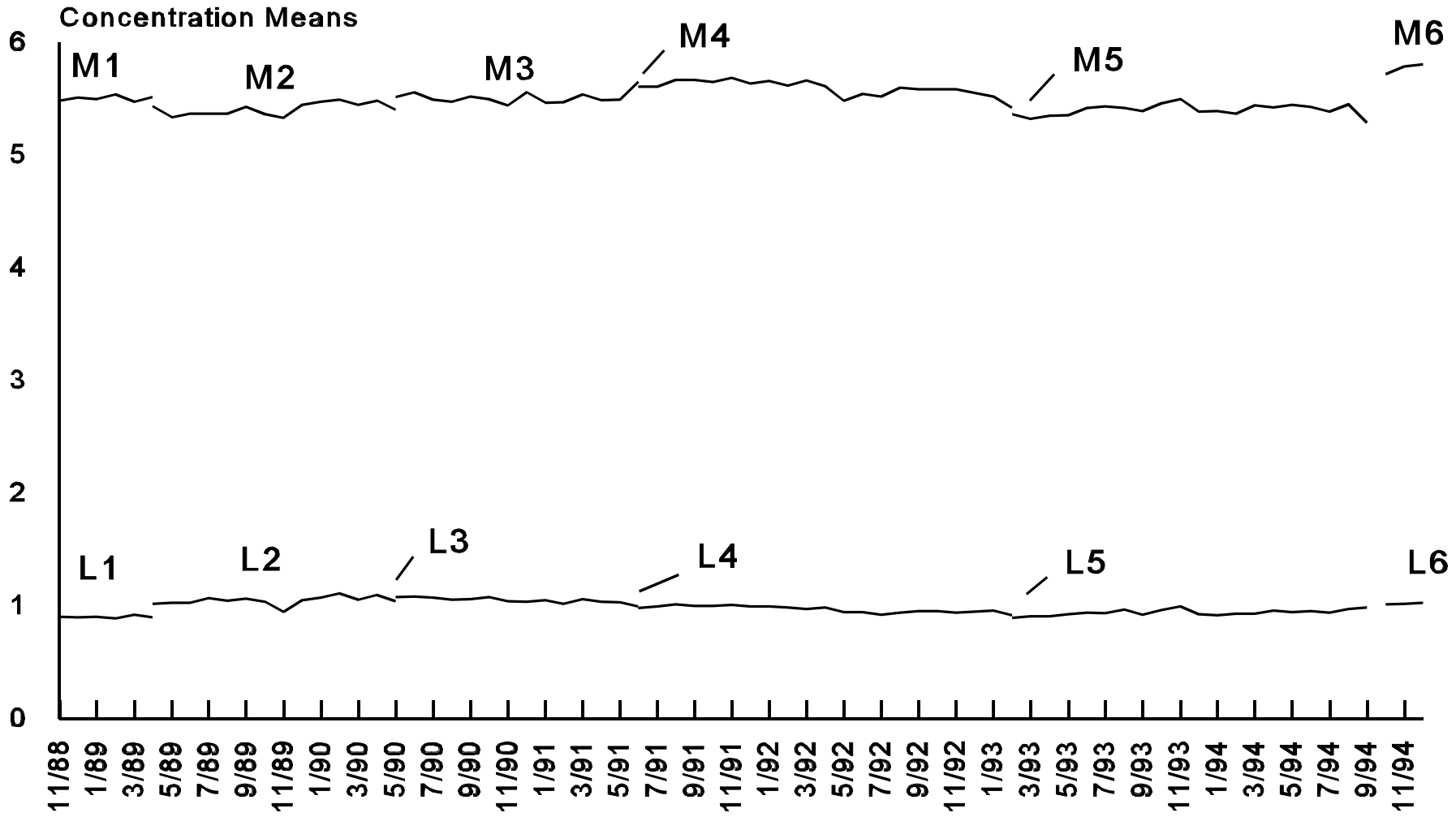
SUMMARY STATISTICS FOR
URINARY CREATININE LOW/MEDIUM POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| L1 | 11/04/88 - 04/20/89 | 0.90866 | 0.07017 | 7.72278 | 127 |
| L2 | 04/26/89 - 05/15/90 | 1.06311 | 0.07161 | 6.73591 | 309 |
| L3 | 05/22/90 - 06/03/91 | 1.05732 | 0.06465 | 6.11433 | 314 |
| L4 | 06/05/91 - 02/22/93 | 0.97960 | 0.05115 | 5.22168 | 446 |
| L5 | 02/23/93 - 09/22/94 | 0.94395 | 0.05243 | 5.55430 | 430 |
| L6 | 10/07/94 - 12/12/94 | 1.02245 | 0.04684 | 4.58104 | 49 |
| M1 | 11/04/88 - 04/20/89 | 5.50270 | 0.09483 | 1.72333 | 111 |
| M2 | 04/26/89 - 05/15/90 | 5.42035 | 0.11841 | 2.18455 | 285 |
| M3 | 05/22/90 - 06/03/91 | 5.50000 | 0.09879 | 1.79618 | 292 |
| M4 | 06/05/91 - 02/22/93 | 5.60335 | 0.11076 | 1.97661 | 418 |
| M5 | 02/23/93 - 09/22/94 | 5.40193 | 0.09839 | 1.82147 | 414 |
| M6 | 10/07/94 - 12/12/94 | 5.77059 | 0.08785 | 1.52238 | 51 |

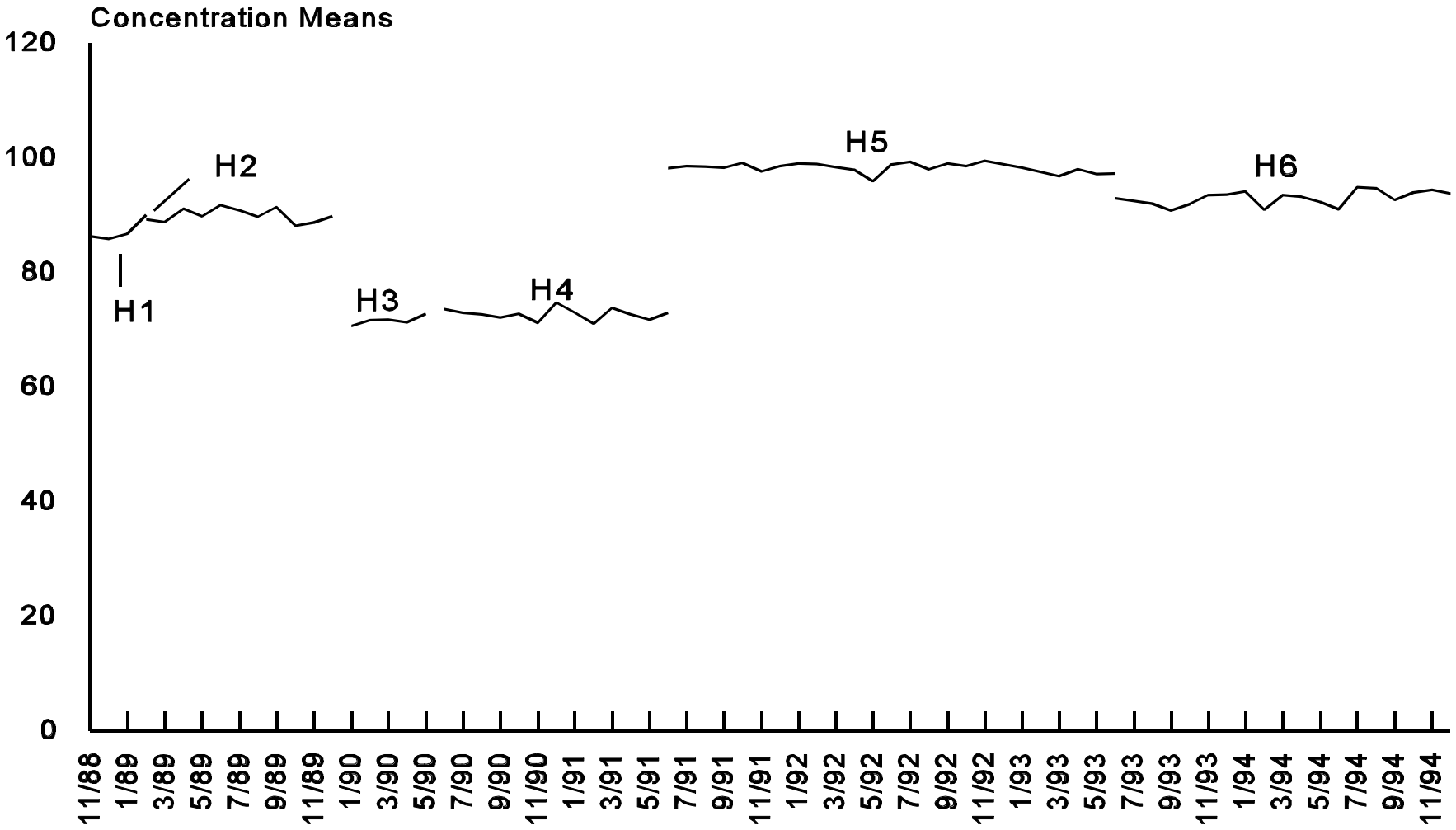
# Urinary Creatinine Monthly Means - Low/Medium Pools



SUMMARY STATISTICS FOR
URINARY CREATININE HIGH POOLS
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| H1 | 11/04/88 - 02/07/89 | 86.4615 | 2.08290 | 2.40905 | 26 |
| H2 | 02/09/89 - 12/28/89 | 89.7373 | 2.34038 | 2.60804 | 118 |
| H3 | 01/03/90 - 05/31/90 | 71.7451 | 1.45387 | 2.02643 | 51 |
| H4 | 06/06/90 - 06/03/91 | 72.7514 | 2.05437 | 2.82383 | 185 |
| H5 | 06/05/91 - 06/11/93 | 98.1824 | 2.18920 | 2.22973 | 340 |
| H6 | 06/15/93 - 12/12/94 | 93.0075 | 2.34160 | 2.51765 | 268 |

# Urinary Creatinine Monthly Means - High Pools



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Luteinizing hormone (LH) is measured by using an immunoradiometric assay (LH MAIAclone from Serono Diagnostics, Ciba-Corning, East Walpole, MA) (1-19). Samples, standards, and controls are reacted with a mixture of monoclonal antibodies to LH. An antibody labelled with $^{125}$I attaches quickly to a unique site on the molecule. A second monoclonal antibody linked to fluorescein binds at a discrete site on the LH molecule forming a sandwich. At the end of the incubation, antifluorescein coupled to a magnetic solid phase (MAIA) is added in excess, and rapidly and specifically binds to the monoclonal antibody complex, then is precipitated in a magnetic field, eliminating the need for centrifugation (1-15, 17-19).

The concentration of antigen is directly proportional to the radioactivity bound to the separation reagent. By measuring the bound fraction (separation reagent pellet) of each standard, sample, and control in a gamma counter calibrated to detect $^{125}$I, one can determine the concentration of LH in the samples and controls.

In women, LH is secreted by the pituitary gland and circulates in the blood. Its secretion and levels are under positive and negative feedback controls. The rise of estradiol prior to ovulation triggers a surge of LH secretion, which is the positive feedback. Continued high levels of estrogen and certain pituitary or hypothalamic diseases cause a decrease in LH levels. A decrease in estrogen secretion by the ovary results in high levels of LH, which is the negative feedback and is the cause for the high levels of LH in postmenopausal women.

## 2. SAFETY PRECAUTIONS

This assay employs $^{125}$I as a tracer, and all necessary radiation safety considerations for isotope management and disposal must be observed according to the radiation safety guidelines. In addition, all personnel must successfully complete the training course *Radiation Safety in the Laboratory* or demonstrate having received equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eyewear, and labcoat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines.

The Serotest quality control serum and antigen in these kits have been prepared from human serum. Consider these reagents a potential biohazard and handle them with the same precautions as applied to any serum or plasma specimen. The CDC/NIH health manual, *Biosafety in Microbiological and Biomedical Laboratories*, 1984 contains additional information on handling potentially hazardous biological materials.

LH RIA test kits contain approximately 6.0 µCi radioactive iodine ($^{125}$I) in the form of conjugated antigen or antibody. Any laboratory that uses RIA kits must hold a current NRC Certificate of Registration and meet to all of the storage, handling, and disposal requirements.

Sodium azide is used as a preservative in the Serono LH MAIAclone kit's reagents at a final concentration of not more than 0.2 g/dL. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush the drain with a large volume of water to prevent azide build-up.

Material safety data sheets (MSDS) for sodium azide, thimerosal, Tris buffer and sodium hypochlorite are available through the Environmental Health Safety Office of the University of Massachusetts Medical Center.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III Mobile Examination Center (MEC) contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" high-density (HD) floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. After the data is calculated and the final values are approved by the reviewing supervisor for release, all results are entered onto the NHANES diskettes by using the program provided by National Center for Health Statistics (NCHS).

c. After the results are entered on those diskettes, back-up copies are made and stored in locked areas.

d. The original diskettes containing analytical results are mailed to NCHS.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. No special instruction such as special diets or fasting is necessary.

b. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers. Specimens are allowed to clot at room temperature and centrifuged. Serum is transferred to 2-mL polypropylene screw-top vials and frozen at ≤-20 °C. Once a week, an overnight courier carries batches of frozen serum to the laboratory in a styrofoam-insulated shipping container with dry ice.

c. Serum specimens are stable up to 24 hours at 4-8 °C. For longer storage, store the serum at ≤-20 °C in glass or plastic vials; ensure that the vials are tightly sealed to prevent desiccation of the sample.

d. The optimal amount of serum is 0.5 mL to 2.0 mL. Specimen volumes of less than 0.4 mL are unacceptable.

e. Avoid repeated freeze-thaw cycles.

f. Specimens should generally arrive frozen.

g. Residual samples are frozen at ≤-20 °C.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) LKB - Clinigamma Counter, model 1272-001, single-well (LKB Instruments Co., Gaithersburg, MD).

(2) Eppendorf repeating pipet, 100- to 500-µL (Brinkman Instruments, Westbury, NY).

(3) Gilson Pipetman, 10- to 100-µL (Rainin Instrument Co, Woburn, MA).

(4) Vortex Genie mixer (Scientific Industries, Inc., Bohemia, NY).

(5) MAIA magnetic separator and rack (Serono Diagnostics, Ciba-Corning, Walpole and Medford, MA).

b. **Materials**

(1) Disposable 12- X 75-mm polypropylene tubes (Quality Scientific Plastics, Petaluma, CA).

(2) Milli-Q type I deionized water system (Millipore Co, Stoughton, MA).

(3) Eppendorf Combitip syringes, cat. no. P5063-29 (Brinkmann Instruments).

(4) Flask, volumetric, 100-mL, (Fisher Scientific Co, Fairlawn, NJ).

(5) Serono Diagnostics LH MAIAclone immumoradiometric assay magnetic solid-phase kit containing $^{125}$I anti-LH reagent, LH separation reagent, LH standards, LH wash buffer concentrate, and Serotest (Ciba-Corning).

(6) Serotest lyophilised quality control serum (Ciba-Corning).

c. **Reagent Preparation**

(1) $^{125}$I Anti-LH Reagent
8.1 mL per vial containing fluorescein and $^{125}$I-labelled mouse monoclonal antibodies to LH, <296 kBq (<8 µCi) per vial, in phosphate buffer containing normal sheep serum, bovine serum albumin (BSA), inert dye and 0.2 g/dL sodium azide ($NaN_3$). Ready to use. Store at 4-8 °C until the expiration date on the vial label; avoid exposure to direct sunlight.

(2) LH MAIAclone Separation Reagent
33.0 mL per vial, containing sheep antiserum to fluorescein covalently bound to magnetic particles in Tris buffer with BSA and $NaN_3$, 0.1 g/dL. Ready to use. Mix thoroughly, but gently, to ensure that the magnetic particles are in uniform suspension. Do not use a magnetic stirrer. Store at 4-8 °C until the expiration date on the vial label. *Do not freeze*.

(3) MAIAclone Wash Buffer Concentrate
12.5 mL per vial containing Tris buffer and $NaN_3$, 0.8 g/dL. Before use, dilute the contents of the vial to a total volume of 100 mL with distilled or deionized water. Mix well. Store at 4-8 °C until the expiration date on the vial label.

(4) Serotest Lyophilised Quality Control Serum
Each vial contains human serum with thimerosal, 0.01 g/dL. Reconstitute with 1.0 mL distilled or deionized water. Allow to stand at room temp for 30 min. Mix gently before use. After reconstitution, store at 4-8 °C for 2 days, or aliquot and store ≤-20 °C for up to 30 days.

d. **Standards Preparation**

(1) LH MAIAclone Standard, 0 mIU/mL
3.0 mL per vial containing bovine serum with $NaN_3$, 0.2 g/dL. Ready to use. Mix by gentle inversion prior to use. Store at 4-8 °C until the expiration date on the vial label.

(2) LH MAIAclone Calibration Standards 0.5, 2, 10, 25, 100 and 200 mIU/mL
Packaged 1.0 mL per vial and precalibrated at 0.5, 2, 10, 25, 100 and 200 mIU/mL of LH per mL (1st IRP 68/40) in bovine serum with $NaN_3$, 0.2 g/dL. Ready to use. Mix by gentle inversion prior to use. Store at 4-8 °C until the expiration date on the vial label.

e. **Preparation of Quality Control Materials**

(1) Serotest Lyophilised Quality Control Serum
Each vial contains human serum with thimerosal ($C_9H_9HgO_2SNa$), 0.01 g/dL. Reconstitute with 1.0 mL distilled or deionized water. Allow to stand at ambient temp for 30 min. Mix gently before use. After reconstitution, store at 4-8 °C for 2 days, or aliquot and store at ≤-20 °C for up to 30 days.

(2) In-house Controls With Low and High Levels of LH
Prepared in-house (University of Massachusetts Medical Center, Worcester, MA). Blood was obtained from healthy men, 21-40 years old, and the serum pooled. Aliquots of 2.0 mL were pipetted into glass vials, capped and stored at ≤-70 °C. These provide control serum with low levels of LH.

Blood was obtained from healthy postmenopausal women and the serum pooled. Aliquots of 2.0 mL were pipetted into glass vials, capped and stored ≤-70 °C. These provide control sera with high levels of LH.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve (0.5-200 mIU/mL standards)**

The LKB Gamma Counter has an automated data reduction system used to construct the LH MIAIclone calibration curve. The gamma counter automatically plots corrected counts per minute versus log concentration of standards on a spline curve run with each assay. An $^{129}$I analogue standard is present with every assay to provide an indication of machine stability. This counter has preventive maintenance every year.

b. **Verification**

(1) Standards are calibrated against World Health Organization, first International Reference Preparation (IRP) for human LH (68/40). A 1.0 mIU Serono standard equals 1.0 mIU first IRP 78/549.

(2) The luteinizing hormone assay uses an $^{129}I$ ($^{125}I$ analogue) calibration standard at the beginning of each run as a sample. The radioactive material of this standard consists of $^{129}I$ in crystalline form, dispensed as a water solution of sodium iodide into a hollow polyester resin mold. The water was then evaporated and the sodium iodide powder was sealed with more polyester to prevent the release of free iodine.

The absolute activity of the standard is calibrated by comparison with the reference standards of $^{129}I$-labelled sodium iodide supplied by the National Bureau of Standards, USA. The standard reference material number 4949 is certified to have an estimated accuracy of ±3.0%. (LKB-Wallac, Gaithersburg, MD). This standard is measured as an unknown in every run and compared with theoretical decay charts to verify instrument specificity and efficiency.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) For each assay, prepare the following groups of tubes and place in the MAIA rack:

- 1 Total Counts tubes (optional for QC)
- 1 $B_0$ tubes (zero concentration of antigen)
- 1 tubes for each standard concentration
- 2 tubes for each serum, plasma, and control sample

Allow reagents to warm to 20-25 °C and mix gently before using.

b. **Instrument Setup for the Gamma Counter**

(1) Power should be on at all times. Set parameter groups as shown in Table 1.

c. **Assay Procedure**

(1) Pipet 100 µL of zero standard into the $B_0$ tubes and 100 µL of the remaining standards, sample, or Serotest into the appropriately labelled tubes.

(2) Pipet 100 µL of $^{125}I$ anti-LH reagent into each tube. Gently vortex all the tubes, or shake the entire rack using a side-to-side motion. Seal and remove the total counts tubes.

(3) Incubate the assay for 1 hour at room temperature.

(4) Pipet 200 µL of thoroughly mixed LH MAIAclone separation reagent into each tube using a repeating pipet and gently vortex or shake the entire rack using a side-to-side motion to mix all tubes. During dispensing, the separation reagent should be swirled occasionally to ensure uniformity.

(5) Incubate without further mixing for 5 min at room temperature.

(6) Slide the rack of tubes into the MAIA magnetic separator and allow the particles to sediment magnetically for 2 min. Make sure all tubes are in contact with the surface of the separator.

(7) Decant the supernatant from all tubes in the rack by smooth inversion of the MAIA magnetic separator. Place the inverted separator on absorbent paper and tap the base of the separator firmly to dislodge any droplets of liquid adhering to the sides of the tubes.

(8) Return the separator to an upright position and add 0.5 mL of diluted wash buffer to each tube. It is not necessary to remove the rack from the separator.

(9) Thoroughly vortex each tube and replace in the rack. Adequate vortexing is essential if good precision is to

be achieved. NOTE: A multi-vortex mixer may be used if available. Remove the rack from the separator, place on the multi-vortex mixer, and mix. Slide the rack or tubes back into the MAIA magnetic separator.

Table 1
Gamma Counter Set-up for LH Assay

| | Parameter | Setting |
|---|---|---|
| | PARAMETERS | 13 |
| 01 | PROGRAM MODE | 01 |
| 02 | ID: LH MAIA | |
| 03 | TIME-S (SEC) | 0060 |
| 04 | TIME-U (SEC) | 0060 |
| 05 | IS (I 125) | 05 |
| 06 | METHOD | 06 |
| 07 | REPLICATES | 02 |
| 08 | PRINTOUTS | 3,4,5,8,12 |
| 09 | FACTOR 1 | +1.000E+02 |
| 10 | FACTOR 2 | +6.000E+01 |
| 11 | FIRST PAT. NO | 0001 |
| 12 | FIRST POS. NO | 0001 |
| 13 | FREE FRACTION | (1/0) 0 |
| 14 | MAX ERROR% | +1.000E+01 |
| 20 | POS-CODE | 0001-BLAN |
| 21 | POS-CODE | 0002-TOTA |
| 22 | POS-CODE | 0003-REFR |
| 23 | POS-CODE | 0004-0.5 |
| 24 | POS-CODE | 0005-2 |
| 25 | POS-CODE | 0006-10 |
| 26 | POS-CODE | 0007-25 |
| 27 | POS-CODE | 0008-100 |
| 28 | POS-CODE | 0009-200 |
| 29 | POS-CODE | 0010-UNKS |
| 30 | POS-CODE | 0011-UNKS |
| 31 | POS-CODE | 0000- |

(10) After vortexing the final tube, check to see that all tubes are in contact with the surface of the separator. Wait for 2 min to allow MAIA particles to sediment magnetically.

(11) Decant the supernatant from all tubes in the rack by smooth inversion of the MAIA magnetic separator. Place the inverted separator on absorbent paper and drain for 5 min. Firmly tap the tubes to dislodge any droplets of liquid adhering to the sides of the tubes.

(12) Count each tube for 60 sec in a counter calibrated to detect $^{125}I$.

(13) Look for the READY prompt. If the READY prompt is not displayed, press letter "O", press ENTER, and wait until instrument displays "READY."

(14) Put the code rack and sample racks on the right side conveyor and press "A," or put the sample racks on the conveyor without the code rack and press "A##" (where ## is the parameter group number) and enter.

(15) The first results should be printed out after the first sample has been counted.

(16) If samples have been loaded incorrectly, press letter "O," ENTER, and wait until the counter displays "READY." Go to first step. If unable to get "READY" in the first step and the counter doesn't take any commands, shut off the counter for 30 sec, turn it on again, and immediately (in less than 1 sec) press "C" and enter. The computer will reset and ask for date and time (NOTE: Day, Month, Year, Military Time). The counter has permanent memory, so parameters will not be lost if they have been saved.

(17) A standard curve must be run in each assay but not necessarily in each rack.

(18) The counting time for statistical accuracy may vary depending on counter efficiency and tracer age.

e. **Recording of Data**

(1) Quality Control Data

Serotest and other control serum results should fall within the confidence ranges established in each laboratory. Control ranges obtained at Serono are printed on the Controltest Certificate that accompanies each kit. Enter control serum results onto the NHANES diskettes using the program provided by NCHS.

(2) Analytical Results

Counts per minutes (CPM) for 100 µL of $^{125}I$ Anti-LH reagent should be approximately 150,000 when freshly iodinated anti-LH reagent is used. Counts bound in the absence of antigen are less than 750 CPM when freshly iodinated $^{125}I$ anti-LH reagent is used.

Use the "NHANES Analytical Worksheet" to record the specimen results from the laboratory notebook.

Enter both the quality control data and the analytical results on the floppy diskettes provided by the NHANES III Survey.

Use a laboratory notebook to set up batch runs. Information recorded must contain date, run number, analyte, shipper number, name of analyst, sample ID, sample volume used, and the calculated result for LH in mIU/mL.

If a sample is missing, write "NOSAX." If a sample is not satisfactory, write "UNSAX" in blank.

f. **Replacement and Periodic Maintenance of Key Components**

(1) The LKB gamma counter automatically self-calibrates to include efficiency checks and background corrections. A report is generated listing the efficiency and background measurements.

(2) As noted, the LKB gamma counter receives preventive maintenance on a yearly basis to include a thorough cleaning and lubrication, calibration, check of the electronics and mechanical devices, and replacement of defective parts, if warranted.

g. **Calculations**

(1) The LKB gamma counter has a software program as an integral component. The gamma counter automatically plots corrected CPM versus log concentration of standards on a spline curve run with each assay. The unknowns are reported as mIU/mL.

(2) The program calculates the average CPM for each pair of assay tubes. The average CPM of the blank tubes is substracted from all counts to obtain the corrected CPM. The sample concentration is obtained by interpolation of sample counts on the spline curve. If samples were run diluted, multiply results from the curve by the appropriate dilution factor to obtain final results.

h. **Special Procedure Notes**

(1) Use of the 0.5 mIU/mL standard is optional. The detection limit of the assay, defined as the concentration of LH equivalent to the mean CPM of 20 replicates of the zero standard plus 2 standard deviations is typically 0.3 mIU/mL. Use the 0.5 mIU/mL standard to ensure a better fit of the curve (lower CV) at the lower end of the curve.

(2) Gentle but complete vortexing (with the multiple-touch method) is essential for accurate test results.

(3) Thouroughly mix the magnetic antibody suspension before use to ensure a uniform suspension of magnetic particles. After pipetting into every 10 to 20 tubes, swirl the vial to resuspend particles. Do not use a magnetic stirrer to mix the LH MAIAclone separation reagent.

(4) A standard curve must be run in each assay.

(5) The counting time for statistical accuracy may vary depending on counter efficiency and tracer age. No tracer should be used past its expiration date.

## 9. REPORTABLE RANGE OF RESULTS

Reportable results are expressed as 0.15-200.00 mIU/mL.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in epidemiological health studies. The method has proven to be accurate, precise, and reliable. The primary standards used are provided by the kit manufacturer. Estimates of imprecision can be generated from results of the long-term quality control pool.

"Bench" quality control specimens are used in this analytical method. The control specimens are inserted by the analyst in each analytical run so that judgements may be made on the day of analysis. The results are assessed by running these samples through the complete analytical process. The data from these materials are used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Each assay includes control serum provided by Serono Diagnostics and in-house controls containing low levels and high levels of LH. These controls are run in duplicate and are randomly scattered throughout the assay. The in-house controls are prepared in sufficient quantity to provide serum samples for all the assays for 5 years. The pools are aliquoted into small vials and stored at $\leq$-70 °C. Vials will be removed as needed to provide controls so there will be no thawing and refreezing. Serum gonadotropin will remain stable for 10 years when stored in this fashion.

Quality control limits are established for each pool. An analysis of variance is performed for each pool after 30 or more characterization runs have been performed.

Table 2
Precision and Accuracy as Demonstrated by QC Materials Used During NHANES III

| Pool | Mean (mIU/mL) | 95% limits | 99% limits | N Runs | Total CV (%) |
|---|---|---|---|---|---|
| LH-Low | 2.87 | 2.69-3.05 | 2.74-3.01 | 36 | 13.8 |
| LH-High | 27.94 | 27.59-28.29 | 27.47-28.41 | 36 | 3.7 |

The in-house controls are used to control for drift and for interassay variations. The system is declared "out of control" if any of the following events occur:

For the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two or more pools fall either both above or both below the 95% limit.
- Two successive run means for a single pool fall either both above or both below the 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

For the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

Other analytical aspects monitored include:

- Five percent of all samples are repeated in different assays for LH.
- CPM for 100 µL of $^{125}$I Anti-LH Reagent should be approximately 150,000 when freshly iodinated Anti-LH Reagent is used.
- Counts bound in the absence of antigen (non-specific binding) are less than 750 when freshly iodinated $^{125}$I Anti-LH Reagent is used.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. Prepare new reagent and test with control serum.

b. If the system should be declared "out of control," the entire run should be repeated. If the "out of control" condition still exists, a new kit should be used and the autodiluter evaluated for pipetting precision and accuracy. Specimens for that analytical run should be reassayed after the system has been reverified to be "in control."

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

There are no known interfering substances. The highest concentration measurable without dilution is 200 mIU/mL. The lowest level is 0.15 mIU/mL.

## 13. REFERENCE RANGES (NORMAL VALUES)

Table 3
LH Normal Values

| | Mean (mIU/mL) | Range (mIU/mL) |
|---|---|---|
| Normally Menstruating Females: | | |
| Follicular Phase | 4.9 | 1.8 - 13.4 |
| Mid-cycle Peak | 35.1 | 15.6 - 78.9 |
| Luteal Phase | 3.9 | 0.7 - 19.4 |
| Postmenopausal Females | 25.8 | 10.8 - 61.4 |

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable for this procedure.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens should remain at room temperature during preparation and testing. Otherwise, the serum is stored at ≤-20 °C.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods for performing this test for NHANES III. In case of system failure, store all specimens at ≤-20 °C until the system is functioning. Serum samples are indefinitely stable at ≤-70 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable for this procedure.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

We recommend that records (including related QA/QC data) be maintained for 7 years beyond the duration of the survey. Only numerical identifiers (e.g. NCHS ID numbers) should be used.

For the NHANES III study, residual samples are stored at ≤-20 °C for 1 year after analysis, then returned to the NCHS serum repository at Rockville, MD.

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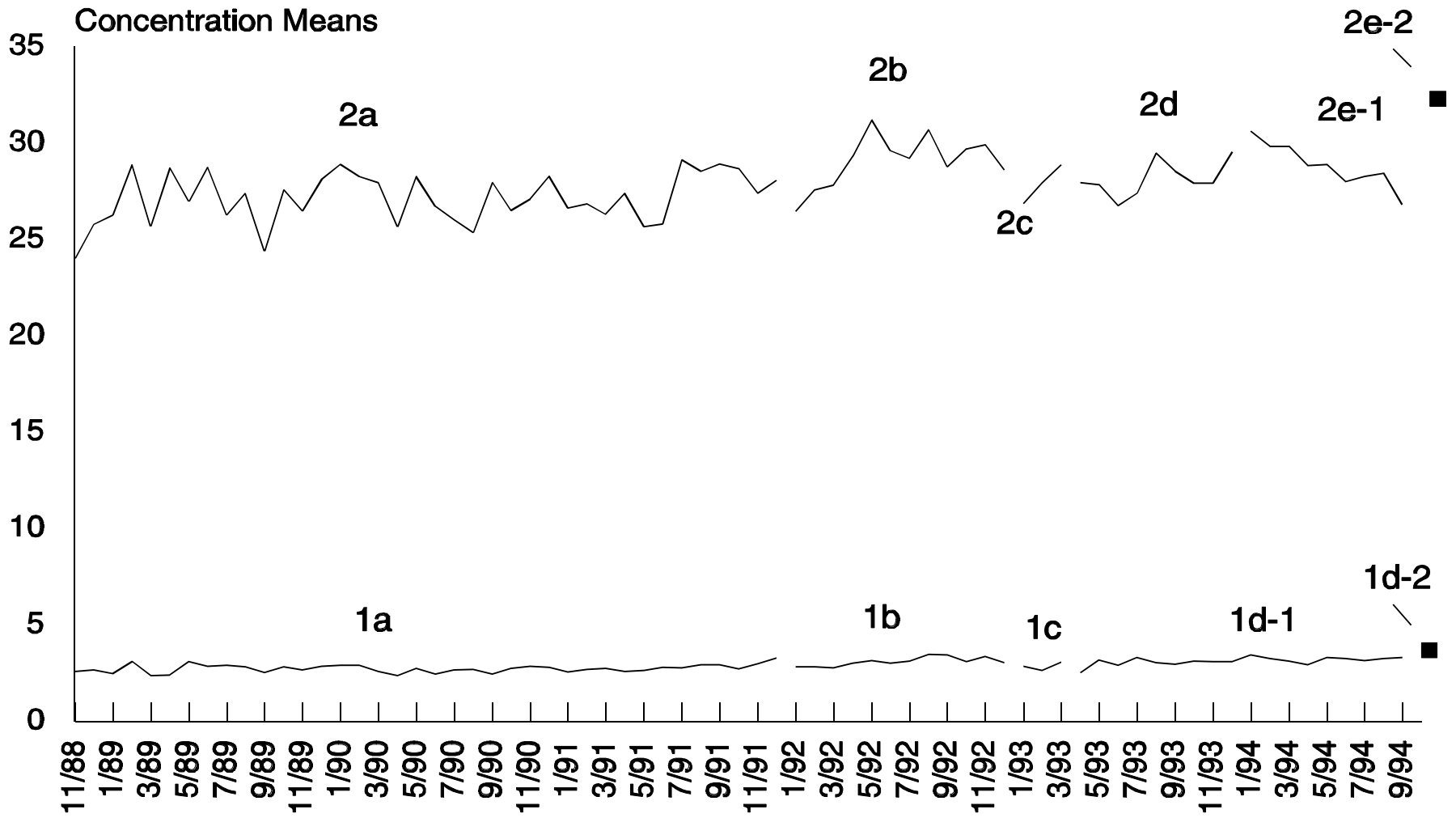
SUMMARY STATISTICS FOR
LH
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 1a | 11/03/88 - 12/17/91 | 2.7438 | 0.40203 | 14.6521 | 429 |
| 1b | 01/16/92 - 12/22/92 | 3.1042 | 0.37125 | 11.9594 | 128 |
| 1c | 01/14/93 - 03/30/93 | 2.8620 | 0.36973 | 12.9185 | 30 |
| 1d-1 | 04/02/93 - 09/30/94 | 3.1454 | 0.28824 | 9.1637 | 222 |
| 1d-2 | 10/01/94 - 10/20/94 | 3.7167 | 0.14720 | 3.9604 | 6 |
| 2a | 11/03/88 - 12/17/91 | 27.1699 | 1.80667 | 6.6495 | 419 |
| 2b | 01/16/92 - 12/22/92 | 28.9947 | 1.61738 | 5.5782 | 81 |
| 2c | 01/14/93 - 03/30/93 | 27.9182 | 0.96739 | 3.4651 | 22 |
| 2d | 04/02/93 - 12/29/93 | 28.1750 | 1.59367 | 5.6563 | 96 |
| 2e-1 | 01/21/94 - 09/30/94 | 28.7594 | 1.57122 | 5.4633 | 106 |
| 2e-2 | 10/01/94 - 10/20/94 | 32.0000 | 0.56921 | 1.7788 | 6 |

# LH Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Follicle stimulating hormone (FSH) is measured using an immunoradiometric assay obtained as a kit (FSH MAIAclone, Serono Diagnostics, Ciba-Corning, East Walpole, MA) (1). Samples, standards and controls undergo reaction with a mixture of monoclonal antibodies to FSH. An antibody labelled with $^{125}$I attaches quickly to a unique site on the molecule. A second monoclonal antibody linked to fluorescein binds at a discrete site on the FSH molecule, forming a sandwich (2).

At the end of the incubation, antifluorescein coupled to a magnetic solid phase (MAIA) is added in excess. This mixture rapidly and specifically binds to the monoclonal antibody complex and forms sediment in a magnetic field, eliminating the need for centrifugation.

The concentration of antigen is directly proportional to the radioactivity bound to the separation reagent. By measuring the bound fraction (separation reagent pellet) of each standard, sample, and control in a gamma counter calibrated to detect $^{125}$I, researchers can determine the concentration of FSH in the samples and controls (1-4).

In women, FSH is secreted by the pituitary and circulates in the blood (3,4). Its secretion and levels are under negative feedback control (5,6). Continued high levels of estrogen or inhibin cause a decrease in FSH as do certain pituitary or hypothalamic diseases (7,8). A decrease in estrogen secretion by the ovary results in high levels of FSH. This is negative feedback and is the cause for the high levels of FSH in postmenopausal women (9).

## 2. SAFETY PRECAUTIONS

Because this assay employs $^{125}$I as a tracer, observe all necessary radiation safety considerations for isotope management and disposal according to the guidelines of radiation safety. In addition, all personnel must successfully complete a training course, *Radiation Safety in the Laboratory*, or demonstrate equivalent instruction. Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eye wear, and lab coat during all steps of this method because of both infectious and radioactive contamination hazards. Place all plastic and glassware that contacts serum other than that which is contaminated by the radioactive tracer in a plastic autoclave bag for disposal. Dispose of all radioactive waste and contaminated material according to radiation safety guidelines.

FSH radioimmunoassay (RIA) test kits contain <30 microcuries (µCi) of radioactive iodine ($^{125}$I) in the form of conjugated antigen or antibody. Any laboratory using RIA kits must hold a current NRC Certificate of Registration and conform to all of the storage, handling and disposal requirements set by the NRC.

The Serotest controls and standards in these kits have been prepared from human serum. Consider these reagents a potential biohazard and handle them with the same precautions as you would any serum or plasma specimen. The CDC/NIH health manual, *Biosafety in Microbiological and Biomedical Laboratories, 1984* contains additional information on good laboratory practices for handling biological materials.

Sodium azide is used in the Serono FSH MAIAclone kit reagents at a final concentration of not more than 0.2 g/dL. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. After disposing of reagents containing sodium azide, flush the drain with a large volume of water to prevent azide buildup.

Material safety data sheets (MSDS) for sodium azide, Tris buffer, thimerosal, and sodium hypochlorite are available through the Environmental Health Safety Office of the University of Massachusetts Medical Center.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" high density (HD) floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.

b. After the data is calculated and the final values are approved by the reviewing supervisor for release, all results are entered onto the NHANES diskettes by using the program provided by National Center for Health Statistics (NCHS).

c. After the results are entered on diskettes, back-up copies are made and stored in locked areas.

d. The original diskette containing analytical results is mailed to NCHS.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. No special instruction such as special diets or fasting is necessary.

b. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers. Specimens are allowed to clot at room temp and centrifuged. Serum is transferred to 2-mL polypropylene screw-top vials and frozen at ≤-20 °C. Each week, batches of frozen serum samples are placed in a styrofoam-insulated shipping container with dry ice and sent to the laboratory by an overnight courier.

c. Serum specimens are stable up to 24 hours at 4-8 °C. For longer periods, store the serum at ≤-20 °C in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

d. The optimal amount of serum is 0.5 mL to 2.0 mL. Specimen volumes of less than 0.4 mL are unacceptable.

e. Avoid repeated freeze-thaw cycles, which may compromise specimen integrity.

f. Specimens should generally arrive frozen.

g. Residual samples are frozen at ≤-20 °C.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) LKB - Clinigamma counter, model 1272-001, single-well (LKB Instruments Co., Gaithersburg, MD).

(2) Eppendorf repeating pipet, 100- to 500-µL (Brinkman Instruments, Westberg, NY).

(3) Gilson Pipetman, 10- to 100-µL (Rainin Instrument Co, Woburn, MA).

(4) Vortex Genie mixer (Scientific Industries, Inc., Bohemia, NY).

(5) MAIA magnetic separator and rack (Serono Diagnostics, Ciba-Corning, Walpole and Medford, MA).

b. **Other Materials**

(1) Serono Diagnostics FSH MAIAclone immunoradiometric assay magnetic solid phase kit containing $^{125}$I anti-FSH reagent, FSH separation reagent, FSH standards, FSH wash buffer concentrate, and Serotest (Ciba-Corning, East Walpole, MA).

(2) Serotest Lyophilized Quality Controls (Serono Diagnostics, Ciba-Corning).

(3) Disposable 12- X 75-mm polypropylene tubes (Quality Scientific Plastics, Pataluma, CA).

(4) Milli-Q type I deionized water system (Millipore Co., Stoughton, MA).

(5) Eppendorf Combitip syringes, cat. no. P5063-29 (Brinkmann Instruments, Westbury, NY).

(6) Flask, volumetric, 100-mL, (Fisher Scientific Co, Fairlawn, NJ).

c. **Reagent Preparation**

Each Serono FSH MAIAclone kit contains reagents for assaying 150 tubes. Mix the reagent contents gently to avoid foaming. Reagents are stable until the expiration date, which is printed on the label. The recommended storage temperature for all reagents is 4-8 °C.

(1) $^{125}$I Anti-FSH Reagent
5.4 mL per vial, containing fluorescein and $^{125}$I-labelled mouse monoclonal antibodies to FSH, <370 kBq (<10 µCi) per vial, in phosphate buffer containing normal sheep serum, bovine serum albumin (BSA), inert dye, and sodium azide ($NaN_3$) 0.2 g/dL. Ready to use. Store at 4-8 °C until the expiration date on the vial label. Avoid exposure to direct sunlight.

(2) FSH MAIAclone Separation Reagent
33.0 mL per vial containing sheep antiserum to fluorescein covalently bound to magnetic particles in Tris buffer with BSA and $NaN_3$, 0.1 g/dL. Ready to use. Mix thoroughly, but gently, to ensure that the magnetic particles are in uniform suspension. Do not use a magnetic stirrer. Store at 4-8 °C until the expiration date on the vial label. Do not freeze.

(3) MAIAclone Wash Buffer Concentrate
12.5 mL per vial containing Tris buffer and $NaN_3$, 0.8 g/dL. Before use, dilute the contents of the vial to a total volume of 100 mL with distilled or deionized water. Mix well. Store at 4-8 °C until the expiration date on the vial label.

d. **Standards Preparation**

(1) FSH MAIAclone Standard, 0 mIU/mL
3.0 mL per vial containing bovine serum with $NaN_3$, 0.2 g/dL. Ready to use. Mix by gentle inversion prior to use. Store at 4-8 °C until the expiration date on the vial label.

(2) Calibration Standards

FSH MAIAclone Standards - 0.5, 2, 10, 25, 75 and 150 mIU/mL
Standards come packaged in 1.0 mL volumes and precalibrated at 0.5, 2, 10, 25, 75 and 150 mIU/mL of FSH per mL (2nd IRP 78/549) in bovine serum with $NaN_3$, 0.2 g/dL. Ready to use. Mix by gentle inversion prior to use. Store at 4-8 °C until the expiration date on the vial label.

e. **Preparation of Quality Control Materials**

(1) Serotest Lyophilized Quality Controls
Each vial contains human serum with thimerosal ($C_9H_9HgO_2SNa$), 0.01 g/dL. Reconstitute with 1.0 mL distilled or deionized water. Allow to stand at 20-25 $^0$C for 30 min. Mix gently before use. After reconstitution, store at 4-8 °C for 2 days, or aliquot and store ≤-20 °C for up to 30 days.

(2) In-house Controls with Low and High Levels of FSH
Prepared in-house (University of Massachusetts Medical Center, Worcester, MA). Blood was obtained from healthy men, 21-40 years old, and the serum was pooled. Two-mL aliquots were pipetted into glass vials, capped, and stored at ≤-70 °C. These pools provide control serum with low levels of FSH.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve (0.5-150 mIU/mL standards)**

The LKB Gamma Counter has an automated data reduction system used to construct the FSH MIAIclone calibration curve. The gamma counter automatically plots corrected counts per minute versus the log concentration of standards on a spline curve run with each assay. An $^{129}$I standard is present with every assay to provide an indication of instrument stability.

b. **Verification**

(1) Standards of 0.5, 2, 10, 25, 75 and 150 mIU/mL
Calibrated against World Health Organization, second International Reference Preparation of (IRP) for human FSH (2nd IRP 78/549). A 1.0 mIU Serono standard equals 1.0 mIU of 2nd IRP 78/549.

(2) $^{125}$I Analogue ($^{129}$I) Calibration Standard
The radioactive material of this standard consists of $^{129}$I in crystalline form, dispensed as a water solution of sodium iodide into a hollow polyester resin mould. The water is then evaporated and the sodium powder sealed with more polyester to prevent the release of free iodine.

The absolute activity of the standard is calibrated by comparison with the reference standards of $^{129}$I-labelled sodium iodide supplied by the U.S. National Institute of Standards and Technology. The standard reference material number 4949 is certified to have an estimated accuracy of ±3.0%. This standard is measured as an unknown in every run and the results compared with values on theoretical decay charts to verify instrument specificity and efficiency.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Allow the frozen samples, Serono control, and two in-house controls to reach 20-25 $^{0}$C. Invert gently to mix.

(2) Allow the standards, antibody, separation reagent, and wash buffer concentrate to reach 20-25 $^{0}$C.

(3) For each assay, prepare the following groups of 12- x 75-mm polypropylene tubes and place them in the MAIA rack:

- 2 total counts tubes
- 2 $B_0$ tubes (zero concentration of antigen);
- 2 tubes for each standard concentration;
- 2 tubes each for a serum or plasma, and control sample.

(4) The testing sequence: total counts (TC), standards, one set of controls, specimens, second set of controls.

b. **Sample Preparation**

(1) First analyze all samples undiluted.

(2) Load samples in racks according to the protocol.

c. **Instrument Setup of Gamma Counter**

Power should be on at all times. Set parameters as shown in Table 1.

d. **Operation of Assay Procedure**

(1) Pipette 100 µL of zero standard into the $B_0$ tubes and 100 µL of the remaining standards, sample, or Serotest into the appropriately labelled tubes.

(2) Pipette 100 µL of $^{125}$I anti-FSH reagent into each tube. Gently vortex all the tubes, or shake the entire rack using a side-to-side motion. Seal and remove the total counts tubes.

(3) Incubate the assay for 1 hour at 20-25 °C.

(4) Pipette 200 µL of thoroughly mixed FSH MAIAclone separation reagent into each tube using the repeating pipet. Gently vortex or shake the entire rack using a side-to-side motion to mix all tubes. During dispensing, the separation reagent should be swirled occasionally to ensure uniformity.

(5) Incubate without further mixing for 5 min at 20-25 °C.

Table 1
Gamma Counter Parameters Protocol

| | Parameter | Setting |
|---|---|---|
| | PARAMETERS | 14 |
| 01 | PROGRAM MODE | 01 |
| 02 | ID: FSH MAIA | |
| 03 | TIME-S (SEC) | 0060 |
| 04 | TIME-U (SEC) | 0060 |
| 05 | IS (I 125) | 05 |
| 06 | METHOD | 06 |
| 07 | REPLICATES | 02 |
| 08 | PRINTOUTS | 3,4,5,8,12 |
| 09 | FACTOR 1 | +1.000E+02 |
| 10 | FACTOR 2 | +6.000E+01 |
| 11 | FIRST PAT. NO. | 0001 |
| 12 | FIRST POS. NO. | 0001 |
| 13 | FREE FRACTION | (1/0) 0 |
| 14 | MAX ERROR% | +1.000E+01 |
| 20 | POS-CODE | 0001-BLAN |
| 21 | POS-CODE | 0002-TOTA |
| 22 | POS-CODE | 0003-REFR |
| 23 | POS-CODE | 0004-0.5 |
| 24 | POS-CODE | 0005-2 |
| 25 | POS-CODE | 0006-10 |
| 26 | POS-CODE | 0007-25 |
| 27 | POS-CODE | 0008-75 |
| 28 | POS-CODE | 0009-150 |
| 29 | POS-CODE | 0010-UNKS |
| 30 | POS-CODE | 0000- |

(6) Slide the rack of tubes into the MAIA magnetic separator and allow the particles to sediment magnetically for 2 min. Make sure all tubes are in contact with the surface of the separator.

(7) Decant the supernatant from all tubes in the rack by smoothly inverting of the MAIA magnetic separator. Place the inverted separator on absorbent paper and tap the base of the separator firmly to dislodge any droplets of liquid adhering to the sides of the tubes.

(8) Return the separator to an upright position and add 0.5 mL of diluted wash buffer to each tube. It is not necessary to remove the rack from the separator.

(9) Thoroughly vortex each tube and replace each in the rack. Adequate vortexing is essential if good precision is to be achieved. Note: A multivortex mixer may be used if available. Remove the rack from the separator, place on the multi-vortex mixer, and mix. Slide the rack or tubes back into the MAIA magnetic separator.

(10) After vortexing the final tube, check to see that all tubes are in contact with the surface of the separator. Wait for 2 min to allow MAIA particles to sediment magnetically.

(11) Decant the supernatant from all tubes in the rack by smoothly inverting the MAIA magnetic separator. Place the inverted separator on absorbent paper and drain for 5 min. Firmly tap the tubes to dislodge any droplets of liquid adhering to the sides of the tubes.

(12) Count each tube for 60 sec in a counter calibrated to detect $^{125}$I.

(13) Look for a READY prompt. If READY prompt is missing, press letter "O." Press ENTER and wait until instrument displays READY.

(14) Put code rack and sample racks on the right side conveyor and press "A," or alternatively, put the sample racks on the conveyor without the code rack and press A## (where ## is the parameter group number) and enter.

(15) The first results should be printed out after the first sample has been counted.

(16) If the samples were incorrectly loaded, press letter "O," enter and wait until the counter displays READY. Go to first step. If unable to get READY in the first step and the counter doesn't take any commands, shut off the counter for 30 sec, put it back on and immediately (in less than 1 sec) press "C" and enter. The computer will reset and ask for date and time(NOTE: Day, Month, Year, Military Time). The counter has permanent memory so parameters will not be lost, assuming they have been saved.

(17) Run a standard curve in each assay.

(18) Although 60 sec is the normal counting time, counting time for statistical accuracy may vary depending on the counter's efficiency and the tracer's age. Do not use the tracer past its expiration date.

e. **Recording of Data**

(1) Quality Control Data

Serotest and other control serum test results should fall within the confidence ranges established in each laboratory. Control ranges obtained at Serono are printed on the Controltest Certificate that accompanies each kit. Results of the control serum tests are entered into the diskettes using the program provided by NCHS.

(2) Analytical Results

Results for 100 µL of $^{125}$I Anti-FSH reagent should be approximately 265,000 counts per minute (CPM) when freshly iodinated anti-FSH reagent is used. In the absence of antigen (or nonspecific binding) results should be approximately 900-1,700 CPM when freshly iodinated $^{125}$I anti-FSH reagent is used.

Use the "NHANES Analytical Worksheet" to record the specimen results from the laboratory notebook.

Enter both the QC data and the analytical results on the floppy diskettes provided by the NHANES III Survey.

Use a laboratory notebook to set up batch runs. Information recorded must contain the date, run number, analyte, shipper number, name of the analyst, sample ID, sample volume used, and calculated result for FSH in mIU/mL.

If a sample is missing, write "NOSAX" in blank. If a sample is not satisfactory, write "UNSAX" in the blank.

f. **Replacement and Periodic Maintenance of Key Components**

(1) The LKB Gamma Counter receives preventive maintenance on a yearly basis including a thorough cleaning and lubrication, calibration, check of the electronics and mechanical devices, and replacement of defective parts, if warranted. An $^{129}I$ standard is present with every assay to provide an indication of instrument stability.

(2) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) The LKB Gamma Counter has a software program as an integral component. The gamma counter automatically plots CPM versus the log concentration of standards on a spline curve run with each assay. The unknowns are reported as mIU/mL.

(2) The program calculates the average CPM for each pair of assay tubes. The average CPM of the blank tubes is subtracted from all counts to obtain the corrected CPM. The sample concentration is obtained by interpolation of sample counts on the spline curve. If diluted samples were run, multiply results from the curve by the appropriate dilution factor to obtain final results.

h. **Special Procedure Notes**

(1) The use of the 0.5 mIU/mL standard is optional. The detection limit of the assay, defined as the concentration of LH equivalent to the mean CPM of 20 replicates of the zero standard plus 2 standard deviations is typically 0.3 mIU/mL. Use the 0.5 mIU/mL standard to ensure a better fit of the curve (lower CV) at the lower end of the curve.

(2) Gentle but complete vortexing (use of the multiple touch method) is essential for accurate test results.

(3) The magnetic antibody suspension must be thoroughly mixed before use to ensure a uniform suspension of magnetic particles. Mix the suspension after dispensing each 10-20 tubes.

(4) *Do not use a magnetic stirrer to mix the FSH MAIAclone Separation Reagent.*

(5) A standard curve must be run in each assay.

(6) The counting time for statistical accuracy may vary depending on counter's efficiency and tracer's age. No tracer should be used past its expiration date.

## 9. REPORTABLE RANGE OF RESULTS

Reportable results are expressed as 0.15-150 mIU/mL.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this protocol has been used for several years in epidemiological health studies. The method has proven to be accurate, precise, and reliable. The primary standards used in this assay are provided by the kit manufacturer. Estimates of imprecision can be generated from long-term QC pool results.

"Bench" QC specimens are used in this analytical method. The control specimens are inserted by the analyst in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. The results are assessed by running these samples through the complete analytical process. The data from these materials are used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Each assay includes control serum provided by Serono Diagnostics and in-house controls containing low levels and high levels of FSH. These controls which are run in duplicate, are randomly scattered throughout the assay. The in-house controls are prepared in sufficient quantity to provide serum samples for all the assays for 5 years. The pools are aliquoted into small vials and stored at $\leq$-70 °C. Vials will be removed as needed to provide controls so there will be no

thawing and refreezing. Serum gonadotropin will remain stable for 10 years when stored in this fashion (9).

QC limits are established for each pool. An analysis of variance is performed for each pool after 30 or more characterization runs have been performed.

a. The precision and accuracy as demonstrated by QC materials used during this survey are shown in Table 2.

Table 2
Precision and Accuracy

| Pool | Mean (mIU/mL) | 95% limits | 99% limits | No. of Runs | Total CV |
|---|---|---|---|---|---|
| FSH-Low | 5.64 | 5.47 -5.81 | 5.41-5.87 | 36 | 8.9% |
| FSH-High | 80.8 | 79.97-81.65 | 79.68-81.94 | 36 | 3.1% |

b. The in-house controls are used to control for drift and for interassay variations. The system is declared "out-of-control" if any of the following events occur:

(1) On the Means Chart:

- A single run mean for one or more pools falls outside the upper or lower 99% limit.
- The run means for two or more pools fall either both above or both below the 95% limit.
- Two successive run means for a single pool fall either both above or both below the 95% limit.
- Eight successive run means for a single pool fall either all above or all below the center line, establishing a trend.

(2) On the Range Chart:

- A single within-run range falls above the upper 99% limit.
- The within-run ranges for two or more pools fall above the upper 95% limit.
- Two successive within-run ranges for a single pool fall above the upper 95% limit.
- Eight successive within-run ranges for a single pool fall above the center line.

c. Other analytical aspects monitored:

- 5% of all samples are repeated in different assays for FSH.
- The CPM for the freshly iodinated $^{125}$I anti-FSH reagent should be in the range of 265,000 CPM, falling off slightly as the reagent ages.
- The nonspecific binding tube should have 900-1700 CPM with fresh $^{125}$I anti-FSH reagent.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. New reagent will be prepared and tested with control serum.

b. If the system is declared "out of control," the entire run is repeated. If the "out of control" condition still exists, a new kit should be used and the autodiluter evaluated for pipetting precision and accuracy. Specimens for that analytical run should be reassayed after the system has been reverified to be "in control."

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

There are no known interfering substances. The highest concentration measurable without dilution is 150 mIU/mL. The lowest level is 0.3 mIU/mL. Imprecision in the procedure may be caused by intermittently imprecise micropipettors.

**13. REFERENCE RANGES (NORMAL VALUES)** (15,16)

Table 3
Reference Ranges

| | Mean (mIU/mL) | Range (mIU/mL) |
|---|---|---|
| Normally Menstruating Women | | |
| Follicular Phase | 6.4 | 3 - 12 |
| Mid-cycle Peak | 12.8 | 8 - 22 |
| Luteal Phase | 4.3 | 2 - 12 |
| Postmenopausal Women | 72.4 | 35 - 151 |

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable to this procedure.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Keep the specimens at 20-25 °C during preparation and testing. Otherwise, store the serum at ≤-20 °C.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no acceptable alternative methods for performing this test for NHANES III. In case of system failure, store all specimens at ≤-20 °C until the system is functioning. For long term storage, freeze samples at ≤-70 °C. Serum samples are indefinitely stable at these temperatures.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable for this procedure.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

We recommend that records, including related QA/QC data, be retained for 7 years beyond the duration of the survey. Only numerical identifiers (e.g., NCHS ID numbers) should be used.

For the NHANES III study, residual samples are stored at ≤-20 °C for 1 year after analysis, then returned to the NCHS serum repository at Rockville, MD.

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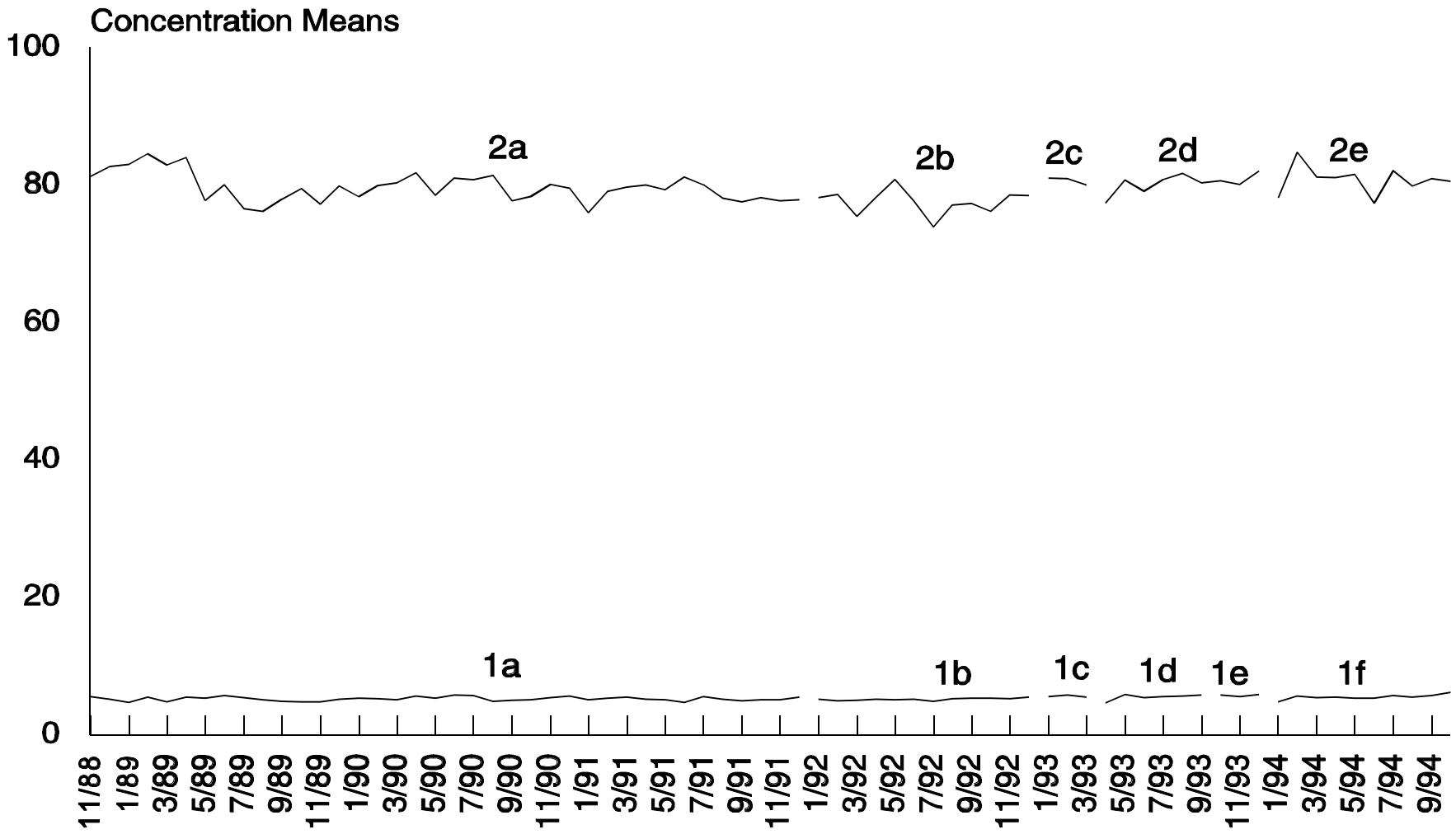
SUMMARY STATISTICS FOR
FSH
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 1-A | 11/03/88 - 12/17/91 | 5.2591 | 0.66540 | 12.6524 | 425 |
| 1-B | 01/16/92 - 12/22/92 | 5.2182 | 0.38368 | 7.3526 | 130 |
| 1-C | 01/14/93 - 03/30/93 | 5.6780 | 0.41776 | 7.3576 | 30 |
| 1-D | 04/02/93 - 09/28/93 | 5.6222 | 0.51834 | 9.2195 | 80 |
| 1-E | 10/12/93 - 12/29/93 | 5.7682 | 0.31385 | 5.4410 | 44 |
| 1-F | 01/21/94 - 10/20/94 | 5.5596 | 0.40696 | 7.3198 | 114 |
| 2-A | 11/03/88 - 12/17/91 | 79.5375 | 3.21633 | 4.0438 | 413 |
| 2-B | 01/16/92 - 12/22/92 | 77.6189 | 2.70784 | 3.4886 | 95 |
| 2-C | 01/14/93 - 03/30/93 | 80.6080 | 2.68823 | 3.3349 | 25 |
| 2-D | 04/02/93 - 12/29/93 | 80.5439 | 2.58465 | 3.2090 | 107 |
| 2-E | 01/21/94 - 10/20/94 | 81.0637 | 3.23538 | 3.9912 | 113 |

# FSH Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Urinary iodine is measured by using the reduction-oxidation reaction between ceric and arsenite catalyzed by iodide. Therefore, the iodine concentration is proportional to its catalytic activity. Samples of urine, controls, and iodate standard are first digested with chloric acid, then measured spectrometrically at 420 nm with a Technicon AutoAnalyzer. Calculations are based on an iodine standard curve. The final concentration of urinary iodine is expressed in micrograms per deciliter and is easily converted to micrograms iodine per gram urine creatinine. Iodine contamination must be carefully avoided throughout all procedures. All digestions are performed in a perchloric acid fume hood (1-3).

Urinary iodine provides a marker for dietary iodine intake. This assay was used to determine the relationship of urinary iodine excretion to thyroid function.

## 2. SPECIAL SAFETY PRECAUTIONS

Wear gloves, lab coat and safety glasses while performing the analysis. Place disposable plastic, glass, and pipet tips contaminated with urine in a biological autoclave bag and autoclave. When work is completed, wipe all work surfaces with 10% sodium hypochlorite solution.

Dispose of all biological samples and diluted specimens in a biological autoclave bag at the end of the analysis.

*Special care should be taken when handling and dispensing concentrated sulfuric acid. Always remember to add acid to water. Sulfuric acid is a caustic chemical capable of causing severe eye and skin damage. Wear metal-free gloves, lab coat and safety glasses. If the sulfuric acid comes in contact with any part of the body, quickly wash with copious quantities of water for at least 15 min.*

*Chloric acid digestions should be performed in a perchloric acid fume hood. When the digestions are completed, use the water wash-down system provided with the hood. Wash down the hood for 15 min in order to remove perchlorate salts. Buildup of these salts could create conditions under which an explosion could occur. Do not mix acids with solvents or allow any evaporation during the digestion process.*

Material safety data sheets (MSDSs) for chloric acid, arsenious acid, sulfuric acid, ceric ammonium sulfate, sodium chromate, potassium iodate, perchloric acid, arsenic trioxide, potassium chlorate, sodium chloride, and sodium hypochlorite are available through the Environmental Health Safety Office of the University of Massachusetts Medical Center.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. After the data are calculated and the final values are approved by the reviewing supervisor for release, all results are entered onto the NHANES disks by using the program provided by National Center for Health Statistics (NCHS).

b. Back-up copies are made made from the NHANES disks and stored in locked areas.

c. The NHANES disks containing analytical results are mailed to NCHS.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Specimens are from a random collection of urine. A 24-hour collection may also be used.

c. The optimal amount of specimen is 5 to 10 mL. Specimens <1.0 mL are unacceptable.

d. Random urine specimens are collected with sterile collection cups. Specimens are transferred to 15-mL Falcon screw-top vials and frozen at ≤-20 °C. Frozen urine samples are sent in batches in styrofoam-insulated shipping containers with dry ice to the laboratory weekly via an overnight courier.

e. Specimen stability has been demonstrated at both 5 °C and ≤-20 °C.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Technicon "AutoAnalyzer" continuous-flow analyzer (Technicon Instrument, Inc., Tarrytown, NY).
- Sampler, model 1
- Proportioning pump, model 1
- Colorimeter, model 1, and 420-nm filters
- Technicon recorder, model (TC) T1-1PH5X560-51-T24-T132X
- 10-mm flowcell
- 70-ft glass coil

2) Temperature-controlled water bath (P.M., Tamson, N.V., Holland).

3) Sartorius analytical balance, model 1801 (Sartorius Corporation, Edgewood, NY).

b. **Other Materials**

(1) Perchloric acid, $HCl0_4$ (70%-72%), ACS grade (Fisher Scientific Co., Fairlawn, NJ).

(2) Sulfuric acid ($H_2SO_4$), ACS grade (Fisher).

(3) Arsenic trioxide ($AS_2O_3$), ACS grade (Fisher).

(4) Sodium chromate ($Na_2CrO_4$), ACS grade (Fisher).

(5) Potassium chlorate ($KCl0_3$), purified (J.T. Baker Co., Phillipsburg, NJ).

(6) Ceric ammonium sulfate ($(NH_4)_4Co(SO_4)_4 \bullet 2H_2O$) (G. Frederick Smith Chemical Co., Columbus, OH).

(7) Sodium chloride (NaCl), ACS grade (Fisher).

(8) Potassium iodate ($KIO_3$), ACS grade (Sigma Chemical Co., St. Louis, MO).

(9) Ultrapure deionized water (Millipore Corporation, Bedford, MA).

(10) 40-mL Pyrex heavy-duty centrifuge tubes (Fisher).

(11) 2.0-mL disposable conical-bottom polystyrene sample cup for AutoAnalyzer system, cat. no. 02-544-19 (Fisher).

(12) Manifold pump tubing for AutoAnalyzer system, cat. nos. 14-190-109, 14-190-110, 14-190-111, 14-190-122 (Fisher).

(13) PVC transmission tubing, cat. no. 116-0528-010 (Fisher).

(14) Glass mixing coils, cat. no. 105-0082-000 (Fisher).

(15) H Connectors, cat. no. 116-0203-000 (Fisher).

(16) Cactus glass fittings and connectors, cat. no. 116-0211-000 (Fisher).

(17) Perforated linear chart paper, cat. no. 13-940-9507 (Fisher).

(18) Epoxy-coated test tube racks, cat. no. 14-793-2 (Fisher).

(19) Hot plate equipped with a special superstructure and sand-bath heaters adapted at the laboratory (University of Massachusetts, Worcester, MA).

(20) Beach sand for sand-bath heaters (Fisher).

(21) Whatman no. 1 filter paper (Fisher).

(22) Codex linear graph paper (Codex Book Co., Norwood, MA).

(23) Colorimeter lamps (Fisher).

(24) 1-L polyethylene bottles (Fisher).

c. **Reagent Preparation**

(1) Chloric acid ($HCl0_3$)
28% (vol/vol) (for digestion). In a 3-L Florence flask, dissolve, by heating, 500 g of potassium chlorate ($KCl0_3$) in 910 mL of distilled water. Turn off heat and remove flask. Then, with constant stirring add, use a separatory funnel to add 375 mL of perchloric acid ($HCl0_4$) at a rate of approximately 15 mL/min. When the solution cools to ambient temperature (20-25 °C), place the flask in a freezer (-10 °C) overnight. The following day, filter the chloric acid by using a Buchner funnel with a Whatman #1 filter paper. Add 1 mL of a 33.3 g/dL sodium chromate solution to 1 L of chloric acid. Prepare weekly; store at 5 °C in 1-L polyethylene bottles.

> *CAUTION: Prepare this acid in a perchloric acid fume hood. Use the water wash-down system provided with the hood. Wash down hood for 15 min in order to remove perchlorate salts. Buildup of these salts could create conditions under which an explosion could occur.*

(2) Arsenious acid
Dissolve, by heating, 10 g of arsenic trioxide ($A_{s2}O_3$), 50 g of sodium chloride (NaCl), 400 mL of 5 N sulfuric acid ($H_2SO_4$) and approximately 1 L of distilled water in a 2-L Florence flask. When the solution cools to 20-25 °C, dilute to 2 L with distilled water. Prepare every 3-4 weeks; store at 20-25 °C in an amber bottle.

(3) 5 N Sulfuric acid
Prepare in an ice-water bath (5 °C). Slowly add 280 mL of concentrated sulfuric acid ($H_2SO_4$) into a 2-L Florence flask containing 1 L of distilled water. Dilute to 2 L when the solution is at 20-25 °C. Prepare monthly; store at 20-25 °C in an amber bottle.

(4) 2.5 N Sulfuric acid
Dilute 1 part 5 N sulfuric acid ($H_2SO_4$) with an equal volume of distilled water. Prepare monthly.

(5) Ceric ammonium sulfate
Dissolve 10 g of ceric ammonium sulfate ($(NH_4)_4Co(SO_4)_4 \cdot 2H_2O$) with 2.5 N sulfuric acid ($H_2SO_4$) in a 1-L volumetric flask. Dilute to volume. Prepare weekly; store at 20-25 °C in an amber bottle.

(6) "Screening" ceric ammonium sulfate solution
Dissolve 16 g of ceric ammonium sulfate ($(NH_4)_4Co(SO_4)_4 \cdot 2H_2O$) with 2.5 N sulfuric acid ($H_2SO_4$) in a 1-L volumetric flask. Dilute to volume. Prepare monthly; store at 20-25 °C in an amber bottle.

(7) Sodium Chromate (33.3 g/dL)
Dissolve 33.3 g of sodium chromate ($Na_2CrO_4$) with distilled water in a 100-mL volumetric flask. Dilute to volume. Prepare weekly; store at 20-25 °C in an amber bottle.

d. **Standards Preparation**

Potassium Iodate Standards ($KIO_3$)
Prepare the stock standard yearly. Working standard solutions are also stable for 1 year.

(1) 1000 µg/mL (stock standard)
Dissolve 168.6 mg of potassium iodate ($KIO_3$) to volume with distilled water in a 100-mL volumetric flask.

(2) 10 µg/mL (intermediate stock standard)
Dilute 1.0 mL of stock standard (1000 µg/mL) to volume with distilled water in a 100-mL volumetric flask.

(3) Working standards
Prepare the following working standards using the 10 µg/mL intermediate stock standard (ISS) diluted with distilled water. Store at 20-25 °C in amber bottles for 1 year.

Table 1
Preparation of Working Standards

| Concentration (µg/mL) | ISS (mL) | Final Volume (mL) |
|---|---|---|
| 0.02 | 1.0 | 500 |
| 0.04 | 2.0 | 500 |
| 0.06 | 3.0 | 500 |

e. **Preparation of Quality Control Materials**

Collect a 24-hour human urine specimen. Mix well and divide the specimen into two equal portions. Analyze for urine iodine content. If necessary, dilute the first half to obtain a dose level of approximately 5.0 µg/dL. Spike the second half with purified iodine standard to create a dose level of approximately 50.0 µg/dL. Store the controls in 1-L amber bottles at 4-8 °C. Label and date each bottle. The solution is stable indefinitely. (Or aliquot the pools into 2.0-mL vials and freeze at ≤-20 °C indefinately.)

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration**

A calibration curve is constructed on linear graph paper by using the percent transmission (%T) values of the standards at 0.0, 0.02, 0.04, and 0.06 µg/mL iodine plotted versus their µg (mass) concentrations.

b. **Verification**

Reference materials prepared by the International Council for Control of Iodine Deficiency Disorders were analyzed. These materials, prepared from human urine and spiked with purified iodine, had target values determined by neutron activation analysis. Results of the analysis performed by the UMMS laboratory closely approximated the target values.

No proficiency testing materials are available for this analysis.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) For information regarding the range of linearity and how to handle results outside of the range, refer to the calculations section of this document (Sect. 8.g.).

(2) Allow frozen urine specimens and quality control specimens to reach 20-25 °C. Mix manually by inversion for 10 sec.

(3) While the specimens are reaching 20-25 °C, prepare lab notebook work sheets. Set sand-bath heaters to digesting temperature. Turn on the perchloric acid fume hood, the Technicon AutoAnalyzer temperature water bath, the recorder and the colorimeter.

### b. Sample Preparation

There are 16 sample tube spaces in the sand-bath heaters: each set consists of 10 unknowns, 2 controls, and 4 standards. These sets are run singly and reanalyzed by the same process the next day.

(1) First day

(a) Pipet 200 µL of urine specimens, 200 µL of the Level 1 urine control and 100 µL of the Level 2 urine control into their appropriately numbered tubes. Pipet 1.0 mL of the 0.02, 0.04, and 0.06 µg/mL iodate standards into the numbered standard tubes. (The 0.0 µg/mL tube is a blank.)

(b) Add 3.0 mL chloric acid to all tubes.

(c) Digest unknown specimens, controls, and standards at 105-110 °C using the sand-bath heater. Digestion is completed in approximately 2-2.5 hours. The end point of the digestion is marked by the formation of red chromium trioxide crystals.

(d) Remove all tubes from the sand bath-heaters and allow them to cool down to 20-25 °C.

(e) Add 2.1 mL arsenious acid to all tubes and mix.

(f) Transfer approximately 2.0 mL of the test sample solution to AutoAnalyzer cups and place the cups on the sampler.

(g) Prior to allowing the samples to proceed through the AutoAnalyzer system for the color reaction, screen test the residual portion of the test sample in the digestion tube to identify those samples containing grossly elevated iodine content. This step is done to avoid contamination of the AutoAnalyzer system. For the screen test, add 1 drop of the "screening" ceric ammonium sulfate reagent to each tube. If the yellow color fades to colorless (clear) within 1.5 min, the iodine content is too elevated to measure. Remove the samples from the sampler and discard them. Further dilute the samples and repeat the test.

(h) After the screening procedure is complete, analyze samples with the AutoAnalyzer system automatically at a sampling rate of 40 samples per hour with the temperature of the water bath set at 32 ±0.1 °C .

(i) Record the percent transmission of the standards, controls, and the unknown specimen peaks from the chart recorder.

(j) Calculate the concentrations of the controls and the unknown specimens.

(2) Second day

Repeat the analysis. On the basis of the concentrations of the unknown specimens obtained on the first day, use either a smaller or larger sample volume in order to confirm and obtain more accurate values on the calibration curve. For example, if the iodine concentration of the urine specimen is less than 0.01 µg/mL use a 1.0- or 2.0-mL sample volume. For those specimens ranging from 0.01 to 0.05 µg/mL, use 0.2 mL; and for

those reading above 0.05 to 0.06 µg/mL use 0.1 mL. For grossly elevated specimens, dilute urine specimens 10-fold or more with distilled water. Micropipettes may be used in place of dilutions. Compare the second day results with the first day results. If the results agree within 10%, average them and report the average. If the difference between the two results is greater than 10%, repeat the analyses a third time before reporting the result.

c. **Instrument Setup for the Technicon AutoAnalyzer**

(1) Set the water bath temperature at 32 °C.

(2) Turn on the recorder and the colorimeter, and allow them to warm up for at least 20 min.

(3) Thirty min prior to adding samples to the sampler module, start the proportioning pump to pump distilled water through the system for 10 min. Adjust the colorimeter for 100% transmission and 0% T baseline. (To get 0% T baseline, insert a blank aperture plug in front of the lamp in the colorimeter.)

(4) Place transmission lines into the ceric reagent (2.5N $H_2SO_4$) and distilled water bottles. Pump the reagents throughout the system for 20 min to establish a steady baseline (usually around 10% T).

(5) Turn on the chart drive. Check the quantity of the chart paper. Also check the level of ink in the chart paper styli. Add paper or ink as necessary.

d. **Operation**

(1) Turn on the sampler switch to begin the AutoAnalyzer run. Turn on the chart drive switch and the colorimeter lamp. The first four peaks before the unknowns are the calibration curve, and the last two peaks are the controls.

(2) After the run is completed, place all manifold transmission lines into a distilled water bottle and pump distilled water through the AutoAnalyzer system for 30 min to clean the system.

(3) After the system is clean, turn off the AutoAnalyzer system.

e. **Recording of Data**

(1) Quality Control (QC) Data
Use the "NHANES III Analytical Worksheet" to record the run QC data. Also record the QC data in the Lab Quality Control notebook. The Lab QC notebook contains QC charts, a daily log of QC control values, an out-of-limits log, a monthly summary of the mean and standard deviations (SDs) of control values and a preventive maintenance log. Enter the QC data on the floppy diskettes provided by NHANES III.

(2) Analytical Results

(a) Use the "NHANES Analytical Worksheet" to record the specimen results from the laboratory notebook.

(b) Enter the analytical results on the floppy diskettes provided by NHANES III.

(c) Use a laboratory notebook to set up batch runs. The information recorded must include the date, run number, analyte, shipper number, analyst's name, sample ID, sample volume used, percent T, µg of iodine read off the calibration curve, and the calculated result for urinary iodine in µg/dL.

(d) If a sample is missing write "NOSAX" in the blank. If a sample is not satisfactory, write "UNSAX" in the blank.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Maintain a supply of spare colorimeter lamps for immediate availability. Reorder when only two are left in reserve.

(2) Keep a supply of chart recorder paper on hand. Reorder when the reserve supply has only two rolls.

(3) Maintain an extra supply of AutoAnalyzer cups in reserve.

(4) Replace proportioning pump manifold with new tubing monthly or as needed.

(5) Replace transmission tubing every 6 months or as needed.

(6) Oil motors and movable parts of AutoAnalyzer pump every 6 months.

(7) Clean the colorimeter flow cell with Alconox every 2 weeks or as needed, and then rinse it with deionized water.

g. **Calculations**

(1) To calculate the calibration curve and the specimen concentration, use the Codex linear graph paper (10 divisions per inch both ways).

(2) Calculate the iodine concentration (in µg\mL) of the specimens and controls from the slope and the Y-intercept of the linear regression equation of the standard curve. Correct the final iodine concentration for the sample volume used in the analysis. For specimens with non-elevated concentrations, this calculation is:

Initial Concentration (µg/dL) x 5 (for a sample diluted 1:5) x 100

For specimens with elevated concentrations, add an additional dilution factor to account for further dilutions.

(3) This method is linear to 0.06 µg/mL (60 µg/dL).

## 9. REPORTABLE RANGE OF RESULTS

Verify results by using larger sample volumes for any urine samples with values less than 0.01 µg/mL and by diluting any urine samples with values greater than 0.06 µg/mL.

## 10. QUALITY CONTROL (QC) PROCEDURES

The method described in this procedure has been used since 1965 in the Boston Medical Laboratory, Boston, MA; in 1985, it was transferred to the University of Massachusetts Medical Center in Worcester, MA. It has been used by a reference laboratory nationally and internationally for environmental and health studies. The method has been published in *Clinical Chemistry* (1) and has proven to be accurate, precise, and reliable. Estimates of precision can be generated from long-term QC pool results.

"Bench" QC specimens are used in this analytical method. The control specimens are inserted by the analyst in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. The results are assessed by running these samples through the complete analytical process. The data from these materials are used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Two levels of QC pools are used. These pools are prepared in sufficient quantity to last throughout the survey. The targets chosen are in the low range (approximately 5 µg/dL) and high range (approximately 50 µg/dL).

QC limits are established for each pool. An analysis of variance is performed for each pool after 30 or more characterization runs have been performed.

The precision and accuracy of representative QC pools used during the survey are shown in Table 2.

After the standards and bench QC materials are analyzed, the long-term QC charts for each control materials are consulted to determine if the system is in control. The chart plots the means and compares them with the 99% confidence limits. The system is out of control if any of the following events occur:

- One or more QC samples fall outside the confidence limits.
- Both controls from a single set fall outside the range limits.
- The means from eight successive runs fall either all above or all below the mean center line.

If the run is declared "out of control," the system (instrument, calibration standards, etc.) is investigated to determine the root of the problem before any analysis of specimens occurs.

Table 2
Precision and Accuracy

| Pool | Mean | 95% Limits | 99% Limits | Total CV | N |
|---|---|---|---|---|---|
| L1UC3 | 5.40 | 5.0-5.8 | 4.60-6.20 | 14.8 | 30 |
| L2UC4 | 49.4 | 47.9-50.9 | 46.4-52.4 | 6.1 | 30 |

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. Repeat the assay the following day. If the QC studies meet the acceptable criteria and the unknown specimen results agree within 10% of the previous results, report the mean.

b. If the repeat assay does not meet the acceptable QC criteria, do not report the results. Instead, take the following steps:

(1) Prepare fresh calibration standards and run the entire calibration curve using the freshly prepared standards.

(2) Check the AutoAnalyzer system for leaks and poor air bubble patterns.

(3) Install new proportioning pump manifold and transmission lines.

(4) Prepare fresh urine control samples and reanalyze them with previously analyzed urine specimens having acceptable results.

If the steps outlined above do not result in correction of the "out-of-limit" values for the QC materials, consult the supervisor for other appropriate corrective actions. Document all actions and decisions taken during this time. No analytical results should be reported for runs not within statistical control.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

External contamination with iodine or mercury will limit the accuracy of urinary iodine levels.

## 13. REFERENCE RANGES (NORMAL VALUE)

Urinary iodine excretion depends on dietary iodine intake, but estimated ranges are 4-40 µg/dL.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Urinary iodine levels above 1000 µg/dL.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens may reach and remain at 20-25 °C during analysis. Stringent precautions should be taken to avoid external contamination by iodine. Store samples at 4-8 °C for short term storage, or at ≤-70 °C for long term storage. Urine samples are indefinitely stable at these temperatures.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If the AutoAnalyzer system fails, we recommend that laboratoris use a manual spectrophotometric method of analysis until the AutoAnalyzer is restored to functionality. If long-term interruption (greater than 4 weeks) is anticipated, store urine samples at ≤-20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Notify the supervising physician at NCHS by FAX if urinary iodine results are greater than 1000 µg/dL.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard records (e.g., electronic records, mainframe data files, laboratory notebooks, floppy disks) should be used to track specimens. We recommend that records, including QA/QC data and duplicate records, be maintained for 7 years. Only numerical identifiers (e.g., case ID numbers) should be used.

## REFERENCES

1. Benotti J, Benotti N. Protein bound iodine, total iodine and butanol extractable iodine by partial automation. Clin Chem 1963;9:408.

2. Benotti J, Benotti N, Pino S, Gardyna H. Determination of total iodine in urine, stool, diets and tissue. Clin Chem 1965;11:932.

3. Sandell E, Kolthoff JM. Microchim Acta 1937;1:9.

## ACKNOWLEDGMENTS

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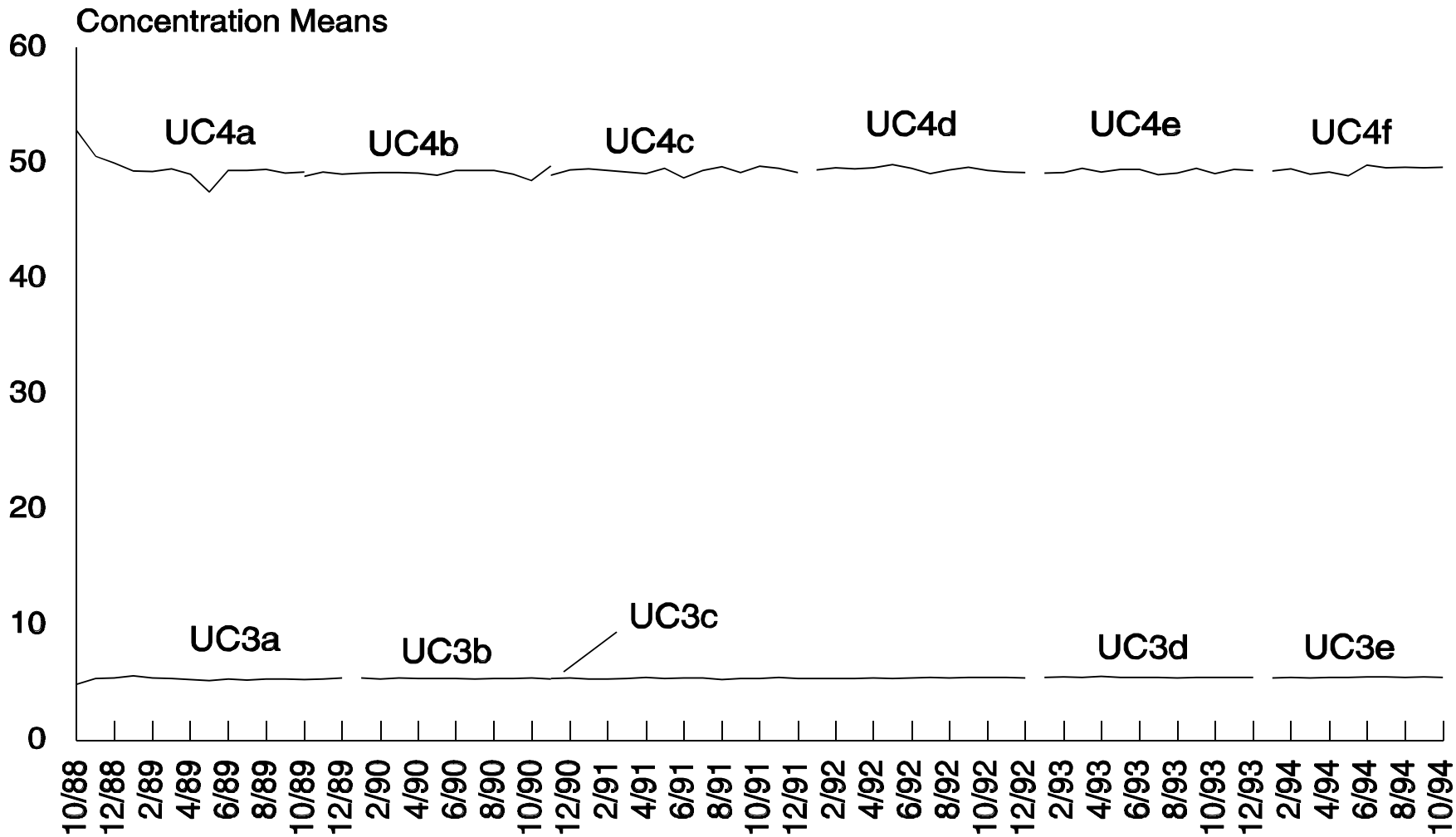
SUMMARY STATISTICS FOR
URINARY IODINE
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| UC3a | 10/31/88 - 12/27/89 | 5.3502 | 0.37642 | 7.03569 | 1114 |
| UC3b | 01/08/90 - 11/13/90 | 5.3916 | 0.32255 | 5.98250 | 789 |
| UC3c | 11/14/90 - 12/31/92 | 5.4084 | 0.32503 | 6.00979 | 2035 |
| UC3d | 01/12/93 - 12/30/93 | 5.4828 | 0.29678 | 5.41306 | 951 |
| UC3e | 01/03/94 - 10/28/94 | 5.4730 | 0.30421 | 5.55836 | 722 |
| UC4a | 10/31/88 - 10/23/89 | 49.2860 | 2.33827 | 4.74428 | 902 |
| UC4b | 10/24/89 - 11/13/90 | 49.1120 | 1.70991 | 3.48166 | 991 |
| UC4c | 11/14/90 - 12/30/91 | 49.2979 | 1.51538 | 3.07393 | 1081 |
| UC4d | 01/13/92 - 12/31/92 | 49.4778 | 1.45043 | 2.93147 | 946 |
| UC4e | 01/12/93 - 12/30/93 | 49.3271 | 1.42623 | 2.89137 | 957 |
| UC4f | 01/03/94 - 10/28/94 | 49.3861 | 1.31863 | 2.67004 | 707 |

# Urinary Iodine Monthly Means



## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The thyroid stimulating hormone (TSH) assay is a chemiluminescence immunometric assay utilizing a mouse monoclonal antibody to TSH immobilized on a polystyrene bead and a goat polyclonal antibody to TSH conjugated with an acridinium ester (1). A serum sample is added to a tube followed by the chemiluminescence-labeled antibody reagent, after which an antibody-coated bead is added to the reactants. The monoclonal and polyclonal antibodies bind the TSH in the sample to form a sandwich-complex. Free-labeled antibody is separated from the labeled antibody bound to the bead by aspiration of the reaction mixture and subsequent washing. The tubes containing the washed beads are placed into a luminometer, which automatically injects reagents that initiate the chemiluminescence reaction. The light is quantitated by the luminometer and expressed in relative light units (RLU). The amount of bound labeled antibody is directly proportional to the concentration of TSH in the sample. A standard curve is generated by plotting the RLUs versus the respective concentration of TSH for each standard on logarithmic scales. The concentration of TSH in the unknown sample is determined directly from the standard curve.

The measurement of TSH is used in the diagnosis and management of thyroid dysfunction. Elevated serum TSH levels are known to increase as a result of a rare TSH-secreting pituitary tumor (secondary hyperthyroidism). Primary hyperthyroidism (e.g., Graves disease, thyroid adenoma, or nodular goiter) is associated with depressed levels of TSH. In combination with T4, T3, and binding protein measurements, a highly sensitive TSH assay may obviate the need for confirmatory thyrotropin releasing hormone (TRH) challenge testing (2,3).

## 2. SAFETY PRECAUTIONS

Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions; wear protective gloves, eye wear, and lab coat during all steps of this method because of infectious contamination hazards. Place all plastic and glassware contaminated with serum in a plastic autoclave bag for disposal. Wash countertops with a 10% sodium hypochlorite (bleach) solution after completing the assay.

Material Safety Data Sheets (MSDS) for sodium chloride, TRIS-hydrochloric acid, sodium azide, thimerosal, and sodium hypochlorite are available through the laboratory office.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES III mobile examination center (MEC) contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 5¼" high density (HD) diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to assay.

b. The integrity of specimen and analytical data generated by this method is maintained by proofreading all transcribed data and storing data in multiple computer systems.

c. The data system generates a cumulative computerized laboratory log of all specimens received; batch receipt dates, any updates, all details associated with specimen handling, manipulation, or analysis; and any problems as they occur.

d. Documentation for system maintenance is contained in printed copies of data records.

e. The results from the assay are transcribed from the dedicated instrument printouts to the assay sheets and computer record. All entries are proofread. Batch data will be transmitted by both IBM DOS floppy diskettes with their report form printout to the National Center for Health Statistics (NCHS). The final test values for each specimen will be identified with the dates of analysis, technical operators involved, whether repeat determinations were required and if so, for what reason.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Serum is the preferred sample. Do not use plasma. Serum specimens may be collected in regular red-top or serum-separator Vacutainers.

c. Minimum sample volume is 400 µL.

d. The appropriate amount of serum is dispensed into a Nalge cryovial or other plastic screw-capped vial labelled with the participant's ID.

e. Specimens collected in the field should be frozen and shipped on dry ice by overnight mail. Once received, they are stored at ≤-20 °C until analyzed. Serum left over after analytical aliquots are withdrawn should be refrozen at ≤-20 °C.

f. Specimens are stored at 2-8 °C for up to 48 hours.

g. For longer storage, freeze samples at -10 to -30 °C. Specimens may be stored in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.

h. Avoid repeated thawing and freezing of samples.

i. Mix each thawed sample completely before analysis.

j. Recovery studies have indicated that neither icteric, moderately lipemic, or hemolyzed serum cause any variation in assay results. Avoid using grossly hemolyzed or grossly lipemic samples.

k. Prior to use, allow all samples to come to 20-25 °C and mix by gentle inversion.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Berthold Auto-CliniLumat LB 952 T (Berthold Systems Inc., Pittsburgh, PA).

(2) Multi-tube vortexer, cat. no. 58816-115 (VWR Scientific, San Francisco, CA).

(3) Orbital shaker, model 2001 (VWR Scientific).

(4) Allegro Aspirwash II, 19-probe tube washer (Nichols Institute Diagnostics, San Juan Capistrano, CA).

(5) Eppendorf pipet, 100- to 1000-µL, cat. no. 022 44 020 9 (Brinkman Instruments, Inc., Westbury, NY).

(6) Eppendorf pipet, 10- to 100-µL, cat. no. 022 44 010 1 (Brinkman Instruments, Inc.).

(7) Eppendorf pipet, 100-µL, cat. no. 022 44 180 9 (Brinkman Instruments, Inc.).

(8) Eppendorf pipet, 2- to 20-µL, cat. no. 022 44 005 5 (Brinkman Instruments, Inc.).

b. **Other Materials**

(1) Nichols Institute Diagnostics 100- and 500-test kits, cat. no. 40-2160 and 40-2161, respectively (Nichols Institute Diagnostics, San Juan Capistrano, CA).

(2) 3.5-mL, 55- x 12-mm polypropylene tubes, cat. no. 55.484 (Sarstedt Laboratories, Newton, NC).

(3) 5-mL, 75- x 12-mm polypropylene tubes, cat. no. 55.476 (Sarstedt Laboratories).

(4) 5-mL, 75- x 12-mm borosilicate glass tubes, cat. no. 60825-550 (VWR Scientific).

(5) Push-in stoppers for 12- x 55-mm and 12- x 75-mm polypropylene tubes, cat. no. 65.809.505, white (Sarstedt Laboratories).

(6) 100% nitrile rubber gloves (Best Manufacturing, Menlo, GA).

(7) Latex gloves, style 312 (Perry X-AM, Smith & Nephew Perry, Massilon, OH).

(8) Biohazard disposable bag, cat. no. 13166 (VWR Scientific).

(9) Bleach, 10% sodium hypochlorite solution (NaOCl) (any vendor).

(10) Kim-Wipe lintless tissues (Kimberly Clark Corp., Roswell, GA).

(11) Singlefold towels, 227-04 (Fort Howard Corp., Green Bay, WI).

c. **Reagent Preparation**

Kit materials are supplied ready-to-use by the manufacturer (Nichols Institute Diagnostics). (Volumes given are for 100-test.)

(1) TSH Antibody coated beads (Reagent A)
One bottle, containing 100 TSH mouse-monoclonal antibody-coated polystyrene beads and desiccants.

(2) Acridinium ester labeled TSH antibody solution (Reagent B)
One vial containing 10 mL of chemiluminescence-labeled TSH goat-polyclonal antibody.

(3) Wash solution (Reagent W)
One bottle with 50 mL of wash solution containing phosphate buffered saline solution with surfactant.

(4) Trigger set
Trigger 1 solution contains hydrogen peroxide in dilute acid and Trigger 2 solution contains dilute sodium hydroxide.

(5) Performance controls
The performance controls should be stored in a non-defrosting freezer at ≤-20 °C upon receipt. Prior to use, thaw one set of vials (performance controls 1 and 2) at 20-25 °C and mix by gentle inversion.

Bring the performance controls to 20-25 °C for 24 hours before making the first measurement.

Once the set is thawed, store it at 20-25 °C for further use. Performance controls are stable for 4 weeks from date of thaw when stored at 20-25 °C.

Each performance control set contains sufficient volume to perform 15 performance checks. Do not combine or interchange performance controls with different lot numbers.

Indications of possible deterioration are the presence of particulate matter in the liquid reagents and/or a significant deviation from previous data. Performance controls are stable until the designated expiration date on the vial when stored unopened at ≤-20 °C.

(a) Performance Control 1
Four vials, each containing 4 mL of a chemiluminescence solution. Prior to opening, mix by gentle inversion. Carefully recap the vials after each use.

(b) Performance Control 2
Four vials, each containing 4 mL of a chemiluminescence solution. Prior to opening, mix by gentle inversion. Carefully recap the vials after each use.

d. **Standards Preparation**

TSH Kit Calibration Standards
Supplied in 4-mL vials, ready-to-use (Nichols Institute Diagnostics). Contains one (two) each of seven vials, labeled 'C' through 'I', human TSH in equine serum with sodium azide as preservative; refer to vial labels for exact concentration. Vial C contains 4 mL; vials 'D' through 'I' contain 2 mL each.

These standards are calibrated by Nichols against the World Health Organization's Second International Reference Preparation (80/558) for TSH (National Institute for Biological Standards and Control, Hertfordshire, England). One mU/L obtained using the TSH assay is equivalent to one mU/L of WHO 2nd IRP (80/558) for TSH.

e. **Preparation of Quality Control Materials**

In-house controls of low, medium, and high levels of human serum TSH (USC Endocrine Services Laboratory, Los Angeles, CA).

In-house quality control pools are prepared in the low, medium and high ranges of the curve. These quality control pools are made from selective pooling of previously analyzed, unused participant serum, aliquoted into appropriately small quantities to minimize any influence of repeat freezing and thawing. Pools are coded and identified by the pooling date. These serum pools, stored at $\leq$-20 °C, are considered to have a shelf life of 2 years.

Sufficient material is always pooled and aliquoted to ensure a supply of quality control material sufficient to last more than 1 year under normal work volumes. New pools are constructed and their normal ranges established from 10 individual assay runs, not less than 3 months prior to the exhaustion of the current pool. The ranges for each control are documented in the Quality Control Book. When more than 10 unknowns are run in a single assay, measure the low, medium, and high controls after the calibrators and after the last unknown sample. When fewer than ten unknowns are run in a single assay, measure the low, medium and high controls after the last unknown sample.

This laboratory has previously determined that serum pools are a better quality control material than commercially available, modified, lyophilized serum-based material. Pooled serum is a matrix identical to the test serum sample and better reflects when a run is "out of control."

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration**

The Berthold Auto-CliniLumat has full data-reduction capabilities. This preprogrammed data-reduction program entitled NICHOL-TSH generates slopes, intercepts, correlation coefficients, ($r^2$), and plotted and fitted curves. The $r^2$ for each curve should be 0.98 or higher.

A calibration curve is constructed by using a LOG/LOG transformation prior to the cubic spline fit. The average RLU for each standard is plotted on the Y-axis versus the corresponding concentration of TSH for each standard on logarithmic scales. The calibration curve is displayed.

b. **Verification**

The TSH standards are prepared by Nichols and are calibrated against the World Health Organization's second international reference preparation of TSH (2nd IRP 80/558), available from the National Institute of Biological Standards and Controls, London, UK. These standards are run daily.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Unpack samples immediately after arrival, check the vials for possible damage during transport, and check the NCHS number of each sample against the packing list for accuracy. Perform the inventory quickly to prevent thawing, and then store the samples at ≤-20 °C. Prepare testing batches of 50 samples to be thawed and assayed.

(2) Identify each batch with the box number that will be used throughout all analyses to identify the batch.

(3) Analyze batches of 50 serum specimens or fewer in a single assay. Analyze batches containing more than 50 specimens in two separate assays to avoid assay drift.

(4) Prior to use, allow reagents to reach 20-25 °C and mix by gentle inversion.

b. **Sample Preparation**

(1) Allow specimens to thaw at 20-25 °C for 1 to 2 hours.

(2) When specimens are thawed, mix thoroughly by vortexing.

**c. Instrument Setup**

(1) **Berthold AutoCliniLumat**

(a) Assay programming for the Berthold AutoCliniLumat Immunoluminometric (ILMA) assay: LOG/LOG transformation is used prior to the spline fit.
Measuring time per tube: 2.0 sec.

(b) Load the tubes as shown in Table 1.

Table 1
Tube Loading Order for TSH Assay

| Tube | Replications | Dose (mU/L) |
|---|---|---|
| Wash Tubes | 2 | -- |
| Empty position | 1 | -- |
| STD 1 | 2 | 0.000 |
| STD 2 | 2 | 0.010 |
| STD 3 | 2 | 0.055 |
| STD 4 | 2 | 0.530 |
| STD 5 | 2 | 5.5 |
| STD 6 | 2 | 11.0 |
| STD 7 | 2 | 52.0 |
| UNK (unknowns) | | |

(c) Turn on the printer and check the printer paper, making sure there is enough for the entire run. If necessary, replace paper.

(d) Check the supply of the trigger solutions, and replace the solutions if necessary. Also check the tubing

for visible leaks, crimps, precipitates, or discoloration.

(e) Press EXIT until the ready screen appears with the main menu.

(f) Press MEASURE to indicate the number of samples for which there is sufficient reagent supply. If new reagent bottles are to be used, press LOAD REAGENT and enter the number of injections available. The current supply is calculated through automatic counting down from this initial value during the measurement. Press CONTINUE to get to the next display.

(g) Select CALCULATED type of measurement.

(h) Select "1" for "No. Of Different Assays."

(i) Enter "1" for "Enter 1st Protocol No."

(j) Select YES for 01: "Enter Protocol OK? TSH."

(k) Select NO for "Use Last Standardization?"

(l) Select YES for "Stop For Changing Standard Curve."

(m) Enter the box number being analyzed at the GENERAL NOTE screen.

(n) Select NO for "Print Loading List", load samples, close the door and press START. The instrument will move the sample tubes to the measuring position. In the beginning, a 5-fold wash cycle is performed. While the samples are being measured, the type of samples that are being transported to the measuring position is displayed.

(o) The standard curve is calculated and displayed. Press PLOT to print a graphic plot of the curve.

(p) The controls and unknown samples are then measured in the order specified in the protocol.

(q) Measure the RLU for 2.0 sec on the luminometer using the preprogrammed data reduction program entitled "NICHOL-TSH".

(r) One blank position following the last tube loaded, the chain moves forward by 16 positions to allow removal of old tubes; then the door is unlocked automatically.

(2) **Allegro Aspirwash II**

The TSH assay wash specifications are stored on a program card designed to perform a specified wash protocol and has been validated in accordance with instrument functional performance specifications.

(a) Insert the TSH program card.

(b) Set the power switch to ON and wait for the washing stations to attain HOME position.

(c) Verify that the check value on the intake tubing is submerged in wash solution and there is sufficient solution to prime the wash station for the complete run.

(d) To prime the washer:

i. Press and hold the RUN button until two beeps are heard. The PRIME/FLUSH program will initiate and run continuously until the RUN button is pushed again.

iii. When the tubing is clear of air bubbles, press and hold the RUN button for two beeps.

(e) To start the wash:

i. Load the assay tubes to be washed into the appropriate rack in full rows to match your system configuration. An entire row of tubes must be completely filled or completely empty.

ii. The assay tube sensor will detect an empty row of tubes, preventing the system from dispensing fluid. The sensor cannot detect a partially filled row. Therefore, to prevent accidental dispensing, do not leave any empty positions in a row of tubes.

iii. Place the rack on the washing station frame.

iv. Press RUN for one beep to start the wash protocol. When the wash protocol has been completed, the washing station will return to the home position, stop, and resume the IDLE mode.

d. **Operation of Assay Procedure**

(1) Label 12- x 75-mm borosilicate glass tubes in duplicate for standards 'C' through 'I' and participant samples.

(2) Pipet 200 µL of the appropriate standard or participant sample directly to the bottom of each tube.

(3) Add 100 µL of labeled antibody to the bottom of each tube. Vortex each tube gently; avoid foaming.

(4) Gently add one antibody-coated bead to each tube using a bead dispenser. Tilting the test tube rack while adding the beads reduces splashing.

(5) Incubate all tubes on a rotator at 180 (±10) rpm for 2 hours at 20-25 °C.

(6) Add 0.5 mL of wash solution to each tube. Wash the beads in an automated wash station, using 2 mL of deionized water according to instructions in Section 8.c. Repeat three times. If a manual wash method is used, aspirate the wash solution before adding deionized water. The beads should be counted within 2 hours of washing.

(7) Count each tube for 2 sec in the luminometer using trigger 1 and trigger 2 solutions.

(8) Discard measured tubes appropriately as chemical waste.

e. **Recording of Data**

(1) Quality Control Data
The USC Endocrine Clinical Laboratory uses the Bio-Rad QC program. The program is set up to allow real-time data entry, providing the user with immediate validation of the actual participant results reported. Data are evaluated and screened on the basis of the multirule system formulated by Westgard. The control data are recorded in the Quality Control Log for NHANES and interpreted for acceptability of assay. Any problem with quality control runs and corrective action taken is indicated on the "Corrective Action Log" and in the computer when QC result entry is made. The laboratory technical director supervisor checks the individual test results worksheet and compares the original to the computer printout to detect clerical errors before releasing participant reports.

(2) Analytical Results
Worksheets are prepared from the transmittal from NHANES. Each of the duplicate results and the mean result are recorded on this worksheet. For results below the detection limit of the method, record "less than 0.01 mU/L." For results greater than the highest standard, dilute the specimens appropriately reassay them.

f. **Replacement and Periodic Maintenance of Key Components**

(1) **Berthold AutoCliniLumat**

(a) A performance check is performed on the luminometer each day of use. This includes the luminometer performance controls. Criteria for acceptability are shown in Table 2.

Table 2
Luminometer Performance Criteria

| | Limits |
|---|---|
| Background count (BC) | <150 RLU |
| Precision, PC1 and PC2 | <5% CV |
| Net dynamic range PC2-BC / PC1-BC | 7-13 |

(b) The printer cartridge is replaced as needed.

(c) Additional periodic maintenance of this equipment is performed by the service representative.

(2) **Micro-pipettors**

All micro-pipettors that are used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

(3) **Allegro Aspirwash II**

(a) Cleaning Fluid Paths

i. Daily: Replace the wash solution with 500 mL deionized water and run the prime/flush cycle to purge the remaining wash solution from the system. Replace the wash solution before resuming wash protocols.

ii. Monthly: Flush the system with 0.1 mol/L NaOH, followed by distilled water as directed in the operator's manual.

(b) Cleaning Manifold Probes

i. Daily: Rinse the outside of the probes with distilled water and wipe dry to prevent mineral deposits.

ii. Monthly: Insert the probe-cleaning wire into the probe for the full length of the tube.

(c) Cleaning/Maintaining the Instrument

i. Daily: Check aspirate/dispense probes for evenness and adjust if necessary each day.

ii. Monthly: Use a soft cloth to clean the optical tube sensor located on the base of the robotic arm. Check the sump for leaks and replace if necessary. Check the intake tubing for damage and replace if necessary.

(d) Replacing Other Components
Remove/replace the dispenser syringe, the intake tubing kit, the waste tubing kit with sump, and the manifold tubing and probes as scheduled.

g. **Calculations**

The Berthold Auto-CliniLumat has full data-reduction capabilities and calculates the following values:

(1) The average RLU for each set of duplicate standard tubes.

(2) The standard curve (which is prepared by plotting the average RLU for each standard on the Y-axis versus the corresponding scales). A cubic spline curve fit is used to interpolate between the standard points.

(3) The controls and sample RLU values (which are read from the standard curve to obtain their corresponding concentration of TSH in mU/L).

h. **Special Procedure Notes -- USC Modifications**

(1) Allow all reagents to come to 20-25 °C before use.

(2) If the Luminometer is out of order and an assay has already been set up, the assay may be saved overnight by: a) reading it at the Clinical Research Center, or (b) adding 300 µL of trigger 1 after the wash step (#9) in the assay procedure. Cover the tubes with parafilm and let stand at 20-25 °C, to read next day in repaired luminometer.

(3) For diagnostic purposes, TSH values should be used in conjunction with other test results, the overall clinical presentation to the physician, and all other appropriate information.

(4) The use of borosilicate glass tubes is recommended for optimum performance and reproducibility due to their inherent low luminescence background and low non-specific binding characteristics.

(5) Samples with discrepant results should be verified by repeat assays.

(6) Since the entire chemiluminescent reaction is completed in 2 sec, each bead can only be read once. It is recommended that the luminometer performance controls are a part of daily luminometer performance checks.

**9. REPORTABLE RANGE OF RESULTS**

a. The normal range is 0.39 - 4.6 mU/L.

b. The working range of the assay is 0.01 - 50.00 mU/L.

c. All values <0.01 mU/L are reported as <0.01 mU/L.

d. All specimens with values >50 mU/L are diluted 1:5 or 1:10 by using the A (0.0 mU/L) standard and reassayed. The resulting value is multiplied by the dilution factor used.

e. Results with a BKG (background) flag next to a TSH value displayed on the result printout are not to be used. "BKG" means that the background for that particular tube was greater than the assay threshold (e.g., 50 RLUs). The value of the other duplicate tube may be used; the laboratory manager or the technical supervisor must be notified of both values.

f. When a quantity nonsufficient (QNS) sample is received and the TSH has been run only at a 1:2 dilution using the "A" (0.0 mU/L) standard used as the diluent, the resulting value is multiplied by two and the result reported. However, if the resulting value of the dilution gives a result of "<<<"; then the result is reported as <0.02 mU/L.

g. Report poor duplicate results on the TSH as follows:

- If both duplicates are between 0.01 and 0.03 mU/L, report the mean.
- If one is <0.01 mU/L and the other is 0.01 mU/L, report <0.01 mU/L.
- If one is <0.01 mU/L and the other is >0.01 mU/L, repeat the analysis.
- If both duplicates are between 0.3 and 5 mU/L, report the mean providing there is <1.0 mU/L difference between the duplicates.
- If both duplicates are >5 but <10 mU/L, report the mean providing there is <2.0 mU/L difference between the duplicates.
- If both duplicates are >10 mU/L, report the mean providing there is <5.0 mU/L difference between the duplicates.

h. Record the master kit lot number and the initials of the technical person running the assay on the worksheets.

**10. QUALITY CONTROL (QC) PROCEDURES**

As of 1987, this was considered by many thyroid specialists to be the most functionally sensitive commercial method available. It offers the best diagnostic accuracy both for measuring values in the normal range and for detecting conditions of subclinical thyroid hormone excess encountered in this survey. Because this method was the "state-of-the-art", it was retained with only minor modifications over the 6-year course of NHANES III.

The USC Endocrine Clinical Laboratory uses the Bio-Rad QC program. This program is set up to allow real-time data entry, providing the user with immediate validation of actual participant results. Data is evaluated and screened on the basis of the multi-rule system formulated by Westgard. This system helps maximize laboratory resources by decreasing unnecessary assay rejection and simultaneously increases the program's sensitivity in detecting errors. The lab QC manager generates QC reports on a monthly basis for individual laboratories (e.g., Levy-Jennings).

The USC Thyroid Clinical Laboratory applies the floating mean principle. If a trend or shift occurs, the problem is investigated and corrected. A new mean is calculated on the basis of the last 2 months' data by using a fixed mean until a new floating mean is established (4,5).

Two types of quality control systems are used in this analytical method. Within one system, "Sample QC", 5% of specimens are randomly selected and analyzed either within-assay or between-assay for quality assurance purposes. If the deviation between duplicates is greater than 10%, the specimen is reanalyzed. With the other system, "bench" quality control specimens are placed before and after all specimens to be analyzed.

An internal quality control program monitors the accuracy and precision of laboratory performance on a daily basis. Bench quality control specimens are inserted by the analyst two times in each analytical run (a set of consecutive assays performed without interruption) so that judgements may be made on the day of analysis. Three separate serum pools having low, medium, and high range values for the analyte are inserted in each assay. Each of three pools are run twice in each assay -- once at the beginning and once at the end of the run. That is, specimens with unknown values are "sandwiched" between the quality control pools to ensure that the assay run has not been subjected to methodologic drift.

If the stock of these controls becomes low, another batch is prepared in time to analyze it concurrently with the current quality control materials. The new controls are used only after their means and ranges are established following a minimum of 10 characterization runs. Sometimes, more runs are used to update control means and ranges if 10 runs are deemed to be inadequate to represent the overall characteristic of the method over time. All updates of control means and ranges are performed after approval from NCHS.

In addition, the assay mean (the mean of all the values falling within the normal limits for that analyte) will be used as an additional medium-range quality control.

The USC Endocrinology (Thyroid) Laboratory, a state-licensed facility, is externally monitored and certified by the College of American Pathologists, an interlaboratory comparison (proficiency testing) system designed to compare a laboratory's performance with those of other laboratories. This monitoring program is particularly critical since this laboratory is actively engaged in a number of research projects related to the monitoring of thyroid tests conducted on serum from HIV-infected participants.

a. Determining the acceptability of control results:

(1) Analyze samples of three different control levels. Make one measurement on each control material each time when testing for statistical control. Record these observations.

(2) Accept the run when both control observations are within the mean ±2 SD and report participant results. When at least one control observation is mor than 2 SD outside the mean, hold the participant results and inspect the control data further using additional control rules.

(3) Inspect control data within the run:

- Reject the run when one control observation is more than 3 SD outside the mean. Do not report participant results.
- Reject the run when both control observations are more than 2 SD outside the mean. Do not report participant results.
- Accept the run if one control is more than 2 SD outside the mean but less than 3 SD outside the mean if other controls are within ±2 SD.

(4) Inspect control data across runs:

- Reject the run when the previous observation on the same control level exceeded the same mean by more than 2 SD. Do not report participant results.

- Reject the run when the last 10 consecutive observations fall on the same side of the mean. Do not report participant results.

(5) Accept the run when none of the above rules indicate a lack of statistical control. Report participant results.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. If the analytical run is out of control take the following actions:

(1) Determine the type of errors occurring (random, systematic, or both) on the basis of the control rules being violated. A review of control data on all control levels will help detect errors occurring throughout the concentration range tested by these controls. A review of control data on a single control level (within control materials) will help detect errors in a particular concentration range.

(2) Refer to a troubleshooting guide to inspect the components of the method or instrument that contribute to the type of error observed.

(3) Correct the problem, then reanalyze the participant samples and control samples, testing for statistical control by the same procedures. Do not include data from previous out-of-control situations in cumulative data. If results exceeded the 3 SD limits, indicate corrective action taken on the "Corrective Action Log" and in the computer when the QC result entry is made.

(4) Consult the laboratory manager or technical supervisor for any decision to report data when there is a lack of statistical control.

b. The laboratory manager or technical supervisor may decide to report the data even when there is lack of statistical control in the following situations:

(1) The control problem can be shown to be due to the control materials themselves.

(2) The control problem can be shown to have resulted from an isolated event that would not have affected the rest of the run (e.g., an interchange of two samples or a clerical transcription error).

(3) The control problem occurs in a concentration range different from the concentrations of the participant samples. The method is in control in the range of the participant samples.

(4) The size of the analytical error is judged to be small relative to the medical usefulness requirements. For example, small shifts of the analytical mean relative to medical needs may be ignored when associated with reagent changes.

(5) Participant data can be released even if quality control results fall outside pre-established statistical limits, so long as participant care is not compromised.

c. Everyday the laboratory technical director supervisor checks the cumulative summary of the results for each participant specimen for clinical appropriateness. The supervisor checks the individual test results worksheet and compares them with the computer printout to detect clerical errors before releasing participant reports.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

a. All samples with a TSH concentration greater than 55 mU/L should be diluted with the "A" (0.0 mU/L) standard and reassayed. The resulting value should then be multiplied by the appropriate dilution factor.

b. The TSH kit shows no interfering high-dose "hook" effect from 0 to 500 mU/L of TSH. Samples between 50 and 500 mU/L will have RLU values greater than the 55 mU/L standard.

c. Recovery studies have indicated that neither icteric, lipemic, or hemolyzed serum causes any variation in assay results.

**13. REFERENCE RANGES (NORMAL VALUES)**

The normal reference range is 0.39-4.60 mU/L (6).

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

All samples with TSH concentrations less than 0.1 mU/L or greater than 15.0 mU/L and with positive results for the antimicrosomal antibody or antithyroglobulin antibody assay are reported to NCHS.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens should be allowed to reach 20-25 °C during testing. Specimens can be refrigerated at 4-8 °C overnight for the TSH analysis. After analysis, specimens are immediately refrozen at ≤-20 °C and thawed only for repeat testing.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no alternative methods for this assay but the laboratory also maintains a backup instrument in the USC Clinical Research Center. Specimens may be stored at 2-8 °C for up to 48 hours, or they may be stored frozen at ≤-20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Results are recorded on a floppy diskette provided by the NHANES MEC. Records of quality control data, specimen results, dilutions, and repeat tests are maintained in the laboratory.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record keeping means (e.g., computer data files and printed copies) should be used to track specimens. We recommend that records, including related QA/QC data, be maintained for 6 years, and that duplicate records be kept. Only numerical identifiers (e.g., NCHS ID numbers) should be used.

Residual specimens are stored at ≤-70 °C for 1 year and returned to the NCHS specimen repository at Rockville, MD.

**19. QUALITY CONTROL SUMMARY STATISTICS AND GRAPHS**

The qc summary statistics and graphs won't be released until the actual TSH data is released.

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Coulter Counter Model S-PLUS JR with Coulter histogram differential, hereafter referred to as the Model S-PLUS JR, is a quantitative, automated hematology analyzer. It is intended for determining the following 16 hematologic parameters:

- White Blood Cell (WBC) or leukocyte count $*10^3$
- Lymphocyte percent (LYMPH percent), %
- Mononuclear cell percent (MONO percent), %
- Granulocyte percent (GRAN percent), %
- Lymphocyte number (LYMPH #) $*10^3$
- Mononuclear cell number (MONO #) $*10^3$
- Granulocyte number (GRAN #) $*10^3$
- Red Blood Cell (RBC) or erythrocyte count $*10^6$
- Hemoglobin (Hb) concentration, g/dL
- Hematocrit (relative volume of erythrocytes) (Hct), %
- Mean Corpuscular (erythrocyte) Volume (MCV), fL
- Mean Corpuscular (erythrocyte) Hemoglobin (MCH), pg
- Mean Corpuscular (erythrocyte) Hemoglobin Concentration (MCHC), g/dL
- Red Cell (erythrocyte volume) Distribution Width (RDW), %
- Platelet (PLT) or thrombocyte count $*10^3$
- Platelet distribution width (PDW)
- Relative volume of thrombocytes (PCT)
- Mean Platelet (thrombocyte) Volume (MPV), fL

The Coulter method of cell counting and sizing is based on the detection and measurement of changes in electrical resistance produced by a particle suspended in a conductive liquid traversing a small aperture. When cells are suspended in the conductive diluent, they function as discrete insulators. When a dilute suspension of cells is drawn through a small cylindrical aperture, the passage of each individual cell momentarily increases the resistance of the electrical path between two submerged electrodes, one located on each side of the aperture. An electrical pulse, suitable for counting and sizing, results from the passage of each cell through the aperture.

The purpose of the Model S-PLUS JR is to differentiate normal blood, with all normal system-generated parameters, from the blood of patients who need additional studies of any of these parameters. These studies might include further measurements of cell size and platelet distribution, biochemical investigations, histogram differentials, or any other definitive test that would help resolve the discrepancy.

## 2. SPECIAL SAFETY PRECAUTIONS

Consider all serum samples received for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses while handling all human blood products. Place all disposable plastic, glass, and paper (pipet tips, accuvettes, gloves, etc.) that contacts serum in a biohazard autoclave bag. Dispose of all biological samples and diluted specimens in a biohazard bag at the end of the analysis. Keep these bags in appropriate containers until they are sealed and autoclaved. Wipe down all work surfaces with 10% bleach solution when work is finished.

The material safety data sheets (MSDSs) for sodium hypochlorite, Isoton III diluent, Coulter Clenz, Lyse S III differential lysing reagent, and S-Cal calibrator are kept in the *Working Safely with Hazardous Chemicals* manual in the Mobile Examination Center (MEC). All Coulter reagent concentrations represent nonhazardous levels.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The Coulter S-PLUS JR data terminal (DT) is interfaced directly to the MEC automated system (VAX).

b. Transmit all results collected during a session from the Coulter DT to the VAX at the end of the session. When the transmission is complete, review the results from the VAX to determine the completeness and the accuracy of the transmission. If the results are inaccurate or incomplete, repeat the transmission procedure. If, after you repeat the procedure, the results are still inaccurate or incomplete, inform the MEC manager.

c. If the results of the transmission are acceptable, transfer the Coulter results from the VAX to the Laboratory Data Base Management System (Oracle). When the Coulter data is in Oracle, access the hematology data entry screen to confirm that duplicate Coulter determinations have been performed for every sample person (SP) who has given a blood or urine sample; if duplicate determinations were not done, explain why.

d. Once a week, print a shipping transmittal of hematology data and transmit the data file to a floppy diskette. Mail to the CDC Serum Bank in Atlanta, GA.

e. Edit the data and average the duplicate determinations. After each NHANES stand is completed (i.e., when all clinical evaluations and analyses from each mobile survey site are complete), the values will be transmitted to the NCHS mainframe computer along with the other NHANES III data.

f. Files stored on the network or CDC mainframe are automatically backed up nightly to tape by the CDC Data Center staff.

**4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION**

a. Samples are collected following NHANES specimen collection criteria.

b. No special instructions such as fasting or special diets are required.

c. Specimen type: whole blood with $K_3$EDTA anticoagulant.

d. The optimal amount of specimen is 1.0 to 1.5 mL.

e. Use a 2- or 3-mL lavender-top $K_3$EDTA tube for sample collection.

f. Clotted specimens are unacceptable for this test.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Before performing the Coulter S-PLUS JR assays on the EDTA samples, follow these instructions to prepare a differential blood smear:

a. Use two 1- x 3-in glass slides.

b. Label one slide with the NCHS sample identification number as it appears on the EDTA blood tube.

c. Place a drop of blood about 2-3 mm in diameter about 1 inch from the end of the labeled, clean dry slide.

d. Use the second glass slide as a "pusher." Place this slide at an angle of 30° to 40° to the specimen slide, and then draw back the pusher slide to make contact with the blood.

e. Allow the blood to spread toward the sides of the pusher slide; then push it smoothly and lightly toward the opposite end of the specimen slide, drawing the specimen behind it in a thin film.

f. A satisfactory smear is narrower than the slide. It is smooth, with no ridges, waves, holes, or finger-like projections at the end. When held to the light, it has a feathery edge.

g. Repeat the above procedure to prepare a duplicate slide.

h. Allow the smears to air dry.

i. Blood smears are stored in numbered slide boxes and mailed to the CDC Serum Bank at the end of the stand.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. **Instrumentation**

(1) Model S-PLUS JR (Coulter Electronics, Hialeah, FL), a modular system consisting of a pneumatic power supply, main unit, data terminal, and printer/plotter.

(2) Damon IEC microhematocrit centrifuge, model MB (Baxter Scientific Products, Columbia, MD) that has the following features: Maximum speed: 14,000 rpm, maximum RCF: 12700 x g, maximum capacity: 24- x 75-mm MHCT tubes, timer: 15 min, and electrical requirements: 115V, 50/60 Hz.

(3) IEC microhematocrit reader, model 2201 (Baxter).

(4) Adams Nutator model 1105 specimen mixer (Clay Adams Division, Becton Dickinson, Parsippany, NJ).

b. **Other Materials**

(1) Accuvettes dilution vials (Curtin Matheson Scientific, Inc., Jessup, MD).

(2) S-Cal Calibrator (Coulter). Refrigerate at 4-8 °C until depletion or until the expiration date. Expired vials of S-Cal Calibrator should not be used.

(3) Tool kit (Coulter).

(4) Printer/plotter paper (Coulter).

(5) Capillary hematocrit tubes (Curtin Matheson).

(6) Clay sealant (Curtin Matheson).

c. **Reagent Preparation**

Isoton III diluent, Coulter Clenz, and Lyse S III diff lytic reagent are supplied by Curtin Matheson Scientific, Inc. ready to use. Store at 20-25 °C.

d. **Standards Preparation**

No calibration curve is generated by the user as part of this assay method.

e. **Preparation of Quality Control Preparation**

4C Plus Cell Controls, high, normal, and low levels (Curtin Matheson). This control material is ready to use; refrigerate at 4-8 °C until depletion or until the expiration date. Do not use expired vials.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

*Keystrokes described in the following sections are for the Coulter S-PLUS JR with version XXX.X software. These keystrokes may not correspond with the same menu items on other Coulter instruments.*

a. **Calibration**

Perform full calibration procedures at the beginning of every stand and after replacing any component that involves the dilution characteristics or the primary measurements. To maintain the Model S-PLUS JR within optimum operational tolerances,

(1) Clean the blood sampling valve and apertures as described in Section 5 of the Product Reference Manual.

(2) Check the level of the reagents.

(3) Perform daily startup procedures as described in the Product Reference Manual (Part 2, sec 3).

(4) Conduct a reproducibility check using the STARTUP:REPRODUCIBILITY function.

(a) Ten replicate samples of one specimen must meet the following requirements:

- They must be from a donor who is not receiving drugs, has normal hematologic parameters and normal erythrocyte, thrombocyte, and leukocyte morphology.
- The specimen must be stored in the proper amount of EDTA anticoagulant.
- The vacuum collection tubes in which the samples are contained must be filled properly.

(b) At the data terminal (DT), access the PRIME mode; prime by "cycling" two normal whole-blood samples.

(c) Access the STARTUP:REPRODUCIBILITY mode, delete any previous results, and cycle 10 consecutive samples.

(d) Review the displayed results. Verify that the average coefficient of variation (CV) does not exceed the limits for the parameters listed in table 1.

Table 1
CV Limits

| Parameter | Maximum Acceptable CV (%) |
|---|---|
| WBC | 3.0 |
| RBC | 2.0 |
| Hb | 1.0 |
| MCV | 2.0 |
| RDW | 3.2 |
| PLT | 4.0 |
| MPV | 5.0 |
| LY# | 5.0 |
| MONO# | 10.0 |
| GRAN# | 5.0 |

(5) Conduct a carryover check using the STARTUP: CARRYOVER, PRIMARY MODE function. Follow the instructions on the display and verify that DATA: ACCEPT appears.

(6) Conduct an electronic subsystems check. Perform the following procedures to verify that the power supply voltages are within their acceptable ranges.

(a) Press DIR to access the Directory.

(b) Press and release ADV until DVM appears.

(c) Observe the initial voltage displayed and use ADV to review the power supply voltages. Verify that the displayed values are within the acceptable ranges given in Table 2.

Table 2
Acceptable Power Supply Voltage Ranges

| Display | Acceptable Range, Volts (DC) |
|---|---|
| HBL | 7.00 ±0.25 |
| +5V supply | 5.00 ±0.20 |
| +15V supply | 15.00 ±0.50 |
| -15V supply | -15.00 ±0.50 |

(d) Verify that the WBC bath contains clean, bubble-free diluent. If the Hb BLANK reading is not within tolerances, use DIR and ADV to access the Hb BLANK ADJUST option. (Refer to manual heading 3.4, Keypad.) Press SEL when the option appears on the display.

(e) Verify that the reading is 7.00 ±0.25 volts (DC). If the reading is out of tolerance, adjust the value by pressing 7, 0, 0, and ENTER. Allow 30 sec for the adjustment to be made before continuing.

(f) When SELECT FUNCTION appears on the display, the adjustment has been completed. Reaccess the Hb BLANK ADJUST or DVM option to verify that the reading is 7.00 ±0.25 volts (DC). If it is not, call the Coulter Service Center.

b. **Verification**

(1) S-Cal calibrator

Use the S-Cal calibrator to perform the final, precise calibration of the Model S-PLUS JR. After S-CAL calibration, verify calibration as described in the S-CAL Kit package insert.

Calibration is required for WBC, RBC, Hb, MCV, and PLT. The Coulter representative calibrates MPV. (See manual heading 6.4.7, "MPV Calibration with Latex.") Hct, MCH, MCHC, RDW, LYMPH #, MONO #, GRAN #, and LYMPH, MONO, and GRAN do not require calibration.

Before beginning, read the procedures on the package insert supplied with the S-CAL Kit. Use the S-CAL Kit, its package insert, and these instructions to calibrate WBC, RBC, Hb, MCV, and PLT.

When using the DT's calibration program, remember to move the cursor past the last digit entered in order to store the value.

(a) From the CALIBRATION MENU, select option 3, CALIBRATION PARAMETER SELECTION, to select the parameters to be calibrated: WBC, RBC, Hb, MCV, and PLT.

(b) Transfer to the DT the average calibration factors that are now in the main unit's memory as follows:

(i) From the CALIBRATION MENU, select option 1, RESULTS AND HISTOGRAMS.

(ii) On the main unit's keypad, press F, 1, 3, and ENTER. XMIT CAL FACTORS displays.

(iii) After the DT displays the average CAL factors, press PLOT on the DT keyboard. This prints a hard copy of the average CAL factors.

(c) From the CALIBRATION MENU, select option 4, OLD CALIBRATION FACTORS. Verify that these factors match the values printed; if not, enter them at this time. Press RETURN.

(d) Select the CALIBRATION MENU option 7, REFERENCE TABLE.

(i) If the table displays data from a previous run, go to option 8 and delete them.

(ii) On the REFERENCE TABLE enter the assigned values from the package insert supplied with your S-CAL Kit. Remember to move the cursor past the last digit.

(e) Select the CALIBRATION MENU option 5, CALIBRATION BATCH TABLE; if the table displays data from a previous run, go to option 6 and delete them.

(f) Prepare the S-CAL calibrator according to the instructions on the package insert.

(g) Select the DT PRIME mode. Cycle two samples of S-CAL calibration to prime the instrument.

(h) Select the CALIBRATION MENU option 1, RESULTS AND HISTOGRAMS, and cycle 10 samples of S-CAL calibrator. Between each cycle, mix S-CAL calibrator gently by inversion. The results are accumulated in the CALIBRATION BATCH TABLE. Monitor the display during each cycle.

- If any sample is unacceptable (for example, a short sample), use option 2 to delete it. The sample can also be deleted at the end of the 10 runs. Follow the CLEAR directions on the bottom of the CALIBRATION BATCH TABLE.
- The backlighted area at the bottom of the histogram display indicates the quantity of samples that have been cycled.

(i) Select the CALIBRATION MENU option 5, CALIBRATION BATCH TABLE. Inspect the 10 sets of results for trending and precision.

*Do not calibrate MCV if RBC % DIFF is out of range.*

(j) Use the CALIBRATION MENU option 3, CALIBRATION PARAMETER SELECTION, to eliminate any out-of-range parameters as follows:

(i) Cancel all parameter selections.

(ii) Select only parameters with acceptable criteria.

(iii) Return to option 5, CALIBRATION BATCH TABLE.

(k) If all the percent differences are within limits, plot the display or record the new calibration factors. Transfer the new calibration factors to the Main unit as follows:

(i) On the main unit keypad, press DIR.

(ii) Press ADV until CALIBRATE displays.

(iii) Press SEL and enter the SUPERVISOR code 8, 4, 2, and ENTER.

(vi) Use the numeric keypad to enter the new calibration factors. Press ADV to advance to the next parameter. When finished, press ENTER. Even if no change is necessary, use ADV to advance through all parameters; otherwise, the changes are not recorded.

(v) Follow the procedure in steps 2 and 3 to transfer the now old calibration factors to the DT for use in the next calibration.

(m) To verify calibration:

(i) Repeat steps d through g.

(ii) Select the CALIBRATION MENU option 5, CALIBRATION BATCH TABLE. The values in the FAC % DIFF row must be those shown in Table 3.

Table 3
Calibration Verification

| Parameter | Limit, % |
|---|---|
| WBC | ≤1.25 |
| RBC | ≤0.7 |
| Hb | ≤0.78 |
| MCV | ≤1.18 |
| PLT | ≤2.7 |

If any parameter is not within these limits, call the Coulter Service Center.

(2) Verification of the Hematocrit

Run a manual hematocrit once during each session or twice daily as a verification of the results obtained with S-PLUS JR. Also run manual hematocrits for all SPs if the S-PLUS JR is dysfunctional. To obtain the hematocrit, centrifuge a sample of EDTA blood at 10,000 rpm for 5 min. After centrifugation, read the volume of red cells as compared with the total volume of the sample measured on the microhematocrit reader.

Enter the results of both runs on the hematology data entry screen. If the results of both determinations do not match each other within ±1 or the Coulter HCT results within ±3, repeat the test and enter the results of the second two determinations on the hematology data entry screen.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) See that Coulter parameters are set as shown in Table 4.

(2) On the Main unit, check that the proper test number is entered. If necessary, press DIR, SEL, and ADV. If the test number is not correct, enter a number from the keypad and press ENTER. If the number is correct, press ADV.

b. **Sample Preparation**

(1) Place 2- or 3-mL lavender-top tubes on the Nutator mixer.

(2) Mix the sample well.

(3) Check the sample for clots. If clots are present, discard the sample.

(4) Analyze each sample within 20 min of collection. Do not wait until the end of the session to run the samples.

> *If it has been more than 1 hour since a blood sample was cycled through the instrument, you must prime it by cycling one sample of normal whole blood before resuming sample analysis.*

c. **Instrument Setup**

(1) Start up at the beginning of a stand

(a) Connect the waste container:

(i) Remove the protective cover from the pickup tube assembly.

(ii) Place the pickup tube assembly straight into the new waste container, and screw the pickup tube into position.

(iii) Check that the tubing to and from the new container is properly connected. (See Figure 5.4. of the Operations Manual.)

Table 4
Parameter Settings for Coulter Model S-PLUS JR

| Parameter | Settings |
|---|---|
| Power: | |
| Input | 115 ±10 vac, 50/60 Hz,3A 230 ±10 vac, 50/60 Hz,3A 100 ±10 vac, 50/60 Hz,3A |
| Temperature | 15.6-35 °C |
| Pneumatics: | |
| Vacuum | 20" Hg (minimum) |
| Pressure | 60 ± 2 psi |
| 30 PSI | 30 ± 1 psi |
| 5 PSI | 5 ± 0.25 psi |
| Recommended anticoagulant | $K_3$EDTA |
| Sample volume | 100 µL |
| Hemoglobin measurement: | |
| Wavelength | 525 nm |
| Bandwidth | 60 nm |
| Aperture length: | |
| RBC | 50 µm diameter, 60 µm L 100 µm |
| WBC | diameter, 75 µm L |

(b) Connect the diluent, detergent reagent, and lytic reagent containers as follows:

(i) Verify that the new reagent does not contain precipitate, turbidity, particulate matter, or unusual color. If any of these is evident, do not use the reagent.

(ii) Verify that the new container has been stored at 20-25 °C and that its contents have not been transferred from the original container.

(iii) Record in the Coulter Reagent Log the new container's lot number, expiration date, today's date, and technician ID number.

(iv) Remove the protective cover from the pickup tube assembly.

(v) Being careful not to touch the pickup tube or contaminate it in any other way, place the pickup tube assembly straight into the new container and screw it into position.

*Keep reagent lines from contact with electrical lines. Such contact could cause elevated background counts.*

(vi) Check that the tubing to and from the new container is properly connected. (See Figure 5.4 of the Operation Manual.)

(vii) Place another reagent container adjacent to the container just connected. This eliminates the possibility of the reagent being shaken prior to connection, thereby avoiding the introduction of bubbles into the diluter.

(viii) If a reagent or waste alarm locks up the instrument after five consecutive alarms, turn off the power to the main unit, then reset the instrument.

(c) Remove spacers from pinch valves.

(d) Power-Up procedure:

(i) Press the POWER button on the channelizing module (located on the right side) and then press the POWER button on the main unit (See Figure 3.1 of the Operations Manual). The POWER buttons will illuminate. Once the channelizing module power button is pressed, one can use the main unit power button to power up and power down both the main unit and the channelizing module. A flashing display accompanied by four beeps indicates that the pneumatic power supply is off. (Do not turn the pneumatic power supply on until you are ready to perform the daily startup procedures.) After four flashes, the DATE prompt appears.

(ii) Enter the date and test number, or press RES1 and RES2 simultaneously to reset the unit. The display changes to SELECT FUNCTION.

(iii) Allow the main unit to warm up 30 min before running patient samples or controls.

(iv) Press the POWER button on the DT.

(v) Open the pneumatic power supply front door; press the POWER button.

(vi) Check that the gauges indicate the required values. The correct readings are listed in Table 5.

Table 5
Gauge Pressures

| Gauge | Pressure |
|---|---|
| Vacuum | 20" Hg (minimum) |
| Pressure | 60 ±2 psi |
| 30 PSI | 30 ±1 psi |
| 5 PSI | 5 ±0.25 psi |

(vii) Close the front door of the pneumatic power supply.

(viii) Open the dilutor front door.

(a) Move the OPTIC LAMP switch to the ON position.

(b) Verify that the top of the red indicator in the electronic manometer is within the green operating range.

(c) Close the dilutor front door.

(ix) Prime the reagents:

(a) Press STARTUP to activate the SWEEP FLOW function by pressing F, 0, 3 and ENTER. Press DRAIN and then press RINSE.

(b) For the lytic reagent, press DRAIN until the baths are empty; press and release LYSE until the lytic reagent supply line and pump are completely filled and free of bubbles. Press DRAIN and then press RINSE.

(c) Turn on the printer/plotter and place on line.

(d) Access the Startup Menu on the data terminal by pressing 3, ENTER, 5, ENTER. To enter the lot numbers and expiration dates of reagents into the DT, from the main menu, press 2, ENTER, then 3, ENTER.

(e) Perform the daily startup procedures (Section 8.c.2.).

(f) Perform the calibration procedures (Section 8.c.3.).

(g) Document the performance of the daily and stand startup procedures in the Scheduled Cleaning Procedures and Operational Checks Log; be sure to record the date and tech ID number.

(h) Keep a copy of the printout from each of the stand startup procedures in the MEC Laboratory files and send the copies to NCHS at the end of the stand.

(2) Daily Startup Procedures

(a) Set the date and test number.

Press DIR to display the first directory option. Press SEL and enter the month, day, and year, then press ADV and enter the test number. When finished, press ENTER.

(b) Open the pneumatic power supply front door, press the POWER button and check that the gauges indicate the required values. The correct readings are the samwe as in Table 5.

(c) Close the pneumatic power supply front door and open the diluter front door.

(d) Move the OPTIC LAMP switch to the ON position.

(e) Verify that the top of the red indicator in the electronic manometer is within the green operating range.

(f) Close the dilutor front door.

(g) Turn on the printer/plotter and place it on-line.

(h) From the MAIN MENU on the DT, press 2, ENTER, and press 1, ENTER. Enter three-digit operator ID number.

(i) From the MAIN MENU on the DT, press 3, ENTER; 1, ENTER; and then again 1, ENTER.

(j) On the Main Unit, verify that SELECT FUNCTION appears on the display.

(k) On the Main Unit keypad, press START UP to observe the automatic startup cycle, and verify that results are acceptable. A system check and background count are automatically run at the end of the STARTUP sequence. Press PLOT on the DT keyboard. Save the printout for the MEC files to be sent to NCHS at the end of the stand.

(l) If reagent lot information is required from the STARTUP MENU on the DT, press 3 and ENTER. Then press PLOT to obtain a printed copy. Save the printout for the MEC files to be sent to NCHS at the end of each stand.

(m) Prime the instrument by cycling a normal, whole-blood sample as follows:

(i) On the DT, access PRIME from the MAIN MENU; then press 6 and ENTER.

(ii) Hold the mixed priming sample to the aspirator tip with the tip submerged in the sample.

*Do not remove the sample until the display reads WIPE TIP.*

(iii) Press the WHOLE BLOOD button and hold the sample in position until the display changes from ASPIRATING to WIPE TIP.

*Do not exert force on the aspirator tip; it may cause it to bend.*

*Remove the sample container and be clear of the area around the aspirator tip before the display indicates BACKWASH.*

(iv) With a vertical motion, wipe the aspirator tip with a clean, lint-free tissue.

(n) Observe the dilutor during the rest of the cycle.

(o) Record the performance of daily startup procedures in the Scheduled Cleaning Procedures and Operational Checks Log. Locate the column headed "Startup Procedures." Record the date you performed the procedures and your tech ID number.

(3) Daily Calibration/Verification with S-Cal Calibrator

Verify the Coulter S-PLUS JR calibration using the S-Cal Calibrator at the beginning of each session after the daily startup procedures. The purpose of this verification is to monitor the accuracy of the Coulter S Plus JR and to correct for the variation in the results produced by the three instruments at three different locations over a 6-year period. Results of the daily calibration verification with the S-Cal Calibrator are reported to Coulter headquarters via telephone and compared with results obtained by Coulter Electronics instruments in Hialeah, FL. The results from each Coulter S-PLUS JR in the MEC laboratories must be within specified limits before the laboratory may run SP specimens. Correction factors are derived for each instrument and applied to the SP results to approximate zero bias in the data.

The S-Cal Calibrator is cycled through the Coulter 10 times, results are plotted, and the data are reported to Coulter Electronics. Results are reported on weekdays only. When daily calibration verification is performed on weekends, the results are saved and reported to Coulter on the next weekday. When the results of the verification of the calibration are within the specified limits, the startup procedures are continued with the 4 C Plus Quality Control Check. On weekends, the startup procedures are continued automatically.

Follow the steps listed below to perform the daily verification and reporting procedures:

(a) Remove a vial of S-Cal Calibrator from the refrigerator and allow it to reach 20-25 °C. Label the vial with the date opened.

(b) Select the DT PRIME mode. Cycle one sample of S-Cal Calibrator to prime the instrument.

(c) From the Coulter DT, select the Main Menu and press 4 and ENTER.

(d) Delete previous values in CONTROL FILE 9. Return to the Main Menu.

(e) Access the CONTROL DATA FILE SELECTION display: press 2 and ENTER, then 4 and ENTER.

(f) Select FILE 9 for daily S-Cal calibration. Set the Batch size equal to one.

(g) Enter the lot number and the expiration date of the S-Cal Calibrator. Return to the Main Menu.

(h) Press 4 and ENTER, then 1 and ENTER. Select the SYSTEM READY FOR CONTROL FILE 9.

(i) Mix S-Cal gently and cycle 10 times, remixing between each sample.

(j) Verify that the values for each parameter are within the limits specified on the assay sheet.

(k) Print the RBC Profile and the WBC-Platelet Profile tables.

(l) Refer to the printed copy when reporting the results by phone to a Coulter Electronics service representative.

(m) Report the service number of the Coulter S-PLUS JR; the S-Cal lot number; the day, date, month and year; the mean and standard deviation; and the number of runs for WBC, RBC, Hb, MCV, PLT, and MPV.

(n) If all the reported parameter values are within limits, the Coulter representative will approve proceeding with the analysis of controls and SP samples. If the values are not acceptable, the Coulter representative may request that the calibration verification be repeated to recalibrate the Coulter.

(o) When all of the parameters receive an "assurance" response, the daily calibration verification is finished.

(p) Keep a copy of the RBC Profile and WBC-Platelet Profile Tables in the MEC files to be sent to NCHS at the end of each stand.

d. **Operation of Assay Procedure**

(1) From the SAMPLE ANALYSIS MENU, access the LOAD LIST via 6, ENTER. Use this function to record the laboratory's worklist. This display includes the following:

(a) DATE [1]. This field displays the date the sample was cycled, as set on the main unit. To edit, move cursor to command line **DATE** field, and press the down arrow. Use left and right arrows to reach desired position.

(b) LAB NUMBER [2]. There is space available for six digits.

- The AUTO-NUMBERING option is enabled and the label number, once set, is automatically generated sequentially. Do not edit a lab number while cycling because that can disrupt the numbering process.
- When storage space for fewer than 30 samples remains, a warning message beneath the LO/HI/DATE fields indicates the number of remaining locations. When the storage space is full, the system does not accept additional entries.
- The lab number can be edited from either the LOAD LIST or REVIEW/EDIT.
- After a lab number and ID number have been entered, erase these fields using CLEAR. This does not delete the line; a blank field is valid and results are assigned to that line. To delete the line, assign it a fictitious lab number and delete as described in Output Options available via LOAD LIST, heading 2.9, Daily: LOAD LIST of the Operations Manual.

(c) ID NUMBER [3]. There are 11 spaces available for the examinee identification number. A decimal point entered as part of the ID number is displayed as a dash. If no ID number is assigned, the display in the ID NUMBER field is blank. This field may be edited.

*Samples must be cycled in the same sequence as they are assigned on the LOAD LIST.*

(d) Verify that the printer/plotter is on and ready to print.

(e) Verify that the instrument is primed.

(f) From the MAIN MENU, press 1, SAMPLE ANALYSIS.

(g) Verify that SELECT FUNCTION appears on the display.

(h) From the SAMPLE ANALYSIS MENU, press 1 and ENTER.

(i) Gently remix the sample.

(j) Hold the sample up to the aspirator tip with the tip submerged toward the bottom of the sample container.

*Do not remove the container from the aspirator tip until the display changes from ASPIRATING to WIPE TIP.*

(k) Press the WHOLE BLOOD button to begin the operating cycle.

*Remove the sample's container and be clear of the area around the aspirator tip at the time the display indicates BACKWASH.*

(l) Remove the sample's container as soon as WIPE TIP displays. Use a clean, lint-free tissue to wipe the excess sample from the aspirator tip.

(m) Observe the diluter and the display during the remainder of the operating cycle.

(n) Check the display at the end of the final count period for a voting matrix. Its presence indicates a voteout of parameter results from one or more of the count periods. Voteouts are indicated by asterisks.

(o) Monitor the display for any alarms or warnings at the end of the diluter cycle. Review the results using the hematology reference ranges provided in Section 13. If the results are out of limits, rerun the specimen. If the results are still outside the action limits, report the results to the physician immediately.

(p) When the results are printed, remove them from the printer.

(q) If a voting matrix appears in the display, press RES1 and RES2 simultaneously to restore the SELECT FUNCTION display. After 3 sec, the SELECT FUNCTION display appears automatically.

(r) Repeat the entire procedure for each sample. If there is an insufficient sample for a second run, save the data from the first run. Document that the specimen volume was inadequate for a duplicate run.

(s) Use the chart below to determine whether or not the duplicate values for RBC, WBC, Hb, and PLT are within the stated precision limits. If they are, continue to step (t). If not, rerun the sample in duplicate. If the results are still outside the limits, resample the 4C Plus control before analyzing the sample again.

RBC: $0.2 \times 10^6$
WBC: $0.4 \times 10^3$
Hb: 0.2 grams %
PLT: $\pm$5%

(t) The $X_B$ analysis is enabled. When 20 samples have accumulated, an alarm will sound, signaling that a batch has been completed. Press any key to stop the alarm. Print the current $X_B$ values for the RBC profile and the WBC - X-PLT profile. Store the printouts in the MEC laboratory files. Ship them to NCHS at the end of the stand.

(e) **Recording of Data**

(1) Quality Control Data

Perform quality control checks using 4C Plus control material at the beginning of each session to verify that the instrument produces accurate results.

Every time a new lot number of 4C Plus control material is opened, the operator must plot graphs of the daily values for each level of control of the previous lot numbers, clear the control files of the previous lot number data, and enter the assay and limitation values for the new lot numbers as follows:

(a) To enter assay and limitation values, access the CONTROL DATA: ASSAY VALUES AND LIMITS displays via 4, ENTER from the DATA ENTRY MENU. Select the file number. For each control file, enter the following data. To store the data, remember to press ENTER or an arrow key after the data is entered.

(i) FILE # [1] (1 through 9).

(ii) CONTROL TYPE [9]. Refer to the legend on the display and enter the number corresponding to the control type for the file for entering data. For example, to enter NORMAL as the control type, press 1, ENTER; NORMAL appears after CONTROL TYPE. (File 1=Normal, File 2=Low, File 3=High).

If 4 is pressed, the space next to CONTROL TYPE is a blank.

(iii) LOT # [8]. Enter the lot number of the control. If the lot number is less than seven digits, enter leading zeros to fill the extra spaces.

(iv) EXP. DATE [7]. Enter the six-digit expiration date of the control using zero for a blank. The slashes appear automatically when the date is stored in memory via ENTER or an arrow key.

(v) BATCH SIZE [6]. Enter 2 for the number of control samples to be included in a batch. All controls are run in duplicate.

(vi) The HOSP ID, DATE, and OPR [2] number are displayed, but not entered. HOSP ID appears as entered via DATA ENTRY, option 1. DATE corresponds to the date that any entry in a particular file was last changed. OPR indicates the number of the operator who made that change.

(vii) ASSAY VALUE [5]. Enter the assay value of each parameter, from the assay sheet supplied with the control. For the differential parameters, enter values for both percent and number; change mode via SYSTEM CONFIGURATION, as described in Lab Action Limits.

(viii) SAMPLE LIMIT [4]. Enter the control sample's limits for each parameter. Use the cell control's assay sheet as a guide.

(ix) BATCH LIMIT [3]. Use the same limits used for SAMPLE LIMIT.

(b) To plot graphs, access the graph options from MAIN MENU by pressing 4, ENTER, and then 10, ENTER. Plot all five graph options for each control level. Store the plots in the MEC laboratory files until the end of the stand and then send them to NCHS.

(c) To clear the control files, access the MAIN MENU and press 4, ENTER, then 11, ENTER. Select the file number and delete old data.

Prime the S-PLUS JR with one sample of normal whole blood before running control materials. Label

each vial of control with the date opened. Use the following procedures to run controls.

(d) From the DT MAIN MENU, press 4 and ENTER to access the CONTROL DATA MENU.

(e) Press 1 and ENTER to access the control DATA FILE SELECTION display.

(f) Follow the directions on the D5 screen to select the file for the lot number of the control.

(g) Press ENTER. The SYSTEM READY FOR CONTROL FILE (#) message will appear.

(h) Cycle each control and monitor the results as they appear on the display. Run each control in duplicate. Cycle the controls in this order: low, normal and high. An H or L (high or low) appears to the right of any parameter value not within the control sample limits. CB LIMIT (IN, OUT) indicates whether the last control batch was within the batch limits.

(i) The parameter results are automatically stored in the CONTROL FILE.

(j) The results will be plotted automatically on the Coulter printer-plotter. Save the histograms in the MEC files to be sent to NCHS at the end of the stand.

(2) Analytical Results

The Coulter S-PLUS JR data terminal (Coulter DT) is interfaced directly to the MEC automated system (VAX). Transmit all SP results collected during a session from the Coulter DT to the VAX at the end of the session. When the transmission is complete, review the results from the VAX system to determine the completeness and the accuracy of the transmission. If the results are inaccurate or incomplete, repeat the transmission procedure. If, after repeating the procedure, the results are still inaccurate or incomplete inform the MEC manager. If the results of the transmission are acceptable, transfer the Coulter results from the VAX system to the Laboratory Data Base Management System (Oracle). When the Coulter data are in Oracle, access the Hematology Data Entry Screen to confirm that duplicate Coulter determinations have been performed for every SP who has given a blood or urine sample; if duplicate determinations were not done, enter a comment to explain the reason.

f. **Replacement and Periodic Maintenance of Key Components**

Refer to the maintenance section of the Coulter S-PLUS JR operations manual for cleaning, bleaching, and maintenance procedures. Record technician initials and the date the procedure was performed in the Scheduled Cleaning Procedures and Operational Checks Log. For optimum instrument performance, perform the Daily Startup Procedures after doing any of the maintenance procedures.

g. **Calculations**

The Coulter Counter Model S-PLUS JR with Coulter histogram differential is a quantitative, automated hematology analyzer and is intended for determining 16 hematologic parameters. The instrument automatically measures, computes, derives, or expresses these parameters as the coefficient of variation.

(1) The directly measured parameters are WBC, RBC, and hemoglobin (Hb).

(2) The derived parameters with their formulas are:

(a) MCV: derived from the RBC histogram

(b) PLT: derived from the PLT histogram

(c) MPV: derived from the PLT histogram

(d) Lymph percent: derived from the WBC histogram

(e) Mono percent: derived from the WBC histogram

(f) Gran percent: derived from the WBC histogram

(3) The computed parameters with their formulas are:

(a) hematocrit: (RBC x MCV) ÷ 10

(b) MCH: 10 x Hb/RBC

(c) MCHC: 100 x Hb/Hct

(d) Lymph #: (Lymph percent ÷ 100) x WBC

(e) Mono #: (Mono percent ÷ 100) x WBC

(f) Gran #: (Gran percent ÷ 100) x WBC

(4) RDW: The size distribution of the RBC populations expressed as the coefficient of variation of red cell distribution.

h. **Special Procedure Notes**

There are no special procedure notes for this assay method.

## 9. REPORTABLE RANGE OF RESULTS

Table 6 shows the ranges that Coulter Electronics cites for the Model S-PLUS JR (1).

Table 6
Ranges and Limits for Coulter S-PLUS JR

| Parameter | Linearity Range | Limits (whichever is greater) |
|---|---|---|
| WBC x $10^3$ cells/µL | 0 to 99.9 | 0.4 or 3.0% |
| RBC x $10^6$ cells/µL | 0 to 7.00 | 0.04 or 2.0% |
| Hb g/dL | 0 to 25 | 0.2 or 2.0% |
| MCV fL | 50.0 to 200.0 | 3.0 or 3.0% |
| PLT x $10^3$ cells/µL | 0 to 999 | 10 or 7.0% |
| MPV | 5 to 20 | 1.0 or 3.0% |

Linearity only affects the measured parameters, not the calculated parameters such as the indices.

## 10. QUALITY CONTROL (QC) PROCEDURES

The NHANES III QC system monitors each of the parameters indicated. Each parameter will be monitored carefully, through a QC program that is reliable and easily understood, implemented, documented, and surveyed. Each QC program should also incorporate periodic surveillance methods and achieve precision and reproducibility.

The QC system includes both internal and external surveillance and monitors the following five parameters:

- Clerical Entries
  Includes properly documented acknowledgement of transmittal and receipt of specimens (i.e., "logging in"), proper labeling of all specimens, correct assignment of laboratory values to the proper S.P. ID number, and maintenance of proper records from all specimens for future reference.
- Reagents and Materials
  Include confirmation of the values of commercial standards and controls before they reach the bench and the proper labeling of all reagents, particularly those prepared in the laboratory; the ensurance that all reagents in use

are not outdated; the possession of an adequate supply of current reliable reagents; the proper calibration of equipment, such as pipettes; and the proper washing of glassware.

- Techniques
  Include continued ensurance that all personnel performing an assay understand the principles underlying a particular assay and the proper technique for that assay; that all personnel use the same technique for a particular assay; that there is ready access to a current technique manual, and that periodic reviews are undertaken to ensure that personnel use the most current and reliable techniques.
- Bench Performance
  Includes the use of controls and standards for each assay performed; a technique based on sound statistical principles, which allows the technologist performing the assay to detect error outside of previously determined limits before reporting data; documentation of daily bench performance for detection of less obvious error (particularly those that tend to accumulate over time, so-called "drift"), and established procedures to be followed wherever error is found to exceed previously determined limits.
- Instrumentation
  Includes periodic preventive maintenance of all instruments in use in the laboratory and documentation that each instrument is maintaining a previously determined level of each performance at each check.

The reliability of analytical values obtained with a procedure often depends on the quality of the standards and the calibration procedure employed. The College of American Pathologists (CAP) suggests that automated instruments be calibrated by using multiple analytical whole blood specimens or a certified, stabilized whole-blood type preparation. The International Committee for Standardization of Hematology (ICSH) requires that a calibrator "be based on or traceable to a reference preparation or material" (2).

Coulter Electronics provides the S-Cal calibrator, which is used to calibrate the Coulter S-PLUS JR at the beginning of each stand and to verify calibration daily. The S-Cal calibrator values for WBC, RBC, Hb, MCV and PLT parameters are derived by replicate analysis of fresh whole blood samples on serial S-PLUS and S series instruments. These assigned values are traceable to reference methods with an accuracy to within $\pm$ 2%.

The 4C Plus Coulter Counter Cell Control is a modified whole blood hematology reference control prepared from fresh human blood. The control has seven stable values. When used with Coulter's blood diluent, Isoton III, it serves as a check on the accuracy of dilution, WBC counts, RBC counts, and hemoglobin determinations. Mean assay values include such variables as:

- Day-to-day variation
- Different analysts
- Different instruments
- Different glassware
- Different batch numbers of test reagents
- A representative sampling, 3% of the production run of 4C.

The allowable limit of variation from the mean value, $\pm$2 standard deviations, represents the precision obtained from routine work.

4C Plus must be run at the beginning of each session to monitor the precision of the instrument, reagents, and technologists.

A planned maintenance program for the Coulter S-PLUS JR includes scheduled cleaning procedures and operation checks that are performed as appropriate on a daily, weekly or monthly basis and documented in the Scheduled Cleaning Procedures and Operations Checks Log of the Quality Control Notebook. Use of Coulter reagents is monitored and documented in the Coulter Reagent Log of the Quality Control Notebook. Any malfunction or repair of Coulter S-PLUS JR equipment is documented in the Coulter Action Log.

The following printouts for the MEC files are to be sent to NCHS at the end of the stand: background check, system check, carryover check, table of average factors, histograms, calibration batch table, 4C control values, and X bar tables.

Four times a year, NCHS receives CAP samples for hematology proficiency testing. Each quarterly shipment includes two 3-mL whole blood specimens. Duplicate Coulter determinations are performed on each whole blood sample. Record the results on the CAP forms provided. Send the completed forms to CAP Surveys Program Support.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Recalibration is necessary when replacing any component that involves the dilution characteristics (such as the blood sampling valve) or the primary measurements (such as an aperture).

If recalibration appears necessary, but no component affecting calibration has been replaced, do NOT recalibrate the instrument. First, check the control sample and then call the Coulter Zone Service Center.

Although the Model S-PLUS JR is relatively insensitive to ambient temperature changes, the calibration should be performed within 20-25 °C. If the ambient temperature varies by >10 °C, then verification and possible recalibration is necessary.

In the normal process of tracking data for an extended period of time, Coulter or NCHS may make a specific decision to recalibrate a given parameter. Never adjust to a specific value for an individual sample.

If any problems or malfunctions are encountered while performing the procedures in this section, see Section 5 of the Coulter Counter Product Reference Manual. If the corrective procedures fail to eliminate the problem, call the Coulter Zone Service Center.

Document the performance of unscheduled calibration procedures in the Coulter Action Log. Record the date, the condition of the Coulter and Tech ID number whenever a situation arises that requires the unscheduled performance of the calibration procedures. Record the date, the action taken, and the technician ID whenever the calibration procedures are performed.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

This method is limited to human whole blood samples. This instrument provides a 3-cell differential (granulocytes, monocytes, and lymphocytes). Extreme temperatures and high humidity can cause instrument malfunction.

The publication *How to Handle Abnormal Blood Results for Coulter Counter Instruments with Histogram Differential* lists interfering substances, parameters that can be affected by those interferences, and the procedures to use when trying to resolve abnormal results (3). It is important to note that not every parameter is always affected. The section numbers below refer to this manual.

Be aware that the data from a substance may reflect multiple interfering factors. Decide how to handle such a specimen, since a particular procedure corrects only the associated interference.

(a) Abnormal BUN, Glucose, or Na levels could affect the MCV. See procedure in Section 5.27.

(b) Abnormal WBCs could affect lymphocytes, monocytes, and granulocytes. See procedure in Section 5.6A.

(c) Abnormally small WBCs could affect white count and lymphocytes, monocytes, and granulocytes. See procedure in Section 5.22.

(d) Clumped platelets could affect white count and lymphocytes, monocytes, granulocytes, RBC, MCV, RDW, platelet count, and MPV. See procedure in Section 5.17.

(e) Cryofibrinogen and cryoglobulins crystals could affect white count, lymphocytes, monocytes, granulocytes, RBC, hemoglobin, platelet count, and MPV. See procedure in Section 5.19.

(f) Elevated WBC count could affect RBC, hemoglobin, MCV, RDW, platelet count and MPV. See procedure in Section 5.3B.

(g) Fragile WBCs could affect white count, lymphocytes, monocytes, granulocytes, platelet count, and MPV. See procedure in Section 5.22

(h) Giant platelets could affect white count, lymphocytes, monocytes, granulocytes, RBC, MCV, RDW, platelet count, and MPV. See procedure in Section 5.16.

(i) Hemolyzed specimen could affect RBC, hemoglobin, platelet count, and MPV. See procedure in Section 5.24.

(j) Lipemic specimen could affect MCV. See procedure in Section 5.25.

(k) Micromegakaryocytes could affect white count, lymphocytes, monocytes, granulocytes. See procedure in Section 5.29.

(l) Nucleated RBCs could affect white count, lymphocytes, monocytes, granulocytes, and hemoglobin. See procedure in Section 5.18.

(m) Platelet satellites could affect white count, lymphocytes, monocytes, granulocytes, platelet count and MPV. See procedure in Section 5.23.

(n) RBC agglutination could affect white count, lymphocytes, monocytes, granulocytes, RBC, hemoglobin, MCV, RDW, platelet count and MPV.. See procedure in Section 5.20.

(o) RBC fragments could affect RBC, MCV, RDW, platelet count, and MPV. See procedure in Section 5.21.

(p) RBCs resistant to lysing could affect white count, lymphocytes, monocytes, granulocytes, and hemoglobin. See procedure in Section 5.26.

(q) Rigid RBCs could affect MCV and RDW. See procedure in Section 5.28.

(r) Small RBCs could affect RBC, MCV, RDW, platelet count, and MPV. See procedure in Section 5.21.

(s) WBC fragments could affect white count, lymphocytes, monocytes, and granulocytes, platelet count, and MPV. See procedure in Section 5.22.

## 13. REFERENCE RANGES (NORMAL VALUES)

Table 7
Hematology Reference Ranges (1)

| Parameter | Range |
|---|---|
| WBC (x $10^3$/µL) | 3.0-11.7 |
| RBC (x $10^6$/µL) | 3.7-5.8 |
| Hb (g/dL) | 10.9-17.3 |
| Hct (%) | 32.5-49.5 |
| MCV (fL) | 73.0-101.0 |
| MCH (pg) | 25.0-34.3 |
| MCHC (g/dL) | 31.2-37.0 |
| RDW (%) | 11.8-14.8 |
| PLT (x $10^3$/µL) | 200,000-400,000 |
| MPV (fL) | 6.5-12.5 |
| LYMPH (%) | 20.5-51 |
| MONO (%) | 1.5-9.5 |
| GRAN (%) | 42-75 |

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

The physician in the MEC reviews each participant's hematology results before she/he leaves the MEC. Laboratory

referral values will focus on, but are not limited to Hb, WBC, and PLT. The reference ranges have been set for those values that require an immediate phone call from the MEC physician to the participant's medical provider and those that are abnormal but do not require a phone call to the provider.

Table 8 shows the laboratory reference levels for physician referral requiring telephone follow-up to a medical provider.

Table 8
Hematology Results Requiring Telephone Followup (Level I)

| Test | Abnormal Range |
|---|---|
| Hb | All ages: <6.5 g/dL. No upper value set |
| WBC | All ages: <1,500; >20,000 |
| PLT | All ages: <50,000; >600,000 |

A Physician's Referral Letter of Findings containing results requiring early medical care is sent to the participant's medical provider. Table 9 shows the laboratory reference levels for a Physician's Referral Letter of Findings.

Table 9
Hematology Results Requiring Physician's Referral Letter (Level II)

| Test | Age (years) | Abnormal Range, Female | Abnormal Range, Male |
|---|---|---|---|
| Hb, g/dL | 1-2: white | <10.5; >13.6 | <10.5; >13.6 |
| | 1-2: black | <9.7; >12.8 | <9.7; >12.8 |
| | 3-14 | <10.9; >14.5 | <11.1; >14.7 |
| | 15+ | <11.3; >15.7 | <12.9; >17.3 |
| WBC | 1-2 | <6,000; >17,500 | <6,000; >17,500 |
| | 3-14 | <3,300; >11,700 | <3,500; >11,000 |
| | 15+ | <3,000; >11,300 | <3,300; >11,300 |
| PLT | 1+ | <200,000; >400,000 | <200,000; >400,000 |

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

The specimens are kept at 20-25 $^{o}$C during the testing process.

## 16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no alternative methods for this method. The lavender-top tubes are stored at 4-8 $^{\circ}$C until the instrument is restored to functionality.

## 17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

(a) Follow the procedures to review the laboratory values for referrals to outside medical care.

(1) Check the Coulter print-out for participant identification. The participant's NCHS number should appear on the print-out.

(2) Review the laboratory values for each participant and compare them with the reference ranges in Section 14.

(3) For Level I and II referrals, follow detailed instructions in the Physician's Procedure Manual.

(4) For Level I referrals, the provider of the participant's source medical care is called. Complete the Referral and Log Telephone Contact screens in the MEC automated system, the Physician's Log, the Incident/Emergency Report, and the MEC Emergency Recording Form.

(5) For Level II referrals, a Physician's Referral Letter of Findings containing results requiring early medical care is sent to the participant's medical provider. Complete the Referral and Log Referral Letter screens in the MEC automated system and the Physician's Log.

(6) Copies of all forms are sent to NCHS.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard records (e.g., computer data files and hard copies) should be used to track specimens. We recommend that records, including related QA/QC data, be retained for 6 years, and that duplicate records be kept. Only numerical identifiers should be used (e.g., NCHS ID numbers).

19. **Quality Control Summary Statistics and Graphs**

**Because these were assayed controls with short 4-6 week expiration dates no statistics were accumulated for qc summary statistics or monthly qc graphs.**

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Homocysteine (HCYS) is an amino acid formed during the conversion of methionine to cysteine. Metabolism of HCYS involves two metabolic pathways: 1) transulfuration with vitamin $B_6$ cofactor and 2) remethylation to form methionine, for which a folic acid derivative is the methyl donor and vitamin $B_{12}$ is the cofactor (1). The assay used measures total HCYS, both reduced and oxidized forms, thus the term homocysteine is appropriate.

HCYS in serum is dissociated from nonrelevant proteins and other disulfides by reduction with tributylphosphine (TBP). The proteins are precipitated by using 0.6 M perchloric acid (PCA), and the sulfhydryl amino acids then react with the fluorescent reagent, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBDF). HCYS is quantified by using reverse-phase high-performance liquid chromatography (HPLC) and fluorescence detection (2).

Hyperhomocysteinemia, a condition of disrupted HCYS metabolism, is an important health risk factor. One of the main objectives of this study is to determine the distribution of HCYS concentration and the prevalence of elevated HCYS in the United States among white, African-American, and Hispanic men and women in six adult age groups.

## 2. SAFETY PRECAUTIONS

All safety procedures and regulations must be observed according to the guidelines set forth by Tufts University. Lab safety manuals outlining these policies are located in the laboratory. All employees are required to attend an employee safety training seminar prior to working in a laboratory environment.

Consider all serum specimens received for analysis potentially positive for infectious agents, including HIV and the hepatitis B virus. We recommend the hepatitis B vaccination series for all analysts working with whole blood or serum. Observe universal precautions: Wear lab coats, safety glasses, and protective gloves during all steps of this method.

> *Exercise particular care when dealing with TBP because of its explosive/corrosive nature. When ordering, it must always be transported by truck. When drawing it from the bottle, purge the syringe with nitrogen. Prior to withdrawing any TBP, first insert an equal or greater volume of nitrogen into the bottle.*

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Information from an analytical run is gathered and handled by Waters Expertise Chromatography Software, Version 3.1 (July 5, 1993). The data are stored on the main VAX cluster (Digital Inc.) of the Human Nutrition Research Center at Tufts, and a hard copy of the chromatogram and results are kept on file in the laboratory. Data will be transferred to a SPSS data file so that the results may be organized and statistical analysis performed. QC data are recorded daily in the laboratory logbook and then transferred to a QC file in SPSS.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

EDTA plasma and serum from fasting subjects, are suitable specimens; however, plasma is preferred. To prevent HCYS values from becomig falsely elevated, separate plasma from the red blood cells immediately after the samples are collected. Specimens should be stored frozen in cryotubes at $\leq$-70°C until assayed.

For the NHANES project, freshly collected plasma samples were not available; therefore, serum samples were collected and processed according to the survey S.O.P. Analyses prior to our receipt of leftover serum necessitated in thawing the samples one to three times. However, the samples were sent frozen and have been kept at -80°C until analysis for HCYS.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. PREPARATION OF REAGENTS, CALIBRATORS (STANDARDS), CONTROLS, AND ALL OTHER MATERIALS; EQUIPMENT AND INSTRUMENTATION

a. **Instrumentation**

(1) Waters 717+ autosampler (Waters Chromatography, Division of Millipore Corp., Milford, MA).

(2) Waters 510 solvent delivery systems (Waters Chromatography).

(3) Fluorescence detector, model no. 98000 (Applied Biosystems, Foster City, CA).

(4) Waters Expertise chromatography software, Version 3.1 (July 5, 1993) (Waters).

(5) Keystone Scientific ODS Hypersil column, 4.6- x 250-mm, cat. no. 255-33 (Keystone Scientific Inc., Bellfonte, PA).

(6) Eppendorf micro-centrifuge (Brinkman).

(7) Waterbath (Blue M Electric Co., Blue Island IL).

(8) pH meter (Corning Inc., Corning, NY).

(9) SP vortex mixer (Baxter, Stone Mountain, GA).

b. **Materials**

(1) Tributylphosphine, reagent grade, approx. 90% (Sigma Chemical, St. Louis, MO).

(2) Dimethyl formamide, reagent grade (Sigma).

(3) Perchloric acid, A.C.S. certified reagent (Aldrich Chemical, Milwaukee, WI).

(4) Disodium ethylenediamine tetraacetate (EDTA), A.C.S.-certified reagent (Fisher Scientific, Springfield, NJ).

(5) Boric acid, A.C.S. certified reagent (Fisher).

(6) 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBDF), reagent grade (Sigma).

(7) Sodium acetate, reagent grade (Sigma).

(8) Sodium hydroxide, reagent grade (Sigma).

(9) Methanol, HPLC grade (Fisher).

(10) Glacial acetic acid, HPLC grade (Aldrich).

(11) DL-Homocysteine, reagent grade (Sigma).

(12) Hydrochloric acid, A.C.S. certified (Fisher).

(13) Eppendorf premium flex-tubes, cat. no. 22 36 411-1 (Brinkman).

(14) Injection vial with a microvolume insert, cat. no. 200 668 (Sun Chromatography).

(15) Double-distilled water.

(16) HA filter (Millipore).

c. **Reagent Preparation**

(1) Mobile phase

(a) Solution 1. Add 11.5 mL of glacial acetic acid to 1 L of deionized distilled $H_2O$.

(b) Solution 2. 0.20 M sodium acetate trihydrate. Dissolve 27.22 g of sodium acetate in 1 L of $ddH_2O$ to obtain a final concentration of 0.20 M.

Solutions 1 and 2 may be stored refrigerated for at least 1 month.

(c) Working mobile phase. Measure the pH of 1 L of the glacial acetic acid solution (prepared in step (a) above). Gradually add, while stirring, the sodium acetate solution (prepared in step (b) above) until a pH of 4.0 is obtained. Add 20 mL of methanol per liter of mobile phase to the bottom of the filtration flask. Filter using an HA filter (Millipore). The mobile phase is stable for about 1 week at 20-25 °C, and for about 1 month at 4-8 °C.

(2) 2.5 M Boric acid, 4 mM EDTA -- pH 9.5 and 10.5

(a) pH 9.5. Dissolve 0.3723 g of EDTA in 200 mL of $ddH_20$. Add 38.69 g of boric acid. Adjust the pH to 9.5 by adding NaOH pellets (30±7 pellets). Bring the volume to 250 mL with $ddH_20$ in a volumetric flask.

(b) pH 10.5. Dissolve 0.7445 g of disodium EDTA in 400 mL of $ddH_2O$. Add 77.3875 g of boric acid. Adjust the pH to 10.5 by adding NaOH pellets (30±7 pellets). Bring the volume to 500 mL with $ddH_2O$ in a volumetric flask.

Over time, a precipitate will form in each of these solutions, but it will not interfere with the assay. These reagents are stable for at least 3 months at room temperature.

(3) 0.6 mol/L perchloric acid (PCA) with 1mM EDTA solution
Add 186 mg of disodium EDTA to 200 mL of $ddH_2O$ and stir until dissolved. Add 24.6 mL of 70% PCA to the EDTA solution, mix, and adjust the volume to 500 mL. This reagent can be stored at room temperature, and it is stable for at least 3 months at 20-25 °C.

(4) SBDF reagent
On the day of analysis, dissolve 5 mg of 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBDF) in 5-mL of pH 9.5 boric acid in a 15-mL Corning tube.

(5) 10% tributylphosphine (TBP) in dimethyl formamide (DMF)
Prepare this reagent the day of the run after the samples are aliquotted. It is a reducing agent that cleaves all the disulfide compounds into free sulfhydryls. (CAUTION: TBP is extremely air-sensitive. To remove 1 mL of TBP from the bottle, fill a 3-mL syringe with 1.5 mL of He or $N_2$ and inject this gas through the bottle septum. Then withdraw 1 mL of the solution from the bottle.)

Removing the 1 mL of TBP in this manner maintains a positive pressure in the bottle, preventing air from contacting the TBP. Add the 1 mL of TBP in the syringe to 9 mL of DMF in a 25-mL Erlenmeyer flask that has been flushed with He or $N_2$. The flask need not be capped at this time, but should be covered with foil to protect the contents from the light. Mix this solution gently by swirling.

d. **Standards Preparation**

(1) Stock HCYS Standard -- 1.0 mMol/L
Dissolve 0.02684 g of DL-homocysteine (MW 268.4, Sigma) in 100 mL of 1N HCI (Sigma). Dispense 1-mL aliquots of stock into 1.5-mL cryo tubes and store at -80°C. These standards are stable for at least 6 months at -80°C.

(2) Working HCYS Standard -- 40 nMol/mL
To prepare a working standard on the day of the assay, dilute 0.5 mL of stock standard with $ddH_20$ in a 25-mL volumetric flask.

e. **Preparation of Quality Control (QC) Materials**

Three quality control pools are prepared from pooled serum: pools with normal (unspiked), mildly elevated, and highly elevated HCYS concentrations. The concentration of the medium-spiked pool will be ≥15 nMol/mL. This level could suggest coronary heart disease in patients. The high-spiked QC pool will be >30 nMol/mL, a level indicative of folate, vitamin B12, or vitamin B6 deficiency. To prepare the two spiked pools, add enough stock standard (see Standards Preparation) to the pooled serum to bring its concentration to the desired level. For example, for the mildly elevated pool, add 0.75 mL of the stock per 100 mL of the serum pool, and for the highly elevated pool, add 1.33 mL of the stock per each 100 mL of the pool. Aliquots of 300-500 µL are prepared and stored at -80°C in cryotubes and are stable for at least 6 months.

(f) **Establishment of Acceptable Limits for Quality Control Materials**

Acceptable QC ranges are established by calculating the mean HCYS concentration for each of the three levels of QC materials over 30 days of analyses. The low and high limits of each of the three ranges are defined as ±2 SD of the mean of the 30 data points. All new batches of quality control materials must be overlapped with previous batches to maintain consistency and interassay agreement.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Reference materials such as those from the National Institute of Standards and Technology (NIST) are not available for HCYS assays. However, samples are occasionally sent to other laboratories for cross-validation studies, and the results are kept on file. Linearity is established every 6 months on the basis of three individual five-point standard curves, (i.e. 5, 10, 20, 30 and 40 nMol/mL). The results, plots, and statistics are recorded and filed. Subsequently, the system is calibrated every run with a 40 nMol/mL external HCYS standard. Peak height is recorded to monitor system performance. A 20 nMol/mL standard is also analyzed after calibration in order to monitor system performance and to verify the calibration.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

Set HPLC conditions as shown in Table 1.

Table 1
HPLC Parameters

| Parameter | Setting |
|---|---|
| Flow rate (mL/min) | 1.2 |
| Run time (min) | 8.0 |
| Fluorescence detection wavelengths (nm) | Excitation = 385, Emission = 515, Filter = 470 |
| Injection volume (µL) | 40 |
| Linear range (nMol/mL) | 2.0-200.0 |

b. **Sample Preparation**

(1) Allow samples to thaw at room temperature (20-25 °C).

(2) Vortex the samples.

(3) Add 100 µL of sample, quality control or standard to a 1.5 mL polypropylene Eppendorf flex-tube.

(4) Add 10 µL of the TBP solution to each sample using a repeater pipette.

(5) Vortex each sample vigorously.

(6) Cap the vials and place the samples in a refrigerator for 30 minutes.

(7) Add 100 µL of PCA solution and vortex the samples until samples are thoroughly precipitated.

(8) Centrifuge the samples in the Eppendorf microfuge for 4 min.

(9) Add 80 µl of the supernatant to 160 µl of the pH 10.5 boric acid in an injection vial with a microvolume insert.

(10) Add 80 µL of the SBDF reagent to the samples. Cap the vials and vortex the contents.

(11) Incubate the samples at 60°C for 60 min in a water bath. The samples will turn bright yellow over the course of the incubation.

(12) Place the vials into a 96-position autosampler carousel. Commence injection after samples have returned to room temperature.

Note: Prepared samples are stable for about 36 hours at 4°C.

c. **Instrument Preparation**

(1) Program the detector to the following settings:

- Emission wavelength = 515 nm, 1.0 AUFS
- Excitation wavelength = 385 nm, 1.0 AUFS

(2) Place fresh mobile phase in the reservoir. The pump is controlled by the Waters Expertise software. Use the isocratic method program. Do not shut the pump off between runs.

(3) Set the aquistion parameters as follows:

- Run time: 8.0 min
- Flow rate: 1.2 mL/min
- Trigger: SIM

(4) Set the integration parameters as follows:

- Scaling factor: 1.00000 e + 03
- Label: mV
- Channel: 1
- Peak width: 15 sec
- Threshold: 250 µV/sec
- Min height: 800 µV
- Min area: 0 µV

The retention time of the peaks will vary with factors such as lamp age and column type and age. The retention times under the current conditions are as follows:

- Cysteine: 3.7 min
- HCYS: 5.2 min

d. **System Maintenance**

Flush the column with 10% methanol:90% dd$H_2O$ once per week. Check the guard columns two to three times per week for build-up. Monitor pressure fluctutations that can also indicate column degradation. Perform routine preventive maintenance on the pump, including replacing worn seals, faulty check valves, and other consumables. Occasionally check the autosampler for proper sample delivery, (i.e., consistent volume). Purge the autosampler before every run. Allow 15 minutes for the detector to calibrate and stabilize. (The detector and pump are automatically programmed to shut down after every run in order to avoid unnecessary lamp use and the introduction of air into the system). Allow the column to equilibrate to the new mobile phase for 45 min. (The entire system is connected to mini/micro computer regulators to keep fluctuations in current to a minimum.)

e. **Calculations**

All calculations are performed by the computer which uses a single point, 40 nMol/mL, calibration.

*concentration of unknown = (40 nMol/mL ÷ peak height of HCYS standard)(peak height of HCYS unknown)*

## 9. REPORTABLE RANGE OF RESULTS

Any samples demonstrating a HCYS concentration of less than 3 nMol/mL or greater than 30 nMol/mL as determined by single analysis should be rerun. HCYS values of less than 5 nMol/mL, although not clinically significant, are thought to exhibit poorer reproducibility than values closer to the singular point of calibration (40 nMol/mL).

## 10. QUALITY CONTROL (QC) PROCEDURES

Secimens from the three levels of QC pools are analyzed at the beginning and end of each analytical run and between each set of 43 samples. A value for each specimen from each pool are recorded daily in a logbook after the analyst has determined that the value falls within the established limits for that pool (within 2SD of the mean). Monthly Levey-Jennings plots are generated separately for each level of QC in order to display trends of distribution around the previously established mean and within the 2SD limits.

An analytical run is accepted if the values for two out of the three levels of QC pools fall within the established range for that pool; separate ranges for QC specimens assayed at the beginning and end of each run have not been implemented. The general working rule is that if a greater than 6% coefficient of variation (CV) exists between the first and last QC run, the run is recalibrated and the samples are reanalyzed.

Table 2
Examples of Acceptable Ranges for Homocysteine Bench QC Pools

| QC Level | Mean (nMol/mL) | Range (nMol/mL) | CV (%) |
|---|---|---|---|
| Low | 8.81 | 7.33-10.29 | 6 |
| Medium | 20.37 | 17.87-22.87 | 6 |
| High | 44.60 | 39.78-49.42 | 6 |

Each serum sample is analyzed once for HCYS. Five percent of the samples (1 out of 20) are randomly selected and reanalyzed for a check of reproducibility.

Table 3
Representative Coefficients of Variation

| | Mean (nMol/mL) | Std Dev | N | CV (%) |
|---|---|---|---|---|
| Intra-assay | 27.9 | 0.9 | 20 | 3.4 |
| Interassay | 27.3 | 1.6 | 20 | 5.9 |

Table 4
Representative Recovery of High Control

| Expected (nMol/mL) | Mean (nMol/mL) | N | CV (%) | Recovery (%) |
|---|---|---|---|---|
| 30 | 28.9 | 4 | 3.4 | 96 |

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

a. Acceptance of Calibration

Record the peak height of the 40 nMol/mL standard in a logbook and compare it with the previously obtained peak

heights for calibration. In addition, analyze a 1:2 dilution of the 40 nMol/mL standard (20 nMol/mL standard) following the calibration in order to monitor system performance and to verify the calibration. If the difference between the most recently obtained peak height and the mean of the previous peak heights is greater than 25%, re-inject the standard. Do not analyze samples until the proper calibration has been established. If an acceptable peak height is obtained, resume the analytical run.

b. Corrective Maintenance of Instrumentation

If the peak height of the 40nMol/mL standard remains variable, check the flow rate of the mobile phase and the pump pressure and the status of the guard column on the HPLC system. Correct any problematic areas according to guidelines established by Waters Chromography. System operation is judged to be optimal when an injection of the 40 nMol/mL standard results in an acceptable peak height according to the criteria above.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Strict sampling conditions are necessary when plasma homocyseine is assayed: the concentrations of free and total HCYS in plasma increases over time if blood is stored uncentrifuged after being sampled (3).

Note: The samples analyzed for this subset of NHANES III, phase II, were not optimally collected plasma samples but were instead residual serum samples originally analyzed for folate and vitamin B12 and refrozen at -70 °C until shipment to the Tufts-USDA facility. The serum samples were harvested within 1/2 to 1 hour of collection and promptly frozen. Hemolyzed samples were not analyzed.

## 13. REFERENCE RANGES (NORMAL VALUES)

Table 5
HCYS Normal Ranges by Percentile from Framingham Heart Study Data

| Percentile | HCYS (nMol/mL), among adults <65 years old | HCYS (nMol/mL), among people 67-69 years old |
|---|---|---|
| 5th | 5.5 | 6.0 |
| 10th | 6.2 | 6.3 |
| 90th | 13.0 | 14.0 |
| 95th | 15.0 | 16.4 |

Percentiles were established from the Framingham Heart Study data derived from the Framingham Elders Cohort (for individuals 67-96 years old), and the Framingham Offspring Cohort (for adults <65 years old). In both cohorts, the normal distribution was restricted to those individuals (n=80 for the elders and n=163 for the offspring) with optimal vitamin status (defined as plasma levels of folate, vitamin B6, and vitamin B12 above the 70th percentile for each vitamin).

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable. These results will not be reported to the survey participants.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens reach and maintain 20-25 °C during testing. After testing, the specimens are stored at ≤-70 °C.

## 16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no alternate methods for performing this analysis. If the analytic system fails, the specimens should be refrigerated at 4-8 °C until the analytic system is restored. If long-term interruption is anticipated, refreeze specimens at ≤-70 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Results are transmitted to NCHS electronically and in printed form at periodic intervals.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Specimens are logged in the "Specimens Received Notebook." Specimen IDs are entered in the Tufts laboratory computer record and in the analyst's lab notebook. The computer record becomes the basis of the data report to NCHS.

**19. Quality Control Summary Statistics and Graphs**

**Because homocysteine was approved as a surplus sera project testing isn't complete. Therefore, summary statistics and graphs weren't available to meet printing deadlines.**

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

In order to better define the prevalence of the antibodies to rubella virus and varicella-zoster virus (VZV) in the U.S. population, a subset of the serum samples collected during the NHANES III from October 1, 1988, through September 30, 1994 will be tested by enzyme immunoassay (EIA).

These EIA tests have been developed by the staff of the Immunoserology Unit of the California State Department of Health Services (CSDHS) Viral and Rickettsial Disease Laboratory (VRDL). The procedures described below are the standardized protocols of the VRDL's in-house EIA tests for serodiagnosis of rubella and varicella-zoster viral infections (1-4).

In the indirect EIA, a suitable antigen material (i.e., solubilized varicella-zoster virus) is coated on the wells of a 96-well microtiter plate which is subsequently incubated with a diluted test specimen. If the specimen contains antibody to the antigen, the antibody will form complexes with the antigen on the coated plate. After unreacted serum components are washed from the plate, an antibody-enzyme conjugate is added to the wells and incubated. The conjugate consists of anti-human IgG covalently coupled to the enzyme alkaline phosphatase. The conjugate will react with the antigen-antibody complex on the surface of the well resulting in a sandwich of well-antigen-antibody-antibody-enzyme. If the test specimen does not contain IgG antibody to the antigen, the conjugate will not bind to the well surface and will be removed by washing. The presence of enzyme in the complex is determined by adding an enzyme substrate (indicator system) to the well and incubating the sample while a color reaction occurs. The enzyme substrate reaction will result in a yellow-colored product, which is measured in a spectrophotometer adjusted to a wavelength of 405 nm with a side band adjusted to 630 nm.

The level of antibody providing resistance to infection with the rubella virus has been established using hemagglutination inhibition (HAI or HI) procedures. A publication from the Centers for Disease Control and Prevention states that "any level of detectable antibody should be considered presumptive evidence of immunity" (5). Field evaluations have shown that the results from the VRDL EIA test for rubella are equivalent to HAI results in determinations of immune status.

Rubella (German measles) is generally a mild viral disease. However, if contracted during the first trimester of pregnancy, the virus may produce a severe infection in the fetus resulting in multiple abnormalities referred to as congenital rubella syndrome. Additional consequences of rubella infection may include spontaneous abortion of the fetus, miscarriage, or stillbirth. Women of childbearing age infected with rubella virus represent a significant public health problem because of the fetal congenital defects associated with this disease.

Varicella-zoster virus (VZV) is the etiologic agent of chickenpox (varicella) and shingles (zoster). The major role of serological diagnosis of varicella-zoster virus antibodies is to determine the immune status of susceptible individuals in high-risk groups such as immunocompromised patients, hospital employees, transplant recipients, and pregnant women.

## 2. SAFETY PRECAUTIONS

Consider all serum specimens for analysis potentially positive for infectious agents including HIV and the hepatitis B virus. Observe universal precautions. Wear gloves, lab coat, and safety glasses while handling all human blood products and infectious virus. Place disposable plastic, glass, and paper (pipet tips, reaction wells, gloves, etc.) that contacts blood or virus in a biohazard autoclave bag or discard pan to be autoclaved. Use disposable plastic-backed paper for all work surfaces. Treat viral antigens that have been inactivated as if they are infectious.

The Laboratory Safety Guidelines of the Viral and Rickettsial Disease Laboratory are followed by all personnel in the laboratory.

Material safety data sheets (MSDSs) for sodium chloride, disodium phosphate, potassium phosphate, sodium azide, Tween-20, magnesium chloride, diethanolamine, hydrochloric acid, paranitrophenyl phosphate, trisodium phosphate, Tris(HCl), EDTA, disodium deoxycholate, Nonidet P-40, bovine albumin, and phosphate-buffered saline are maintained in the laboratory.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The data on the NHANES disks are read into the ORACLE database, and specimen listings are checked for duplicate, inconsistent, or missing data. Run sheets are generated corresponding to specimen location and read into LOTUS 123 spreadsheet files.

b. The data captured from the microplate reader using MIRROR software is merged with corresponding runsheets in LOTUS 123 and read into ORACLE.

c. The final form of data can be a flat ASCII file, a LOTUS worksheet file, an ORACLE DUMP file, or another format as required.

d. Routine backup procedures include 1) daily backup of each day's test data on 3.5" floppy disks; 2) weekly backup of the hard disk; and 3) archival on 3.5" floppy disks.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Serum is the preferred sample. Do not use plasma. Serum specimens may be collected by using regular red-top or serum-separator Vacutainers. Collect and separate specimens aseptically.

c. Store specimens at 4-8 °C for up to 5 days, or at ≤-20 °C if longer storage is required.

d. Do not use grossly hemolyzed or microbial-contaminated specimens.

e. Specimens containing cellular debris or red blood cells should be clarified prior to use.

f. To test for immunity status, one need use only a single specimen. Specimens used to test for immunity status following exposure must be collected within 7 days of the exposure.

To test for acute or recurrent infection, both acute-phase and convalescent-phase specimens are required. The acute-phase specimen should be collected within 7 days of onset of symptoms, and the convalescent-phase specimen 14 days or more after onset of symptoms.

g. Specimen stability has been demonstrated for 1 year at ≤-20 °C.

h. Specimens collected in the field should be frozen, then shipped on dry ice by overnight mail. Once received, they should be stored at ≤-20 °C until analyzed. Samples are thawed prior to testing and vortexed to ensure thorough mixing. Specimens remaining after analytical aliquots are withdrawn are refrozen at ≤-20 °C.

i. Samples thawed and refrozen several times are not compromised.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Bio-Tek model EL312E microplate reader, automated dual wavelength 405 nm/630 nm (Bio-Tek Instruments, Inc., Winooski, VT).

(2) IBM PS/2 or IBM-compatible computer (with a minimum 286 CPU) used for specimen entry, and to record and analyze data from plate reader (IBM, Research Triangle Park, NC).

(3) Bio-Tek model EL403, 96-well microplate washer with plastic carboys (2), tubing, and traps (Bio-Tek Instruments, Inc.).

(4) VWR Model 1520 nonhumidified incubator, 37 °C (±1) (VWR Scientific, San Francisco, CA).

(5) Fan, household type, to dry 96-well microplates (Patton HV-18C, Ace Hardware, Berkeley, CA).

(6) Rainin EDP-Plus EP-25 and EP-250 electronic digital pipette capable of dispensing 1 µL to 250 µL, and tips (Rainin Instrument Co., Inc., Emeryville, CA).

(7) Labsystems Finnpipette digital, 12-channel, AS-8043 and Finntip 300 µL tips AS-9341, (Applied Scientific, South San Francisco, CA).

(8) S/P Brand Ultrasonic Cleaner (Baxter Scientific, Atlanta, GA).

**b. Materials**

(1) NUNC strip well plates, NUNC cat. no. 4-69949 or AS cat. no. 72110 microplates, 96-well, polystyrene, flat-bottom, high binding capacity (Applied Scientific).

(2) ICN/Flow plastic microplate sealers, cat. no. 76-401-05 (ICN/Flow Biochemicals, Inc., Costa Mesa, CA).

(3) ICN/Flow 96-well tube racks compatible with microplate format, and 1-mL polypropylene microplate dilution tubes compatible with multichannel pipettor tips, cat. no. 61-225-00 (ICN/Flow Biochemicals, Inc.).

(4) Costar plastic disposable multichannel pipette reagent reservoirs, cat. no. 4870 (Costar, Van Nuys, CA)

(5) Kapak pouch sealer, cat. no. 11214-108, for polyester bags (VWR Scientific).

(6) Kapak polyester, heavy duty, heat sealable, 8" x 10" or 6-1/2" x 8" bags, cat. nos. 11214-491 and 11214-527 (VWR Scientific).

(7) Humidity indicating cards (Multiform Desiccants, Inc., Buffalo, NY).

(8) Minipax desiccants, cat. no. 02-00040AG15 silica gel desiccator packs, 5-g (Multiform Desiccants, Inc.).

(9) KayDry plastic-backed paper cut for use as blotting pads, cat. no. 52857-120 (VWR Scientific).

(10) Rainin pipet tips, 1- to 250-µL and 50- to 300-µL (Rainin Instrument Co.).

(11) Glassware: 1000-, 500-, 200-, and 100-mL bottles for storing solutions; 1000-, 400-, 250-, 100- and 50-mL beakers; 1000-, 500-, 250-, 100-, and 50-mL graduated cylinders; and 1000-, 500-, 250-, 100-, and 50-mL Erlenmeyer flasks (Baxter Scientific Products, Hayward, CA).

(12) pH standard buffers, 4.0, 7.0, 9.0 (Beckman Instruments, Palo Alto, CA).

(13) 2-mL capped polypropylene storage vials (Sarstedt, Newton, NC).

(14) Heavy-duty paper wipes (any vendor).

(15) Latex disposable gloves (various sizes and manufacturers).

(16) Magnetic stirrer and assorted size stirring bars (Van Waters & Rogers, San Francisco, CA).

(17) Metler balance (Van Waters & Rogers).

(18) pH meter and electrode (Beckman Instruments, Palo Alto, CA).

(19) Multi-tube vortexer (Van Waters & Rogers).

(20) Test tube racks made in house (California State Department of Health Services, Viral and Rickettsial Disease Laboratory, Berkeley, CA).

(21) Disposable glass or plastic pipettes, 10-, 5-, 2-, and 1-mL (Baxter Scientific Products).

(22) 60-min adjustable timer (Van Waters & Rogers).

(23) BHK-21 cells (American Type Culture Collection (ATCC) Rockville, MD).

(24) Rubella virus, RV strain (ATCC).

(25) Growth medium containing 10% fetal bovine serum (FBS).

(26) Maintenance medium containing 2% inactivated FBS

(27) Sodium bicarbonate,($Na(HCO)_2$) (Sigma Chemical Co., St. Louis, MO).

(28) VZV-infected human fetal diploid lung (HFDL) cells

(29) Uninfected HFDL cells

(30) Sodium chloride (NaCl), cat. no. SX0420-1 (EG Science, Gibbstown, NJ).

(31) Disodium phosphate ($Na_2HPO_4$), cat. no. S-9390 (Sigma).

(32) Potassium phosphate ($KH_2PO_4$), cat. no. P-3786 (Sigma).

(33) Double-distilled water.

(34) Sodium azide ($NaN_3$) cat. no. SX0299-1 (EG Science).

(35) Tween-20, cat. no. P-1379, (Sigma).

(36) Magnesium chloride ($MgCl_2.6H_20$) cat. no. 2444, (J.T. Baker, Irvine, CA).

(37) Concentrated hydrochloric acid (HCl) (12 N), cat. no. HX0603-3 (EG Science).

(38) Diethanolamine $HN(CH_2CH_2OH)_2$ (2,2'-iminodiethanol) cat. no. D-8885 (Sigma).

(39) Paranitrophenyl phosphate substrate (PNPP), cat. no. 104 (Sigma)

(40) Conjugate, IgG, cat. no. A-5403 (Sigma).

(41) Trisodium phosphate (TSP) ($Na_3PO_4.12H_2O$) (Sigma).

(42) Tris(HCl), cat. no. T-3253 (Sigma).

(43) Ethylenediaminetetraacetic acid (EDTA), cat. no. EX0550-5 (EG Science).

(44) Disodium deoxycholate, cat no. DX 115 (Matheson, East Rutherford, NJ).

(45) Sodium chloride (NaCl), cat. no. SX0420-1 (EG Science).

(46) Nonidet P-40 (NP-40) (Particle Data Lab NP-40, Elmherst, IL).

(47) Aprotinin, cat no. A-6279 (Sigma).

(48) Casein (Hammersten quality), cat. no. 1082C-1 (Research Organics, Cleveland, OH).

(49) Bovine albumin (Armour Fraction V) 98% pure, cat. no. AL135 (Spectrum, Rondo, CA).

(50) Sodium hydroxide (J.T. Baker, Phillipsburg, NJ).

(51) Household bleach (any vendor).

(52) WHO CDC 1000 International Unit (IU) Rubella/Varicella Positive Serum, (Scientific Resources Program, National Center for Infectious Diseases, National Centers for Disease Control and Prevention, Atlanta, GA).

(53) Eythylene glycol (J.T. Baker).

(54) Dry ice (any vendor).

(55) Fetal Bovine Serum (FBS) cat. no. A-1111-L (Hyclone Labs)

(56) Eagle's Minimum Essential Media (MEM) with Earl's salts 1x, without L-Glutamine, with sodium bicarbonate, 1500 mg/L, cat. no. 11096-021 (Life Technologies, GibcoBRL, Grand Island, NY).

(57) L-glutamine 200 mM (100x), cat no. 25030-024 (GibcoBRL).

**c. Reagent Preparation**

Unless otherwise specified, all chemicals are American Chemical Society "Reagent Grade." Reagents are prepared with double-distilled water.

(1) Preparation of cell culture media

Growth medium containing 10% FBS can be prepared in the laboratory using Eagle's MEM with Earl's salts 1x, L-glutamine, and FBS. Maitenance media can be similarly prepared subsituting 2% FBS for 10% FBS.

(2) Preparation of rubella EIA antigen

(a) Trypsinize BHK-21 cells and infect them with rubella virus (RV strain) with a multiplicity of at least 1 plaque forming unit (pfu) per cell in suspension. Incubate for 1 hour with gentle shaking.

(b) Seed approximately 7-8 x $10^6$ cells per T-150 bottle with growth medium containing 10% fetal bovine serum (FBS).

(c) After 48 hours, replace the growth medium with a maintenance medium containing 2% inactivated FBS.

(d) After an additional 48-hour incubation, dislodge the cell monolayer with sterile glass beads into the medium, and adjust the pH to 8.5-8.6 by adding 8.8% sodium bicarbonate.

(e) Clarify the medium by centrifuging it at 6,000 x g for 30 min at 4-8 °C.

(f) Resuspend the pellet in a small volume of growth medium, freeze-thaw the medium two times, clarify it at 6,000 x g for 30 min, then add supernatant to the rest of medium. Discard the pellet.

(g) Centrifuge the supernatant to pellet the virion at 78,000 x g for 2 hours. Discard the supernatant.

(h) Resuspend the pellet in 1/50 to 1/60 of the original volume, depending on the pellet size, in EIA solubilizing buffer to dissolve and inactivate the virus.

(i) To dissociate viral aggregates, sonicate the solubilized antigen at 10-15 sec intervals, for a total of 90 sec.

(j) Prepare uninfected control BHK-21 antigen in the same manner as the infected antigen.

(k) Titrate the RV and control antigen in the 96-well microtiter plate to determine which dilution is to be used in the test proper. For the present study, a dilution of 1:400 for RV and control antigen was determined to be appropriate.

(l) To avoid repeated freezing and thawing of antigen, store it at ≤-70 °C in 0.25-mL aliquots, which will be used for coating one batch of 20 plates.

(3) Preparation of varicella-zoster virus EIA antigen

(a) Prepare virus-infected monolayers by mixing VZV-infected human fetal diploid lung (HFDL) cells with

uninfected HFDL cells at a ratio of 1:3.

(b) Harvest the cell monolayer when the cells show 3-4+ CPE, usually within 72-96 hours.

(c) Using sterile glass beads, dislodge the cell monolayer into the medium.

(d) Centrifuge the medium and cells at 1,000 x g for 10 min. Save the supernatant and pellet.

(e) Freeze/thaw the pellet in ethylene glycol and dry ice 3 times.

(f) Centrifuge the supernatant from (d) at 78,000 x g for 1 hour. Remove the supernatant from this tube and discard.

(g) To dissolve and inactivate the virus, re-suspend the pellets from all tubes (including freeze/thaw pellet) in EIA solubilizing buffer.

(h) Sonicate the solubilized antigen at 10-15 sec intervals for total of 90 sec to dissociate viral aggregates.

(i) Prepare uninfected control HFDL antigen in the same manner as the infected antigen.

(j) Titrate the VZV and control antigen in 96-well microplates to determine which dilution will be used in the test proper. For the present study, a dilution of 1:800 for VZV and control antigen was determined to be appropriate.

(k) To avoid repeated freezing and thawing of antigen, store it at ≤-70 °C in 0.125-mL aliquots for coating one batch of 20 plates.

(4) Coating of viral antigen and control antigen to the 96-well microplate

*Important: All new lots of viral and control antigen must be titrated for optimal dilution before being used for coating.*

(a) Color code the microplates prior to coating them with antigen.
(Example: red for Rubella, yellow for VZV).

(b) Add 100 µL of the working dilution of viral antigen and control antigen to each 96-well plate (control antigen in odd strips/viral antigen in even strips). Dry the microplate 3-5 hours at 37 °C using an electric fan blowing an air stream over the surface.

(c) Store microplates as follows: Package each dried microplate in a heat-sealed plastic bag. Include a desiccant and humidity indicator in each bag. Store the plates at 4 °C. The coated microplates are stable for at least 6 months. Do not use them if the humidity indicator has changed from blue to red, indicating the presence of excess moisture.

(5) Phosphate buffered saline (PBS), 10X stock, pH 7.2-7.4
Combine 85.0 g of sodium chloride, 5.65 g of disodium phosphate, and 1.35 g of potassium phosphate. Add double-distilled water to bring the final volume to 1000 mL. Store the solution at 20-25 °C for no longer than 1 month. Discard the solution if it becomes contaminated.

(6) Sodium azide, 10 g/dL
Weigh out 10 g of sodium azide. Add double-distilled water to yield a final volume of 100 mL. Clarify the solution if necessary, using a 0.45-µm filter. Store the solution at 20-25 °C. When discarding solutions with azide, autoclave them to break down the azide. If autoclaving is not possible, pour them down the sink followed by a large volume of water. Store the solution indefinitely.

*Use gloves and a mask when weighing azide.* ***Do not inhale dust****.*

(7) Tween-20, 10% (v/v)
Add 1 mL of 10 g/dL sodium azide solution to 10 mL of Tween-20. Add double-distilled water to yield a final volume of 100 mL. Store the solution at 20-25 °C for no more than 6 months.

(8) Magnesium chloride solution, 0.5 mol/L with 20 g/dL sodium azide
Combine 20.0 g of sodium azide (See step 2 above) with 10.2 g of magnesium chloride. Add double-distilled water to yield a final volume of 100 mL. Store the solution at 20-25 °C indefinitely.

(9) 1 N hydrochloric acid (HCl)
Add 50 mL of concentrated HCl (12 N) to approximately 400 mL of double-distilled water. Add double-distilled water to yield a final volume of 500 mL. Store the solution at 20-25 °C for no more than 1 week.

(10) Diethanolamine (DEA) buffer, pH 9.8
In a 4-L beaker, add 1 L double-distilled water and begin stirring. Add 340 mL diethanolamine. Rinse the graduated cylinder with 1 L double-distilled water and add the water to the beaker. Add another 400 mL of double-distilled water. While stirring, adjust the pH of the solution to 9.8 with 1 N HCl (approximately 300 to 600 mL 1 N HCl is needed). Record the volume of 1 N HCl used. Add 3.5 mL of stock solution containing 0.5 mol/L magnesium chloride and 20 g/dL sodium azide. Use double-distilled water to bring the final volume to 3.5 L, and ensure that the final pH is 9.8; adjust the pH if necessary. Store DEA at 4-8 °C for no more than 12 months.

(11) Paranitrophenyl phosphate substrate (PNPP)
Obtain PNPP as preweighed capsules in amounts suitable for one run. Store PNPP at ≤-20 °C. Discard it if the color has changed from cream-colored to yellow. (Tablets may be used, but they take longer to dissolve).

(12) Conjugate -- IgG
This is an anti-human IgG (gamma chain specific) $F(ab')_2$ fragment labeled with alkaline phosphatase. Store at 4-8 °C until the expiration date given by the manufacturer. Titrate each lot of conjugate to determine dilution for use.

(13) Enzyme stopping reagent -- trisodium phosphate (TSP)
Weigh out 20.0 g of TSP. Add double-distilled water to yield a final volume of 1000 mL. Store in a glass bottle at 20-25 °C for no longer than 12 months.

(14) Solubilizing buffer -- Tris/saline/EDTA solubilizing buffer, pH 9.0
Used for virus solubilization and inactivation. Combine 1.21 g of Tris(HCl), 0.83 g of EDTA, 1.00 g of disodium deoxycholate, 8.00 g of NaCl, and 1 mL/L Nonidet P-40 (NP-40). Adjust the pH to 9.0. Bring to a final volume of 1 L using double-distilled water. Add 1% Aprotinin as protease inhibitor. Sterilize by membrane filtering through a 0.22-µ filter. Store at 4-8 °C.

(15) 5% casein in phosphate buffered saline
Specimen diluent stock (blue), conjugate diluent stock (red). Tare a 1-L beaker and add to it:

- 40.0 g of casein (Hammersten quality)
- 1.6 g of sodium azide **(Be careful not to inhale dust)**
- 620 mL of double-distilled water
- 80 mL of 10X PBS
- 4 mL of 10% Tween-20

Stir for 2 hours. Adjust the pH with 1 N NaOH to 7.40. Add double-distilled water to yield a final volume of 800 mL. Stir at 4 °C overnight.

On the next day warm the solution to 56 °C and stir until it cools to 37 °C. Adjust the final pH to 7.40. Centrifuge the solution for 30 min at 900 x g in 200-mL flat bottom bottles (3000 RPM in the Beckman J-6). Remove the top debris layer. Remove the supernatant fluid carefully without contaminating it with pellet material. Add 50 µL Schilling food coloring (red or blue) to each 100 mL. Store at 4-8 °C for no longer than 6 months.

(16) 5 g/dL bovine serum albumin (BSA) in phosphate buffered saline (PBS)
Mix the following ingredients in the order given:

- 10 mL of 10x PBS
- 89 mL of double-distilled water
- 5 g of bovine albumin (Armour Fraction V)
- 1 mL of 10% sodium azide

Stir until the BSA is dissolved. Filter using a 0.22-µm filter. Store at 4 °C indefinitely.

(17) Phosphate buffered saline, 1X (PBS)
Dilute the 10X phosphate buffered saline stock solution 1:10 in double-distilled water. Store the solution at 20-25 °C. Prepare fresh as needed.

(18) Phosphate buffered saline - Tween 20 (PBS-T)
Add 5.0 mL of the 10% Tween-20 solution per L of PBS and mix well. This working solution may be used for 2 weeks. Store at 20-25 °C. PBS-T is used for washing plates, making the working solution of serum diluent, and making the working solution of conjugate diluent.

(19) Serum diluent stock
Dilute one part of 5 g/dL casein blue with 5 parts of PBS-T. 500 µL of stock is needed for each participant or control serum specimen. Prepare weekly. Store at 4 °C.

(20) Conjugate diluent stock
Dilute one part of 5 g/dL casein red with 5 parts of PBS-T. Prepare fresh daily and warm to 20-25 °C before use.

(21) Viral antigen and control antigen
Use viral antigens and matching noninfected cell control antigens prepared for EIA. The noninfected cell antigen is to be used as the nonspecific antigen control. Store the antigens at ≤-70 °C. **Aseptic techniques must be adhered to for the preparation and growth of viral antigen.**

(22) Working conjugate dilution
Dilute the conjugate in red conjugate diluent. Use the dilution previously determined by titration. Prepare no sooner than 60 min prior to use.

(23) Substrate
Warm the DEA buffer to 20-25 °C for the day's run. Add 1 g PNPP substrate per mL of DEA buffer used. Pour in preweighed powder from capsule. Discard the empty capsule. Prepare no sooner than 60 min prior to use.

(24) 10% Hypochlorite
Add 300 mL bleach to 2700 mL of double-distilled water. The solution is stable for 1 week when stored at 20-25 °C.

(25) 0.01 N NaOH
Dilute 0.8 g NaOH with 2.0 L of double-distilled water. Place the solution into an empty dispense bottle.

(26) 0.01 N HCl
Dilute 1.6666 g of 12 mol/L HCl with 2.0 L of double-distilled water. Store at 20-25 °C; use as needed.

**d. Standards Preparation**

Rubella standard
Dilute WHO or CDC 1000 International Unit (IU) Serum to contain 10, 40, and 100 IU in 5 g/dL BSA.

**e. Preparation of Quality Control (QC) Materials**

Select a panel of serum samples that have previously been tested and determined to be high positive, low positive, or negative for rubella and varicella-zoster.

Use individual serum samples or pooled serum. Add 10 µL of a 10 g/dL sodium azide solution per ml of serum (final dilution of sodium azide is 1:1,000). Store the serum at 4-8 °C or ≤-20 °C. These QC materials are stable

indefinitely, unless contamination occurs.

Individual serum samples and serum pooled for controls are screened for HBs antigen and antibody to HIV before pooling. Samples testing positive should be eliminated or labeled accordingly.

Controls included on each plate:
RUBELLA -- 10, 40, & 100 IU standard, negative, low positive
VARICELLA -- standard, high positive, low positive, negative.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### a. Calibration Curve

No calibration curve is generated by the user as part of these assay methods.

### b. Verification Procedure

Perform the following verification procedure after installing a new Bio-Tek EL312E Microplate Reader, whenever the lamp is changed, and periodically thereafter to ensure that the reader is performing optimally.

(1) Connect the printer directly to the reader with a communication cable.

(2) Turn on the reader and printer.

(3) To test the reader's display, press SHIFT and A simultaneously. (The SHIFT key is the blue dot between SERIAL OUT and ESCAPE.) The display will cycle as if the reader was just turned on.

(4) To test the printer function and the printer-reader interface, press SHIFT and B simultaneously. The printer will respond by printing all characters and numbers.

(5) Perform the blank carrier test.

(a) Set the reader to a single wavelength.

(b) Perform a read with no plate in the carrier. The acceptable values are 0.000 (±0.001) for all wells.

(6) To test the reader operational verification (photodetectors, lamp, and amplifier), press SHIFT and H simultaneously. This is also called the self-test. To pass, the reference channel and calibration test must print "PASS."

(7) To check the reader-to-computer communication, press SHIFT and SERIAL OUT simultaneously. Perform the following steps:

(a) Use the PROMPT key to scroll through the parameters. Use the OPTION key to change the parameters.

The correct settings for BVS Version 4.12 are:

- 2400 BAUD
- 8 CHARACTER BITS
- DISABLE PARITY
- ODD PARITY
- EOLCHAR=CR-LF
- EOT CHAR(HEX)1A

(b) If any parameters are changed, press SHIFT and 0 (zero) simultaneously to store the changes.

(8) Press ENTER. The display should now read READY FILE1.

(9) To check the file parameters perform the following steps:

(a) To select the desired file, press the FILE NUMBER, then press ENTER.

(b) Use the PROMPT key to scroll through the parameters.

(c) Use the OPTION key to select the desired option.

The correct parameters for file1 are:

- **READY**FILE 1
- READ-AND-EJECT
- NO DELAY IN READ
- DUAL WAVELENGTH
- FILTER W1 = 450
- FILTER W2 = 630
- EXTENDED MODE
- OFF
- NO SHAKNG
- NO FACTOR DATA
- PRINT HEADING
- PRINT DENSITIES
- NO LIMITS
- NO RANGES
- 8 X 12 FORMAT
- NO RS232 OUTPUT
- NO ID
- FULL BLANK MAP
- SECTOR NUMBER 1
- ENABLE READING
- S-01 BLK-01
- @_ -_(Press CLEAR to empty the spaces)
- NO EDIT TEXT
- **READY**FILE I

(d) Press ENTER when finished, then press ENTER again.

(10) Press SETUP to obtain a printout of settings.

(11) Initial the preventive maintenance checklist located in the preventive maintenance module.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Preliminaries

(1) Prepare run sheets weekly:

Enter the numbers of the controls and sample specimens into the computer to generate plate maps for testing specimens as follows:

(a) Get a disk with ".prn" files from the Data Unit and copy the ".prn" files using XTREE into the C:\ZRV\1CUR subdirectory. Insert the disk into the disk drive and type XT and press ENTER. Type L, then A, and then tag all the ".prn" files. Press Ctrl-C to copy tagged files as *.* to C:\ZRV\1CUR. Type Q to exit XTREE.

(b) Start Lotus by typing 123 and pressing ENTER.

(c) Retrieve the rubella master worksheet: /F R 00RVMAST.WK1.

(d) Press ALT C to run a macro that clears the worksheet data ranges and then allows the operator to rename the run and type in the identification of the repeat specimen. The macro then continues to print the plate map and save the worksheet under the new run name (i.e., RUB220.WK1). In EDIT mode,

backspace the appropriate number of spaces and type in the correct run number, followed by ENTER. Type the repeat number into the repeat well (RW). Type the BOX LOC and press ENTER, type the BOXNUM and press ENTER, and type the SPECNUM and press ENTER. The specimen numbers are combined automatically; check the first specimen number against the number on the original list. The plate map is printed, and the worksheets saved, automatically.

(e) After the map is printed, the repeat well has to be marked with a red square, and the BOXLOC, BOXNUM, and SPECNUM must be written at the bottom of the map with the appropriate repeat run (4 runs later) pencilled in next to it.

(f) Retrieve the V-Z master worksheet: / F R 00VZMAST.WK1

(g) Press ALT C to run a macro that clears the worksheet data ranges, then allows the operator to rename the run. The macro then continues to import the specimen numbers from the Rubella runsheet (of the same number), to print the plate map, and to save the worksheet under the new run name (i.e., VAR220.WK1).) Correct the run number in EDIT mode; then backspace the appropriate number of spaces and type in the correct run number and press ENTER. The specimen numbers are combined, and the worksheets are saved, automatically. Check the first specimen number against the number on the rubella map.

(2) Pull specimens to be run (weekly):

(a) Remove pertinent boxes from the walk-in freezer.

(b) Remove specimens from boxes and check their numbers against the "extract list" or map; then place the specimens in blocks according to runsheet maps.

(c) Place repeat specimens from earlier runs in an appropriate location in the current block.

**b. Sample Preparation**

(1) For each participant specimen and control sample to be run, use the Finnpipette digital multichannel pipette, to add 249 µL of serum diluent 2 times (498 µL total) to 1-mL microplate tubes in the 96-well rack.

(2) Using the Rainin EDP electronic digital pipette, add 2.5 µL of each participant serum or control sample to tubes containing 498 µL of diluent for a final 1:200 dilution.

(3) Vortex all tubes.

(4) Dilutions may be made the day before the test and the solutions stored overnight at 4-8 °C.

(5) Warm dilutions to 20-25 °C in 24 °C water bath for 10 min prior to use.

**c. Instrument Setup of Bio-Tek EL312E Spectrophotometer**

(1) Set up the plate reader:

(a) Turn on the plate reader (switch at the back).

(i) The display will say READY.

(ii) Choose FILE -> FILE 5 by pressing 5.

(iii) Open the door (for the plate to read) by pressing STOP.

(iv) Insert plate: put the left corner first, press against the spring until the back right corner can be seated, and check to see that the individual strips are not raised.

(b) Set up the computer by placing the reading disk in drive A.

**d. Operation of Assay Procedure**

(1) Prepare the plate washer according to the manufacturer's instructions:

(a) Empty waste bottles.

(b) Fill the water and wash fluid reservoirs.

(c) Turn on the washer, pump, and pressure delivery module.

(d) Check the pressure on the pressure delivery module.

(e) Rinse the washer with deionized water by pressing RINSE and START.

(f) Prime the washer with deionized water by pressing PRIME and START.

(g) Check the fluid delivery levels by pressing DISPENSE and START and observing the microplate well volumes.

(h) Check the fluid aspiration by pressing ASPIRATE and START and observing the dryness of the microplate well.

(i) Check the wash program by pressing WASH and START and observing the process.

(j) Replace the water reservoir with the wash fluid reservoir

(k) Prime the washer with wash fluid by pressing PRIME and START.

(l) Turn off the washer with the toggle switch at the rear of the instrument.

(2) Remove the required number of appropriate (rubella or varicella-zoster coated) 96-well microplates and serum dilutions from the 4-8 °C refrigerator and allow them to reach 20-25 °C before beginning the test.

(3) Wash the plates three times prior to use to remove excess antigen coating.

(4) Follow the MAP runsheet. Using a multichannel pipette, transfer 100 µL of each diluted control serum and participant's serum to the appropriate wells coated with viral antigen and control antigen.

(5) Cover the plates with plastic sealers and incubate them for 60 min in a 37 °C incubator. In order to maintain timing, stagger the plates at 3-min intervals.

(6) Prepare the working dilution of conjugate in the conjugate diluent buffer.

(7) After 1 hour, wash each plate in the plate washer five times.

(8) Using a multichannel pipette and reservoirs, add 100 µL of the conjugate dilution to each well. Maintain 3-min separation.

(9) Cover the plates with plastic sealers and incubate them for 60 min in a 37 °C incubator.

(10) Prepare the substrate working solution.

(11) After 1 hour, remove the plates from the incubator, remove the seals, and wash as in step 6. Do not allow the plates to dry. Washed plates may be held inverted until all plates have been washed.

(12) Add 100 µL substrate to each well.

(13) Cover and incubate the plates in the 37 °C incubator for 30 min.

(14) After 30 min, remove the plates, remove the seals and add 100 µL of the stopping solution (TSP) to each well.

(15) Read the microplates on the microplate reader set on 405 ng/630 ng (reference) with no blank and capture data from the reader into the computer using Mirror Software.

(a) Read the plates.

(i) Type DIAG RUBxxx where xxx represents the run number of the plate being read.
(ii) When the screen displays "Waiting for string "^Z," press START on the plate reader.

(b) Check the computer screen to ensure that the data have been saved on a floppy disk. Prompt should read:

C:\COMM>COPY STARX.PRN A:\rubxxx.PRN
1 File(s) copied

Check to see that the current run number is in the filename, then press PRINT SCREEN.

(c) Repeat steps a and b for each run.

(d) Turn off reader:

(i) Press START to close the reader door.
(ii) Turn off the switch quickly.

(16) Clean the microplate washer according to the manufacturer's instructions. Prepare the washer for overnight storage:

Decontaminate the waste bottles by adding one scoop of dry sodium hypochlorite to each bottle and leaving them overnight. Turn on the washer, pump, and pressure delivery system perform "overnight maintenance" by pressing MAINT, OPT UP, and START and the washer will rinse, prime, and leave the washer tips immersed in deionized water.

(17) Discard the microplate strips in a proper discard pan for autoclaving and discarding, keeping the plastic frames for future use.

**e. Recording of Data**

(1) Quality control data

(a) The QC data are captured from the microplate reader by using MIRROR software. The reader automatically calculates the control results.

(b) Prepare a log of control results for each run, including the date tested; the run number; the mean net absorbance for each standard and control; and a notation when an individual control or run is unsatisfactory.

(2) Analytical results

(a) Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file on a 5¼" high-density (HD) floppy diskette. These disks are read into the ORACLE database, and specimen listings are checked for duplicate, inconsistent, or missing data. Run sheets are generated corresponding to the location of specimens and read into LOTUS 123 spreadsheet files.

(b) The data captured from the microplate reader using MIRROR software are merged with corresponding runsheets in LOTUS 123 and read into ORACLE.

(c) After the data are checked and corrected, a back-up copy of the diskette and the lab report are made. The original diskettes and report are sent to NCHS in an anti-static floppy disk mailer.

**f. Replacement and Periodic Maintenance of Key Components**

(1) Bio-Tek EL312E spectrophotometer

The Bio-Tek EL312E microplate reader is basically maintenance free. Remove the dust from the inside of the reader with canned air as needed. Preventive maintenance is performed on the spectrophotometer quarterly. The manufacturer's representative performs function checks using the Bio-Tek calibration plate to ensure the accuracy, reproducibility, linearity, and alignment of the Bio-Tek EL312E Microplate Reader. The Test Plate reading procedure consists of selecting a wavelength to test, then performing three readings of the plate at the desired wavelength. The statistical variance between the readings is then calculated.

(2) Bio-Tek EL403 microplate washer

Preventive maintenance is performed on the microplate washer bimonthly: bleach and NaOH solutions are used to disinfect the system and remove protein buildup. The following tasks are performed by the laboratory personnel according to the manufacturer's specifications:

(a) Twice Monthly Decontamination

Decontaminate the microplate washer by pumping 10% bleach followed by double-distilled water. Clean the inlet filter in the back of the washer using soapy water. Rinse thoroughly and reinstall the filter. Consult the instrument manual for detailed instructions.

Initial the preventive maintenance checklist located in the preventive maintenance module.

(b) Quarterly ACID/Base Maintenance

Rinse the system with 0.01N NaOH followed by 0.01N HCl as described in the instrument manual. Perform OVERNIGHT MAINTENANCE using double-distilled water.

Initial the preventive maintenance checklist located in the preventive maintenance module.

(3) Pipettors

All micropipettors that are used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with the manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations** (performed automatically by the computer program)

(1) For each control and test specimen, calculate the absorbance of the antigen well minus the absorbance of the control well (AG-NS value) and the ratio of the antigen well to the control well (AG/NS).

(2) Calculation of index values (rubella & varicella)

(a) Cut-off calculations:

RUBELLA - The mean AG-NS value of the duplicate 10 IU standards is used as the cut-off value.

VARICELLA - The cut-off value is 0.1.

(b) Calculate the O.D. INDEX by dividing the AG-NS value by the cut-off value for each serum.

$$\text{O.D. INDEX} = \frac{\text{Viral antigen ABS - control antigen ABS}}{\text{Cut-off value}}$$

(3) Calculation of International Units (IU) (Rubella)

(a) Perform a regression analysis and calculate a standard curve using the duplicate AG-NS values of the

10, 40, and 100 IU standards and their squares.

(b) Predict the IU value for each control average and test sample from the standard curve.

An O.D. INDEX of $\geq$1.0 indicates the presence of antibody. An O.D. INDEX of <1.0 indicates that antibody was not detected. An International Unit value of $\geq$10 is considered significant for rubella.

**9. REPORTABLE RANGE OF RESULTS**

a. Rubella

(1) The index value result range is 0 to 30.

(2) The International Unit range is 0 to >200 IU.

b. Varicella

(1) The index results range is 0 to 30.

**10. QUALITY CONTROL (QC) PROCEDURES**

a. Evaluation of control results

(1) Rubella: Evaluate the control wells and AG-NS value for each control serum specimen. Evaluate the $r^2$ value and predicted IU values for controls.

(2) Varicella: Evaluate the control wells and AG-NS values for each control serum specimen.

(a) Prepare a log of control results for each run including the date tested; the run number; the mean net absorbance for each standard and control; and a notation when an individual control or run is unsatisfactory.

(b) Calculate the mean and standard deviation for each control on the basis of the first 40 acceptable runs with a lot of antigen.

(c) Prepare a Levey-Jennings plot for each control using the mean from step (b) above and two and four standard deviations. Plot the controls in batches of 40 runs. Plot all runs. The plots are referenced to the control log by log number.

(d) Select at random one serum specimen from each run for repeat testing (2.56%). Repeat both rubella and varicella tests in the following day's runs.

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

a. When all control AG-NS values fall within the 2 SD limits and the $r^2$ and IU values are within limits, accept the run and report participant results.

b. If one or more of the controls exceed the 2 SD limits, inspect the control data and the participant results. It may be necessary to recalculate the results or reject the run and not report participant results. Inform the supervising microbiologist.

c. If two or more control observations fall outside the 4-SD limits, reject the run and do not report results. Repeat the run.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

a. Any reaction of a participant's serum specimen with the control (NS) antigen >0.200 O.D. units is suspect. Calculate the AG/NS ratio to determine if results can be released.

b. High nonspecific reactions should be individually evaluated to determine if they are influencing the interpretation of

results. If they are, the test is unsatisfactory. The serum should be adsorbed and retested by EIA and by immunofluorescense assay (IFA).

c. Samples with AG-NS ratio <2:1 should be individually evaluated for nonspecific reactions.

**13. REFERENCE RANGES (NORMAL VALUES)**

The normal ranges of these antibodies in the U.S. population will be determined after analysis of the NHANES III data.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens reach and maintain 20-25 °C during testing. After analysis, the specimens are stored at ≤-20 °C.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There are no alternate methods for performing this analysis. If the analytic system fails, the specimens should be refrigerated at 4-8 °C until the analytical system is restored. If long-term interruption is anticipated, refreeze specimens at ≤-20 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Negative rubella results are hand-entered into a list and sent by facsimile to the National Center for Health Statistics on a weekly basis.

All test results data are compiled in the ORACLE system and sent on a 3.5" floppy diskette and hard copy format to the National Center for Health Statistics on a regular basis as data collection at each location is completed.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (e.g., electronic, mainframe, data files, laboratory notebook, floppy diskettes) are used to track specimens. We recommend that records be kept for 6 years. The samples are packaged and mailed to the NCHS serum repository in Rockville, MD, where they are stored at ≤-20 °C for 1 year after analysis. Only numerical identifiers are used. All personal identifiers are kept masked and available only to the project coordinator in order to safeguard confidentiality.

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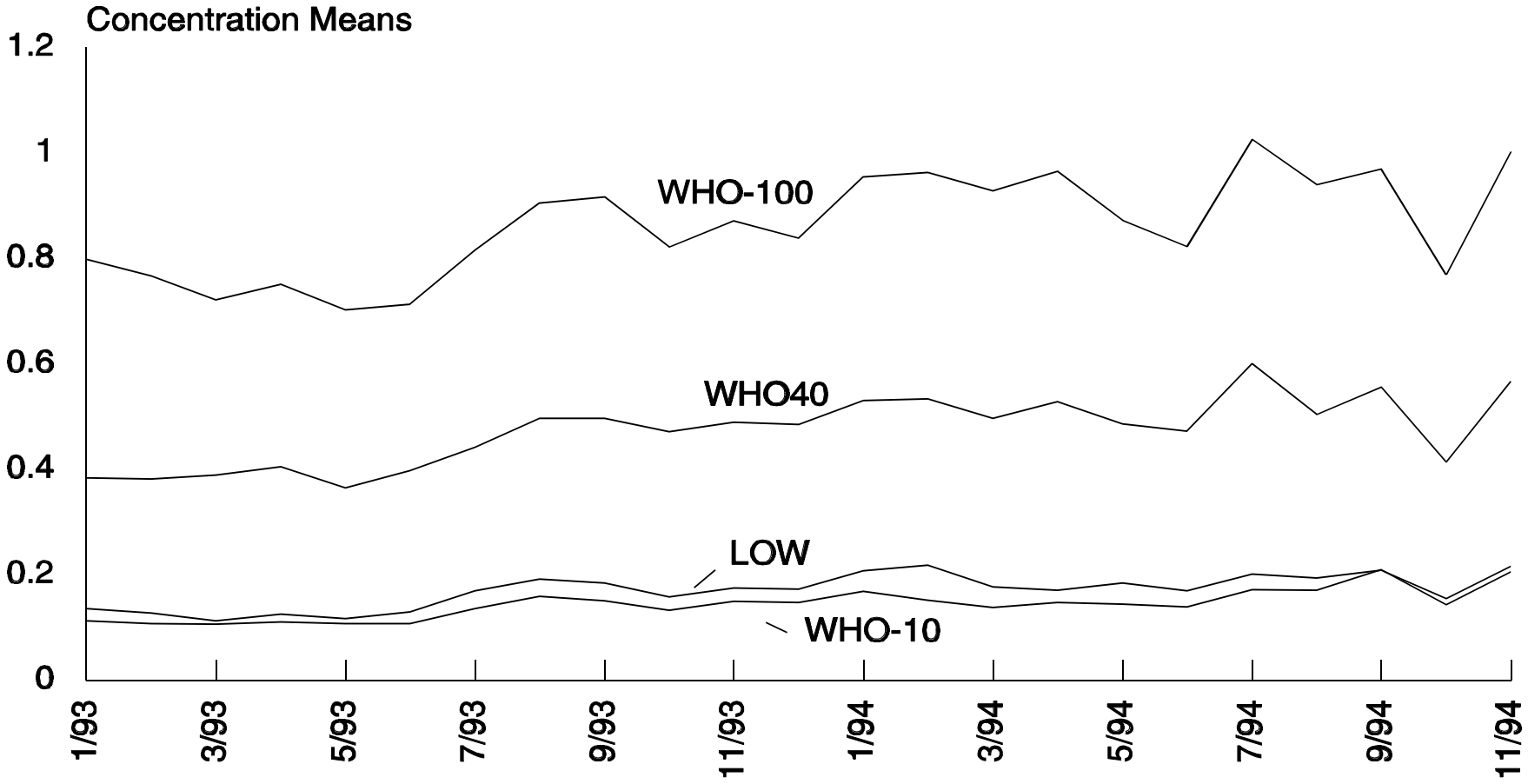
SUMMARY STATISTICS FOR
RUBELLA
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| LOW | 01/11/93 - 11/04/94 | 0.14976 | 0.03935 | 26.273 | 543 |
| NEG | 01/11/93 - 11/04/94 | 0.00535 | 0.00740 | 138.368* | 545 |
| W-10 | 01/11/93 - 11/04/94 | 0.12712 | 0.03157 | 24.836 | 545 |
| W-40 | 01/11/93 - 11/04/94 | 0.43287 | 0.07626 | 17.618 | 545 |
| W-100 | 01/11/93 - 11/04/94 | 0.80748 | 0.11357 | 14.064 | 545 |

* Since the monthly means of the negative pool are consistently at or near 0.00, the coefficient of variation becomes very unstable and should be disregarded as a meaningful statistic.

# Rubella Monthly Means




Note: The plot for the negative (NEG) control pools shows no significant departure from zero and was omitted from the chart for the purpose of graphic clarity.

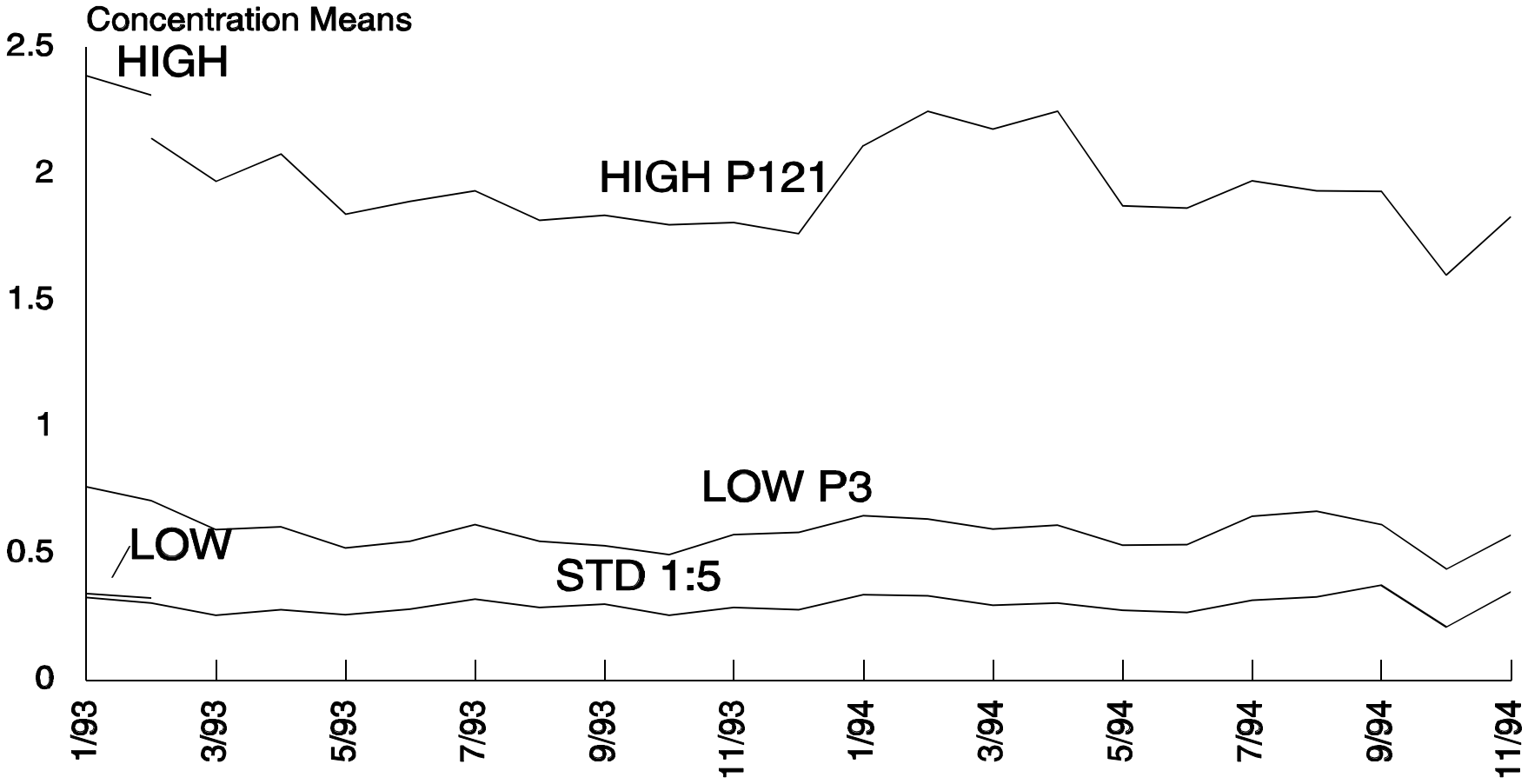
SUMMARY STATISTICS FOR
VARICELLA
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| STD15 | 01/11/93 - 11/04/94 | 0.29394 | 0.04397 | 14.960 | 545 |
| LOW | 01/11/93 - 02/24/93 | 0.33193 | 0.04867 | 14.661 | 115 |
| HIGH | 01/11/93 - 02/24/93 | 2.34043 | 0.15766 | 6.736 | 115 |
| HP121 | 02/26/93 - 11/04/94 | 1.94123 | 0.20734 | 10.681 | 430 |
| NEG | 01/11/93 - 11/04/94 | 0.00439 | 0.01129 | 257.074* | 545 |
| LOP3 | 01/11/93 - 11/04/94 | 0.61472 | 0.09772 | 15.897 | 545 |

**

** Since the monthly means of the negative pool are consistently at or near 0.00, the coefficient of variation becomes very unstable and should be disregarded as a meaningful statistic.

# Varicella Monthly Means




Note: The plot for the negative (NEG) control pools shows no significant departure from zero and was omitted from the chart for the purpose of graphic clarity.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The test procedure is a solid-phase enzyme immunoassay technique called an indirect enzyme immunoassay (EIA). Diluted test samples are placed into the toxoplasma antigen-coated wells of the microplate. Antibodies will bind to the Toxoplasma antigen. Unbound antibody and other serum proteins are removed by washing. Peroxidase-labelled monoclonal antibody specific for human gamma chain immunoglobulin (IgG) is added. The monoclonal antibody binds to the IgG antibody-Toxoplasma antigen-complexes attached to the microplate wells. Unbound conjugate is removed by washing. A solution of peroxidase substrate and chromogen ortho-phenylene diamine (OPD) initiates a color reaction stopped by addition of an acid. The enzymatic reaction, read as optical density on a spectrophotometer set at 492 nm, is proportional to the quantity of *Toxoplasma gondii* IgG antibody present in the test sample. The results are calculated using a standard curve, and expressed as IU/mL.

Toxoplasmosis, caused by the parasite *Toxoplasma gondii*, is usually an asymptomatic infection with few serious after-effects. However, people with acquired immune deficiency syndrome (AIDS) may develop life-threatening central nervous system disease. Infection during pregnancy may also cause severe congenital abnormalities. Because parasitic infection is very difficult to diagnose, the detection of Toxoplasma-specific antibody is an acceptable means of confirming infection (1-4).

## 2. SAFETY PRECAUTIONS

Wear gloves, lab coat, and safety glasses while handling all human blood products. Place disposable plastic, glass, and paper (pipet tips, gloves, microtiter plates, etc.) that contact serum samples in a hazardous waste container prior to autoclaving. Wipe down all work surfaces with 10% sodium hypochlorite (bleach) solution when work is finished. We recommend that you use the foot pedal on the Micromedic Digiflex pipettor to avoid any skin contamination.

> *Avoid skin and mucous membrane contact with the substrate buffer, chromogen (OPD), and stopping solution due to a risk of toxicity, irritation, burns, and chronic effects.*

Material safety data sheets (MSDSs) for sulfuric acid, hydrochloric acid o-phenylenediamine and sodium hypochlorite are kept in the *Working Safely with Hazardous Chemicals* manual.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. The integrity of specimen and analytical data generated by this method is maintained by computer-assembled data, and data stored in multiple computer systems. Data files, containing the date, analytical run ID, and specimen analytical results by specimen ID, are stored on two Dell 310 hard drives and two sets of 3 1/2" high-density (HD) diskettes kept at different locations. Specimen tracking is accomplished by using a laser bar code reader controlled by a dBASE III Plus custom-written file system.

b. Routine backup procedures include archival on 3 1/2" HD diskettes after each run. One set of diskettes is stored offsite.

c. Documentation for system maintenance is contained in hard copies of data records for 5 years.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. No special instructions such as fasting or special diets are required.

b. Specimen type: may be serum or plasma.

c. The optimal amount of specimen required is 0.5 mL to allow for repeat testing; the minimum volume is 20 µL (0.02 mL).

d. Specimen stability has been demonstrated for 5 years at $\leq$-20 °C.

e. The criterion for unacceptable specimen volume is <0.02 mL.

f. Do not use hyperlipemic or hemolyzed serum.

g. Specimen handling conditions require separation of serum/plasma from cells and storage of the specimen at ≤-20 °C until time of analysis. Samples thawed and refrozen are not compromised.

**5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES**

Not applicable for this procedure.

**6. EQUIPMENT AND INSTRUMENTATION MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS**

a. **Instrumentation**

(1) MAXline microplate reader with IBM AT computer equipped with SOFTmax software for reader control and data analysis, and printer (Molecular Devices Corp., Menlo Park, CA).

(2) Laser handgun scanner, wedge reader, power pack, and PC IRL software (INTERMEC, Norcross, GA).

(3) Custom-designed dBASE III software program to integrate the data from both the microplate reader and the laser scanner (Division of Parasitic Diseases, Center for Infectious Diseases, CDC, Atlanta, GA).

(4) Micromedic Digiflex Automatic pipettor equipped with 2000-µL dispensing syringe, 40-µL sampling syringe, 0.75-mm tip, and foot pedal (ICN Biomedical, Costa Mesa, CA).

(5) Titertek pipette, 12-channel variable-volume, 50- to 250-µL (ICN Biomedical).

(6) Water bath, 37 °C (Daigger Scientific, Manassas, VA).

(7) Vortex Genie mixer (Fisher Scientific, Atlanta, GA).

(8) Magnetic stirrer and stirring bars (Baxter Scientific Products, Atlanta, GA).

b. **Other Materials**

(1) Platelia Toxo IgG kit (Sanofi Diagnostics Pasteur, Chaska, MN).

(2) Deionized water (Millipore, Bedford, MA).

(3) 25-, 50-, 100-, and 1000-mL graduated cylinders (Corning Glass Works, Corning, NY).

(4) 50-mL plastic beaker for preparing working substrate (Any vendor).

(5) 1000-mL screw-cap glass Wheaton bottle (Wheaton Industries, Millville, NJ).

(6) 96 Titertube micro tubes, racked (Bio-Rad Laboratories, Richmond, CA).

(7) Pipette tips (1- to 200-µL) for Titertek, racked (Marsh Biomedical Products, Rochester, NY).

(8) Kay-Dry paper towels and Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).

(9) Vinyl examination gloves (Travenol Laboratories, Inc., Deerfield, IL).

(10) 10% sodium hypochlorite (bleach) solution (any vendor).

(11) 1.0-mL disposable plastic pipets (Falcon, Oxnard, CA).

(12) Test tube racks, 13-mm, for specimen vials (any vendor).

c. **Reagent Preparation**

All reagents are supplied in the Platelia Toxo IgG kit. Store all reagents at 4-8 °C.

(1) Wash solution concentrate (10X) . 100 mL
Tris sodium chloride buffer (pH 7.6), 1% Tween-20 (polyoxyethylene-20 sorbitan monolaurate), and 0.01% thimerosal ($C_9H_9HgO_2SNa$). Dilute to working solution with deionized water.

(2) Conjugate concentrate (50X), 0.6 mL
Murine monoclonal antibody to human gamma chains coupled with horseradish peroxidase. Dilute with assay diluent.

(3) Assay diluent
2 80-mL bottles, each containing Tris sodium chloride buffer (pH 7.6), bovine serum albumin (BSA), phenol red, and 0.01 g/dL thimerosal.

(4) Stopping solution
12 mL; 4 N $H_2SO_4$; ready to use. CAUTION:CORROSIVE.

(5) Chromogen tablets
12 30-mg tablets, each contain o-phenylenediamine • 2 HCL (OPD).

> *CAUTION: Toxic, potential carcinogen. Avoid contamination with skin and mucous membranes. Use plastic tweezers to handle tablets.*

(6) Inactivated Toxoplasma gondii antigen (RH strain)
Coated on one 96-well microplate. Allow microplate to reach 20-25 °C before opening sealed bag. Remove only the required number of strips. Unused strips can be stored up to 1 month at 4-8 °C if bag is tightly sealed with tape.

(7) Substrate buffer
120 mL containing 0.05 M (pH 5.6) citric acid and sodium citrate, 0.03% (v/v) hydrogen peroxide, and 0.01 g/dL thimerosal .

(8) Working washing solution (WWS)
Dilute 100 mL of washing solution concentrate (WSC) in 900 mL of deionized water for each microtiter plate. Mix thoroughly. Between uses, store the diluted wash solution at 4-8 °C for a maximum of 28 days. Before each use, examine for evidence of microbial contamination. Discard if solution becomes cloudy or develops a foul odor, or if mold colonies are seen.

(9) Working conjugate (WC)
Dilute 0.5 mL of 50X conjugate concentrate in 25 mL diluent for each plate 15 min before the end of the first incubation period. Mix thoroughly and use immediately.

(10) Substrate-chromogen solution (SCS)
Add 1 OPD Tablet for each 10 mL of substrate buffer 5 min prior to the end of the second incubation period. Protect solution from light until ready to use. Once prepared, use within 10 min.

d. **Standards Preparation**

(1) Calibration standards are supplied ready to use by Sanofi Diagnostics Pasteur, as 0, 6, 60, and 240 IU/mL. The 0 IU calibrator contains human serum negative for T. gondii IgG antibody with 0.01 g/dL thimerosal. The 6-, 60-, and 240-IU calibrators contain human serum positive for T. gondii IgG antibody with 0.01 g/dL thimerosal.

The standards are calibrated by Sanofi Diagnostics Pasteur (Copenhagen, Denmark) to the World Health Organization's "Toxo 60" serum in International Units (IU).

(a) Using the Micromedic Digiflex, dilute standards 1:101 by aspirating 5 µL of 0-, 6-, 60-, and 240-IU standards and flushing them into microtubes with 500 µL diluent. Add 200 µL of the diluted 60 IU standard to 200 µL diluent to obtain a 30 IU standard. Place diluted 0-, 6-, 60-, and 240-IU standards in rack in positions A1, B1, C1, and D1, and place the 30-IU standard in position E1.

(b) Using the Micromedic Digiflex, dilute two quality control (QC) samples 1:101 by adding the 5-µL sample to 500 µL of diluent; place the samples in a microtube rack in positions F1 and G1.

e. **Preparation of Quality Control Materials**

In-house QC pools are prepared in the low concentration ranges of the curve. These QC pools are made from previously-analyzed participant serum samples that have been pooled selectively. The QC pools are then aliquoted into appropriately small quantities to minimize any influence of repeat freezing and thawing. Pools are coded and identified by the pooling date. These serum pools, stored at $\leq$-20 °C, are considered to be stable indefinately.

Sufficient material is pooled and aliquoted to ensure a supply of QC material sufficient to last the length of the NHANES III project.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. **Calibration Curve**

(1) A calibration curve is constructed by using the measured absorbance value of the 0-, 6-, 30-, 60-, and 240-IU standards plotted versus concentration. The 4-parameter curve fit should be chosen in Softmax software.

(2) The calibration curve should be displayed in the "Show curve" window. If the correlation coefficient is >0.98, the test is acceptable, and test results may be printed.

b. **Verification**

(1) To validate the assay, the manufacturer states that the following criteria must be met:

1) $\frac{\text{OD B1}}{\text{OD A1}} > 2.0$

2) OD D1 > 0.8

Where A1, B1, and D1 refer to microtiter plate wells containing the 0-, 6-, and 60-IU standards, respectively, and OD represents the optical density.

(2) The 2 QC samples in positions A6 and A7 should be >10 IU but <21 IU.

(3) The standards are supplied by Sanofi Diagnostics Pasteur and are calibrated to the World Health Organization's "Toxo 60" serum in International Units.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. **Preliminaries**

(1) Place the specimens in test tube racks in following positions:

(a) Calibrators in A1, B1, C1, D1, and E1.

(b) QC specimens in F1 and G1.

(c) Unknowns 1-89 in A2-H12.

(2) Using the laser scanner, follow the instructions of the TOXO program to read the barcode of each specimen in the plate configuration. Choose BARCODE from PC Main Menu, then B. NHANES PENDING, then GUN/WAND/PC KEYBOARD. Enter Test Date, Plate Name HMM/DD/YY1, and Kit lot number. Scan the specimen barcode in the appropriate plate position. Steps (1) and (2) may be performed prior to the test day.

(3) Bring reagents and serum specimens to 20-25 °C before use.

(4) Prime the Micromedic Digiflex pumps with the serum diluent.

(5) Label the microwell strips A-H according to their position in the plate holder.

(6) Prepare the working washing solution (WWS).

b. **Sample preparation**

(1) Vortex each sample.

(2) Using the Micromedic Digiflex, dilute the specimens and controls 1:101 by adding 5 µL sample to 500 µL diluent in the appropriate tube of the 96 racked microtubes.

c. **Instrument setup for the Vmax plate reader.**

(1) Turn on both the computer and the Vmax reader.

(2) Choose SOFTMAX from the PC main menu, then choose F8 Protocals. Place the light bar on TOXOGNEW protocol, press F3 to recall file, answer RECALL PLATE ANALYSIS ONLY?, N. Answer RECALL COMPLETE PROTOCOL?, Y. Press F2 for MAIN MENU, then F3 for INSTRUMENT SETUP, and then enter the following parameters:

- Plate name: Hmmddyy1
- Mode: Endpoint L1-L2
- Wavelength L1: 490
- Wavelength L2: 650
- Data display: Analyzed
- Std curve: 4-para
- Automix, Autocal, Autodisk, Autoprint: ON

d. **Operation of Assay Procedure**

(1) Wash microplate strips once with WWS. Invert the microplate and gently tap on the absorbent paper to remove remaining liquid.

(2) With the 12-channel Titertek pipette, add 200 µL of diluted samples to the appropriate wells corresponding to the sample position read by the laser gun. Cover the microplate with adhesive film.

(3) Incubate the microplate at 37 ±2 °C in the water bath for 60 ± 5 min.

(4) Prepare the working conjugate (WC) 15 min before the end of the first incubation period.

(5) Wash the microplate three times using WWS. Invert the microplate and gently tap on the absorbent paper to remove remaining liquid.

(6) Add 200 µL of conjugate solution to each well with the 12-channel Titertec pipet. Cover the microplate with adhesive film.

(7) Incubate the microplate at 37 ±2 °C for 60 ± 5 min.

(8) Prepare substrate-chromogen solution 5 min prior to the end of the second incubation period. Protect from light until ready to use.

(9) Wash the microplate four times using WWS. Invert the microplate and gently tap on the absorbent paper to remove remaining liquid.

(10) Add 200 µL of substrate-chromogen solution to each well with the Titertec pipet.

(11) Incubate the microplate in the dark at 20-25 °C for 30 ± 2 min.

(12) Add 100 µL of stopping solution to each well with the Titertek pipet.

(13) Wipe the underside of plate wells dry with a lint-free tissue to remove condensation.

(14) Press F5 to read the microtiter plate.

(15) Press F1 to show the curve, F4 to print the curve, F5 to show the report, F4 to print the report, and F10 to export the data.

(16) Enter the export path, C:\BARCODE, and export the file name, HMMDDYY1.

(17) Press F2 to return to the main menu and F10 to exit to DOS.

(18) At PC Main Menu, choose BARCODE, next MERGE ID's AND VMAX IU VALUES, then B. NHANES PENDING. Enter the Vmax report name (HMMDDYY1.001) and the plate name to be merged (HMMDDYY1).

(19) To print a report, choose BARCODE, REPORTS, then D. COMPLETED NHANES, and B. SPECIMENS BY PLATE/WELL.

(20) Return to BARCODE on the main menu, choose UTILITIES, and then BACKUP DATA TO DISKETTE; insert the MASTNH3.BAK disk.

e. **Recording of Data**

(1) Quality Control Data
For each run, enter the following on the master sheet in the NHANES III binder: kit lot number, expiration date, plate ID, IU values of both QC samples, the absorbance value of B1 divided by the absorbance value of A1, and the absorbance value of D1.

(2) Analytical Results
The raw and analyzed data are printed automatically under the SOFTmax program. The printed report should be filed in the NHANES III data binder. The results are merged with the IDs as explained in sections 8.e.18-19. and printed. This printed report is filed in the NHANES III binder.

f. **Replacement and Periodic Maintenance of Key Components**

(1) Vmax lamp: a spare lamp should be available. Order another if the spare is used for replacement.

(2) Inspect and replace the Digiflex sampling tip as needed.

(3) All micropipettors used in testing clinical specimens should be checked for calibration every 6 months. Pipettors that do not conform to specifications should be autoclaved and sent out for recalibration in accordance with manufacturer's recommendations. Calibration records should be kept for each pipettor by serial number.

g. **Calculations**

(1) The SOFTmax program uses a 4-parameter curve fit to calculate the calibration curve. The unknown value is read off the curve in IU. The SOFTmax program generates a plotted curve and provides curve ststistics and correlation coefficients.

The correlation coefficient should be $\geq 0.975$.

h. **Procedure Notes**

The substrate-chromogen solution should be colorless to very pale yellow. Discard it if it is yellow-orange.

**9. REPORTABLE RANGE OF RESULTS**

a. Samples with results <6 IU are reported as negative for the presence of antibodies to toxoplasma.

b. Samples with optical density readings ≥6 IU are reported positive for the presence of *T. gondii*.

c. Results >240 IU are reported as ">240" to indicate strongly positive.

**10. QUALITY CONTROL (QC) PROCEDURES**

The method described in this protocol has been used for several years in epidemiological health studies. The method has proven to be accurate, precise, and reliable. The calibration standards used in this assay are provided by Sanofi Diagnostics Pasteur and are calibrated to the World Health Organization's "Toxo 60" serum. Estimates of imprecision can be generated from long-term QC pool results.

"Bench" QC specimens are used in this quantitative method. The control specimens are inserted by the analyst in each plate so that judgements may be made on the day of analysis. Results are obtained by running these samples through the complete testing procedure. The data from these materials are used in estimating methodological imprecision and in assessing the magnitude of any time-associated trends.

Each assay includes in-house controls containing low levels of antibodies to toxoplasma. These controls, which are run in duplicate, are randomly scattered throughout the assay. The in-house controls are prepared in sufficient quantity to provide serum samples for all runs for the length of NHANES III project. The pools are aliquoted into small vials and stored at ≤-20 °C. Serum antibodies to toxoplasma will remain stable indefinitely when stored in this fashion.

QC limits are established for each pool. An analysis of variance is performed for each pool after 30 or more characterization runs have been performed.

Table 1 shows the precision and accuracy of a representative pool used for NHANES III.

Table 1
Precision and Accuracy

| n | Mean | 95% limits | 99% limits | Runs | Total CV |
|---|---|---|---|---|---|
| 62 | 14.95 | 14.2-15.7 | 14.0-15.9 | 31 | 19.26 |

Control samples are placed in each microtiter plate in wells F1 and G1. After the results of the QC specimens are determined, the long-term QC charts are examined to determine if the system is "in control."

**11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA**

Consult with the supervisor for appropriate corrective actions. Check to see that plate reader is operating properly. If necessary, reanalyze all specimens.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

Do not use hyperlipemic or hemolyzed serum.

**13. REFERENCE RANGES (NORMAL VALUES)**

The prevalence of chronic toxoplasmosis among adults will vary significantly in different populations.

The kit manufacturer recommends that all titers of <6 IU be considered NEGATIVE and all titers ≥6 IU be considered positive.

A positive result is an indication of past infection with *Toxoplasma gondii*.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

Not applicable.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Allow specimens to reach and maintain 20-25 °C during analysis. Otherwise, store them at ≤-20 °C.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

This laboratory does not have an alternative method for performing this test. Specimens will be stored at ≤-20 °C until testing resumes.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

Not applicable.

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record-keeping means (e.g., electronic-dBase, mainframe data files, floppy disc) should be used to track specimens. The records will be maintained for 5 years, and duplicate records will be kept off-site in electronic format. Excess serum is stored in NCHS, CDC.

**19. QUALITY CONTROL SUMMARY STATISTICS**

Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Lp(a) is measured immunochemically by using an enzyme-linked immunosorbant assay (ELISA). The sample is added to a microtiter plate with wells that have been precoated with a monoclonal antibody (capture antibody) to apo(a), the unique protein contained in Lp(a). Lp(a) in the sample is thus bound to the plate-bound antibody. Excess sample is removed, the well is washed extensively, and a polyclonal antibody to apo (a) (a detection antibody) to which a marker enzyme (alkaline phosphatase) is covalently bound, is added. The detection antibody binds to the plate-bound Lp(a). Following removal of excess detection antibody, the well is washed extensively. A substrate (p-nitrophenyl phosphate) for the marker enzyme is added, the plate is incubated, and the reaction is stopped by adding NaOH. The amount of yellow p-nitrophenol produced is proportional to the Lp(a) concentration. The reaction sequence is as follows:

Monoclonal Ab + Lp(a) -----> Mab-Lp(a) (immobilized complex)

Mab-Lp(a) + polyclonal Ab-marker enzyme ----->Mab-Lp(a) - polyclonal Ab-marker enzyme complex

Complex + p-nitrophenyl phosphate -----> p-nitrophenol

Lp(a) concentrations above 30 mg/dL are associated with increased risk for corony heart disease (CHD), stroke, and probably peripheral vascular disease (1-4). The pathophysiologic mechanisms underlying the associations are unclear, but it is currently thought that Lp(a) may interfere with thrombolysis (1). The association with CHD is independent of other risk factors, and Lp(a) itself appears to be metabolized independently of other lipoproteins. Lp(a) concentrations are unrelated to the concentrations of other serum lipids and lipoproteins (1).

## 2. SAFETY PRECAUTIONS

### a. Blood Handling

The improper handling of blood samples from patients with infectious hepatitis or HIV can lead to infection of personnel who collect, handle, analyze, or otherwise use the samples. Transmission can occur by ingestion, contact, or inhalation, and personnel must exercise care when handling blood samples. Never pipette samples by mouth. Avoid contact with the serum. Cover any scratches or cuts on fingers and hands very carefully before handling plasma. Always use gloves when handling the samples. Store all samples in sealed containers, and minimize the generation of aerosols by not leaving samples open to the atmosphere longer than necessary.

The infectious potential of hepatitis has been estimated to be 30 times that of HIV: therefore, take the usual precautions for handling blood specimens to prevent hepatitis infection as a guide to prevent AIDS infection as well. All personnel should adhere to the CDC Universal Precautions for Prevention of HIV Infection in Health Care Settings (5,6).

### b. Sample Spillage

Disinfect the contaminated area with a 1:10 dilution of household bleach in water. Discard any wipes used to clean spilled samples in a red biohazard bag.

### c. Daily Safety Precautions

All personnel working in the laboratory must wear eye protection, gloves, and buttoned laboratory coats. Remove gloves when answering the telephone or when leaving the immediate work area. Dispose of all used gloves, vials, pipettes and other items that contact specimens in a biohazard box lined with a biohazard bag. Clean all work benches at the end of each day with fresh bleach solution and cover the lab bench with plastic-backed white paper.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Analytical data are downloaded from the automated analyzer (see Section 6) to a computer and then to computer-generated NHANES logs. When all analyses for specimens are completed and the results have been reviewed, the NHANES logs are sent via diskette to NCHS. The QC data are sent to NCHS on a quarterly basis. Any "panic" or critical values are communicated to NCHS via telephone or FAX.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. There is little current information on the effect that fasting has on the measurement of Lp(a).

b. Lp(a) can be measured in plasma or serum.

c. The sample volume required for the "Macra" Lp(a) test is 10 µL. Adequate sample volume should be provided to allow repeat analysis if necessary.

d. Two-mL plastic screw-top Nalgene cryovials are used for sample shipment and storage. Another acceptable container for shipment and storage of serum is a glass serum bottle equipped with a rubber stopper and sealed with an aluminum seal.

e. Serum should be stored at ≤-20 °C for no longer than 4 weeks. For long term storage (> 4 weeks) samples should be maintained at -70 °C. A Lp(a) storage stability study conducted by the Johns Hopkins Lipid Research Laboratory indicated a decrease in Lp(a) value after 9 to 12 months storage at -70 °C.

f. Specimen handling procedures

(1) On-site collection

(a) Collect blood in a red-top Vacutainer or other evacuated glass collection tube.

(b) Allow the blood to stand for 45 min at room temperature to allow complete clotting and clot retraction. A shorter period may result in incomplete clotting and the formation of secondary clots later. During the clotting period, leave the collection tube sealed.

(c) Centrifuge the samples at 1500 x g for 30 min at 4 °C. It is preferable to use a refrigerated centrifuge for this purpose, but an unrefrigerated centrifuge can be used if necessary. If an unrefrigerated centrifuge is used, place the samples into an ice bath immediately after centrifuging and maintain them at 2-4 °C thereafter.

(d) Keep samples frozen at -20 °C until they are shipped to the laboratory. If a shipment must be delayed longer than 4 weeks, keep the specimens at -70 °C. If specimens were thawed and refrozen prior to shipment or analysis, note that on the transmittal form.

(e) Ship samples on dry ice via overnight courier service. Do not ship samples to arrive on a weekend or holiday without making prior arrangements with the laboratory.

(2) Samples collected away from the laboratory should generally arrive frozen. If analyses cannot be performed immediately,do not thaw the specimens. Instead keep them frozen at -20 °C for up to 4 weeks. After 4 weeks, store them at -70 °C. The maximum time for -70 °C storage of specimens is undetermined. Refrigerated samples may be used if they are brought promptly from the collection site.

g. Little is currently known about the effects of various conditions such as turbitity or hemolysis on Lp(a) analysis. Dilute any samples deemed too turbid by mixing them with saline (1 part sample, 1 part saline by volume).

h. On the log sheet, note any unsatifactory specimens received by the lab along with the problem codes. If necessary, call the originators to inform them of the problem. Such problems can include cracked vials, inadequately sealed vials, empty vials, gross hemolysis, and thawed samples.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

### a. Instrumentation

(1) Finnpipette multi-channel pipet (Labsystems, Needham Heights, MA). Used to dispense colorimetric reagent and stop solutions. This pipet is calibrated by Premier Technologies, Inc. every 6 months.

(2) Dyna Washer II plate washer (Dynatech, Inc., Chantilly, VA). Used to wash the test wells at the indicated times. The wash pins and tubing are cleaned or replaced on a regular maintenance schedule.

(3) Vmax kinetic microplate reader with Softmax v 2.01 (Molecular Devices, Inc., Menlo Park, CA). Used to read the absorbance rates in the test wells after all steps are completed. The absorbance readings are saved as a file on the computer (386 DX, 33 Mhz), which is linked to the plate reader and is used to calculate the Lp(a) concentrations.

(4) Rotomix type 50800 rotator (Thermodyne, Dubuque, IA).

### b. Materials

(1) Lp(a) enzyme-linked immunosorbant assay (ELISA) kit (Strategic Diagnostics, Newark, DE).

### c. Reagent Preparation

(1) Wash buffer (20X)
Prepare the wash buffer by diluting 50 mL of concentrate with enough deionized water to bring the final volume to one liter. Allow any crystals in the concentrate to dissolve at room temperature and then mix the solution thoroughly. Store the diluted wash buffer at 20-25 °C for 2 weeks, or at 2-8 °C for 3 months.

(2) Color developing solution
Prepare fresh color developing solution 20 min prior to use by dissolving one ortho-phenylenediamine (OPD) tablet in 5 mL of colorimetric reagent for every 40 test wells.

### d. Standards and Control Materials

Because standards and control materials are pre-diluted 1:201 they need not be diluted with sample diluent.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibrate the plate reader by reading a blank template and a marked template, both of which are supplied by the manufacturer. Check the readings against the acceptable operation parameters specified by the manufacturer. Perform this calibration with the regular maintenance schedule. If these readings are not within the specified parameters for each test well, check the settings and repeat the calibration procedure.

b. Verify calibration by analyzing the six standards in triplicate along with the samples. Use the regression information (stated values vs calculated values) to determine the acceptability of calibration linearity. The $R^2$ of the regression equation should be 1.0 ±0.1.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### a. Receipt of Samples

Log in arriving samples by batch according to the shipping transmittal sheet that accompanies the samples. Record the receipt date and the sample condition (OK, thawed, missing, etc.) using the appropriate sample condition code on the transmittal sheet. Transfer the samples to a -70 °C freezer for storage until the time of analysis.

### b. Instrument Settings

- Wave length - 490 nm

- $\lambda_1$ only
- Read Mode - optical density

c. **Procedure**

(1) Allow reagents to come to room temperature, and mix them thoroughly prior to use.

(2) Remove the plate from the package of Lp(a) test well strips. Return unused test well strips to their original pouch with the desiccant pack and seal the pouch with tape.

(3) Dilute serum or plasma samples 1:201 in 13- x 100-mm test tubes by adding 10 µL of sample to 2.0 mL of sample diluent. Vortex the solution intermittently.

(4) Pipet 100 µL of prediluted standards and controls, in triplicate, into the bottom of the test wells.

(5) Pipet 100 µL of diluted samples, in duplicate, into the bottom of the remaining wells.

(6) Incubate the plate at room temperature (18-25 °C) for 1 hour $\pm$2 min on an orbital platform shaker set at 120 $\pm$5 rpm. Set the timer to start timing when the last sample has been added.

(7) While the plate is incubating, type the sample IDs into the computerized run sheet (spreadsheet) along with any applicable dilution factors.

(8) At the end of the incubation period, wash each well four times using the plate washer. Invert the plate and gently tap it on an absorbent pad to remove any excess wash solution.

(9) Add 100 µl of anti-Lp(a)-HRP conjugate into the bottom of each well using the multi-channel pipet.

(10) Incubate the plate at room temperature for 20 $\pm$1 min on a rotator set at 120 $\pm$5 rpm. Set the timer as soon as the plate is placed on the rotator.

(11) Prepare the color-developing solution.

(12) Repeat plate washing (step 8).

(13) Dispense 100 µL of color-developing solution into each well and incubate the plate at controlled room temperature for 20$\pm$1 min on the rotator. The timing of the color development step is critical for the precision and accuracy of the results.

(14) Dispense 50 µL of stopping reagent with a multichannel pipet into each test well.

(15) Measure the absorbance of each well at 490 nm within 15 min of adding the stop reagent.

(16) Download the absorbance reading to the computerized run sheet and compute the Lp(a) concentration, then printout the review.

(17) Record the QC data and verify that the run is acceptable. Examine both the QC pool values and the r values for the regression equation. QC data should be within laboratory limits, and the r value for the regression equation should be $\geq$0.985.

d. **Review of Results**

(1) Because each sample is measured in duplicate, the difference between the two values is checked by computer for acceptability. For samples with concentrations from 0 to 40 mg/dL, the acceptable range between values is $\leq$ 7 mg/dL; for samples with concentrations from 41 to 80 mg/dL, the acceptable range is $\leq$12 mg/dL; for concentrations >80 mg/dL, the acceptable range is 18 mg/dL. These ranges represent the 99% limit for within-run imprecision.

(2) Any samples with concentrations $\geq$ 80 mg/dL must be diluted and reanalyzed because the upper limit of the

standard curve is 80 mg/dL.

## 9. REPORTABLE RANGE OF RESULTS

The reportable range of concentration has not been established for this test. The normal level is accepted as 0 to 30 mg/dL (1).

## 10. QUALITY CONTROL (QC) PROCEDURES

The laboratory monitors the QC of the Lp(a) assay by running two manufacturer control pools (control #1, Lot 104326; control #2, Lot 104327) with ranges of 15-20 mg/dL and 32-48 mg/dL, respectively. In addition, the laboratory runs an in-house pool in the 55-65 mg/dL range.

The control limits for each pool are calculated from the average and standard deviations of the daily means and daily ranges of the analyses of the control pools. Temporary control limits for each pool are calculated from the first 20 run days. Permanent control limits are determined after 50 run days and then used until the supply of the pool is exhausted. Continuity from pool to pool is maintained through at least 20 overlapping runs in which the replacement pool is analyzed concurrently with the current pool. The 20-run temporary limits are established for the replacement pool from this period of overlap. During this period, the acceptability of the analyses is determined on the basis of results for the current pool: that is, the analyses of the current pool must be "in control" before the data are accepted and used to establish control limits for the replacement pool.

Limits for the new pool are calculated and evaluated, and control charts are prepared. Care is taken to ensure that data used in the calculations are only from runs that are "in control" (i.e., that meet established quality control criteria). As soon as acceptable temporary limits are established, control is transferred to the replacement pool, and the original pool is no longer analyzed.

It is important that the data used to calculate control limits be collected during a stable analytical period representative of overall laboratory performance. The daily mean ($\bar{x}$) for a control pool is calculated for each run by averaging the two values for the pool:

$$\bar{x} = \frac{\Sigma \text{ of control values}}{\text{number of control values}}$$

The overall mean for the pool is calculated by dividing the sum of the individual run means by the number of runs (N).

N = 20 run days for temporary limits
N = 50 run days for permanent limits

The standard deviation of the run means (SD) is also calculated for the control pool.

The range (R) for each run is the difference between the highest and the lowest value obtained for the pool in that run. Because duplicates are used, the range is the difference between duplicates. The average range for a series of runs is calculated by dividing the sum of the ranges for the series by the number of runs. Again, N=20 for temporary limits, and N=50 for permanent limits.

The control limits (99%) for the means chart are calculated, and the values rounded to the nearest whole number.

The limits used for the R chart are calculated by multiplying R for the pool by probability factors appropriate for duplicate analyses. The lower limit for the range chart is zero because there is no negative range.

Before the control chart can be used for quality control, it is reviewed to determine that the data have been collected during a stable analytical period. The chart is examined for outliers, for periods of questionable or unstable performance, and for evidence of excessive bias. An outlier will distort the 99% limits if incorporated into the final calculations. An outlier is considered to be any value of $\bar{x}$ that falls outside the 3-SD limits or any value of R that exceeds the upper control limit for R. These values are eliminated, as are values from any questionable period of performance. The values of $\bar{x}$, SD, and the control limits are recalculated, and the charts are evaluated again.

When values from at least 20 acceptable runs are used for the final calculations, the control charts are constructed

according to the criteria listed below. If there are not 20 acceptable runs after unacceptable data have been eliminated, continue analyzing the pool until at least 20 acceptable runs have been completed.

The criteria used in the Control Laboratory were those that served as guidelines for the Lipid Research Clinics Program and are designed to minimize both bias and variability.

Construct Levey-Jennings mean and range charts. Each day, enter the daily mean and range values on the appropriate chart. Keep detailed notes on anything that effects the quality of analyses (personnel changes, reagent problems, changes in instrument components, etc.,).

Values for $\bar{x}$ outside the 3-SD limit or values of R that exceed the (R + 3.27R) limit indicate the run is out of control. The run must be repeated. A value outside the 95% limits but not the 99% limits is interpreted as an indication of possible trouble.

For runs consistently out of control, check the expiration date of calibrator material and standards.

If one QC pool fails to maintain the specified ranges, regardless of whether or not calibrators are within range, the run is declared "out of control" and the analyses are repeated. Any exception to this rule is decided by the lab director, the lab supervisor, and the quality control manager.

## 11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If one or more quality control samples fall outside the ±2 SD range or a within-run control sample shifts >2 SD from its previous value, then take the following steps:

a. Determine the type of errors occurring (random, systematic, or both) on the basis of the control rules being violated.
b. Correct the problem, then reanalyze the participant's samples and control samples, testing for statistical control by the same procedure.

c. Consult the quality control manager and laboratory director for any decision to report data when there is a lack of statistical control. For NHANES III analyses, no data are accepted from "out of control" runs.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Serum or plasma samples with Lp(a) concentrations >80 mg/dL should be diluted into the assay range with sample diluent. The result obtained should be multiplied by the dilution factor.

b. Assay conditions have been established by the manufacturer for operation between 18 and 25 °C. The expected absorbance of the 80 mg/dL standard has been set at 22 °C. Ambient temperatures slightly above or below 22 °C may affect the absorbance, but they should not affect measured values.

c. The following concentrations of potentially interfering substances do not interfere with the ability of the assay to quantitate Lp(a).

- Triglyceride: ≤2500 mg/dL
- Hemoglobin: ≤5 g/L
- Bilirubin: ≤2.5 mg/dL

## 13. REFERENCE RANGES (NORMAL VALUES)

Currently there are no reference ranges for Lp(a).

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

There are no critical call or "panic" values for this assay.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach and maintain ambient temperature during analysis, but they should be returned to 2-8 °C storage as soon as possible. After a run is accepted, return samples to long-term storage at -70 °C.

**16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There is no backup method for the Lp(a) assay. If the analytical system fails, store samples at -20 °C until the system is restored to functionality. If long-term interruption (greater than 4 weeks ) is anticipated, store samples at -70 °C.

**17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

a. When results are downloaded from the Lp(a) spreadsheet, the sample and QC results are reviewed for acceptability. If the run is "out of control," repeat it on the next run day. If the run is judged "in control," accept the run and download the results into the automated NHANES database. If a result exceeds the specified limits (i.e., 80 mg/dL), dilute the sample and repeat the analysis on the next run day. Repeat analyses of individual samples if the range for the duplicate assays exceeds the limits specified above.

b. When the analysis of the specimens is complete, the data are reviewed by the data coordinator or by the senior lab technician (preliminary review). After all necessary repeat analyses are completed, the results are then reviewed by the laboratory supervisor, data coordinator, or laboratory director (final review).

**18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING**

Standard record keeping means (electronic and log accession books) are used to track specimens. All records, including related QC data, are maintained for a minimum of 5 years, in electronic and printed form. Only numerical identifiers are used to identify subjects. All related personal identifiers are maintained by the NCHS coordinator to safeguard confidentiality and are not available to the laboratory.

Residual NHANES III specimens for which analyses have been completed are sent to the NCHS specimen repository in Bethesda, MD, for long-term storage.

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7. Bachorik PS, Lovejoy KL, Carroll MD, et al. Measurement of apolipoproteins A-1 and B during the National Health and Nutrition Examination Survey (NHANES) III. Clin Chem 1994;40:1915-20.

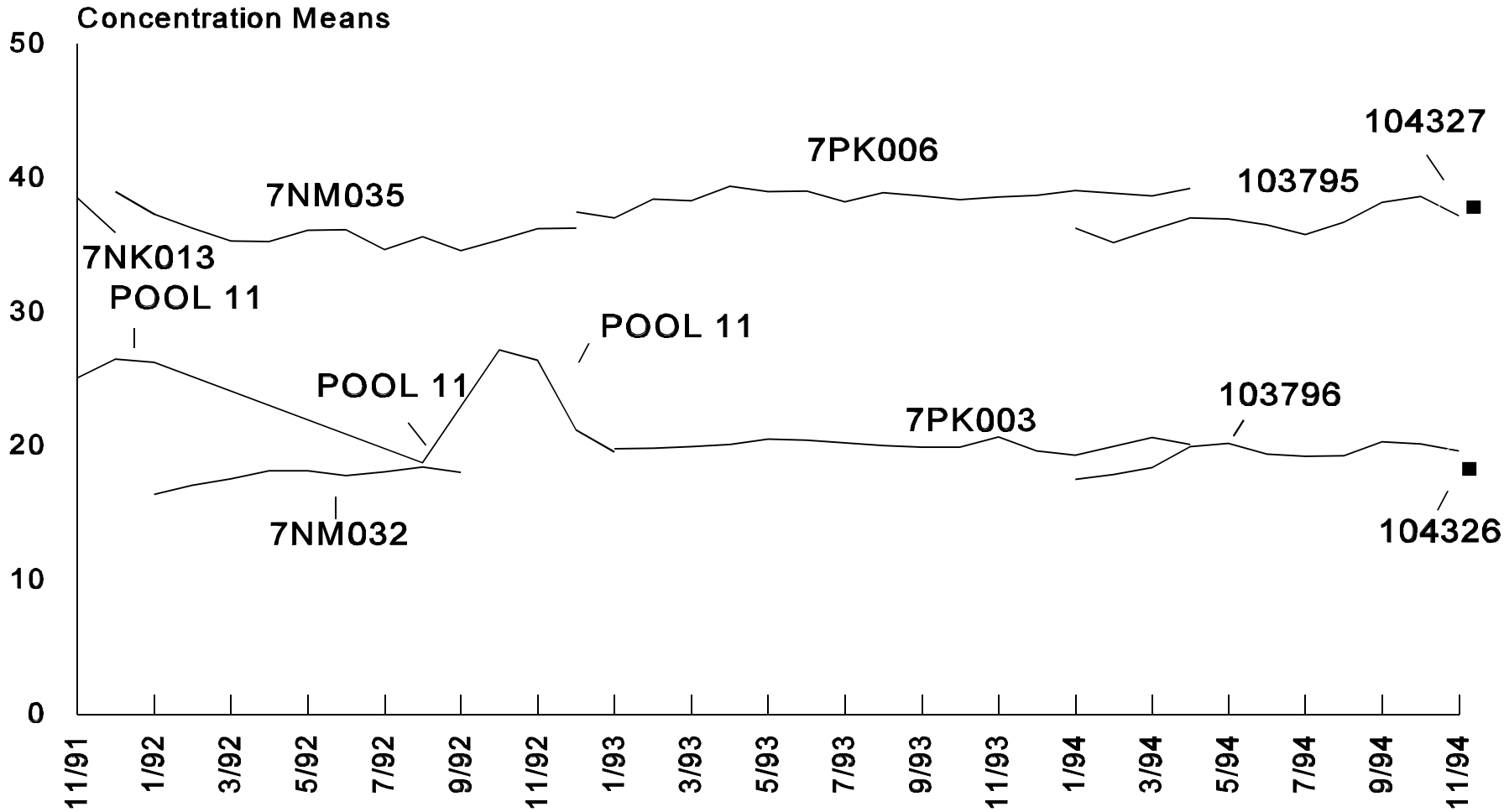
**ACKNOWLEDGMENTS**

We gratefully acknowledge the contributions of Douglas Ahrens, Paul Bachorik, Ph.D., Kathleen Lovejoy, Mary Claire Russell, and Gale Sherman who assisted in developing the methodology, performing the analysis for lipoprotein (a) in the NHANES III study, and preparing the manuscript for this chapter.

SUMMARY STATISTICS FOR
LIPOPROTEIN - A
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| 7NK013 | 11/91 - 12/91 | 37.0556 | 4.02098 | 10.8512 | 36 |
| POOL11 | 11/91 - 01/93 | 24.0903 | 3.67550 | 15.2572 | 144 |
| 7NM032 | 01/92 - 09/92 | 17.8115 | 1.24611 | 6.9961 | 244 |
| 7NM035 | 01/92 - 12/92 | 35.8012 | 2.55151 | 7.1269 | 332 |
| 7PK006 | 12/92 - 04/94 | 38.5795 | 1.76319 | 4.5703 | 459 |
| 7PK003 | 01/93 - 04/94 | 20.0668 | 1.09065 | 5.4351 | 434 |
| 103795 | 01/94 - 11/94 | 36.9064 | 2.58358 | 7.0003 | 374 |
| 103796 | 01/94 - 11/94 | 19.4786 | 1.88356 | 9.6699 | 374 |
| 104326 | 11/94 | 18.5000 | 1.69031 | 9.1368 | 8 |
| 104327 | 11/94 | 38.3750 | 1.99553 | 5.2001 | 8 |

# Lipoprotein - A Monthly Means




NOTE: No samples assayed for Pool 11 during 02/92 - 07/92 and 09/92.

## 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

ApoA-I is the major protein component of HDL constituting approximately 50% of its protein. Under normal conditions, almost all of the apoA-I in serum is found in HDL. ApoB is the major protein component of VLDL and LDL, constituting about half the protein in VLDL and essentially all the protein in LDL. There is one molecule of apoA-I in each apoA-I-containing lipoprotein particle, and apoA-I is therefore a measure of the number of apoA-I-containing lipoprotein particles present. High concentrations of apoA-I are associated with reduced risk for CHD; conversely, high concentrations of apoB are associated with increased risk for CHD. In recent years, most studies which compared apoA-I and HDL-cholesterol, or apoB and LDL-cholesterol as indicators of CHD have revealed that by themselves the Apolipoprotein are somewhat more sensitive indicators of risk than the respective lipoprotein-cholesterol measurements. In addition, since the chemical compositions of the lipoproteins can vary considerably, apoB measurements have been found useful in revealing the presence of high concentrations of apoB-containing particles in patients whose LDL-cholesterol concentrations may be normal or only marginally high (1-3).

## 2. SAFETY PRECAUTIONS

### a. Blood Handling

The improper handling of blood samples from patients with infectious hepatitis or HIV can lead to infection of personnel who collect, handle, analyze, or otherwise use the samples. Transmission can occur by ingestion, contact, or inhalation, and personnel must exercise care when handling blood samples. Never pipette samples by mouth. Avoid contact with the serum. Cover any scratches or cuts on fingers and hands very carefully before handling plasma. Always wear gloves when handling the samples. Store all samples in sealed containers, and minimize the generation of aerosols by not leaving samples open to the atmosphere longer than necessary.
The infectious potential of hepatitis has been estimated to be 30 times that of HIV: therefore, take the usual precautions for handling blood specimens to prevent hepatitis infection as a guide to prevent AIDS infection as well. All personnel should adhere to the CDC Universal Precautions for Prevention of HIV Infection in Health Care Settings (4,5).

### b. Sample Spillage

Disinfect the contaminated area with a 1:10 dilution of household bleach in water. Discard any wipes used to clean spilled samples in a red biohazard bag.

### c. Daily Safety Precautions

Wear eye protection, gloves, and buttoned laboratory coats while working in the laboratory. Remove gloves when answering the telephone or when leaving the immediate work area. Dispose of all used gloves, vials, pipettes and other items that contact specimens in a biohazard box lined with a biohazard bag. Clean all work benches at the end of each day with fresh 10% bleach solution and cover the lab bench with plastic-backed white paper.

## 3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Analytical data are printed then data are transcribed to a log and typed into the computerized log sheet. When all analyses for specimens are completed and the results have been reviewed, the NHANES logs are sent via diskette to NCHS. The QC data are sent to NCHS on a quarterly basis. Any "panic" or critical values are communicated to NCHS via telephone or FAX.

## 4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

a. Fasting status does not affect analysis of apoA-I or apoB. However, since turbidity and chylomicron formation can affect analysis, use of a fasting specimen is preferable.

b. ApoA-I and apoB may be measured in fresh samples or samples that have been frozen for several months.

c. The sample volume required for the Beckman array is 150 µL. Adequate sample volume should be provided for repeat analysis if necessary.

d. One 6-mL plastic screw-top Nalge cryovial is used for sample shipment and storage. A glass serum bottle

equipped with a rubber stopper and sealed with an aluminum seal is also an acceptable shipping and storage container.

e. Store serum at -20 °C for no longer than 4 weeks. For long term storage (> 4 weeks) store samples at -70 °C.

f. Specimen handling procedures

(1) On-site collection

(a) Collect blood in a red-top Vacutainer or other evacuated glass collection tube.

(b) Let the blood to stand for 45 min at room temperature in to allow complete clotting and clot retraction. A shorter standing period may result in incomplete clotting and the formation of secondary clots.

(c) Centrifuge the samples at 1500 x g for 30 min at 4 °C. It is preferable to use a refrigerated centrifuge for this purpose, but an unrefrigerated centrifuge may be used if necessary. If an unrefrigerated centrifuge is used, place the samples into an ice bath immediately after centrifuging and maintain them at 2-4 °C thereafter.

(d) Keep samples frozen at -20 °C until they are shipped to the laboratory. If a shipment must be delayed longer than 4 weeks, keep the specimens at -70 °C. If specimens were thawed and refrozen prior to shipment or analysis, note that on the transmittal form.

(e) Ship samples on dry ice via overnight courier service. Do not ship samples to arrive on a weekend or holiday without making prior arrangements with the laboratory.

(2) Samples collected away from the laboratory should be shipped frozen. If analyses cannot be performed immediately, do not thaw the specimens. Instead, keep them frozen at -20 °C for up to 4 weeks. If storage for more than 4 weeks is required, store samples at -70 °C. The maximum time for -70 °C storage of specimens is undetermined. Refrigerated samples may be used if they are brought promptly from the collection site.

g. Turbidity and chylomicron formation can affect apoA-I and apoB analysis. Samples must be mixed completely and turbid samples are diluted. Dilution is performed by mixing the sample with saline (1 part sample, 1 part saline by volume). The results are calculated accordingly.

h. On the log sheet, note any unsatisfactory specimens received by the lab along with the problem codes. If necessary, call the originators to inform them of the problem. Such problems can include cracked vials, inadequately sealed vials, empty vials, gross hemolysis, and thawed samples.

## 5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

## 6. EQUIPMENT AND INSTRUMENTATION MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

a. Instrumentation

(1) Beckman Nephelometer Automated Array (Beckman Instruments, Inc., Brea, CA).

b. Reagent Preparation (All reagents are supplied ready to use by Beckman Instruments).

(1) Apo Antibody A-1

(2) Apo Antibody B

(3) Buffer

(4) Diluent

(5) Apo diluent

c. Control preparation

(1) ApoA-I control (Beckman Instruments)
Reconstitute the lyophilized control with 1 mL deionized water. Mix the solution thoroughly on a hematology mixer for no less than 30 minutes. Prior to use, dilute the reconstituted material 1:3 with deionized water (1 part control, 2 parts deionized water. This preparation is stable for 2 weeks.

(2) ApoB control (Beckman Instruments)
Reconstitute the lyophilized control with 1 mL deionized water. Mix the solution thoroughly on a hematology mixer for 30 minutes. No further preparation is necessary. This preparation is stable for 2 weeks.

(3) Omega Control
This control is used for apoA-I and apoB analysis. Reconstitute the lyophilized material with 3 mL of the provided diluent. Dispense the diluent slowly against the sides of the vial using a volumetric pipette. Swirl the vial gently until the contents are thoroughly mixed. *Do not vortex*.

d. Calibrator preparation (supplied ready-to-use, Beckman Instruments)

(1) ApoA-I Calibrator
Reconstitute the material with 1 mL deionized water and gently invert the vial until the contents are mixed thoroughly. Prior to use, dilute the reconstituted material again 1:3 with deionized water (one part calibrator material, 2 parts deionized water, by volume).

(2) ApoB Calibrator
Reconstitute the material with 1 mL deionized water and inverted the vial gently until the contents are thoroughly mixed.

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

a. Calibration is retained for 2 weeks and is not lost if the machine is shut off.

(1) Press F2 for Cal status.

(2) Select the analytes to be calibrated by moving the cursor to the desired test and pressing SELECT.

(3) Press F1 to read the antibody cards.

(a) The message "INSERT CARD" will appear.

(b) Insert the antibody cards for apoA-I or apoB.

(c) The message will change to "CARD READ SUCCESSFUL." If unsuccessful, you will be prompted to reinsert the card.

(d) Continue above procedure until both antibody cards have been read into the system.

(4) Move cursor to the buffer lot column and type in the lot number followed by ENTER.

(5) Press F2 for CAL TRAY SET-UP to set calibration tray and enter the calibrator lot numbers.

(6) Press F2 for READ CAL CARDS to read the calibration card.

(7) Insert the "ApoCal" card into the card reader.

(a) Move cursor to APB and press SELECT to deselect APB. The cup is now defined only for the test highlighted.

(b) The Apo calibrator requires a 1:3 dilution for apoA-I and no dilution for apoB calibration, therefore, calibration of both tests from the same sample cup is not possible.

(8) Press F1.

b. If the calibration is unacceptable, check all controls, reagents and calibrators for incorrect lot numbers. Repeat the calibration run. If calibration is still unsuccessful, call the Beckman Instruments Technical Support line.

c. Calibration runs are saved and the control information recorded and kept in a binder which is located in the lab.

d. Calibration is performed every Monday morning.

**8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS**

a. Analyze a batch of 60 samples in one run.

(1) Press F4 for CLR CUR RUN. Press PREV SCREEN to close the window and allow movement into the sample program screen.

(2) From the master screen, press F1, SAMPLE PROGRAM. The cursor will be located at the cup prompt. Press ENTER to highlight the cup number appearing at the prompt, or type in desired cup number.

If a series of cups have identical tests requested they may be programmed together by typing the first cup followed by a hyphen and the last cup number of the series. After the cup has been defined, the cursor moves to the first "sample ID" prompt.

(3) Type in sample ID or select control F4 and press ENTER. The cursor will move to the sex prompt.

(4) Enter PASS SEX and PASS AGE. The cursor will move to the panel prompt.

(5) Enter the panel number and press Enter. Press "1" for A and B, "2" for A and "3" for B.

(6) Press F1, SAVE CUP to store the programming into memory. To delete a cup from the sample program, enter the cup number to be deleted and press CLEAR.

(7) Continue the above routine until all samples are programmed pressing F1 after each sample, including the last one. Press PREV SCREEN.

(8) Press F4 for program summary.

(9) Press PRINT to print program summary.

(10) Load the sample wheels with the sample segments.

(11) Place 150 µL (or 6 drops) of each sample into its corresponding sample well.

(12) Press START to begin the instrument operation. To select a non-standard dilution, press F7, NSTD DIL after the sample ID has been entered. Enter the number of the desired dilution and press SELECT. Press PREV SCREEN to save the dilution.

NOTE: The printed result will require correction for the difference between the standard and the prepared dilution.

To prevent short sample pick-ups, remove any air bubbles or foam present in the sample wells prior to running samples.

b. The runs are determined acceptable or not (in or out of control) by plotting the control means on a Levey-Jennings graph. If out of control, the run is repeated the next run day.

c. There are no reference ranges for apoA-I and apoB. The results are therefore compared for ratios to the lipids analyzed in the same samples. The apoA-I to HDL ratio should be $\geq$2.0 and $\leq$4.0. The apoB to LDL ratio should be between $\geq$0.75 and $\leq$1.25. Values outside these ranges are repeated on the next run day. If the repeat value is within 15% of the original value, the result is considered confirmed and the original value is reported.

## 9. REPORTABLE RANGE OF RESULTS

The reportable ranges of concentrations for these assays vary depending on the method used. Apolipoprotein A-1 and B are currently being prepared for standardization by the International Federation of Clinical Chemistry-World Health Organization (IFCC-WHO). There will be IFCC-WHO references values for manufacturer controls when the process is complete.

## 10. QUALITY CONTROL (QC) PROCEDURES

The laboratory monitors the Quality Control (QC) of the apoA-I and apoB assay by running the 2 manufacturer controls. In addition, the laboratory runs the following: Omega control; lab pool (a laboratory prepared pool); and CDC 1883 (a Sercal pool). These controls are run on each calibration run as well.

The control limits for each pool are calculated from the average and standard deviations of the daily means and daily ranges of the analyses of the control pools. Temporary control limits for each pool are calculated from the first 20 run days. Permanent control limits are determined after 50 run days and then used until the supply of the pool is exhausted.

Continuity from pool to pool is maintained through at least 20 overlapping runs in which the replacement pool is analyzed concurrently with the current pool. The 20-run temporary limits are established for the replacement pool from this period of overlap. During this period, the acceptability of the analyses is determined on the basis of results for the current pool: that is, the analyses of the current pool must be "in control" before the data are accepted and used to establish control limits for the replacement pool.

Limits for the new pool are calculated and evaluated, and control charts are prepared. Care is taken to ensure that data used in the calculations are only from runs that are "in control" (i.e., that meet established quality control criteria). As soon as acceptable temporary limits are established, control is transferred to the replacement pool, and the original pool is no longer analyzed.

It is important that the data used to calculate control limits be collected during a stable analytical period representative of overall laboratory performance. The run mean for a control pool is calculated by averaging the two values for the pool.

The overall mean for the pool is calculated by dividing the sum of the individual run means by the number of runs (N).

N = 20 run days for temporary limits
N = 50 run days for permanent limits

The standard deviation of the run means (SD) is also calculated for the control pool.
The range (R) for each run is the difference between the highest and the lowest value obtained for the pool in that run. Because duplicates are used, the range is the difference between duplicates. The average range for a series of runs is calculated by dividing the sum of the ranges for the series by the number of runs. Again, N=20 for temporary limits, and N=50 for permanent limits.

The control limits (99%) for the means chart are calculated, and the values rounded to the nearest whole number.

The limits used for the R chart are calculated by multiplying R for the pool by probability factors appropriate for duplicate analyses. The lower limit for the range chart is zero because there is no negative range.

Before the control chart can be used for quality control, it is reviewed to determine that the data have been collected during a stable analytical period. The chart is examined for outliers, for periods of questionable or unstable performance, and for evidence of excessive bias. An outlier will distort the 99% limits if incorporated into the final calculations. An

outlier is considered to be any value of that falls outside the 3-SD limits or any value of R that exceeds the upper control limit for R. These values are eliminated, as are values from any questionable period of performance. The values of , SD, and the control limits are recalculated, and the charts are evaluated again.

When values from at least 20 acceptable runs are used for the final calculations, the control charts are constructed according to the criteria listed below. If there are not 20 acceptable runs after unacceptable data have been eliminated, continue analyzing the pool until at least 20 acceptable runs have been completed.

The criteria used in the Control Laboratory were those that served as guidelines for the Lipid Research Clinics program and designed to minimize both bias and variability.

Construct Levey-Jennings mean and range charts. Each day, enter the daily mean and range values on the appropriate chart. Keep detailed notes on anything that effects the quality of analyses (personnel changes, reagent problems, changes in instrument components, etc.,).

Values for outside the 3-SD limit or values of R that exceed the (R + 3.27R) limit indicate the run is out of control. The run must be repeated. A value outside the 95% limits but not the 99% limits is interpreted as an indication of possible trouble.

For runs consistently out of control, check the expiration date of calibrator material and standards.

If one QC pool fails to maintain the specified ranges, regardless of whether or not calibrators are within range, the run is declared "out of control" and the analyses are repeated. Any exception to this rule is decided by the lab director, the lab supervisor, and the quality control manager.

**12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS**

Excessive turbidity can affect analysis. Samples may be diluted 1:2 (one part sample, one part saline, by volume) if necessary.

**13. REFERENCE RANGES (NORMAL VALUES)**

Currently there are no reference ranges available for apoA-I and apoB by a nephelometric method. The results are therefore compared for ratios to the lipids analyzed in the same samples. The apoA-I to HDL ratio should be $\geq$2.0 and $\leq$4.0. The apoB to LDL ratio should be between $\geq$0.75 and $\leq$1.25. Values outside these ranges are repeated on the next run day. If the repeat value is within 15% of the original value, the result is considered confirmed and the original value is reported.

**14. CRITICAL CALL RESULTS ("PANIC VALUES")**

There are no established ranges for "panic values" with this test.

**15. SPECIMEN STORAGE AND HANDLING DURING TESTING**

Specimens may reach and maintain ambient temperature during analysis, but they should be returned to 2-8 °C storage as soon as possible. After a run is accepted, return samples to long-term storage at -70 °C.

**16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS**

There is no backup method for this assay. If the analytical system fails, store samples at -20 °C until the system is restored to functionality. If long-term interruption (greater than 4 weeks ) is anticipated, store samples at -70 °C.

**17. TEST REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)**

a. When results are printed from the nephelometer the sample and QC results are reviewed for acceptability. If the run is "out of control," repeat it on the next day. If the run is judged “in control” accept the run and transcribe the results onto the NHANES log sheet.

b. When the analysis of the specimens is complete including lipid results, the data are reviewed by the data

coordinator or by the senior lab technician (preliminary review). After all necessary repeat analyses are completed, the results are then reviewed by the laboratory supervisor, data coordinator, or laboratory director (final review).

## 18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Standard record keeping means (electronic and log accession books) are used to track specimens. All records, including related QC data, are maintained for a minimum of 5 years, in electronic and printed form. Only numerical identifiers are used to identify subjects. All related personal identifiers are maintained by the NCHS coordinator to safeguard confidentiality and are not available to the laboratory.

Residual NHANES III specimens for which analyses have been completed are sent to the NCHS specimen repository in Bethesda MD for long-term storage.

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1. Bachorik PS, Lovejoy KL, Carroll MD, Johnson CL, Albers JJ, Marcovina SM. Measurement of apolipoproteins A-I and B during the National Health and Nutrition Examination Survey (NHANES III). Clin Chem 1194;40:1915-20.

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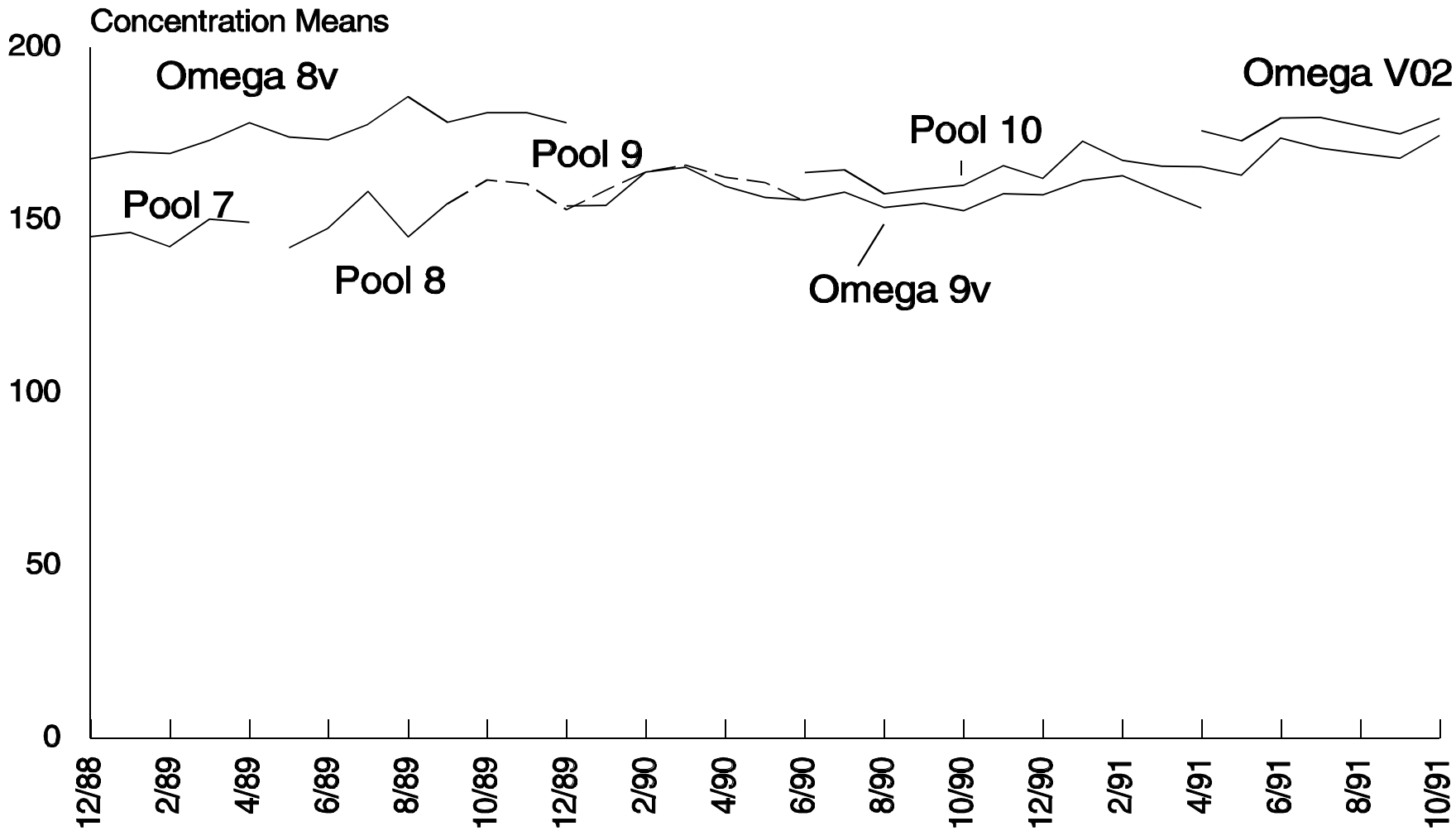
## ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Paul Bachorik, Ph.D., Kathleen Lovejoy, and Richard Foran who assisted in developing the methodology and performing the analysis for Apo (A1) and (B) in the NHANES III study, and preparing the manuscript for this chapter.

SUMMARY STATISTICS FOR
APOLIPOPROTEIN A-1 (RID)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| OM8V | 12/09/88 - 12/01/89 | 173.402 | 10.2443 | 5.90785 | 194 |
| OM9V | 12/27/89 - 04/02/91 | 157.412 | 6.1329 | 3.89611 | 204 |
| OV02 | 04/09/91 - 10/29/91 | 177.349 | 5.6254 | 3.17191 | 83 |
| 7 | 12/09/88 - 04/01/89 | 146.779 | 7.4690 | 5.08859 | 104 |
| 8 | 05/02/89 - 09/05/89 | 150.452 | 11.1321 | 7.39909 | 31 |
| 9 | 09/14/89 - 06/08/90 | 159.543 | 7.3998 | 4.63808 | 92 |
| 10 | 06/15/90 - 10/29/91 | 166.083 | 7.4450 | 4.48267 | 229 |

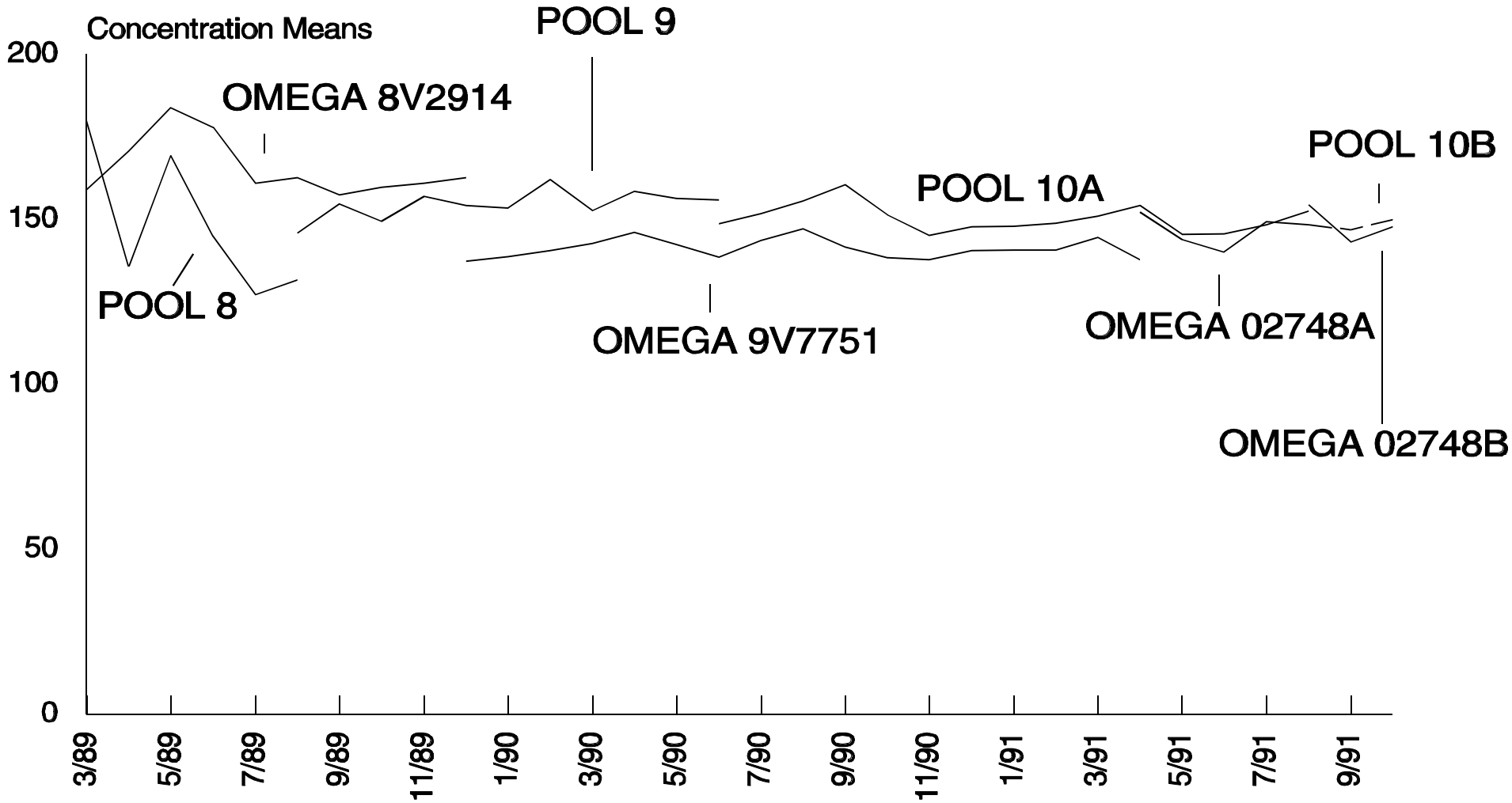
# Apolipoprotein A-1 (RID) Monthly Means



SUMMARY STATISTICS FOR
APOLIPOPROTEIN A-1 (INA)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| OMEGA 8V2914 | 03/28/89 - 12/07/89 | 166.800 | 12.8972 | 7.7322 | 125 |
| OMEGA 9V7751 | 12/12/89 - 04/08/91 | 141.056 | 6.7137 | 4.7596 | 391 |
| OMEGA 02748A | 04/16/91 - 08/21/91 | 146.176 | 7.6198 | 5.2128 | 119 |
| OMEGA 02748B | 08/28/91 - 10/24/91 | 146.160 | 6.7744 | 4.6349 | 50 |
| POOL 8 | 03/28/89 - 08/22/89 | 143.424 | 29.8599 | 20.8193 | 99 |
| POOL 9 | 08/29/89 - 06/06/90 | 154.886 | 8.0058 | 5.1689 | 236 |
| POOL 10A | 06/12/90 - 08/21/91 | 150.115 | 7.9256 | 5.2797 | 391 |
| POOL 10B | 08/28/91 - 10/24/91 | 147.981 | 4.8298 | 3.2638 | 53 |

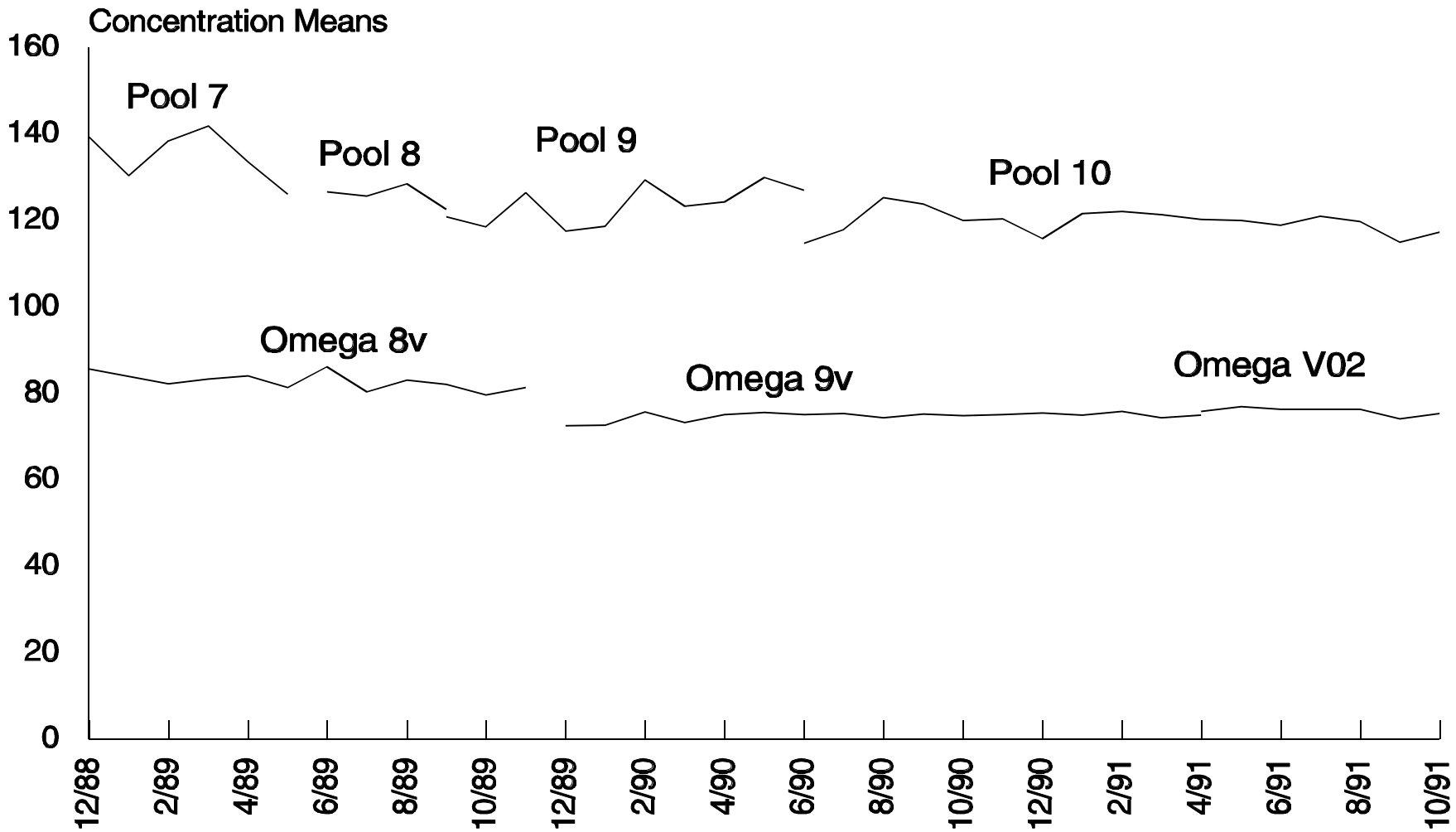
# Apolipoprotein (A-1) by INA Monthly Means



SUMMARY STATISTICS FOR
APOLIPOPROTEIN B (RID)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| OM8V | 12/02/88 - 12/28/89 | 83.536 | 3.90452 | 4.67403 | 220 |
| OM9V | 12/27/89 - 04/02/91 | 74.815 | 2.01458 | 2.69273 | 195 |
| OV02 | 04/09/91 - 10/29/91 | 75.828 | 2.09766 | 2.76635 | 87 |
| 7 | 12/02/88 - 05/19/89 | 136.489 | 9.02804 | 6.61449 | 135 |
| 8 | 06/02/89 - 09/05/89 | 126.316 | 5.83607 | 4.62022 | 38 |
| 9 | 09/14/89 - 06/08/90 | 123.000 | 5.04199 | 4.09918 | 84 |
| 10 | 06/12/90 - 10/29/91 | 119.734 | 4.67860 | 3.90750 | 229 |

# Apolipoprotein B (RID) Monthly Means



SUMMARY STATISTICS FOR
APOLIPOPROTEIN B (INA)
BY POOL

| POOL | DATES OF USE | MEAN | STANDARD DEVIATION | COEFFICIENT OF VARIATION | NUMBER OF OBSERVATIONS |
|---|---|---|---|---|---|
| OMEGA 8V2914 | 03/28/89 - 12/07/89 | 68.207 | 2.93835 | 4.30797 | 193 |
| OMEGA 9V7751 | 12/12/89 - 04/08/91 | 58.460 | 1.64458 | 2.81317 | 361 |
| OMEGA 02748A | 04/16/91 - 08/21/91 | 58.067 | 1.81206 | 3.12063 | 119 |
| OMEGA 02748B | 08/28/91 - 10/24/91 | 80.259 | 2.60757 | 3.24894 | 54 |
| POOL 8 | 03/28/89 - 08/22/89 | 87.217 | 4.73200 | 5.42552 | 92 |
| POOL 9 | 08/29/89 - 06/06/90 | 83.201 | 2.51481 | 3.02258 | 234 |
| POOL 10A | 06/12/90 - 08/21/91 | 85.294 | 2.26500 | 2.65553 | 361 |
| POOL 10B | 08/28/91 - 10/24/91 | 117.925 | 3.23353 | 2.74204 | 53 |

# Apolipoprotein B by INA Monthly Means