United States
Environmental Protection
Agency

EPA Microbiological Alternate Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods

Guidance

April 2004

U.S. Environmental Protection Agency
Office of Water
Engineering and Analysis Division
1200 Pennsylvania Avenue, NW (4303T)
Washington, DC 20460

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DISCLAIMER

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Questions concerning this report should be addressed to:

Robin K. Oshiro
Engineering and Analysis Division (4303T)
U.S. EPA Office of Water, Office of Science and Technology
1200 Pennsylvania Avenue, NW
Washington, DC 20460
oshiro.robin@epa.gov
202-566-1075
202-566-1053 (facsimile)

FOREWORD

This document describes a process for seeking EPA approval of microbiological alternate test procedures (ATPs) or new methods for use in monitoring drinking water, ambient water, and wastewater. This document serves as a supplement to the ATP guidelines at 40 CFR 136.4, 136.5, and 141.27.

With this guidance document, EPA has revised and combined the three existing microbiological ATP protocols to make the ATP process more transparent to applicants while maintaining the same level of data quality. The guidelines in *Quantitative Membrane Filter Methods* (Reference 10.16), *Presence/Absence Liquid Culture Methods for Finished Water* (Reference 10.17), and *Presence/Absence Membrane Filter Methods for Finished Waters* (Reference 10.15) have been incorporated into this single protocol, which is applicable to a wider range of analytes and techniques.

This revised ATP protocol describes a process for conducting side-by-side method comparisons and for conducting quality control (QC) acceptance criteria-based method studies for EPA-approved reference methods with QC acceptance criteria. Additionally, in some cases the revised protocol provides applicants an opportunity to demonstrate comparability by meeting QC acceptance criteria associated with the EPA-approved reference methods for different combinations of analyte and determinative technique.

Under EPA's ATP program, any person may apply for approval of the use of an ATP or new method to test for a regulated analyte. EPA anticipates that the standardized procedures described herein should generally expedite the approval of ATPs, encourage the development of innovative technologies, and enhance the overall utility of the EPA-approved methods for compliance monitoring under the National Pollution Discharge Elimination System (NPDES) permit program and national primary drinking water regulations (NPDWRs).

This document is not a legal instrument and does not establish or affect legal obligations under Federal regulations. EPA reserves the right to change this protocol without prior notice.

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Engineering and Analysis Division (4303T)
U.S. EPA Office of Water, Office of Science and Technology
1200 Pennsylvania Avenue, NW
Washington, DC 20460
oshiro.robin@epa.gov
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SECTION 1.0 INTRODUCTION

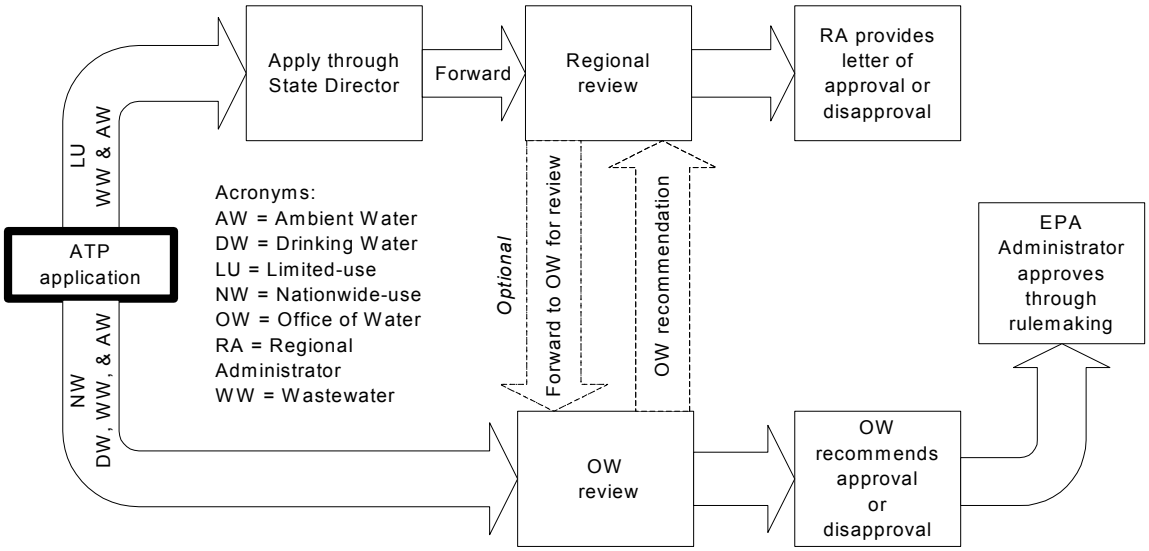
1.1 Background and Objectives

In accordance with the Clean Water Act (CWA) and Safe Drinking Water Act (SDWA), the U.S. Environmental Protection Agency (EPA) promulgates guidelines establishing test procedures (analytical methods) for data gathering and compliance monitoring under National Pollution Discharge Elimination System (NPDES) permits and national primary drinking water regulations (NPDWRs). The approved test procedures can be found in the Code of Federal Regulations (CFR) at 40 CFR Part 136 for wastewater and ambient water and 40 CFR Part 141 for drinking water. In addition, EPA's regulations at 40 CFR 136.4, 136.5, and 40 CFR 141.27, allow entities to apply for Agency permission to use an alternate test procedure (ATP) in place of an EPA-approved reference method. Figure 1.1 below summarizes the ATP or new method review process within EPA. These regulations are the basis for the Agency's alternate test procedure (ATP) program for water methods that is administered by the Office of Water, Office of Science and Technology, Director of Analytical Methods.

An ATP is a modification of an EPA-approved reference method or a procedure that uses the same determinative technique (i.e., the physical and/or chemical process used to determine the identity and concentration of an analyte) and measures the same analyte(s) of interest as the EPA-approved reference method. The use of a different determinative technique to measure the same analyte(s) of interest as an EPA-approved reference method is considered a new method.

Under the ATP program, an organization or individual may apply for approval of an ATP or new method to be used as an alternate to an EPA-approved reference method. The applicant is generally responsible for characterizing method performance of its proposed alternate test procedure prior to submission to the ATP program. EPA can provide assistance to applicants in the development of a study plan to demonstrate comparability with the EPA-approved reference method. Figure 1.1 summarizes the ATP or new method review process within EPA. The Agency reviews the ATP package, approves or disapproves the application, and, for nationwide applications, will generally propose to include successful ATPs in the CFR (unless the ATP is for limited use or constitutes a minor modification-- See Appendix A).

Figure 1-1. Summary of the ATP or New Method Review Process



To make the ATP program more accessible to applicants while maintaining data quality, EPA has revised and combined the three microbiological ATP protocols into this comprehensive protocol. The guidelines in *Quantitative Membrane Filter Methods* (Reference 10.16), *Presence/Absence Liquid Culture Methods for Finished Water* (Reference 10.17), and *Presence/Absence Membrane Filter Methods for Finished Waters* (Reference 10.15) have been incorporated into a single protocol applicable to a wider range of analytes and techniques. Additionally, the revised protocol illustrates how applicants can demonstrate comparability by meeting quality control (QC) acceptance criteria associated with EPA-approved reference methods for which those criteria have been developed.

The ATP program provides laboratories and regulated facilities with an opportunity to enhance compliance monitoring and encourages the use of innovative technologies. Approval for an ATP or new method may be sought when, for example, the alternate procedure reduces analytical costs, overcomes matrix interferences problems, improves laboratory productivity, or reduces the amount of hazardous materials used and/or produced in the laboratory.

Any person or organization may apply to gain approval for the use of an ATP or new method for determination of a specific constituent that is regulated under the NPDES permit program or the NPDWRs. The ATP applicants generally may demonstrate comparability of its proposed ATP or new method with the EPA-approved reference method using the procedures described in this protocol. Other possible method comparison procedures include those provided by organizations such as ASTM (Reference 10.5), AOAC-International (Reference 10.1), and ISO (Reference 10.8).

1.2 Types of Applications

The types of applications submitted may depend on the intended use of the ATP. Methods intended for use in demonstrating compliance with the NPDES permit program (wastewater or ambient water ATPs) may be submitted for approval for limited-use (single laboratory) or for nationwide-use (all laboratories). Because only the Administrator has the authority to approve an alternate analytical technique for SWDA purposes, EPA will generally consider proposed methods intended for use in demonstrating compliance with NPDWRs (drinking water ATPs) that are submitted for approval for nationwide-use only.

1.2.1 Limited Use

The primary intent of the limited-use ATP is to allow use of an ATP or new method by a single laboratory. Limited-use ATPs can be applied to one or more matrix types, excluding drinking water matrices; limited-use applications generally will not apply to Office of Ground Water and Drinking Water (OGWDW) ATP applications (Reference 10.18). If a method developer intends to apply the method to more than one matrix type, method studies should be conducted on each matrix type. Generally, nine different wastewater types should be analyzed to demonstrate the ATP or new method will be applicable to most other matrix types. If method modifications are within the specified flexibility of the EPA-approved reference method and all QC acceptance criteria are met, it generally will not be necessary to submit the modification to the ATP program.

1.2.2 Nationwide Use

The primary intent of a nationwide-use ATP is to allow use of an ATP or new method by all regulated entities and laboratories for one or more matrix types including drinking water. Nationwide-use approval allows vendors to establish that new devices and reagents produce results that are acceptable for compliance monitoring purposes, and allows environmental laboratories across the United States to apply new technologies or modified techniques throughout their chain of laboratories to one or more matrix types. If a method developer intends to apply the method to more than one matrix type, method studies should be conducted on each matrix type. Generally, nine different wastewater types should be analyzed

to demonstrate the ATP or new method will be applicable to most other wastewaters. If method modifications are within the specified flexibility of the EPA-approved reference method and all QC acceptance criteria are met, it generally will not be necessary to submit the modification to the ATP program.

1.3 Types of Studies

The type of study most useful in seeking approval of an ATP or new method generally depends on whether or not the EPA-approved reference methods contain QC acceptance criteria. There are two basic types of studies described in this protocol:

- **Side-by-Side Method Comparison Study.** A side-by-side method comparison study generally consists of parallel testing of an ATP or new method along side an EPA-approved reference method to determine whether the performance of the new or modified method is acceptable compared to the reference method.
- **QC Acceptance Criteria-Based Method Comparison Study.** For EPA-approved reference methods that contain (or are supplemented with) QC acceptance criteria for most combinations of analyte(s) and determinative technique(s), the goal of the study is for the applicant to demonstrate that its ATP or new method is able to meet the QC acceptance criteria of the EPA-approved reference method (or other EPA-specified document) for the applicable combination of analyte and determinative technique through a QC acceptance criteria-based comparison study.

Specific guidelines for the studies can be found in Section 5.0: Quality Control, Section 6.0: Study Design, Section 7.0: Sample Preparation and Analysis, and Section 9.0: Review of Study Results.

1.4 Scope of Alternate Test Procedures

This protocol for demonstration of comparability, submission, and approval of an ATP or new method offers flexibility to modify EPA-approved reference methods. Generally, an applicant should demonstrate and document that the modified method produces results better than or equal to those produced by an appropriate EPA-approved reference method for the applicable combination of analyte and determinative technique.

1.4.1 EPA-Approved Reference Methods

The ATP process is based on comparing the performance of an ATP or new method to an EPA-approved reference method through a side-by-side comparison study or a QC acceptance criteria-based comparison study. Method comparability is demonstrated when results produced by an ATP meet or exceed the performance criteria associated with the EPA-approved reference method. Table 1-1 below lists the EPA-approved reference methods for the analytes covered by this protocol. This table will be updated as necessary as additional pathogens are added to the list or advances in technology merit a change in the EPA-approved reference method. When performing a study, the applicant should use the reference method that uses the same determinative technique (e.g., MF, MPN) as the ATP for the analyte(s) of interest. If the applicant is validating a new method, which generally will use a determinative technique that is not currently approved for use with the analyte(s) of interest, then the applicant should consult EPA prior to commencing the study to determine which is the most appropriate reference method.

Table 1-1. EPA-Approved Reference Methods

Analyte	Method Format[1]	EPA-Approved Reference Method[2]	40 CFR Citation
Total coliforms	MPN	SM 9221B	136.3, 141.21, 141.74
	MF	SM 9222B	
	Presence/Absence	SM 9221D	141.21
Fecal coliforms	MPN	SM 9221E	136.3, 141.21, 141.74, 503.8(b)
	MF	SM 9222D	
E. coli	MPN	SM 9221F	136.3, 141.21, 141.74
	MF	SM 9222G, SM 9213D	
HPC	Pour Plate	SM 9215B	141.74
Fecal streptococcus	MPN	SM 9230B	136.3
	MF	SM 9230C	
Enterococcus	MPN	SM 9230B	136.3
	MF	SM 9230C	
Salmonella	MPN	Kenner and Clark	136.3, 503.8(b)
Enteric virus	Plaque Assay	EPA Document [3]	503.8(b)
Helminth ova	Microscopy	EPA Document [4]	503.8(b)
Aeromonas	MF	USEPA Method 1605	141.40
Coliphage	Plaque Assay	USEPA Method 1601	141.403
	Two-Step Enrichment	USEPA Method 1602	
Cryptosporidium	Filtration/IMS/FA	USEPA Method 1622/1623	136.3, 141.74
Giardia	Filtration/IMS/FA	USEPA Method 1623	

[1]MPN = most probable number, MF = membrane filtration, IMS/FA = immunomagnetic separation/fluorescent antibody

[2]"SM" refers to *Standard Methods for the Examination of Water and Wastewater.* For the edition(s) approved for a specific method, consult the CFR sections referenced in the column headed "40 CFR Citation." (References 10.2, 10.3, and 10.4)

[3] See References 10.12 and 10.13

[4] See References 10.12 and 10.19

1.4.2 Modifications to Sample Preparation Techniques

A sample preparation technique is any technique in the analytical process conducted at the laboratory that precedes the determinative technique (i.e., the physical and/or chemical process by which measurement of the identity and concentration of an analyte is made). Sample preparation techniques include the procedures, equipment, reagents, etc., that are used in the preparation and cleanup of a sample for analysis. Laboratories generally may modify sample preparation techniques, provided the modification is not explicitly prohibited in the EPA-approved reference method that is being modified and provided the modification can be demonstrated to produce results equal or superior to results produced by an EPA-approved reference method for each combination of analyte and determinative technique.

SECTION 2.0 APPLICATION

ATP applications should be submitted in triplicate to EPA to facilitate the review process. The application consists of a completed ATP application form (a sample application form is provided in Appendix D) with any attachments. Electronic submissions are also generally acceptable and often may accelerate the review process.

2.1 Submission Addresses and Approval Authority

A summary of ATP submission information and approval authorities is provided in Table 2-1.

Table 2-1. Submission of Alternate Test Procedure Applications

Level of Use	Applicant	Submit Application To[1]	Approval Authority
Limited Use for Wastewater or Ambient Water	EPA Regional laboratories	EPA Regional Administrator (Regional ATP Coordinator)[2]	EPA Regional Administrator
	States, commercial laboratories, individual dischargers, or permittees in States that do not have the authority to administer Clean Water Act and Safe Drinking Water Act monitoring programs	EPA Regional Administrator (Regional ATP Coordinator)[2]	
	States, commercial laboratories, individual dischargers, or permittees in States that have the authority to administer Clean Water Act and Safe Drinking Water Act monitoring programs	Director of State Agency issuing the NPDES permit[2]	
Nationwide Use for Drinking Water, Wastewater, Ambient Water	All applicants	Director, Analytical Methods, Attn: ATP Program Coordinator, EPA Headquarters	EPA Administrator

[1] See Appendix E for EPA addresses.

[2] The Regional Administrator or the Director of State Agency issuing the NPDES permit may choose to forward limited-use applications to the Director of Analytical Methods, Attn: ATP Program Coordinator for an approval recommendation. Generally, the Regional Administrator or the Director of State Agency issuing the NPDES permit will forward a copy of the approval to the Director of Analytical Methods, Attn: ATP Program Coordinator.

Generally, upon receipt, the application will be assigned an identification number, and a confirmation letter referencing this identification number will be sent to the applicant. The applicant should use the identification number in all future communications concerning the application.

2.2 Application Information

The following information should be provided on the ATP application form (Appendix B):

- Name, mailing address, phone number, and email address of the applicant
- Date of submission of the application
- Method number, title, and revision date of the ATP or new method submitted for review
- The analyte(s) included in the ATP or new method submitted for review

- The matrix or matrices to which the ATP applies
- EPA-approved reference method used for demonstration of comparability
- Type of application (i.e., wastewater, drinking water, ambient water, point source categories regulated at 40 CFR Parts 400-499)
- The level of use desired (i.e., limited use or nationwide use)
- Type of study (side-by-side comparison or QC acceptance criteria-based comparison study)
- Applicant's NPDES permit number, the issuing agency, the type of permit and the discharge serial number (if applicable)

The following items should be submitted as attachments to the initial application:
- Reason for proposing the ATP or new method
- The proposed ATP or new method prepared in standardized format (Section 3.0)
- A method comparison table that gives a side-by-side comparison of the steps of the proposed ATP or new method and the EPA-approved reference method (Section 2.5)
- Method development information
- Study plan for EPA review and comment (Section 4.0)

A study plan is generally not needed with the application if an applicant is unsure whether or not a modification is allowed within the method-specified flexibility. In such cases, the applicant may request that EPA determine the usefulness of a full ATP comparability assessment based on the other information submitted with the application. From this information, EPA can determine whether a full ATP assessment will be helpful, whether the proposed modification is considered to be a minor modification (i.e., employs the same chemistry and/or biological principles as the EPA-approved reference method to determine the presence/absence or to quantify the amount of the target organism in a sample), or whether the proposed modification is considered to be within the specified flexibility of the EPA-approved reference method.

The elements of a complete application are presented in Table 2-2. A list of the information discussed in detail in Sections 2.3 to 2.8 is provided in Appendix C. EPA will generally seek all application information and attachments before the application is considered complete.

Table 2-2. Application Information

Application Information
• Completed application form • Reason for ATP • Method in EPA format • Method comparison table • Method development information • Study plan (to be approved by EPA before proceeding with study) • Study report (final report generally considered part of a complete application)

Note: Although the application process generally begins with the initial submission of ATP materials, the application is not usually considered to be complete until the final study report has been submitted, and all EPA questions on the report have been resolved.

2.3 Reason for ATP

The entity that proposes an ATP should indicate why the ATP is being proposed. Examples include, but are not limited to, the following:
- The ATP improves method performance
- The ATP provides equivalent method components for a lower cost
- The ATP enables laboratories to perform analyses more efficiently

- The ATP successfully overcomes some or all of the interferences associated with the EPA-approved reference method
- The ATP significantly reduces the amount of hazardous wastes generated by the laboratory
- The ATP provides another means for measuring a contaminant (i.e., provides more choices)

2.4 Standard EPA Method Format

In accordance with the standard EPA format advocated by EPA's Environmental Monitoring Management Council (EMMC), methods should contain 17 specific topical sections in a designated order. The 17 sections are listed in Section 3.0 of this document. Additional numbered sections may be inserted starting with Section 11.0, *Procedure*, as appropriate for a particular method. For detailed information on the EPA format for proposed methods, see the Guidelines and Format document (Reference 10.14).

2.5 Method Comparison Table

As part of the application, the applicant should provide a two-column table comparing the proposed ATP or new method to the EPA-approved reference method. The two-column method comparison table should include the number and title of each method, the latest revision date of the proposed ATP, and a detailed discussion of each of the 17 topics specified by the standard EPA method format (as applicable). Each topic should be discussed on a separate row in the method comparison table. The applicant should highlight any differences between the proposed ATP and the EPA-approved reference method.

2.6 Method Development Information

Before EPA reviews the study plan and works with applicants on ATP studies, the applicant should provide data on performance of the modified or new method in the water matrix for which the ATP or new method is being applied. These data may have been generated during method development by the vendor, or through independent tests by third-party laboratories. Examples include, but are not limited to, replicate spiked reagent water or replicate spiked matrix water tests. It is the responsibility of the applicant to provide sufficient data to demonstrate that the ATP or new method performs sufficiently at a preliminary level in the matrix of interest to merit evaluation of an ATP. If sufficient data is not available, EPA may request additional studies be conducted prior to the review of the ATP study plan.

In addition to data, the following descriptive method information will facilitate EPA's evaluation of the ATP application:

- The purpose and intended use of the method
- The analytical basis for the method, noting any relationship of the method to other existing analytical methods and indicating whether the method is associated with a sampling method
- Method limitations and an indication of any means of recognizing cases where the method may not be applicable to specific matrix types (e.g., turbidity greater than 50 NTU)
- The basic steps involved in performing the test and data analysis
- Options to the method, if applicable

This information also will aid EPA in preparing the docket and the preamble for the proposed rule that will be published in the *Federal Register* if EPA proposes to approve the ATP for nationwide use.

2.7 Study Plan

Prior to conducting all studies, the applicant should submit a study design for EPA review and comment. A detailed procedure (Section 3.0) for the new method or the modification should be included as an attachment to the study plan. Generally, EPA will evaluate the study plan to ensure that the appropriate

data quality objectives identified in this protocol are defined and addressed. EPA comments will be incorporated into the study design and this process will be repeated until EPA has approved the study design. Generally, the study plan should contain the elements listed below:

- Background
- Objectives
- Study Design
- Coordination
- Data Reporting

These elements are further described in Section 4.0.

2.8 Study Report

The applicant should conduct a study and provide a comprehensive study report with the ATP or new method application. The study report should include the following elements:

- Background
- Study Objectives and Design
- Study Implementation
- Data Reporting and Validation
- Results
- Data Analysis and Discussion
- Conclusions
- Appendix A - Method
- Appendix B - Study Plan
- Appendix C - Supporting Data
- Appendix D - Supporting References

These elements are further described in Section 9.0.

2.9 Proprietary Information in Applications

All information provided to the Federal government is subject to the requirements of the Freedom of Information Act. Therefore, any proprietary information submitted with the proposed ATP application should be marked as confidential. However, EPA prefers that supporting documentation labeled as confidential business information not be submitted as part of the ATP application. If proprietary information is determined to be essential to the application, EPA staff will request the information and will handle such information according to the regulations in subparts A and B of 40 CFR Part 2.

Specifically, in accordance with 40 CFR §2.203, a business that submits information to EPA may assert a business confidentiality claim covering the information by placing on (or attaching to) the information at the time it is submitted to EPA, a cover sheet, stamped or typed legend, or other suitable form of notice employing language such as *trade secret*, *proprietary*, or *company confidential*. Allegedly confidential portions of otherwise non-confidential documents should be clearly identified by the business, and may be submitted separately to facilitate identification and handling by EPA. If the business desires confidential treatment only until a certain date or until the occurrence of a certain event, the notice should so state. Please be advised, however, that any methods proposed in the *Federal Register* cannot be claimed as confidential business information.

If a claim of business confidentiality is not made at the time of submission, EPA will make such efforts as are administratively practicable to associate a late claim with copies of previously submitted information in EPA files. However, EPA cannot ensure that such efforts will be effective due to the nature of application review that may already be in progress.

SECTION 3.0 METHOD FORMAT

Because alternate test procedures may be approved by EPA as comparable to the reference methods, and may be implemented by multiple laboratories, it is important that the written procedures include all of the information necessary to use the technique in the laboratory, including but not limited to: reagents and equipment, sample collection and preservation procedures, quality control, and a detailed description of the procedure. The information described below should be provided in the method. In addition, EPA recommends the Environmental Monitoring Methods Council (EMMC) format described below be used.

Sections 3.1 through 3.17 provide a list of EMMC method sections and a general description of the type of information that should be included in each section. The date and revision number of the method should be included on the cover page. In addition, the date should be included as a footer on each page of the method. A detailed description of method format guidelines, as well as an example of a formatted method, is provided in Reference 10.14. The detailed information in Reference 10.14 is provided as guidance for the method write-up and as such, specific suggestions for font size, margins, etc. are optional.

3.1 Scope and Application

Include a list of target organisms (by common name), taxonomic group and their CAS registry numbers or other accepted numbering systems (if available), the matrices to which the method applies, a generic description of method sensitivity (the minimum number of organisms the method can detect per unit volume or mass, if known), and the data quality objectives that the method is designed to meet or monitoring programs for which the method was designed to support.

3.2 Summary of Method

Summarize the method in a few paragraphs. The purpose of the summary is to provide a succinct overview of the method procedure to aid the reviewer or data user in understanding the method and how the results are generated. Include a general description of the method procedure, sample volume, type of media used, preparation steps, incubation time and temperatures, and the techniques used for qualitative or quantitative determinations.

3.3 Method Definitions

Provide definitions of terms that are necessary to understand how the method is used or what the results represent. This should include a definition of the target organism or group of organisms, relative to the determinative step of the method. For extensive lists of definitions, this section may simply refer to a glossary attached at the end of the method document.

3.4 Interferences

This section should discuss any known method interferences such as toxic materials, particulates, non-target organisms, etc. If known interferences in the reference method are not interferences in the alternate method, this also should be clearly stated.

3.5 Safety

This section should discuss only those safety issues specific to the method and beyond the scope of routine laboratory practices. Target analytes or reagents that pose specific health, toxicity, or safety issues should be addressed in this section.

3.6 Equipment and Supplies

For critical equipment that may affect the performance of the method, cite the manufacturer, model name, and catalog or product number of the equipment that was used to develop or validate the method; note that equivalent equipment can be used, if applicable. Use generic language for standard laboratory glassware and disposables.

3.7 Reagents and Standards

Provide sufficient details on the concentration and preparation of reagents and standards to allow the work to be duplicated, but avoid lengthy discussions of common procedures. If only pre-prepared proprietary reagents can be used, specify this. Include catalog and/or product numbers where appropriate. Indicate shelf life of packaged materials and special storage specifications.

3.8 Sample Collection, Preservation, and Storage

Provide information on sample collection, preservation, shipment, storage conditions, and holding times. If effects of holding time were specifically evaluated, provide reference to relevant data.

3.9 Quality Control

Describe specific quality control (QC) measures that enable one to establish the sensitivity, specificity, false positive rates, false negative rates, bias, and precision of measurements using the method, and that the measurements are free from contamination. Specific QC measures may include positive and negative controls, duplicate samples, method blanks, and media sterility checks. Indicate which QC measures are appropriate initially, before a laboratory uses the method, and which are appropriate on an ongoing basis. Indicate frequencies for each QC measure and list minimum specifications or acceptance ranges (see Section 5.0). Indicate corrective actions that should be taken when QC measures are not met. Define all terms in method definitions section.

3.10 Calibration and Standardization

Discuss initial calibration specifications for instruments used in the method (e.g., water baths, refrigerators, thermometers, balances, pH meters, microscopes, etc.). Indicate frequency of such calibrations; refer to performance specifications; and indicate corrective actions that should be taken when performance specifications are not met. This section may also include procedures for calibration, verification, or continuing calibration, or these steps may be included in the procedure section.

3.11 Procedure

Provide a detailed description of the sample processing and analysis steps. Avoid unnecessarily restrictive instructions, but provide sufficient detail for manual procedures so that analysts in other laboratories perform the method consistently. Ranges should be provided for temperature requirements, time requirements, etc.

3.12 Data Analysis and Calculations

Identify qualitative and quantitative aspects of the method. List criteria for the identification of target organism(s) and interpretation of results for all steps of the method, including criteria for presumptive and confirmed results. Provide equations used to derive final sample results. Provide discussion of estimating detection limits, recoveries, specificity, false positive/false negative rates, etc., if appropriate.

3.13 Method Performance

Provide detailed information on method performance, including data on precision, bias (for quantitative methods), specificity, detection limits (including the method by which they were determined and matrices to which they apply), statistical procedures used to develop performance specifications (i.e., recovery, precision, specificity, false positive/false negative rates, etc.). Where performance is tested relative to the reference method, provide a summary of the side-by-side comparison of performance versus reference method specifications.

3.14 Pollution Prevention

Describe aspects of this method that minimize or prevent pollution that may be attributable to the reference method.

3.15 Waste Management

Cite how waste is minimized and the proper disposal of samples and waste.

3.16 References

Include source documents, publications, etc.

3.17 Tables, Diagrams, Flowcharts, and Validation Data

Additional information may be presented at the end of the method. Lengthy tables may be included here and referred to elsewhere in the text by number. Diagrams should only include new or unusual equipment or aspects of the method.

SECTION 4.0 STUDY PLAN

Applicants should submit a study design for EPA review, comment, and approval prior to conducting the side-by-side comparison study or the QC acceptance criteria-based comparison study. This process protects the applicant by providing written approval of the study design before resources are spent to conduct the study. The ATP program is intended to be flexible, and thus EPA may modify the study design for a particular proposed method. Data from studies conducted without EPA review and approval may not meet EPA's criteria, and may not adequately address the applicant's study objectives. A detailed procedure (Section 3.0) for the ATP or new method should be included as an attachment to the study plan. EPA will generally evaluate the study plan to verify that the appropriate data quality objectives identified in this protocol are defined and addressed. EPA comments are incorporated into the study design. This review/revision process is repeated until EPA has approved the study design.

Generally, the study design should include the information described in Sections 4.1 through 4.5.

4.1 Background

This section of the study plan should include the following information:

- A statement identifying the ATP as a new method or a modification of a reference method
- The EPA program(s) to which the ATP or new method applies (e.g., drinking water, wastewater, ambient water, point source categories regulated at 40 CFR Parts 400-499, etc.)
- A short (one paragraph) summary of the ATP or new method
- The organization and method number of the reference method if applicable
- A description of the reasons for the extent of the modification, the logic behind the technical approach to the modification, and the result of the modification
- The matrices (e.g., finished water, wastewater, ambient water, etc.), matrix types (e.g., turbidity greater than 10 NTU, etc.), and/or media to which the ATP or new method is believed to be applicable
- A list of the analytes measured by the ATP or new method, including the corresponding CAS registry number (if available) or other identification number

4.2 Objectives

Include a description of the new method or modification, describe the goals of the study, and define data quality objectives.

4.3 Study Design

The following information should be included in the study design:

- Laboratories that will participate in the study (Sections 5.0 and 6.0)
- Number and type of samples to be analyzed (Section 6.0)
- Description of the matrices that will be used (Sections 6.0 and 7.0)
- Description of the spikes that will be used (Section 7.0)
- Description of the spiking procedure (Section 7.0)
- Positive and negative control organisms (Section 5.0)
- Quality control procedures that will be followed (Section 5.0)

4.4 Coordination

Describe how the study will be coordinated, how the spikes will be shipped to the laboratories, and who will compile the data for submission. Data compilation should not be performed by any of the analysts conducting the sample analyses.

4.4.1 Study Management

This section of the study plan should include the following information:
- The organization responsible for managing the study
- The laboratories, facilities, and other organizations that will participate in the study
- A delineated study schedule including, but not limited to, sample collection, start of sample analysis, interpretation of sample results, completion of study, etc.

4.4.2 Technical Approach

This section of the study plan should include the following:
- A description of how sample matrices and participating laboratories will be selected
- A description of how samples will be collected and distributed
- The numbers and types of analyses to be performed by the participating laboratories
- A description of sample spiking procedures
- A description of how analyses are to be performed

4.5 Data Reporting

List the data elements that will be collected and provide sample bench sheets (see Appendix D) that will be used to record raw data during the study. Raw data should be submitted as an attachment to the Study Report (Section 9.0). Address the statistical analysis of the study results that will be performed, if the statistical analyses will differ from those described in Section 8.0. Please note, however, that EPA's evaluation of method performance will generally be based on the statistical analyses described in Section 8.0.

SECTION 5.0 QUALITY ASSURANCE/QUALITY CONTROL

For side-by-side comparison studies in which only one laboratory is performing analyses, the comparability study should be conducted at an independent laboratory and the laboratory should be certified to perform microbiological analyses under EPA's drinking water laboratory certification program. A laboratory with a vested interest in the method, instrumentation, apparatus, reagents, media, or associated kits may not participate in the side-by-side comparison study.

For QC acceptance criteria-based comparison studies, at least three independent laboratories should participate. A laboratory with a vested interest in the ATP also may participate in the study, but in such instances there should be at least three independent laboratories participate and the majority of the laboratories participating are independent laboratories. All laboratories should be certified to perform microbiological analyses under EPA's drinking water certification program, or a comparable certification program, if a laboratory located outside of the U.S. is included. At least three independent laboratories participating in the study should to be certified for microbiological analyses under EPA's drinking water certification program. If more than three laboratories participate, the majority of the laboratories should be certified for microbiological analyses under EPA's drinking water certification program.

5.1 Quality Assurance

The laboratory should have a comprehensive quality assurance (QA) program in place and operating at all times during the performance of the comparability study. General QA program guidance is provided at http://www.epa.gov/quality/qs-docs/r2-final.pdf (Reference 10.11). The laboratory should adhere closely to all QA and quality control (QC) measures in this protocol as well as the QC measures in the method(s). Laboratory QA/QC criteria for facilities, personnel, and laboratory equipment are included in *Standard Methods* 9020-Quality Assurance (Reference 10.4) and the U.S. EPA *Manual for the Certification of Laboratories Analyzing Drinking Water*, *Fourth Edition* (March 1997) (Reference 10.13).

The laboratory should adhere to standard laboratory practices for cleanliness and environment, and to the methods for glassware and apparatus, reagents, solvents, and safety. Additional guidelines regarding general laboratory procedures should generally be followed, as specified in Sections 4 and 5 of the *Handbook for Analytical Quality Control in Water and Wastewater Laboratories*, EPA-600/4-79-019 (Reference 10.19).

5.2 Quality Control

Laboratories participating in a comparability study should perform all QC procedures specified in the methods, except where explicitly stated in the approved study plan. The QC procedures listed in Table 5-1 and described below should be performed, as appropriate, based on the technique used in the method. Other QC procedures may be necessary, based on the approved study plan. Laboratories participating in side-by-side comparison studies or QC acceptance criteria-based comparison studies should perform all QC procedures specified in the methods, except where explicitly stated in the approved study plan. The laboratory should maintain records to define the quality of data that are generated. The laboratory should maintain a record of the date and results of all QC sample analyses. Laboratories should maintain reagent and material lot numbers along with samples analyzed using each of the lots. Laboratories should also maintain media preparation records.

Table 5-1 lists quality control measures for each laboratory participating in side-by-side comparison studies and QC acceptance criteria-based comparison studies. Detailed descriptions of the QC measures are provided in Sections 5.2.1 to 5.2.15. If contamination is detected in any of the blanks or sterility checks described below, the source of contamination should be identified and corrected. The

blank/sterility check and all samples associated with that contaminated blank/sterility check should be reprepared and reanalyzed. Measures taken to eliminate contamination should be reported.

Table 5-1. Quality control measures for each laboratory involved in the study

Quality Control Measure	Frequency	Suggested for Study	
		Side-by-Side Comparison Study	QC acceptance criteria-based Comparison Study
Analyst counting variability (Section 5.2.1)	2 samples per study	✓	✓
Autoclave sterilization verification (Section 5.2.2)	Within one week prior to the start of the study	✓	✓
Dilution/rinse water blanks (Section 5.2.3)	1 per every 20 samples or 1 per day of study, whichever is greater	✓	✓
Incubator/waterbath temperatures (Section 5.2.4)	2 times per day when used in study	✓	✓
Initial demonstration of capability (IDC) (Section 5.2.5) or Initial precision and recovery (IPR) (Section 5.2.6)	Each laboratory participating in the study should generate acceptable IDC/IPR data.		✓
Matrix spike/matrix spike duplicate (Section 5.2.7)	Per approved study plan		✓
Media sterility checks (Section 5.2.8)	1 per each batch of media used in the study or per each test run, whichever is greater	✓	✓
Method blank (Section 5.2.9)	Per approved study plan	✓	✓
Ongoing demonstration of capability (ODC) (Section 5.2.10) or Ongoing precision and recovery (OPR) (Section 5.2.11)	Included as part of the study; not needed as separate QC.		Optional
Positive and negative controls (Section 5.2.12)	1 positive control and 1 negative control for each media/stain used in the study. See 5.2.12 below for more details.	✓	✓
Preparation blanks (Section 5.2.13)	Frequency depends on the type of method. See 5.2.13 below for more details.	✓	✓
Refrigerator/freezer temperatures (Section 5.2.14)	Once per day when used in study	✓	✓
Sample processing equipment sterility checks (Section 5.2.15)	Prior to the analysis of samples. See 5.2.15 below for more details.	✓	✓

5.2.1 Analyst Counting Variability

If the laboratory has two or more analysts, each should count colonies, plaques, or positive wells *on the same plate/tray* from one positive field sample per month. Compare each analyst's count of the colonies, plaques, or wells. Counts should fall within 10% between analysts. If counts fail to fall within 10% of each other, analysts should perform additional sets of counts, until the number of target colonies, plaques, or positive wells counted fall within 10% between analysts for at least three consecutive samples. If there is only one analyst replicate counts should be done and be within 5% of original counts.

5.2.2 Autoclave Sterilization Verification

Autoclave sterilization verification should be performed within one week of the start of the study by placing *Bacillus stearothermophilus* spore suspensions or strips inside glassware. Autoclave at 121°C for 15 minutes. Place *Bacillus stearothermophilus* spore suspensions in trypticase soy broth tubes and incubate at 55°C for 48 hours. Check for growth to verify that sterilization was adequate. If sterilization was inadequate, determine appropriate time for autoclave sterilization. Repeat spore test. The laboratory should have historical data verifying that at a minimum, autoclave sterilization is performed on a monthly basis.

5.2.3 Dilution/Rinse Water Blanks

The laboratory should analyze dilution/rinse water blanks to demonstrate freedom from contamination. An aliquot of dilution/rinse water which is analyzed exactly like a field sample should be analyzed for every day of the study or for every 20 samples, whichever is more frequent, and observed for contamination with agent of interest.

5.2.4 Incubator/Waterbath Temperatures

Incubator or waterbath temperatures should be measured and recorded two times per day when in use. Temperatures should be taken at least 4 hours apart and should be within the range of the desired temperature as specified in each method. Thermometers used to measure "in-use" temperatures should be calibrated yearly against an NIST traceable thermometer.

5.2.5 Initial Demonstration of Capability

Laboratories participating in a QC acceptance criteria-based comparison study should have successfully performed an initial demonstration of capability (IDC) test for the new or modified method under evaluation in the study. An IDC test is performed when QC acceptance criteria are available for the evaluation of precision or recovery, but not both. The laboratory should perform an IDC as specified in the method to demonstrate acceptable performance. The laboratory should complete any additional analyses as specified in the study plan.

For the IDC test, the laboratory spikes and analyzes reference matrix (e.g., reagent water, buffered water, etc.) samples to demonstrate acceptable performance with the method prior to the analysis of field samples. The number of samples involved in the IDC varies by method. If the results of the IDC test meets all IDC acceptance criteria (e.g., RSD, minimum number of samples positive, etc.), system performance will generally be acceptable. If any of the IDC test results fail to meet the acceptance criteria, system performance will generally be unacceptable. In this event, the laboratory should identify and correct the problem and repeat the test. IDC tests should be accompanied by a method blank (Section 5.2.9).

5.2.6 Initial Precision and Recovery

Laboratories participating in a QC acceptance criteria-based comparison study should have successfully performed an initial precision and recovery (IPR) test for the method using the modified version of the method under evaluation in the study. An IPR test is performed when QC acceptance criteria are available for the evaluation of both precision and recovery. The laboratory should perform an IPR as specified in the method to demonstrate acceptable performance. The laboratory should complete any additional analyses as specified in the study plan.

For the IPR test, the laboratory spikes and analyzes reference matrix (e.g., reagent water, buffered water, etc.) samples to establish the laboratory's ability to generate acceptable precision and recovery prior to the analysis of field samples. Using results of the analyses, the laboratory calculates mean percent recovery and relative standard deviation (RSD) of the recoveries for the analyte(s) and compares them with the corresponding limits for the IPR test criteria in the method. If the RSD and the mean percent recovery meet the acceptance criteria, system performance is acceptable and analysis of samples may begin. If the RSD or the mean percent recovery are unacceptable, system performance will generally be unacceptable. In this event, the laboratory should identify and correct the problem and repeat the test. IPR tests should be accompanied by method blank tests (Section 5.2.9).

5.2.7 Matrix Spike and Matrix Spike Duplicate Samples

Matrix spike (MS) and matrix spike duplicate (MSD) samples are spiked matrix water samples analyzed by the laboratory to verify acceptable method performance in the matrix being monitored. During routine performance of the method, MS/MSD samples are analyzed by the laboratory for the first sample of any new matrix that will be monitored, and on the 21st sample thereafter. During a QC acceptance criteria-based comparison study for some methods (e.g., *Cryptosporidium* and *Giardia* methods), the laboratory should analyze MS samples as part of routine laboratory QC, however these analyses may not be necessary during this study because MS/MSD samples using blinded spiking suspensions distributed by the study coordinator will be used for the study.

5.2.8 Media Sterility Checks

Before using newly prepared media, a representative portion of each media batch needs to be checked for sterility. The laboratory should test media sterility by incubating one unit (tube or plate) from each batch of medium specified in the method or per each test run, whichever is more frequent, at the appropriate temperature for the length of the method-specified incubation time and observing for growth.

5.2.9 Method Blank

Method blanks are reagent water blanks or other blanks including but not limited to buffered water, tap water, etc., depending on the method analyzed to demonstrate freedom from contamination. Method blanks should be analyzed at the frequency specified in the approved study plan. For QC acceptance criteria-based comparison studies, the laboratory needs to analyze a method blank with the IPR and IDC tests. During routine performance of the method, the laboratory should analyze at least one method blank per every 20 test samples or per every week. During a QC acceptance criteria-based comparison study for some methods (e.g., *Cryptosporidium* and *Giardia* methods), the laboratory should analyze method blanks as part of routine laboratory QC, but method blanks also should be shipped to the laboratory as double-blind samples by the study coordinator.

5.2.10 Ongoing Demonstration of Capability (ODC) Samples

Ongoing demonstration of capability samples are spiked reference matrix (e.g., reagent water, buffered water, etc.) samples that are analyzed to demonstrate that the analytical system is in control on an ongoing basis.

5.2.11 Ongoing Precision and Recovery (OPR) Samples

Ongoing precision and recovery samples are spiked reference matrix (e.g., reagent water, buffered water, etc.) samples that are analyzed by the laboratory to verify that method performance criteria are being met. During a performance based comparison study for some methods (e.g., *Cryptosporidium* and *Giardia* methods), the laboratory should analyze OPR samples as part of routine laboratory QC because generally OPR samples using blinded spiking suspensions distributed by the spiking coordinator will be used for the study.

5.2.12 Positive/Negative Controls

Positive and negative controls are target and non-target organisms processed to ensure the laboratories are familiar with the identification of the target organism and to ensure that confirmation test results are appropriate.

5.2.12.1 Positive/Negative Culture Controls (Culture-Based Methods)

Positive and negative culture controls refer to cultures that, when analyzed exactly like field samples, produce a known positive or a known negative result, respectively, for a given type of media. One positive culture control and one negative culture control should be prepared and analyzed for every media (including confirmation media) used in the method whenever a new batch of medium or reagents is used, every day of the study, or every 20 samples, or as specified in the method, whichever is more frequent. Each control should be carried through the entire procedure and should exhibit the expected positive or negative result.

5.2.12.2 Positive/Negative Staining Controls (*Cryptosporidium* and *Giardia* Methods)

A positive staining control for *Cryptosporidium* and *Giardia* methods is a slide containing positive antigen or intact *Cryptosporidium* oocysts and *Giardia* cysts, and that is stained using the same procedure as used for field samples or test samples. A negative staining control is a slide containing only phosphate buffered saline (PBS) that is stained using the same procedure as used for field samples or test samples. The laboratory should prepare and examine positive and negative staining controls with each batch of slides the laboratory prepares during the study. Positive staining controls should exhibit acceptable fluorescence and negative staining controls should not exhibit fluorescence.

5.2.12.3 Positive/Negative Staining Controls (Other)

At a minimum, the laboratory should prepare and examine a positive and negative control using the same procedure as used for field or test samples whenever a new batch of media or reagents is used every day of the study or every 20 samples, whichever is more frequent. Each control should be carried through the entire procedure and should exhibit the expected positive or negative result.

5.2.13 Preparation Blanks (PB)

5.2.13.1 Membrane Filter Preparation Blank (PB-MF)

If membrane filtration is used, at the beginning and the end of each filtration series, a PB-MF is performed by filtering 20-30 mL of dilution water through the membrane filter and testing for growth. If the control indicates contamination with the target organism, all data from affected samples should be rejected. A filtration series ends when 30 minutes or more elapse between sample filtrations.

5.2.13.2 Multiple-Tube Fermentation Test Preparation Blank (PB-MTF)

If a multiple-tube fermentation test is used, a volume of sterile buffered water that is analyzed exactly like a field sample should be analyzed for every day of the study or every 20 samples, whichever is more frequent. The preparation blank should be incubated with the sample batch and observed for growth of the target organism. If the control indicates contamination with the target organism, all data from affected samples should be rejected. If buffered water is not used for dilutions, only the multiple-tube fermentation media should be included.

5.2.13.3 Other Preparation Blank (PB-Other)

A volume of sterilized water (e.g., reagent grade as defined in Specification D 1193, Annual Book of ASTM Standards) that is analyzed exactly like a field sample should be analyzed for every day of the study or every 20 samples, whichever is more frequent. The preparation blank should be incubated with the sample batch and observed for growth of the target organism. If the control indicates contamination with the target organism, all data from affected samples should be rejected.

5.2.14 Refrigerator/Freezer Temperatures

Refrigerator and freezer temperatures should be measured and recorded once per day when in use. Refrigerator temperature should be maintained at 1°C to 4°C. Freezer temperatures should be maintained at -15°C to -20°C. Special freezers capable of long-term storage of cultures or virus should be maintained at -70°C to -80°C. Thermometers used to measure "in-use" temperatures should be calibrated yearly against an NIST traceable thermometer.

5.2.15 Sample Processing Equipment Sterility Checks

A representative portion of non-disposable items such as sample containers, blender jars, etc., used to collect or process samples should be checked for sterility prior to use in analyses. To test for sterility add approximately 500 mL (or appropriate volume based on the size of the equipment being used) of a sterile non-selective broth (e.g., tryptic soy, trypticase soy, or tryptone broth) to the non-disposable item and incubate at 35°C ± 0.5°C for 24 hours and check for growth. Depending on the incubation times specified in the method, the length of the incubation time for the sample processing equipment sterility check may be increased.

SECTION 6.0 STUDY DESIGN

This section provides a description of the study design that is important for assessing comparability using either side-by-side method comparison studies (Section 6.1) or QC acceptance criteria-based studies (Section 6.2). The sections below address the number of laboratories, number of matrices, and the number of samples that should be analyzed in the studies.

6.1 Side-by-Side Comparison Studies

Methods are compared using the following parameters:

- **Recovery.** Does the new method have similar, better or worse recoveries of the target organism as the reference method?
- **Precision.** Are the recoveries by the new method significantly less or more variable than the reference method?
- **False positive rate/specificity.** Is the new method significantly more likely or less likely to detect non-target organisms or other sample constituents that would be reported as the target organism by the analyst when compared to the reference method?
- **False negative rate/sensitivity.** Is the new method significantly more likely or less likely to exhibit non-detects for samples with the target organism or to exhibit results that are biased low when compared to the reference method?

To generate these parameters, samples are analyzed by a single laboratory (6.1.1). The number of samples (and matrix types) used in the study are determined using a historical EPA standard (6.1.2.1)

6.1.1 Number of Laboratories

A single laboratory should be used for a side-by-side comparability study. Since the study should be conducted in a single independent laboratory with no conflict of interest; the laboratory selected cannot be the method developer's laboratory and cannot be affiliated with the method developer.

6.1.2 Number of Samples

The following standards generally provide the minimum number of samples that should be analyzed. Additional data are generally acceptable and may be very helpful when reviewing an ATP or new method.

6.1.2.1 Method Comparison Study Design Summary

Table 6.1 provides a summary of the method comparability requirements for nationwide microbiological ATPs and new methods.

Table 6-1. Summary of Side-by-Side Method Comparison Study Design for Nationwide Microbiological Alternate Test Procedures

Regulatory Information						Comparability Study[1]							Specificity Study[2]	
						Sample Source		Natural Source of Organisms (For Spiking into Sample Source)			Verification of Reference Method[7]	Minimum Comparability Results	Minimum Proposed Method Results	
Rule/Program	40 CFR Citation	Matrix	Type of Test	Regulated Analytes	Techniques[8]	Type	Number	Type	Number	Replicates	Typical[5]	Atypical[6]	Reference	Proposed	False Pos	False Neg
National Pollutant Discharge Elimination Program (NPDES)	136.3	Wastewater	Quantitative	Total Coliform	MPN MF	Wastewater[3]	10	n/a	n/a	20	400	Not required	200	200	200	200
				Fecal Coliform	MPN MF											
Total Coliform Rule	141.21	Drinking Water	Presence/ Absence	Total Coliform	MPN MF Enzyme Substrate	Drinking Water (oxidant-free)	1	Wastewater	10	20	200	Not required	200	200	100	100
				Fecal Coliform	MPN MF											
				E. coli	MPN MF Enzyme Substrate											
Surface Water Treatment Rule	141.74	Surface Water	Quantitative	Total Coliform	MPN MF Enzyme Substrate	Drinking Water (oxidant-free)	1	Wastewater[4]	10	20	400	Not required	200	200	200	200
				Fecal Coliform	MPN MF	Drinking Water (oxidant-free)	1	Wastewater[4]								
				Heterotrophic Bacteria	Pour Plate	Drinking Water (oxidant-free)	1	Polluted Surface water[4]								
Guidelines Establishing Test Procedures for the Analysis of Pollutants; Analytical Methods for Biological Pollutants in Ambient Water	136.3	All Recreational Waters	Quantitative	Fecal Coliform	MF	All Recreational Waters	10	Wastewater[4]	1	20	200	Not required	200	200	200	200
		Fresh Recreational Waters	Quantitative	*E. coli*	MF	Fresh Water	10	Wastewater[4]								
				Enterococci	MF	Marine Water	10	Wastewater[4]								
		Marine Recreational Waters	Quantitative	Enterococci	MF	Marine Water	10	Wastewater[4]								

1) For studies conducted in drinking and surface water matrices, the comparability study should be conducted in a laboratory certified under the drinking water laboratory certification program. A method developer having a vested interest in the method, instrumentation, apparatus, reagents, media, or associated kits should not perform the comparability study analyses in the applicant's laboratory.
2) The specificity study may be conducted in the method developer's laboratory. Specificity data is not required for the reference method.
3) For wastewater validation studies, unspiked wastewater samples should be tested. No additional natural sources of organisms are required.
4) For coliform methods, a single drinking water sample source is spiked with 10 different wastewaters to create 10 different test samples. For HPC methods, a single drinking water source sample is spiked with 10 different surface waters (e.g., ambient water, receiving water,
5) For quantitative reference methods, 10 typical colonies (sheen colonies) must be verified per replicate for a total of 400 colony verifications (10 samples x 4 randomly chosen out of 20 replicates x 10 sheen colonies). For presence/absence tests, all positive samples must be verified (up to 200 samples).
6) For presence/absence reference methods, verification of atypical colonies is not required. Method developers may verify atypical colonies to provide additional data.
7) Verification of proposed method is not required.
8) Readers should refer to Section 1.4.1 of the document for a list of approved reference methods.

Number of Matrices

Ten different water/wastewater matrices from geographically diverse locations should be included to obtain, as much as is practical, a good presentation of the wide range of water types with an even wider range of target organisms to which the method should appropriately respond. Each water or wastewater sample should be collected in sufficient volume to complete all replicate analyses of sample or dilution volumes by both the ATP or new method and the EPA-approved reference method. For ambient water studies, the turbidity of at least one matrix should be greater than 10 NTU. Generally, for matrices other than finished drinking water or ambient water, matrix composition will be addressed on a study-specific basis.

Number of Samples and Replicates

Twenty replicate analyses should be performed by each method for each of the 10 matrices for a total of 200 replicate analyses per method. The replicate analyses should be performed on the same day for both the proposed and reference methods.

Verification of Results

For quantitative reference methods, 10 typical colonies must be verified from 4 randomly chosen replicates (of the 20 replicates) of each of the 10 samples for a total of 400 colony verifications. For presence/absence tests, all positive samples should be verified for up to 200 samples. See Section 6.1.3 for additional information pertaining to the verification of results. Calculations for false positive rates and false negative rates are described in Section 8.4.3.

6.1.3 Verification of Results

6.1.3.1 False Positives

To assess whether the false positive rates are significantly different between methods, replicates known to contain non-target organisms that could be falsely identified as the target organism should be analyzed by both the ATP or new method. The determination that the samples do not contain the target organism should be based on a third independent standard method (see Section 7.6) rather than by the EPA-approved reference method being used in the comparison. This is because it should not be assumed that the accepted method has a false positive or negative rate of zero. In side-by-side comparison studies using the EPA standard for determining the number of matrices and the number of samples, at least 200 positive results should be verified for the ATP or new method in order to adequately compare false positive rates.

6.1.3.2 False Negatives

To assess whether the false negative rates are significantly different between methods, replicates known to contain target organisms should be analyzed by both the ATP or new method. The determination that the samples do not contain the target organism should be based on a third independent standard method (see Section 7.6) rather than by the EPA-approved reference method being used in the comparison. This is because it should not be assumed that the accepted method has a false positive or negative rate of zero. In side-by-side comparison studies using the EPA standard for determining the number of matrices and the number of samples, at least 200 negative results should be verified for the ATP or new method in order to adequately compare false negative rates.

6.2 QC Acceptance Criteria-Based Comparison Studies

QC acceptance criteria-based comparison studies are generally conducted to demonstrate that the ATP or new method is able to meet the QC acceptance criteria of the EPA-approved reference method. In some instances, the quality control (QC) acceptance criteria specified in a method may not be sufficient to demonstrate comparability between the ATP and the EPA-approved reference method and a side-by-side comparison study should be conducted. Generally, EPA will make this decision based on review of the application materials.

6.2.1 Number of laboratories

A minimum of three laboratories should be used for a QC acceptance criteria-based comparison study. All three laboratories should meet all QC acceptance criteria in the EPA-approved reference method. If more than three laboratories participate, at least 75% of the participating laboratories should meet all QC acceptance criteria in the EPA-approved reference method.

6.2.2 Number of Matrices

For all QC acceptance criteria-based comparison studies, there should be one matrix per laboratory. For ambient water studies, the turbidity of at least one matrix should be greater than 10 NTU. Generally, for matrices other than finished drinking water or ambient water, matrix composition should be addressed on a study-specific basis.

6.2.3 Number of Replicates per Matrix

The number of replicates per matrix and laboratory should be specific to the QC criteria to which the results will be compared. Generally, the number of reagent results should be 4 and the number of source water results per matrix should be 2.

SECTION 7.0 SAMPLE PREPARATION AND ANALYSIS

7.1 Collection of Samples for Analysis

Each sample should be collected in sufficient volume to complete all replicate analyses by both the ATP or new method and the EPA-approved reference method. Samples should be spiked (if necessary) and analyzed as soon as possible after collection.

7.1.1 Source Water Characterization

Source water characterization information should be collected at the time of sample collection. This information will be useful in characterizing the matrix to identify potential interferences and generally includes, but is not limited to, the following:

- Sample collection location
- Source of water (e.g., ground water, stream, river, lake, etc.)
- Plant treatment processes (if sample is collected from a water treatment facility)
- Temperature
- pH
- Turbidity
- Total organic carbon (TOC)
- Free and total disinfectant residual at time of sample collection
- Heterotrophic plate count (HPC) unless heterotrophic bacteria are the target analytes, in which case total coliforms should be measured

In addition to this information, data on the concentration of potential interferences (e.g., competing bacteria, interfering chemicals, etc.) in the water collected for analysis may be necessary. Known interferences should be discussed in the method and addressed in the study plan. The final results, as well as bench sheets, log sheets, instrument printouts, and any associated quality control analyses should be submitted as part of Appendix C of the study report (Section 9.10).

7.2 Sample Spiking and "Stressing" Procedures for Bacteriological Methods

Depending on the matrix and the analyte of interest, it may not always be necessary to spike samples prior to analysis. Rangefinding analyses should be performed to assess the ambient concentration of target organism(s) in the matrix of interest to determine whether sample spiking will be useful. Samples are collected and dilutions are prepared in order to enumerate the number of organisms in the sample without qualifiers (i.e. less than, greater than, or too numerous to count). In addition, rangefinding can also be used to obtain environmental isolates for use in sample spiking or to determine the concentration of target organism(s) in a spiking suspension or spiked sample.

If samples are spiked, environmental isolates should be used, as pure strains may exhibit different recovery and precision characteristics than natural flora. NELAC (http://www.epa.gov/ttn/nelac/standard/5qs-15-0-jhrev-corr.pdf, Reference 10.9) and ATCC (http://www.atcc.org/SearchCatalogs/faqBacteriology.cfm#Q15, Reference 10.6) recommend that bacterial cultures be transferred monthly and passed no more than five times before returning to the original culture.

Sections 7.2.1 through 7.2.4 below, detail several different procedures for sample spiking and stressing.

7.2.1 Drinking Water: Spiking and Chlorine-Stressing

Drinking water samples should be spiked and stressed with chlorine as described in Sections 7.2.1.1 and 7.2.1.2 below.

7.2.1.1 Drinking Water Spiking

Because finished drinking water does not typically contain the target analyte(s) of interest, finished drinking water should be spiked. For the evaluation of drinking water samples, a single finished drinking water matrix is spiked with target organism(s) from other matrices, such as non-chlorinated secondary sewage effluents or polluted surface waters. Sewage effluent generally has the advantage of providing a wide range of strains, whereas surface waters typically have the advantage of providing organisms more variable in quality. Depending on the study design (Section 6.0), the source of spiking suspensions (i.e., wastewaters) should come from geographically dispersed sites.

To prepare spiked drinking water samples:

(1) Collect at least five liters of each non-chlorinated secondary sewage effluents or polluted surface water to be used as spiking suspensions.

(2) Perform rangefinding analyses as described above on each spiking suspension to determine target organism density as soon as possible after receipt of the sample to ensure that target organism density and diversity are not reduced.

(3) For each spiking suspension, spike a sufficient volume of drinking water with a sufficient volume of spiking suspension (based on rangefinding) to obtain 10^3-10^5 target organisms/100 mL. *Please note*: A single drinking water, with negligible concentrations of oxidants and reductants (e.g., chlorine and sodium thiosulfate, respectively) should be used for the entire study. Refrigerate all spiked drinking waters at 1°C - 4°C for use in preliminary chlorination study and comparability study. High oxidant or reductant levels in the drinking water could interfere with organism stressing.

7.2.1.2 Preliminary Chlorination Study to Determine Appropriate Exposure Time

Microorganisms in the spiked drinking water samples (from Section 7.2.1.1) should be stressed by chlorination at ambient temperatures under conditions similar to those in drinking water treatment facilities. The goal of chlorinating the spiked samples is to simulate drinking water treatment by reducing the number of organisms in the spiked drinking water samples from 10^3-10^5 target organisms/100 mL to 1-10 chlorine-stressed target organisms/100 mL for most probable number methods or to 20 - 100 chlorine-stressed target organisms/100 mL for membrane filtration methods (i.e., a 2-4 log removal).

After chlorination, no two samples are expected to produce the same levels of injured (stressed) target organisms because the disinfection process is impacted by physical and biological factors. These include: the type of spiked drinking water sample to be disinfected (e.g., spiked with sewage effluent or polluted source water), the initial concentration of the target organism(s), the chlorine demand of the spiked sample, the type and concentration of chlorinating agent, the exposure time, the sample mixing , pH, and temperature. As a result, a preliminary chlorination study should be performed to establish the exposure time necessary to reduce the number organisms in the spiked drinking water samples from 10^3-10^5 target organisms/100 mL to 1-10 chlorine-stressed target organisms/100mL (or a 2-4 log removal). During this preliminary chlorination exposure time study, the physical and biological parameters in Section 7.1.1 should be carefully monitored and recorded for each sample.

The exposure time is directly dependent upon the initial concentration of the target organism present, the matrices' chlorine demand, and the form of chlorine present. For testing, the spiked sample is generally exposed to 2.0-2.5 mg total chlorine/L for over a range of times such as 10, 20, and 30 minutes to reduce the density of target organisms from 10^3-10^5 CFU/100mL to 1-10 CFU/100 mL sample. However, the period of exposure of a sample with a low chlorine demand may be significantly shorter than 20-30 minutes.

Suggested Preliminary Chlorination Study

(1) Determine and record the total residual and free residual chlorine concentrations using an EPA-approved N, N diethyl-p-phenylenediamine (DPD) colorimetric method (e.g., Standard Method 4500-Cl^- G) initially, at midpoint, and at the end of the exposure time just prior to dechlorination.

(2) For each exposure time, place 2 L of each spiked drinking water sample (from Section 7.2.1.1) in a glass container.

(3) Add an appropriate volume of a diluted solution of reagent grade sodium hypochlorite (e.g., a 1:20 dilution of 5% (w/v) stock solution), to achieve the desired level of chlorinating agent and stir the sample continuously during exposure to chlorination. If the spiked sample has an appreciable chlorine demand (e.g., spiked with a primary effluent or a sewage sample), add dilute sodium hypochlorite solution until a total residual chlorine level between 2.0 and 2.5 mg/L is maintained in the absence of free chlorine. If a sample has a low chlorine demand, avoid over-stressing or killing the organisms by prolonged exposure to free residual chlorine. The free residual chlorine concentration should not exceed 0.5-1.0 mg/L.

(4) Stop the chlorine oxidation (dechlorinate) at the end of the exposure period by adding 0.8 mL of a 10% (w/v) sodium thiosulfate solution/L sample.

(5) Enumerate the target organism density in an aliquot of the spiked, chlorine-stressed, dechlorinated drinking water sample using the appropriate EPA-approved reference method from Table 1-1.

(6) For each exposure time, repeat Steps 3 - 5, above.

7.2.1.3 Suggested Procedure for Chlorination and Dilution of Samples for Comparability Study

(1) Determine and record the total residual and free residual chlorine concentrations initially, at midpoint, and at the end of the exposure time just prior to dechlorination using an EPA-approved N, N diethyl-p-phenylenediamine (DPD) colorimetric method (e.g., Standard Method 4500-Cl^- G).

(2) Place a sufficient volume of each spiked drinking water in a glass container to perform sufficient repeat analyses at multiple dilutions.

(3) Immediately prior to chlorination, perform enumeration to determine target organism density as described in 7.2.1.1(2). This value should be used to determine the log reduction due to chlorination.

(4) Add reagent-grade sodium hypochlorite to achieve the same concentration, as in the preliminary chlorination exposure study.

(5) To reduce the density of target organisms from 10^3-10^5 CFU/100mL to 1-10 CFU/100 mL, chlorinate each spiked drinking water sample for the appropriate time, based upon the preliminary chlorination exposure study. Stir the sample continuously during the chlorination.

(6) Stop the chlorine oxidation (dechlorinate) at the end of the exposure period by adding 0.8 mL of a 10% (w/v) sodium thiosulfate solution/L sample.

(7) Enumerate target organism density in an aliquot of the spiked, chlorine-stressed, dechlorinated drinking water sample using the appropriate EPA-approved reference method from Table 1-1.

(8) Refrigerate the spiked, chlorine-stressed, dechlorinated drinking water samples at 1°C - 4°C for use in comparability testing.

(9) Read the plates to determine the approximate density of the target organisms. Use these results to estimate the appropriate dilution necessary to reach the target organism density of 1-10 CFU/100 mL.

(10) Evaluate three dilutions of each spiked drinking water sample. Samples should be diluted with the same, original, oxidant-free and reductant-free drinking water, as necessary to reach a target organism density of 1-10 target organisms/100 mL for most probable number methods or 20-100 target organisms/100 mL for membrane filtration methods. Make the dilution and at least two others that bracket the target density, for example, half and double that dilution. One of these dilutions should contain the desired 1-10 target organisms per 100 mL. Immediately conduct the comparability analyses with each sample using these three dilutions.

Please note: For the evaluation of presence/absence methods, the data used for comparison should be from the dilution(s) which produces results closest to an equal number of positive and negative results for the reference method. A 25% to 75% split in responses (in either direction) should be sought. For comparability, the evaluated results for the ATP or new method should be from the same dilution as the reference method. If one of the dilutions does not produce an acceptable split in positive and negative results for the reference method, the applicant should return to the original, spiked sample.

7.2.2 Preparation of Enumerated Spiking Suspension

This dilution scheme is adapted from *Standard Methods for the Examination of Water and Wastewater*, *19th Edition*, Section 9020 B (Reference 10.3). This entire process should be performed quickly to avoid loss of viable organisms. There should be approximately 10^{10} organisms per slant. Therefore, dilution bottles "A" through "E," below contain approximately 10^{10}, 10^8, 10^6, 10^4, and 10^3 organisms per dilution bottle, respectively. Depending on the growing conditions, these numbers may vary. As a result, until experience has been gained, more dilutions should be filtered to determine the appropriate dilution.

Inoculate bacterial culture onto the entire surface of several nutrient agar slants with a slope approximately 6.3 cm long in a 125 × 16 mm screw-cap tube. Incubate for 24 ± 2 hours at 35°C ± 0.5°C.

From the slant that has the best growth, prepare serial dilutions using four dilution bottles with 99 mL of sterile buffered dilution water (bottles A, B, C, and D) and one dilution bottle containing 90-mL of sterile buffered dilution water (bottle E).

Pipette 1 mL of buffered dilution water from bottle "A" to one of the slants. Emulsify the growth on the slant by gently rubbing the bacterial film with the pipette, being careful not to tear the agar. Pipette the suspension back into dilution bottle "A." Repeat this procedure a second time to remove any remaining growth on the agar slant, without disturbing the agar.

Make serial dilutions as follows:

(1) Shake bottle "A" vigorously and pipette 1 mL to bottle "B" containing 99 mL buffer
(2) Shake bottle "B" vigorously and pipette 1 mL to bottle "C" containing 99 mL buffer
(3) Shake bottle "C" vigorously and pipette 1 mL to bottle "D" containing 99 mL buffer
(4) Shake bottle "D" vigorously and pipette 10 mL to bottle "E" containing 90 mL buffer; this should result in a final dilution of approximately 10 organisms / mL. *If it is more convenient for your laboratory, an acceptable alternative to the dilution scheme presented for this step, is to pipette **11 mL** of dilution D into dilution bottle E, which contains **99 mL** of dilution water.*

Filter 1- to 5-mL portions in triplicate from bottles "D" and "E" according to standard membrane filtration methods to determine the number of CFU in the dilutions. The recommended target dilution and spike volume depends on the method and the target analyte(s). Typically, dilutions should be stored at 1°C to 5°C and may be used throughout the day they are prepared, however, storage conditions should be adjusted as necessary since both storage conditions and viability may vary from organism to organism.

7.2.3 Log Phase Growth Curve

Inoculate 100 mL of broth media with a single isolated colony and incubate organisms at optimum temperature. If possible, shake at 200 RPM during incubation.

Take optical density (OD) measurements at 550-600 nm at 30 minute intervals for the first 8 hours and record readings. In addition to OD readings at 30 minute intervals, using aseptic technique, remove a 0.1-mL portion of the culture and make a series of 1:10 dilutions in sterile buffer. Initially plate 0.1 mL of the 1.0, 10^{-1}, and 10^{-2} dilutions in duplicate and incubate overnight at optimum temperature. Count colonies and record data. As the optical density increases, evaluate serial dilutions to accommodate the increased numbers of bacteria (i.e., when the optical density exceeds 1.0 then plate 0.1 mL from dilutions 10^{-1}, 10^{-2}, and 10^{-3}).

Based on OD and the CFU/mL results, the OD of fresh cultures (in log-phase growth) can be used to determine the concentration of bacteria in the tubes by a simple graphic representation of the combined OD and CFU/mL results.

Commercially available McFarland standards may be used to determine the bacterial density instead of actually doing a growth curve within the lab. In order to determine bacterial densities using McFarland standards, the OD of the standards are compared to the OD of the log phase culture.

7.2.4 Commercially Available Enumerated Spikes

One time use, commercially available spiking suspensions may be obtained from a variety of vendors. Prepare spiking suspensions according to manufacturer's instructions. It should be noted that a different spiking volume than that recommended by the manufacturer may be necessary to achieve the target density.

7.3 Spiking Procedures for Virus Methods

7.3.1 Cell Monolayer Propagation

In order to propagate virus stock suspensions cell monolayers should be propagated. The type of assay cytopathic effect (CPE) or plaque assay (PA), being performed determines the size of the flask and the number of days the monolayer incubates prior to use for the assay. Inoculate T75 or T25 flasks with 1 $x10^5$ cells containing cell specific growth medium. Flasks for CPE should incubate for 5-7 days at 37°C ± 0.5°C with 95% relative humidity (and 5% CO_2 concentration, if necessary). One CPE flask should be stained with crystal violet and microscopically checked to ensure a 95% confluent cell monolayer prior to use in the assay. Flasks used for PA incubate for 7-10 days under the same conditions as the monolayers used for CPE. PA flasks should also be checked to ensure 95% confluency before use for assay.

7.3.2 Propagation of Virus Stock Suspension

For (CPE) analysis, utilizing the appropriate cell line (e.g., Buffalo green monkey kidney cells; BGMK), inoculate the cell monolayer with virus and incubate culture flasks at 37°C ± 0.5°C until the entire monolayer has been destroyed by virus replication (approximately 72 - 96 hours). Freeze (to approximately -80°C) and thaw the flasks three times, then pool contents of the flasks and spin at 10,000 x *g* for 30 minutes. Filter supernatant using a 0.2µm pore nylon filter to remove any remaining cell debris. The filtrate is the virus stock suspension.

7.3.3 Titering of the Virus Stock Suspension

Titer the virus stock suspension (from Section 7.3.2) by performing a plaque assay. Inoculate monolayers with 0.2 mL of serial diluted viral stock suspension. After rocking and rinsing the monolayers, add the agar overlay to the monolayer. Incubate the culture flasks for seven days while reading the number of plaques each day for the entire seven days. After the viral stock suspension has been titered, appropriate volumes of the suspension can be used to spike test matrices to obtain plaque forming units (PFU)/mL.

7.4 Spiking Procedures for *Cryptosporidium and Giardia*

Enumerated spiking suspensions are needed for initial and ongoing precision and recovery (IPR and OPR) samples (often referred to as positive controls) and matrix spike (MS) samples. Flow cytometer–sorted organisms are necessary for these spiking suspensions, rather than manual techniques. Flow cytometer–sorted spikes generally are characterized by lower variability than manually enumerated spikes.

Spiking suspensions should be prepared using unstained organisms that have not been irradiated, heat-fixed or formalin-fixed. Immediately before sorting spiking suspensions, initial calibration of the flow cytometer should be performed by conducting a series of sequential sorts directly onto membranes or well slides. These initial sorts should be stained and counted microscopically to verify the accuracy of the system. When sorting the spiking suspensions, ongoing calibration samples should also be prepared and counted at regular intervals. The mean of the ongoing calibration counts should be used as the estimated spike dose. Flow-cytometer-sorted spiking suspensions should be used by the expiration date noted on the suspension. Flow-cytometer-sorted spiking suspensions containing live organisms should be used within two weeks of the preparation date. General procedures for preparing flow-cytometer-counted spikes for *Cryptosporidium* and *Giardia* can be found in EPA Method 1622 and 1623 (April 2001).

A potential commercial source of flow-sorted *Cryptosporidium* and *Giardia* spiking suspensions is:

Wisconsin State Laboratory of Hygiene
Flow Cytometry Unit
2601 Agriculture Drive
Madison, WI 53718
Phone: (608) 224-6260
Fax: (608) 224-6213

The Wisconsin State Laboratory of Hygiene prepares and distributes live *Cryptosporidium parvum* oocysts and *Giardia intestinalis* cysts that have not been treated to reduce viability.

7.5 Analysis of Samples

Samples should be analyzed in accordance with the EPA-approved study plan. *Any deviations from the study plan may suggest the need for additional analyses and potentially, rejection of data generated from the study.* If any deviations from the approved study plan are necessary prior to or during the study, the applicant should consult EPA and receive approval for the modification to the study plan. Deviations from the approved study plan should be documented in the study report (Section 9.0).

Example analysis schemes are provided in Sections 7.5.1 and 7.5.2 below. Generally, the analysis schemes will differ depending on the type of organism and the type of method. The study plan (Section 4.0) should detail the order in which samples will be analyzed.

7.5.1 Side-by-Side Comparison Studies

Method Blank 1
Sample 1 up to Sample 20
Method Blank 2
Positive Control
Negative Control
Method Blank 3
Media Sterility Checks

7.5.2 QC Acceptance Criteria-Based Comparison Studies

Replicate 1 for IPR
Replicate 2 for IPR
Replicate 3 for IPR
Replicate 4 for IPR
Method Blank
Matrix Spike
Matrix Spike Duplicate
Unspiked Matrix Sample

7.6 Verification of Results

The number of positive and negative results to be verified in a side-by-side comparison study is discussed in Section 6.1.3. Sections 7.6.1 to 7.6.3 below discuss the types of independent standards that may be used for the verification of results from bacteriological methods, virus methods, and *Cryptosporidium* and *Giardia* methods, respectively.

7.6.1 Verification of Results from Bacteriological Methods

7.6.1.1 Biochemical Tests that May be Used for Verifications

Oxidase	Citrate
Catalase	Lysine decarboxylase
ONPG	Methyl Red and Voges Proskauer (MRVP)
Indole	Triple sugar iron (TSI)
Coagulase	Lysine iron agar (LIA)
Esculin	Urease

Sugars (e.g., Trehalose, lactose, mannitol, and sorbitol)

It is recommended that multiple biochemical tests be utilized to verify colony identification. The above list of biochemical tests is not exhaustive. The choice of which biochemical tests to use is based on type of organism (i.e., gram stain results) being identified/verified. Biochemical tests used for verification should be discussed in the study plan (Section 4.0). It may be appropriate to perform a gram stain prior to biochemical identification. A description of the biochemical tests listed above, as well as additional tests is provided in *Standard Methods for the Examination of Water and Wastewater,* Method 9225.

7.6.1.2 Commercially Available Biochemical Testing Products

Commercial biochemical test systems incorporate multiple biochemical tests to allow for identification to the genus and/or species level, which may be difficult when using individual biochemical tests prepared in house.

Commercial biochemical test systems are available in two formats: systems that depend on the analyst to manually interpret the results (e.g., API strips, BBL crystal, and enterotube) and systems that automate the interpretation of results (e.g., Vitek and Biolog).

7.6.2 Verification of Results from Virus Methods

Viral protocol testing confirmations tend to be method-specific, therefore a detailed description of the confirmation/verification procedure should be included in the study plan and reviewed on a study-specific basis.

7.6.3 Verification of Results from *Cryptosporidium and Giardia* Methods

Verification of results may not be necessary for *Cryptosporidium* and *Giardia* IFA methods beyond the analyst's microscopic examination of the organism. If verification is necessary, a detailed description of the confirmation/verification procedure should be included in the study plan and reviewed on a study-specific basis.

SECTION 8.0 REVIEW OF STUDY RESULTS

Generally, upon receipt of the applicant's data, EPA will perform the following reviews discussed in Sections 8.1 to 8.5.

1. Assessment of compliance with the approved study plan
2. Data review
3. Data validation
4. Development of descriptive statistics
5. Statistical assessment of method comparability

Methods that are deemed acceptable will generally be recommended for approval (Section 8.6).

8.1 Assessment of Compliance with Approved Study Plan

Generally, EPA will review the study report and associated data to ensure the study was conducted according to the approved study plan. The applicant should explain and justify (possibly with additional studies) any deviations from the study plan. Deviations from the approved study plan that occur without prior approval from EPA may result in the rejection of some or all study data.

8.2 Data Review

Upon receipt of the applicant's data, EPA will generally verify that all raw data described in Section 9.10 are present and complete. Generally, all calculations used in the method will be verified. This may include calculations used for spiking enumeration, preliminary or presumptive stages of the method, and the determination of the final result.

8.3 Data Validation

After verifying data completeness and reviewing of all calculations, EPA will generally verify that all measurements were performed in accordance with the method. This may include, but is not limited to, the following:

- Temperature logs for incubator/waterbath/refrigerator
- Media preparation records
- Sample incubation times
- Associated QC samples (as described in Section 5.0)
    - Method blanks
    - Preparation blanks
    - Sterility checks
    - Positive and negative controls

8.4 Development of Descriptive Statistics

8.4.1 Mean Recovery

To determine if matrix characteristics effect method performance, mean recoveries should be calculated separately for each matrix and method. Mean recoveries should also be calculated for each method over all matrices.

8.4.2 Precision

Precision can be expressed both on an absolute scale (i.e., standard deviation) and on a relative scale (i.e., relative standard deviation). The RSD (sometimes referred to as coefficient of variation) is calculated as the standard deviation divided by the mean, expressed as a percent. For the purpose of summarizing the data, both standard deviations and RSDs should be calculated, and the one which is most appropriate for assessing comparability will be used in analyses. Generally, RSDs are most appropriate for summarizing precision when variability increases as concentration increases.

To give an indication of the effect of multiple matrices on precision, standard deviations should be calculated separately for each matrix and method. Standard deviations also should be calculated for each method over all matrices.

8.4.3 False Positive Rates, False Negative Rates, Sensitivity, and Specificity

False positive (FP) and false negative (FN) rates of approved and reference methods should be evaluated when assessing comparability. An independent standard (described in Section 7.6) may sometimes be necessary to confirm positives and negatives of both the ATP or new method and the EPA reference method. The confirmation methods used should be discussed in the validation study plan.

Generally, performance of the ATP or new method and EPA-approved reference methods will be defined in terms of false positive rates and false negative rates. For the purposes of the ATP protocol, false positive rates and false negative rates are equivalent to (1- Specificity) and (1-Sensitivity), respectively. Specificity is defined as the percent of negative samples correctly identified as negative, and sensitivity is defined as the percent of positive samples correctly identified as positive (see equations on the next page). In order to calculate estimates of false positives, false negatives, sensitivity, and specificity for each method, 2-by-2 tables for each matrix, and over all matrices, should be set up as follows in Table 8-1.

Table 8-1. Standard Format for 2-by-2 Tables

		Independent Standard		
		+	**-**	**Total**
ATP or New Method	**+**	TP_1	FP_1	$TP_1 + FP_1$
	-	FN_1	TN_1	$FN_1 + TN_1$
	Total	$TP_1 + FN_1$	$FP_1 + TN_1$	$TP_1 + FP_1 + TN_1 + FN_1$

		Independent Standard		
		+	**-**	**Total**
EPA-Approved Reference Method	**+**	TP_2	FP_2	$TP_2 + FP_2$
	-	FN_2	TN_2	$FN_2 + TN_2$
	Total	$TP_2 + FN_2$	$FP_2 + TN_2$	$TP_2 + FP_2 + TN_2 + FN_2$

Estimates of sensitivity, specificity, false positive rates, and false negative rates as percentages for the two methods should be calculated as follows:

$$Sensitivity_i = \frac{TP_i}{TP_i + FN_i} * 100\ \%$$

$$Specificity_i = \frac{TN_i}{TN_i + FP_i} * 100\ \%$$

$$False\ positive\ rate_i = \frac{FP_i}{TN_i + FP_i} * 100\ \% = \left(1 - \frac{TN_i}{TN_i + FP_i}\right) * 100\ \% = 1 - Specificity_i$$

$$False\ negative\ rate_i = \frac{FN_i}{TP_i + FN_i} * 100\ \% = \left(1 - \frac{TP_i}{TP_i + FN_i}\right) * 100\ \% = 1 - Sensitivity_i$$

Where F = False, N = Negative, T = True and " i" refers to the specified method (i=1 for new method, i=2 for reference method)

8.5 Statistical Assessment of Method Comparability

8.5.1 Presence / Absence Methods

For presence/absence methods, the chi-square test and Breslow-Day test will generally be used to compare the percent of false positives and false negatives in the proposed method analyses with the percent of false positives and false negatives in the reference method analyses.

Generally, tests for significant differences in false positive and false negative rates between methods will be run using the results summarized in Table 8-1 in Section 8.4. To perform the chi-square and Breslow-Day tests, it may help to first rearrange the data as follows in Tables 8-2 and 8-3:

Table 8-2. False Negative Rate Comparison

		Method		
		New	**Reference**	**Total**
Result	**True +**	TP_1	TP_2	$TP_1 + TP_2$
	False -	FN_1	FN_2	$FN_1 + FN_2$
	Total	TP_1+FN_1	$TP_2 + FN_2$	$TP_1 + TP_2 + FN_1 + FN_2$

Table 8-3. False Positive Rate Comparison

		Method		
		New	**Reference**	**Total**
Result	**False +**	FP_1	FP_2	$FP_1 + FP_2$
	True -	TN_1	TN_2	$TN_1 + TN_2$
	Total	$FP_1 + TN_1$	$FP_2 + TN_2$	$FP_1 + FP_2 + TN_1 + TN_2$

Before running the chi-square tests, positives and negatives should be confirmed using the independent standard method. The chi-square test will be used to determine whether the false positive rate and false negative rate have a statistically significant difference between the ATP or new method and the EPA-approved reference method.

8.5.1.1 Assessing Method Differences (Chi-Square Test)

In order to assess whether the false positive and false negative rates differ between methods, chi-square tests should be run over all matrices; additional tests in each matrix also may be necessary depending on the presence of matrix interactions (see Section 8.5.1.2). For false negative rates, the chi-square test(s) indicate whether the proportions of negative samples correctly identified as negative by the two methods are significantly different, and for false positive rates, the chi-square test(s) indicate whether the proportions of true positive results correctly identified as positive for the two methods are significantly different.

8.5.1.2 Assessing Method/Matrix Interactions (Breslow-Day Test)

In order to assess whether there are false positive and false negative rate differences between methods, it is also necessary to establish if there is a matrix effect on these parameters. The effect of matrices on false positive and false negative rate differences between methods is assessed using the Breslow-Day test. (Reference 10.7). The Breslow-Day test is used to test whether there is an interaction between matrix and method in terms of the likelihood of a false positive result (for specificity) and in terms of the likelihood of a false negative result (for sensitivity). If a significant interaction is found between method and matrix, then the chi-square tests for a difference between methods for that attribute should be done separately for each matrix. Otherwise, separate chi-square tests for each matrix are not necessary, and a single chi-square test can be run using the data from all matrices.

8.5.1.3 Method Comparability Conclusions

A decision on whether the ATP or new method is comparable to the EPA-approved reference method will generally be made based on the results of the false positive and false negative rate comparisons.

Generally, the decision on acceptability of the new method should be made based on the chi-square test using data from all matrices. However, if the results of the Breslow-Day test indicate that there is a significant interaction between method and matrix for false positive and/or false negative rates, then further review should be made for those matrices which yielded a higher false positive and/or false negative rate for the proposed method.

If the results of the chi-square tests indicate that the false positive and false negative rates of the ATP or new method are not significantly different from the EPA reference method, this generally will be interpreted as not having enough evidence to conclude that the performance of the ATP or new method is worse than the EPA-approved reference method. However, if the results of the chi-square test indicate that the false positive and/or false negative rates of the new method are significantly less than that of the EPA-approved reference method, this will generally be interpreted as worse performance and would lead to rejection of the ATP or new method.

8.5.2 Quantitative Methods

8.5.2.1 Testing for Normality

Many of the statistical analyses used to assess differences in recovery and precision between methods require certain assumptions about the data be met. Because the validity of these assumptions affect which statistical tests will be used to assess recovery and precision differences, testing these assumptions is a prerequisite as a first step. There are two assumptions that should be evaluated prior to comparing the two methods statistically. One assumption is that the variability of the replicates is constant for each matrix and method. This assumption is discussed in Section 8.5.2.2. The other main assumption that should be met is that the data follow a normal distribution.

An assessment of normality can be done either graphically or with statistical tests, or both. A normal distribution looks like a bell curve (i.e., symmetric around the mean). A graphical assessment can be done using a histogram, stem-and-leaf or Normal probability plot. Appropriate statistical tests include the Shapiro-Wilk test, D'Agostino test, and Kolmogorov-Smirnov test. These tests are computation intensive, and it may be necessary to use software (e.g., SAS) to run them. The Shapiro-Wilk test is generally inappropriate for testing the normality assumption of a data set with greater than 50 results.

For environmental data (especially field data, though sometimes spiked data as well), the distribution of results will often be positively skewed (i.e., with a few unusually high results). A common corrective action of this that can be utilized is the logarithmic transformation, base e (natural log). If the data are positively skewed, this transformation should be attempted, assuming that none of the results are negative or equal to zero. When zero-valued results are present in a skewed data set, a common approach is to add a small constant to every result prior to transformation. However, the arbitrary choice of this constant will have a large effect on the transformed data (the log of 1 and the log of 0.1 are very different, for example), and this approach is not recommended. The use of non-parametric tests, discussed later in this guidance, are considered to be the appropriate alternatives.

If no zero-valued results are present in a positively skewed data set, then the log-transformed data should be tested for normality. If the log-transformed data do follow a Normal distribution, then all analyses should be done using the log-transformed results.

8.5.2.2 Evaluating Precision

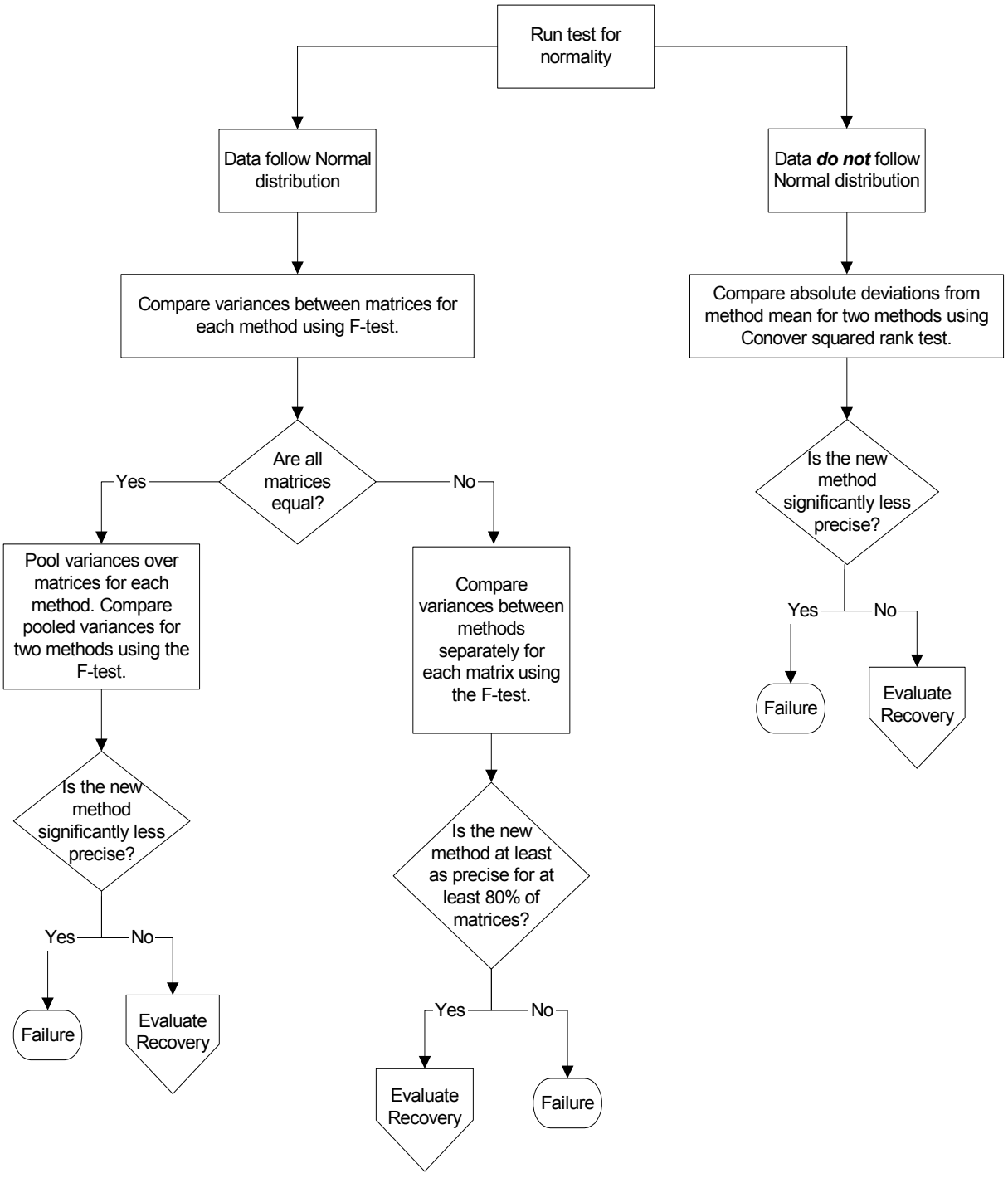
The decision of what statistical test to use to evaluate precision will generally be made on the results of the normality test described in Section 8.5.2.1. If the assumption of normality is met, then the F-test for differences in variance should be used. If the assumption of normality is not met, a non-parametric test

such as the Conover Squared-Rank test should be used. The F-test is based on comparisons of variances (i.e., the squared standard deviations). The Conover Squared-Rank test is based on ranking absolute deviations from the mean. Figure 8-1 provides a summary of the procedure for evaluating precision.

Prior to testing whether there is a precision difference between methods, the precision of the different matrices should be compared for each method.

If the results of the F-test indicate that variances differ significantly by matrix, or if the results of the Conover Squared-Rank test indicate that absolute deviations from the mean differ significantly by matrix, then comparisons of precision between methods should be done separately for each matrix. Otherwise, a single comparison of method precision can be done using the data from all matrices. If the results of the F-test indicate that there is not enough evidence to conclude that the variances differ significantly by matrix, then the pooled within-matrix variance (i.e., the average of the matrix variances for the given method) should be calculated for each method. The F-test should then be used to compare the pooled within-matrix variance for the two methods. If the Conover Squared-Rank test was used and there was not enough evidence to conclude that the mean absolute deviation differed significantly between matrices, then the Conover Squared-Rank test should be used to compare the mean absolute deviations from the overall method mean for the two methods.

Figure 8-1. Evaluating Precision



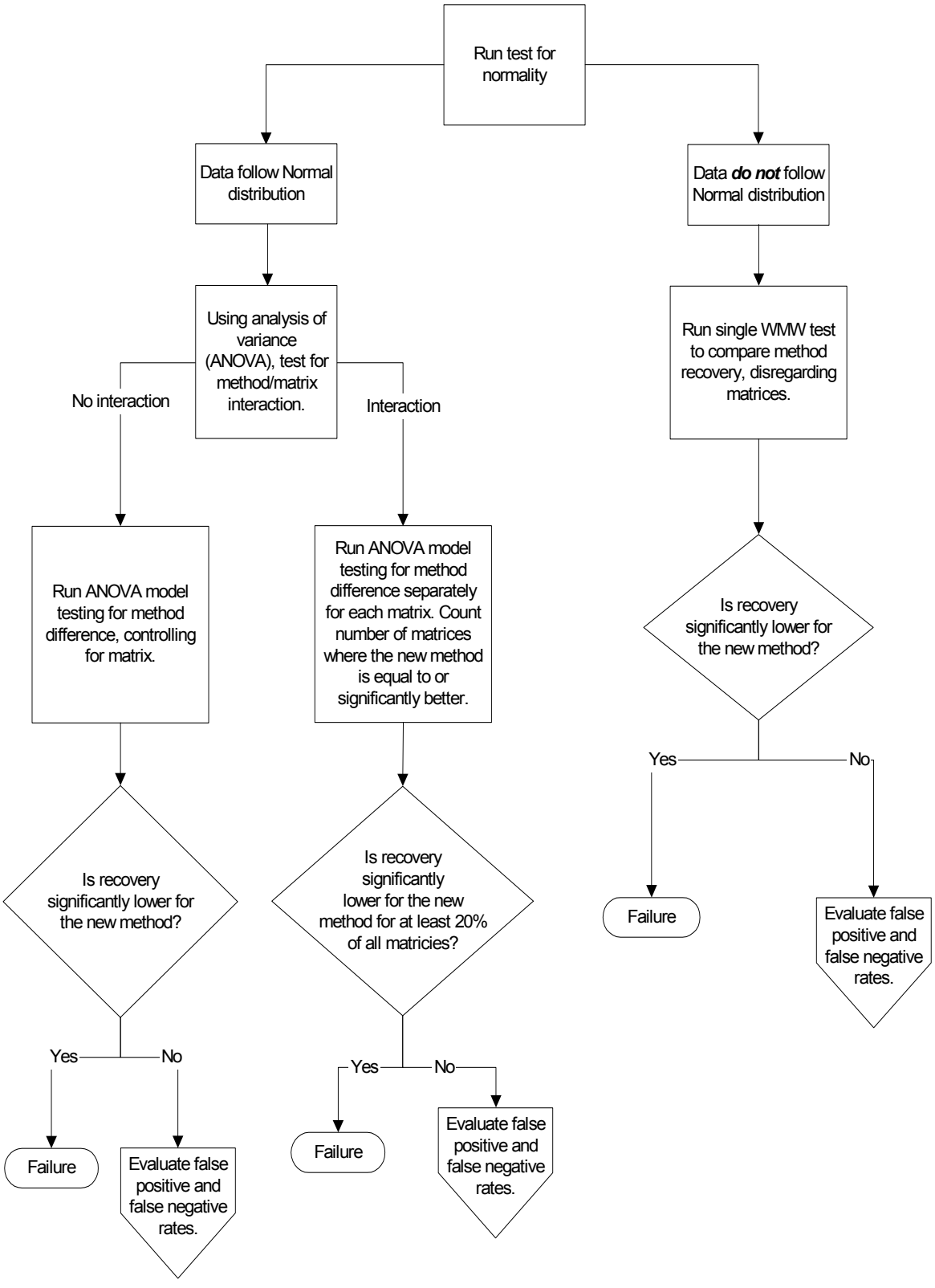
8.5.2.3 Evaluating Recovery

Comparisons of mean recovery of the different methods should be done using either an analysis of variance (ANOVA) model or non-parametric test such as the Wilcoxon-Mann-Whitney (WMW) test. If the assumption of normality is not met, or if the precision differs between methods or matrices based on the precision evaluation described in the previous section, the WMW test should be used. If all assumptions are met, the ANOVA model should be used. Figure 8-2 provides a summary of the procedure for evaluating recovery.

If an ANOVA model is used, method-by-matrix interactions should be tested for significance first using the corresponding ANOVA F-test. If the method-by-matrix interaction is significant, it would mean that the difference between methods is not the same for each matrix. In this case, no conclusion regarding an overall difference between the recovery of methods can be made. If the interaction is not significant, then the test for a significant difference between methods can be run in the ANOVA model, using the F-test for a significant method main effect.

If the WMW test is used, between-matrix and between-laboratory variability cannot be separated from replicate variability. Therefore, significant interactions between method and laboratory or matrix cannot be tested when the WMW test is used. Instead the WMW model only tests for an overall difference between methods. The model can be run separately for each matrix, but the diminished power will limit the value of this approach. It is therefore recommended that a single WMW test be run comparing method recoveries, without stratifying by or controlling for matrix type.

Figure 8.2 Evaluating Recovery



8.5.2.4 False Positive Rates, False Negative Rates, Sensitivity, and Specificity

Comparisons of false positive rates, false negative rates, sensitivity, and specificity should be conducted according to the methods described in Sections 8.5.1.1 and 8.5.1.2.

8.5.2.5 Method Comparability Conclusions

If there is not enough evidence to conclude that there is a significant interaction between method and matrix for recovery, or if there is not enough evidence to conclude that there is a statistically significant difference in precision between matrices for either method, then it can be concluded that any such differences of the method will be consistent. Therefore, lack of sufficient evidence to conclude a significant difference between methods will generally be interpreted as equal or better performance, and might lead to acceptance of the ATP or new method, pending review of false positive and false negative rates. However, a statistically significant test (worse) statistic will be interpreted as worse performance of the ATP or new method, and would generally lead to rejection of the new method.

In cases where significant interactions between matrix and method are found when assessing recovery, or in cases where differences in precision between matrices for at least one method (and therefore a comparison of method precision could not be done over all matrices), some judgment will be necessary in deciding whether the proposed method should be deemed acceptable. The decision should be based on the attribute (i.e., recovery or precision) for each matrix. As a general rule, if there was not enough evidence to conclude that the new method was similar or better than the EPA-approved reference method for that attribute for at least 80% of the matrices used in the study, then the new method can generally be recommended for approval, pending review of false positive and false negative rates.

If there is not enough evidence to conclude that the new method is worse than the EPA-approved reference method for at least 80% of the matrices for both precision and recovery, the false negative rate and false positive rate should next be compared, based on the methods described in sections 8.5.1.1 and 8.5.1.2. If the results of the chi-square test indicate that there is not enough evidence to conclude that the false negative rate or false positive rate of the ATP or new method is worse than that of the EPA-approved reference method, this will be interpreted as equal or better performance and might lead to acceptance of the new methods. However, if the results of the chi-square test indicate that the false negative rate or false positive rate of the new method is significantly greater than that of the EPA-approved reference method, this will generally be interpreted as worse performance for 100% of the matrices used in the study, and would lead to rejection of the ATP or new method.

For example, suppose side-by-side testing is done in one laboratory for ten different matrices. For recovery, there is not enough evidence to conclude that the new method is significantly worse than the EPA-approved reference method for eight of the ten matrices. For precision, there is not enough evidence to conclude a significant matrix effect, and there is not enough evidence to conclude a significant difference between methods. There is not enough evidence to conclude the false negative rate or false positive rate of the ATP or new method is worse than the EPA-approved reference method. Under these circumstances, the ATP or new method may be recommended for approval. However, if there is not enough evidence to conclude that the new method is not worse than the approved method for only seven of ten matrices for recovery, then the ATP or new method will not, generally, be recommended for approval.

8.5.3 QC Acceptance Criteria-Based Comparison Studies

For methods with QC acceptance criteria, necessary calculations and QC criteria are typically provided in the method. Generally, QC criteria will include both recovery and precision specifications. Recovery criteria take the form of a recovery interval for either a single result or mean of multiple results. Precision

criteria will generally be either a maximum standard deviation, RSD, or RPD. Details on calculations that may be necessary for the comparisons are described below.

8.5.3.1 Recovery

Recovery results will generally be necessary for data spiked into both reagent water (IPR, IDC, etc.) and source water (MS, MSD, etc.). For reagent water, percent recovery should be calculated as follows:

$$\%\ Recovery = \frac{Result}{Spike} * 100\ \%$$

where Result is the amount recovered from the sample after spiking, and
Spike is the estimated amount spiked into the sample.

For source water, percent recovery should be calculated as follows:

$$\%\ Recovery = \frac{Result - Background}{Spike} * 100\ \%$$

where Result is the amount recovered from the sample after spiking,
Spike is the estimated amount spiked into the sample, and
Background is the estimated background amount measured in the sample prior to spiking.

For some QC criteria, it may be necessary to calculate the mean of the sample recoveries prior to comparing the results to the criteria. Generally, this will be specified with that given method.

8.5.3.2 Precision

Calculation of an RSD, RPD, or standard deviation is generally necessary for precision criteria. For source water data, the calculation should be based on the recovered amount, rather than the percent recovery. This is because the calculated percentage will be inflated if the background amount is large compared to the total amount recovered. For reagent water-spiked data, where there the background count is zero, the calculation can be made either using the recovered amount or the percent recovery.

Where the precision criteria is to be based on two results, the precision criterion will generally be an RPD. The equation for an RPD is given below:

$$RPD = \frac{|Amount_1 - Amount_2|}{(Amount_1 + Amount_2)\ /\ 2} * 100\ \%$$

Where $Amount_1$ and $Amount_2$ are the two recovered amounts.

Where the precision criterion is to be based on more than two results, the precision criterion will generally be either a standard deviation or RSD. An RSD is calculated based on the equation below:

$$RSD = \frac{SD}{Mean} * 100\ \%$$

Where SD is the standard deviation of all recovered amounts, and
Mean is the mean of all recovered amounts.

8.5.3.3 Presence/Absence Criteria

QC criteria for presence/absence methods may be defined as false positive and/or false negative rates, sensitivity and/or specificity, or as a specified proportion of positive and/or negative results out of a given number of samples. All calculations should be specific to the given situation and should be defined explicitly therein.

8.6 Method Recommendation and Approval

Generally, after completion of the technical and statistical reviews for nationwide-use applications, the Director of Analytical Methods, Attn: ATP Program Coordinator (see Table 2-1 and Appendix E) will prepare a recommendation for approval/disapproval of the ATP or new method and notify the applicant of the recommendation.

If the data evaluation demonstrates that the applicant's method performs at least as well as the EPA-approved reference method, the Director of Analytical Methods, Attn: ATP Program Coordinator will generally recommend approval to the Office of Ground Water and Drinking Water (OGWDW) or appropriate office, which begins the regulation development process. Regulation development includes a *Federal Register* notice proposing to approve an ATP, public comment on the proposed method, and (depending on public comment) a final rule published in the *Federal Register* that approves the method. Generally, the regulation development process may take one year or more.

For limited-use ambient water or wastewater applications the Regional Administrator (see Table 2-1 and Appendix E) will generally prepare a recommendation of approval/disapproval of the ATP or new method and notify the applicant of the recommendation after completion of the technical and statistical reviews.

SECTION 9.0 STUDY REPORT

Laboratories or other organizations responsible for developing ATPs or new methods should document the results of the side-by-side comparison study or QC acceptance criteria-based comparison study in a formal study report that contains the elements described in this section.

The information and supporting data included in the study report should be sufficient to enable EPA to evaluate the performance of the ATP or new method and make a decision on whether it is comparable to the reference method. The applicant is responsible for ensuring that all method-specified criteria are met by the laboratory(ies) involved in the study and that the study report contains all data from the laboratory(ies). A copy of all comparison study data should be maintained at the participant laboratory(ies) or other organization responsible for developing the ATP or new method.

Like the study plan developed and approved by EPA before the study was performed, the study report contains background information and describes the study design. However, the study report also details the process and results of the study, provides an analysis and discussion of the results, and presents study conclusions. The approved study report should also identify and discuss any deviations from the study plan that were made in implementing the study, and the study plan should be appended to and referenced in the study report. Significant deviations from the study plan without prior EPA approval could result in the rejection of the study data.

The study report should be organized into the following sections:
- Background
- Study Objectives and Design
- Study Implementation
- Data Reporting and Validation
- Results
- Data Analysis and Discussion
- Conclusions
- Appendix A - Method
- Appendix B - Study Plan
- Appendix C - Supporting Data
- Appendix D - Supporting References

Details on the information that should be included in each of these sections are provided below, in Sections 9.1 through 9.11.

9.1 Background

This section of the study report should describe the ATP or new method that was tested, and identify the organization responsible for developing the ATP or new method. The background section of the study report should include the following information:
- A method summary
- The organization, method number, and title for the ATP or new method
- The method number or title and publication number (given in 40 CFR parts 136, 141, and 405 - 503) for the EPA-approved reference method that is being used for demonstrating method comparability (i.e., the reference method)
- A description of the nature of the ATP (e.g., alternate media, alternate concentration technique, etc.)

- The matrices, matrix types, and/or media to which the ATP or new method is believed to be applicable
- The analyte(s) measured by the ATP or new method, including corresponding CAS Registry or other identification numbers, when available

9.2 Study Objectives and Design

This section of the study report should identify the overall objectives and data quality objectives of the study and briefly describe the study design. This information should be consistent with the study objectives and design specified in the approved study plan. Any study limitations should be identified. The approved study plan should be appended to the study report.

9.3 Study Implementation

This section of the study report should describe the methodology and approach undertaken in the study. This section should include the following information:

- The organization that was responsible for managing the study
- The laboratories, facilities, and other organizations that participated in the study; describe how participating laboratories were selected; and explain the role of each organization involved in the study
- The type of study performed (i.e., side-by-side or QC acceptance criteria-based comparison study)
- The study schedule that was followed
- A brief description of how sample matrices were chosen, including, for QC acceptance criteria-based comparison studies, a statement of compliance with recommendations for matrix type selection
- A description of any preliminary testing conducted prior to the side-by-side or QC acceptance criteria-based comparison study (e.g., method validation, physical and chemical assessment of the matrices, preliminary range-finding analyses)
- The numbers and types of analyses performed by the participating laboratories
- A description of how samples were collected, distributed, and stored
- The source and strain of the organism used for sample spiking
- If spikes were quantified, a description of how estimated true spike values were determined and provide all supporting data
- The type of water used in the preparation of sample dilutions if not specified by the method (e.g., reagent water, phosphate buffered water, phosphate buffered saline, etc.)
- Any problems encountered with samples, spiking organisms, equipment, etc. and their subsequent resolution
- Any communications with EPA relevant to the study, such as clarification of the study design or approved changes to the study plan
- Any deviations from the study plan and their impact on study performance and/or results

9.4 Data Reporting and Validation

This section of the study report should describe the procedures that were used to report and validate study data. While EPA generally does not use a standard format for analytical data submission, a list of necessary data elements and an example bench sheet may be found in Appendix D of this document.

9.5 Results

This section of the study report presents the study results. Results may be presented in a summary table that lists the recovery or concentration of each sample, by test method, laboratory, and matrix.

For QC acceptance criteria-based comparison studies, results should indicate the QC test associated with each sample (e.g. IPR, method blank, MS/MSD, unspiked matrix sample). Raw data and example calculations should be submitted, and should be included in an appendix to the study report (see Section 9.10.1).

9.6 Data Analysis and Discussion

This section of the study report provides a statistical analysis and discussion of the study results. Recovery, precision, false positive rates, false negative rates, specificity, and sensitivity, as appropriate, should be calculated by test method, laboratory, and matrix, and summarized in a tabular format that includes the mean, standard deviation, and relative standard deviation. The discussion should address any discrepancies between the results and comparability guidelines, or, for QC acceptance criteria-based comparison studies, any discrepancies between the results and the QC acceptance criteria of the EPA-approved reference method.

9.7 Conclusions

This section of the study report should describe the conclusions drawn from the study based on the data analysis discussion. The section should contain a statement(s) regarding achievement of the study objective(s).

9.8 Appendix A - Method

The ATP or new method, prepared in accordance with EPA's Guidelines and Format document (Section 3.0 and Reference 10.14), should be appended to the study report.

9.9 Appendix B - Study Plan

The study plan approved by EPA (Section 4.0) should be appended to the study report.

9.10 Appendix C - Supporting Data

The study report should be accompanied by raw data, quality control information, and example calculations that support the summary results presented in the report.

9.10.1 Raw Data

Appendix C of the study report should include sufficient raw data so that an independent reviewer can verify each determination and calculation performed by the laboratory or the study coordinator. This verification consists of tracing all steps of the method to the final result reported. The raw data are, generally, method-specific and may include but are not limited to the following:

- Sample numbers or other identifiers used by the laboratory
- Sample collection dates and times
- Verification that method-specified QC procedures were met for test samples and all associated QC samples
- Analysis dates and times for all steps in the method
- Sample volume
- Any measurements of ancillary parameters (i.e., temperature, pH, turbidity, percent solids, etc.)
- Results for all intermediate steps in the method
- Preliminary data steps to determine the final result
- Final result

- If appropriate, quantitation reports, data system outputs, and other data to link the raw data to the results reported
- Laboratory bench sheets and copies of all pertinent logbook pages for all sample preparation and cleanup steps, and for all other parts of the determination
- Temperature logs for waterbaths, incubators, refrigerators, etc.
- Media preparation information
- If appropriate, direct instrument readouts and other data to support the final results

Raw data are generally needed for all samples, positive and negative controls, sterility checks, verifications, blanks, matrix spikes and duplicates, and other QC analyses specified in the EPA-approved reference method. Data should be organized so that a microbiologist can clearly understand how the analyses were performed. The names, titles, addresses, and telephone numbers of the analysts who performed the analyses and of the quality assurance officer who verified the analyses should be provided. For instruments involving data systems, raw data on magnetic tape or disk should be made available on request.

9.10.2 Electronic Data Reporting

In addition to the hard copy raw data, applicants should also submit data in electronic format (Excel spreadsheet, or equivalent) so that EPA can to create a database of study results. EPA anticipates that this database will facilitate automated review and statistical analysis of study results. The information included in electronic format may include: laboratory, analyst, method, sample type, sample number, date and time of analysis, volume analyzed, replicate number, raw data, and calculated results. The applicant should discuss an appropriate electronic format with EPA prior to data submission.

9.10.3 Example Calculations

Generally, the study report should provide example calculations that will allow the data reviewer to determine how the laboratory used the raw data to arrive at the final results. Useful examples include both detected analytes and undetected analytes. If the laboratory or the method employs a standardized reporting level for undetected analytes, this should be made clear in the example, as should adjustments for sample volume, etc.

9.11 Appendix D - Supporting References

Hard copies of all references and supporting documentation for the ATP or new method should be attached to the study report as an appendix. The list of references may contain links to web sites, or documents available on-line. However, a hard copy should be submitted with the final study report.

SECTION 10.0 REFERENCES

10.1 AOAC. 1999. Qualitative and Quantitative Microbiology Guidelines for Methods Validation, *Journal of AOAC International*, Vol. 82, No. 2.

10.2 APHA. 1998. *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. American Public Health Association. 1015 15th Street, NW, Washington, DC 20005.

10.3 APHA. 1995. *Standard Methods for the Examination of Water and Wastewater*. 19th Edition. American Public Health Association. 1015 15th Street, NW, Washington, DC 20005.

10.4 APHA. 1992. *Standard Methods for the Examination of Water and Wastewater*. 18th Edition. American Public Health Association. 1015 15th Street, NW, Washington, DC 20005.

10.5 ASTM. 1999. D4855-91: Standard Practice for Comparing Test Methods, ASTM Standards on Precision and Bias for Various Applications. 1999 Annual Book of ASTM Standards: Water and Environmental Technology, Volume 7.02. 100 Barr Harbor Drive, West Conshohocken, PA 19428.

10.6 ATCC. http://www.atcc.org

10.7 Fleiss, J. F. Statistical Methods for Rates and Proportions, 2nd ed., John Wiley & Sons, New York, NY.

10.8 ISO. 2001. CD17994. Water Quality - Criteria for the Establishment of Equivalence Between Microbiological Methods, Final Version, June 15, 2001.

10.9 National Environmental Laboratory Accreditation Conference. 2001. National Environmental Laboratory Accreditation Conference: Quality Systems. Approved May 25, 2001, effective July 1, 2003 unless otherwise noted. Appendix D - Essential Quality Control Requirements, section D.3, pp D-15 to D-16.

10.10 Title 40, Code of Federal Regulations, Sections 136.4, 136.5, and 141.27.

10.11 USEPA. 2001. EPA Requirements for Quality Management Plans. USEPA Office of Environmental Information. EPA/240/B-01/002.

10.12 USEPA. 1999. Environmental Regulations and Technology Control of Pathogens in Vector Attraction in Sewage Sludge. USEPA Office of Research and Development. EPA/625/R-92/013, revised October 1999.

10.13 USEPA. 1997. Manual for the Certification of Laboratories Analyzing Drinking Water: Criteria and Procedures Quality Assurance, Fourth Edition. USEPA Office of Ground Water and Drinking Water. EPA-815-B-97-001.

10.14 USEPA. 1996. Guidelines and Format for Methods to be Proposed at 40 CFR Part 136 or Part 141. USEPA Office of Science and Technology. EPA-821-B-96-003.

10.15 USEPA. 1995a. Presence/Absence Membrane Filter Methods for Finished Waters, USEPA Protocol for Alternate Test Procedures for Coliform Bacteria in Compliance with Drinking Water Regulations, Version 1.2, December 1995. USEPA Office of Research and Development, Cincinnati, OH.

10.16 USEPA. 1995b. Quantitative Membrane Filter Methods, USEPA Protocol for Alternate Test Procedures for Coliform Bacteria in Compliance with Water and Wastewater Regulations, Version 1.0, December 1995. USEPA Office of Research and Development. Cincinnati, OH.

10.17 USEPA. 1995c. Presence/Absence Liquid Culture Methods for Finished Waters, USEPA Protocol for Alternate Test Procedures for Coliform Bacteria in Compliance with Drinking Water Regulations, Version 1.2, December 1995. USEPA Office of Research and Development. Cincinnati, OH.

10.18 USEPA 1989. Memorandum: Analytical Methods for Compliance and Limited Alternate Test Procedures Approvals. December 27, 1989.

10.19 USEPA. 1979. *Handbook for Analytical Quality Control in Water and Wastewater Laboratories*. EPA-600/4-79-019. Environmental Monitoring and Support Laboratory, Cincinnati, OH. March 1979.

APPENDIX A
GLOSSARY

APPENDIX A: GLOSSARY

40 *CFR* part 136—Title 40, part 136 of the *Code of Federal Regulations*. This part specifies approved test procedures for the analysis of pollutants regulated under the Clean Water Act.

40 *CFR* part 141—Title 40, part 141 of the *Code of Federal Regulations*. This part specifies EPA's National Primary Drinking Water Regulations pursuant to the Safe Drinking Water Act; Subpart C of 40 *CFR* part 141 lists analytical methods required for monitoring under the Act.

95% confidence interval— A statistical level indicating a 95% probability that the parameter variable is enclosed within the given data interval.

A

Acceptable version— An acceptable version is a method that is either identical to the approved method or exercises the flexibility explicitly allowed in the method. See "minor modification."

Accuracy— The degree of agreement between an observed value and an accepted reference value. Accuracy includes random error (precision) and systematic error (recovery) that are caused by sampling and analysis.

Aliquot— A representative portion of a sample.

Ambient water— Ambient water refers to any fresh, marine, or estuarine surface water used for recreation; propagation of fish, shellfish, or wildlife; agriculture; industry; navigation; or as source water for drinking water facilities.

Analysis of variance (ANOVA)— A study of the effect of a set of qualitative variables on a quantitative response variable, based on a decomposition of the variance of the response variable.

Analyte— The target organism or class of organisms that are measured by the method.

Analyte of concern— An analyte designated by EPA to adversely affect or have the potential to adversely affect human health, the environment, aesthetics, or the senses. Analytes of concern are listed in approved methods.

Approved method— A testing procedure (analytical method) promulgated at 40 *CFR* parts 136, 141, 405-500, and other parts of the CFR that support EPA's water programs.

Average percent recovery— The average of the recovery, expressed as percent. See recovery.

B

Bias— A systematic or persistent distortion of a measurement process that deprives the result of representativeness; i.e., the expected sample measurement is different than the sample's true value. A data quality indicator. (QAMS)

Blank— See "method blank."

Bulk sample— A large sample that is aliquoted into smaller volumes prior to analyses.

C

Calibration— The process of establishing the relationship between the concentration or amount of material introduced into an instrument or measurement process and the output signal.

Calibration verification— Means of establishing that the instrument performance remains within pre-established limits.

***Code of Federal Regulations*— A codification of the general and permanent rules published in the *Federal Register* by the Executive departments and agencies of the Federal Government.

Comparability test— See side-by-side comparison.

Confidence interval— The numerical interval constructed around a point estimated of a population parameter, combined with a probability statement (the confidence coefficient) linking it to the population's true parameter value. If the same confidence interval construction technique and assumptions are used to calculate future intervals, they will include the unknown population parameter with the same specified probability. (EMMC)

Confirmed counts— Organism counts that have been verified to ensure proper identification.

Conover Squared-Rank test— A nonparametric test for equality of variability, based on the joint squared ranks of deviations from the means. (SPRENT)

Contract laboratory— Private, academic, or commercial laboratory under contract to EPA or other organization to perform testing.

D

D'Agostino test— A statistical test for determining whether a given set of results follow a normal or log-normal distribution. Best used for datasets with at least 50 results. (GILBERT)

Data quality objective— Qualitative and/or quantitative statement of the overall level of uncertainty that a decision-maker is willing to accept in results or decisions derived from environmental data. Data quality objectives provide the statistical framework for planning and managing environmental data operations consistent with the data user's needs. (EMMC)

Determinative technique— The physical and/or chemical process by which measurement of the identity and concentration of an analyte is made.

Differential medium— A solid culture medium that makes it easier to distinguish colonies of the target organism.

Dilution/rinse water blank— An aliquot of dilution/rinse water that is treated exactly like a sample and carried through all portions of the procedure until determined to be negative or positive. The dilution/rinse water blank is used to determine if the sample has become contaminated by the introduction of a foreign microorganism through poor technique.

Discharge—Generally, any spilling, leaking, pumping, pouring, emitting, emptying or dumping (40 *CFR* 109.2; 110.1; 116.3); also, see "discharge of a pollutant" (40 *CFR* 122.2); the medium that is spilled, leaked, pumped, poured, emitted, emptied, or dumped.

Discharge of pollutant— Any addition of any pollutant or combination of pollutants to (1) waters of the U.S. from any point source or (2) to the waters of the contiguous zone or the ocean from any point source other than a vessel or other floating craft which is being used as a means of transportation (40 *CFR* 122.2; 401.11)

Duplicate— A second sample collected from the same sampling point at the same time the original sample is collected and analyzed exactly like the original sample. Duplicate samples can be used as a measure of sample variability.

E

Effluent— A medium that flows out of a point source, e.g., the discharge from a sewage treatment plant.

Enrichment— Using a culture medium to enhance growth of the target organism prior to isolation of that organism.

Explicit flexibility— Modifications that are explicitly allowed in an approved method.

F

F distribution— A type of sampling distribution for a random variable. The ratio of 2 chi-square distributions, each divided by their respective degrees of freedom.

F-test— In an Analysis of Variance, a test for the equality of factor level means (such as for different methods) or for the presence of an interaction between two factors (such as method and matrix). (RICE) (ASTM)

Facility— A plant or group of plants within a single location that is regulated under the CWA and/or SDWA. A single facility may have multiple water supplies, discharges, waste streams, or other environmental media that are subject to compliance monitoring. For example, a single facility within the Pulp, Paper, and Paperboard industrial category may have a direct discharge, an indirect discharge, and an in-process waste stream, all of which are subject to compliance monitoring.

False negative— A target organism incorrectly identified as a non-target organism or not identified at all using the method of interest.

False positive— A non-target organism incorrectly identified as the target organism using the method of interest.

False negative error rate— The proportion of target organisms incorrectly identified as a non-target organism or not identified at all using the method of interest, equal to (1 - Sensitivity). In statistical testing, the rate at which one falsely accepts a statistical hypothesis (such as that a difference between methods does not exist) based on a statistical test, when the hypothesis is actually false. Abbreviated as β, and also referred to as the Type II error rate. (ASTM)

False positive error rate— The proportion of non-target organisms incorrectly identified as the target organism using the method of interest, equal to (1 - Specificity). In statistical testing, the rate at which one falsely rejects a statistical hypothesis (such as that a difference between methods does not exist) based on a statistical test, when the hypothesis is actually true. Abbreviated as α, and also referred to as the Type I error rate. (ASTM)

***Federal Register*—** A daily publication that provides a uniform system for publishing Presidential and Federal agency documents. Documents published in the *Federal Register* make changes to the CFR to keep the CFR current. (OFR)

G

Guidelines and Format— The document titled *Guidelines and Format for Methods to be Proposed at 40 CFR Parts 136 and 141*; available from the National Technical Information Service (NTIS), U.S. Department of Commerce, Springfield, Virginia, 22161 (703-487-4600) as NTIS publication PB96-210448.

H

Histogram— A bar diagram of the distribution of a set of analytical results. The range of values is categorized into sets of subintervals, or bins, and the number of results within each bin are displayed as the height of the bars. (BERRY)

I

Industrial category— A category listed in 40 *CFR* parts 405-503.

Industrial subcategory— A subcategory defined at 40 *CFR* parts 405-503.

Initial demonstration of capability— A test performed to establish the ability to demonstrate control over the analytical system and to demonstrate acceptable performance.

Initial precision and recovery— The analysis of a minimum of four spiked reagent water samples under the same conditions as will be used for analysis of environmental samples. The IPR is used to demonstrate that a laboratory is able to produce reliable results with the method prior to analysis of environmental samples.

Interaction— The situation where the effect of one variable (such as method type) on a dependent variable (such as recovery) is affected by the value of a third variable (such as matrix).

Interference— A positive or negative effect on a measurement caused by a substance other than the one being investigated. (QAD)

Interlaboratory— Occurring in multiple laboratories.

Intralaboratory— Occurring within a single laboratory.

J
K

Kolmogorov-Smirnov test— A statistical test for determining whether a given set of results follow a normal distribution, or any other specified distribution. (GILBERT)

L

Limited use— Use of a method by a single regulated entity or laboratory for analysis of one or more matrix types.

Log-normal— A distribution of a random variable X such that the natural logarithm of X is normally distributed.

Log-phase— Bacterial growth phase in which the logarithm of the bacterial biomass increases linearly with time.

M

Main effect— Situation where a variable (such as method type) has a consistent effect on a dependent variable (such as recovery).

Matrix— The component or substrate that contains the analytes of interest.

Matrix effect— Variability in the analytical performance of a method that can be attributed to the type of sample analyzed.

Matrix spike— A sample prepared by adding a known mass of target analyte to a specified amount of a sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used, for example, to determine the effect of the matrix on a method's recovery efficiency. (QAMS)

Matrix spike duplicate— A replicate of the matrix spike to test precision. The MS/MSD are used in combination to test the precision of an analysis. (QAD)

Matrix type— A sample medium with common characteristics across a given industrial category or subcategory. For example, C-stage effluents from chlorine bleach mills, effluent from the continuous casting subcategory of the iron and steel industrial category, POTW sludge, and in-process streams in the Atlantic and Gulf Coast Hand-shucked Oyster Processing subcategory are each a matrix type. For the purposes of this initiative all drinking waters constitute a single matrix type.

May— This action, activity, or procedural step is neither required nor prohibited.

May not— This action, activity, or procedural step is prohibited.

Measurement quality— Critical level which, if exceeded, is considered to append objective additional, and possibly unacceptable, measurement uncertainty to the corresponding data.

Method— A body of procedures and techniques for performing a task (e.g. sampling, characterization, quantitation) systematically presented in the order in which they are to be executed. (QAD)

Method blank— An aliquot of reagent water or designated matrix that is treated exactly as a sample, including exposure to all glassware, equipment, solvents, and procedures that are used with samples. The method blank is used to determine if analytes or interferences are present in the laboratory environment, the reagents, or the apparatus.

Method-defined analyte— An analyte without a specific, known composition where the analytical result depends totally on the measurement procedure.

Method modification— A change made to an approved method.

Method validation— A process by which a laboratory or vendor establishes the performance of a new method or substantiates the performance of a method modification.

Methods and Criteria— The document titled: *Analysis of Pollutants in Municipal Water and Industrial Wastewater: Test Procedures and Quality Control Acceptance Criteria*; available from the National Technical Information Service (NTIS), U.S. Department of Commerce, Springfield, Virginia, 22161 (703-487-4600) as NTIS publication PB96-210463, and incorporated by reference into this part.

Mid-point response factor— The response factor at the concentration at which calibration is verified.

Minor modification— A modified method that has been reviewed by EPA and has been determined to be technically equivalent to a method approved for use in compliance monitoring. A minor modification employs the same chemistry and/or biological principles as the approved method to determine the presence/absence or to quantify the amount of the target organism in a sample. Supporting data may be necessary to demonstrate that a minor modification will yield results equivalent to those obtained using the approved method but does not require approval as an alternate test procedure through proposal and promulgation in the Federal Register.

Modified method— An approved method that has been modified to change a front-end technique. EPA will judge a modified method to be: 1) an acceptable version or minor modification of a previously promulgated method, which does not require approval as an ATP or 2) a significantly different method which requires an application for an ATP approval.

N

Nationwide use— Use of a method by all regulated entities and laboratories for analysis of one or more matrix types.

Navigable waters— All waters of the United States, including the territorial seas. (40 *CFR* 110.1)

Negative control— A non-target organism processed to ensure the laboratories are familiar with the identification of the target organism and to ensure that confirmation test results are appropriate.

New method— A method that employs a determinative technique for an analyte of concern that differs from determinative techniques employed for that analyte in methods previously approved at 40 *CFR* part 136 or 141.

Nonparametric— A type of statistical analysis for which no assumptions about the underlying distribution of the data are necessary.

Non-selective media— An enrichment media that allows most bacteria to grow.

Normal probability plot— A graphical depiction of the distribution of a set of analytical results. A normal probability plot is a scatterplot depicting the observed results compared to the expected results based on a normal distribution. If the observed data follow a normal distribution, the graph will display a line at a 45 degree angle from the x-axis. Also known as a Q-Q plot.

O

Ongoing demonstration of capability— The laboratory needs to demonstrate that the analytical system is in control on an ongoing basis through the analysis of ODC samples (positive control/positive control duplicate).

Ongoing precision and recovery— A reagent water sample method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified within the method for precision and recovery.

Other approved methods— Promulgated methods that are not designated as a reference method, but continue to carry the same regulatory status.

P

Physical phase— The physical phase of a sample matrix (e.g., air, water, soil).

Positive control— A target organism that is analyzed to ensure that the laboratory is performing the method acceptably and that the media is providing appropriate results.

Power— The probability that a statistical test will conclude that a difference (for example, between methods) exists, when a difference truly does exist. Equal to 1 - β.

Precision— The degree to which a set of observations or measurements of the same property, usually obtained under similar conditions, conform to themselves; a data quality indicator. Precision is usually expressed as standard deviation, variance, or range, in either absolute or relative terms.

The precision obtainable from an environmental measurement method may be estimated from replicate analyses of subsamples taken from the same (homogenous) sample. Generally speaking, the more carefully one executes the various steps of a method and controls the variables affecting the method's capability, the more precise will be the results. The use of a nonhomogeneous sample will compound the precision estimate with the sample variability.

Preparation— Processing performed on a sample prior to analysis, e.g. extraction, concentration, cleanup, etc.

Presumptive counts— Numbers of organisms based on results that have not been confirmed or verified.

Procedures— A set of systematic instructions for performing an activity. (QAD)

Promulgated method— A method that has been published or incorporated by reference into 40 *CFR* parts 136, 141, 405-500, or other parts that support EPA's water programs (i.e., an approved method).

Promulgation— Publication of a final rule in the FR.

Public water system (PWS)— A system for the provision to the public of piped water for human consumption, if such system has at least fifteen service connections or regularly serves an average of at least twenty-five individuals daily at least 60 days out of the year. Such term includes (1) any collection, treatment, storage, and distribution facilities under control of the operator of such system and used primarily in connection with such system, **and (2) any** collection or pretreatment storage facilities not under such control which are used primarily in connection with such system. A public water system is either a "community water system" or a "noncommunity water system."

Q

Quality assurance — An integrated system of activities involving planning, quality control, quality assessment, reporting, and quality improvement to ensure that a product or service meets defined standards of quality with a stated level of confidence. (QAD)

Quality control — The overall system of technical activities whose purpose is to measure and control the quality of a product or service so that it meets the needs of users. The aim is to provide quality that is satisfactory, adequate, dependable, and economical. (QAD)

QC acceptance criteria— Performance specifications developed from validation data and used to control the limits within which an analytical method is operated.

QC acceptance criteria-based comparison study— A study performed to evaluate the performance of a modified method against the quality control acceptance criteria of a reference method.

R

Range finding— Preliminary analyses conducted to assess the ambient concentration of the target organism in a matrix to be used in a study or preliminary analyses of spiked samples involving few, if any, replicates, to assess method performance to identify the spike dose to be used in a study.

Raw data— Data that have not been processed.

Reagent water— Water conforming to Specification D 1193, Annual Book of ASTM Standards, or specifications in Standard Methods 9020B.4.d.

Recovery— The total amount of the analyte found in the sample divided by the amount of the analyte added into the sample as a spike.

Reference method— A method that serves as a standard against which method modifications can be compared.

Regulated entity— Permittees, PWSs, POTWs, and other entities responsible for compliance with provisions of the CWA or SDWA.

Relative percent difference (RPD)— An estimate of the variability of two numbers expressed in relative terms. Calculated as the absolute value of the difference of the two numbers, divided by their mean:

$$RPD = \frac{|A - B|}{\frac{1}{2} * (A + B)} * 100\%$$

Equal to the relative standard deviation of the two numbers multiplied by the square root of 2.

Relative standard deviation (RSD)— The standard deviation expressed as a percentage of the mean (100σ/X); i.e., the coefficient of variation.

Replicate— Multiple samples collected from the same sampling point at the same time and analyzed exactly the same way. Replicate samples can be used as a measure of sample variability.

S

Sample matrix— See "matrix."

Sample preparation technique— Any technique in the analytical process that precedes the determinative technique, including all procedures, equipment, solvents, etc. that are used in the preparation and cleanup of a sample for analysis. Sample preparation techniques do not include conditions and/or procedures for the collection, preservation, shipment, and storage of the sample.

Sensitivity— In presence/absence testing, sensitivity is the proportion of target organisms in the sample that were correctly detected by the method of interest.

Shapiro-Wilk test— A statistical test for determining whether a given set of results follow a normal or log-normal distribution. Best used for datasets with at most 50 results. (GILBERT)

Side-by-side comparison— Parallel testing of a new or modified method and a reference method to determine whether the performance of the new or modified method is acceptable compared to the reference method.

Specificity— In presence/absence testing, the proportion of non-target organisms in the sample that were correctly identified as not being the target organism by the method of interest.

Spike— The process of adding a known amount of target analyte to a sample; used to determine the recovery efficiency of the method. (QAD)

Spiking suspension— Diluted stock suspension containing the organism(s) of interest at a concentration appropriate for spiking samples.

Standard deviation (σ)— The measure of the dispersion of observed values expressed as the positive square root of the sum of the squares of the difference between the individual values of a set and the arithmetic mean of the set, divided by one less than the number of values in the set. For a total of n numbers:

$$SD = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (X_i - Mean)^2}$$

Statistical power— See power.

Stem-and-leaf plot— A graphical depiction of a set of analytical results, that conveys information about the shape of the distribution while retaining the numerical information. The stem-and-leaf plot separates the digits of the values as leaves (the last digit of the values) and stems (the remaining digit of the values). The individual results are grouped according to the stems, and the leaves are listed separately in a format similar to a histogram. (RICE)

Stock suspension— A concentrated suspension containing the organism(s) of interest that is obtained from a source that will attest to the host source, purity, authenticity, and viability of the organisms.

Study plan— A study design submitted for EPA review, comment, and approval prior to conducting the side-by-side or QC acceptance criteria-based method comparability study. This process protects the applicant by providing written approval of the study design before resources are spent to conduct the study. Data from studies conducted without EPA review and approval may not adequately address the applicant's study objectives. A detailed procedure for the ATP or new method should be included as an attachment to the study plan. EPA will evaluate the study plan to verify that the appropriate data quality objectives identified in this protocol are defined and addressed. EPA comments are incorporated into the study design and this review process is repeated until EPA has approved the study design.

Study report— A formal report developed by laboratories or other organizations responsible for developing ATPs or new methods documenting the results of the side-by-side or QC acceptance criteria-based method comparability study. The information and supporting data needed in the study report should be sufficient to enable EPA to evaluate the performance of the ATP or new method and make a decision on whether it is comparable or superior to the reference method. The approved study report should also identify and discuss any deviations from the study plan that were made in implementing the study, and the approved study plan should be appended to and referenced in the study report.

Summary results— Overall study statistics (not sample-specific results).

T

Target organism— The organism the method is designed to detect.

U V

Validate— Reliably assess the performance (bias and precision) of a method in a reference matrix (such as reagent water) and the matrix in which the validated method will be used (such as drinking water, surface water, or municipal wastewater effluent).

Validation, single-laboratory— Assessment of method performance (see "validate") in one laboratory.

Validation, interlaboratory— Assessment of method performance (see "validate") at multiple laboratories.

Variance— A measure of the dispersion of a set of values. The sum of the squares of the difference between the individual values of a set and the arithmetic mean of the set, divided by one less than the number of values in the set. (The square of the sample standard deviation) (QAD)

W

Wilcoxon-Mann-Whitney (WMW) test— A nonparametric analysis used to determine whether the medians from two levels of a given factor (such as method) differ from each other, based on the ranks of all results. (SPRENT)

X
Y
Z

The above definitions are referenced to the following organizations:

ASTM	ASTM D 4855-91. Standard Practice for Comparing Test Methods.
BERRY	Berry, D. A. and B. Lindgren. Statistics Theory and Methods. Wardsworth, Belmonst, CA, 1990.
EMMC	Environmental Monitoring Management Council
GILBERT	Gilbert, R. O. Statistical Methods for Environmental Pollution Monitoring. Van Nostrand Reinhold, New York, 1987.
NELAC QS	National Environmental Laboratory Accreditation Conference, Quality Systems
OFR	Office of Federal Register
QAD	Quality Assurance Division, National Center for Environmental Research and Quality Assurance, Office of Research and Development, USEPA
RICE	Rice, J. A. Mathematical Statistics and Data Analysis, Second Edition. Wadsworth, Belmont, CA, 1995.
SPRENT	Sprent, P. Applied Nonparametric Statistical Methods, Second Edition. Chapman & Hall, London, 1993.

APPENDIX B
ATP APPLICATION FORM

EPA Office of Water Alternate Test Procedure or New Method Preliminary Application Form for Microbiological Analytes		
Applicant Name and Address:		***EPA Use Only*** **ATP Case Number:**
Date Application Submitted:		
Type of Application: *Circle appropriate application type*	Alternate Test Procedure	New Method
Method Number **Title of Method** **Revision Date**		
EPA-Approved Reference Method:		
Analyte(s):		
Applicable Matrices: *Circle all that apply*	Ambient Water Biosolids Drinking Water Wastewater	
Level of Use:	Limited Use	Nationwide
Study Design: *Circle appropriate study design*	Side-by-Side Comparison Study	QC Acceptance Criteria-Based Comparison Study
FOR LIMITED USE APPLICATIONS ONLY		
ID Number of Existing or Pending Permit:		
Issuing Agency:		
Type of Permit:		
Discharge Serial Number:		
Attachments *Check all that apply*		
☐ **Justification for ATP** ☐ **Alternate Test Procedure or New Method (in standard EPA format)** ☐ **Method Comparison Table** ☐ **Study Plan** ☐ **Other:** _____		
Submit Application and Attachments in Triplicate		

EPA Office of Water Alternate Test Procedure or New Method Final Application Form for Microbiological Analytes		
Applicant Name and Address:		***EPA Use Only*** **ATP Case Number:**
Date Application Submitted:		
Type of Application: *Circle appropriate application type*	Alternate Test Procedure	New Method
Method Number **Title of Method** **Revision Date**		
EPA-Approved Reference Method:		
Analyte(s):		
Applicable Matrices: *Circle all that apply*	Ambient Water Biosolids Drinking Water Wastewater	
Level of Use:	Limited Use	Nationwide
Study Design: *Circle appropriate study design*	Side-by-Side Comparison Study	QC Acceptance Criteria-Based Comparison Study
FOR LIMITED USE APPLICATIONS ONLY		
ID Number of Existing or Pending Permit:		
Issuing Agency:		
Type of Permit:		
Discharge Serial Number:		
Attachments *Check all that apply*		
☐ **Justification for ATP** ☐ **Alternate Test Procedure or New Method (in standard EPA format)** ☐ **Method Comparison Table** ☐ **Study Plan** ☐ **Study Report** ☐ **Other:** _____		
Submit Application and Attachments in Triplicate		

APPENDIX C
APPLICATION INVENTORY FORM

1. Completed application form.		
Includes the name and address of the applicant; the date of submission of the application; the method number, title, and revision date; the EPA-approved reference method; the analyte(s) for which the ATP or new method is proposed; the type of application; applicable matrices; study design; level of use; NPDES permit information, if applicable; and the attachments submitted with the application.	Section 2.2 Appendix C	☐
2. Justification for ATP or new method.		
Brief justification for why the ATP or new method is being proposed.	Section 2.3	☐
3. Method in EPA format.		
Scope and application	Section 3.1	☐
Summary of method	Section 3.2	☐
Definitions of method	Section 3.3	☐
Interferences	Section 3.4	☐
Safety	Section 3.5	☐
Equipment and supplies	Section 3.6	☐
Reagents and standards	Section 3.7	☐
Sample collection, preservation, and storage	Section 3.8	☐
Quality control	Section 3.9	☐
Calibration and standardization	Section 3.10	☐
Procedure	Section 3.11	☐
Data analysis and calculations	Section 3.12	☐
Method performance	Section 3.13	☐
Pollution prevention	Section 3.14	☐
Waste management	Section 3.15	☐
References	Section 3.16	☐
Tables, diagrams, flowcharts, and validation data	Section 3.17	☐
4. Method comparison table.		
A two-column table comparing the proposed ATP or new method with the EPA-approved reference method. This table should include the number and title of each method, the latest revision date of the ATP or new method, and a detailed discussion of each of the method sections listed in Section 3.0. Each topic should be discussed in a separate row of the table and the applicant should highlight any differences between the ATP or new method and the EPA-approved reference method.	Section 2.5	☐

5. Study plan.		
Background	Section 4.1	☐
Objectives	Section 4.2	☐
Study design	Section 4.3	☐
Coordination	Section 4.4	☐
Data reporting	Section 4.5	☐
6. Study report.		
Background	Section 9.1	☐
Study objectives and design	Section 9.2	☐
Study implementation	Section 9.3	☐
Data reporting and validation	Section 9.4	☐
Results	Section 9.5	☐
Data analysis and discussion	Section 9.6	☐
Conclusions	Section 9.7	☐
Appendix A: Method	Section 9.8	☐
Appendix B: Approved study plan	Section 9.9	☐
Appendix C: Supporting data	Section 9.10	☐
Appendix D: Supporting references	Section 9.11	☐

APPENDIX D
DATA ELEMENTS AND EXAMPLE BENCH SHEETS

Data Elements

The data elements listed below should be reported on the bench sheets or in the lab notebook for each method, as applicable. EPA will review the information during the data validation process to ensure the method-specific QC measures are met, as agreed to in the approved study plan.

- Laboratory name
- Method number
- Media
- Procedure
- Matrix
- Sample collection date/time
- Dates and times for all method steps associated with holding times or incubation times
- Analyst initials for each processing step in the method
- Presumptive results for all applicable media
- Confirmed/completed results for all applicable media
- All measured volumes
- Dilution information
- Final result per units of measurement

Example Benchsheets

Example bench sheets for the following EPA-approved reference methods are included in this appendix:

- *Aeromonas* (USEPA Method 1605)
- *Cryptosporidium* (USEPA Methods 1622 and 1623)
- *E. coli* (SM 9221F, SM 9222G)
- Enterococci (SM 9230C)
- Fecal coliforms (SM 9221E, 9222D)
- Fecal streptococcus (SM 9230B, SM 9230C)
- *Giardia* (USEPA Method 1623)
- Total coliforms (SM 9221B, SM 9222B)

Note: Additional example bench sheets or electronic copies of the attached bench sheets are available upon request.

Multiple-Tube Fermentation: Total Coliform, Fecal Coliform, *E. coli*
(SM 9221B, SM 9221E, SM 9221F)

Laboratory: _____ **Sample Collection Time:** _____

Sample Collection Date: _____ **Sampling Point:** _____

LTB: Replicate 1

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr / 48 hr LTB read							

Please enter date and time for the following:

LTB start

LTB 24 hr read

LTB 48 hr read,
BGB 24 hr read (from 24 hr LTB),
ECMUG 24 hr read (from 24 hr LTB)

BGB 48 hr read (from 24 hr LTB),
BGB 24 hr read (from 48 hr LTB),
ECMUG 24 hr read (from 48 hr LTB)

BGB 48 hr read (from 48 hr LTB)

LTB: Replicate 2

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr / 48 hr LTB read							

LTB: Replicate 3

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr / 48 hr LTB read							

Comments:

BGB: Replicate 1

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr / 48 hr BGB read (From 24 hr LTB)							
24 hr / 48 hr BGB read (From 48 hr LTB)							

Final tube combination:

Total coliforms/100 mL:

BGB: Replicate 2

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr / 48 hr BGB read (From 24 hr LTB)							
24 hr / 48 hr BGB read (From 48 hr LTB)							

Final tube combination:

Total coliforms/100 mL:

BGB: Replicate 3

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr / 48 hr BGB read (From 24 hr LTB)							
24 hr / 48 hr BGB read (From 48 hr LTB)							

Final tube combination:

Total coliforms/100 mL:

EC-MUG: Replicate 1

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr fecal read (From 24 hr LTB)							
24 hr fecal read (From 48 hr LTB)							
24 hr *E. coli* read (From 24 hr LTB)							
24 hr *E. coli* read (From 48 hr LTB)							

Fecal tube combination: Fecal/100 mL:

E. coli tube combination: *E. coli*/100 mL:

EC-MUG: Replicate 2

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr fecal read (From 24 hr LTB)							
24 hr fecal read (From 48 hr LTB)							
24 hr *E. coli* read (From 24 hr LTB)							
24 hr *E. coli* read (From 48 hr LTB)							

Fecal tube combination: Fecal/100 mL:

E. coli tube combination: *E. coli*/100 mL:

EC-MUG: Replicate 3

	Analyst Initials	Read Temp	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr fecal read (From 24 hr LTB)							
24 hr fecal read (From 48 hr LTB)							
24 hr *E. coli* read (From 24 hr LTB)							
24 hr *E. coli* read (From 48 hr LTB)							

Fecal tube combination: Fecal/100 mL:

E. coli tube combination: *E. coli*/100 mL:

Membrane Filtration: mEndo/NA-MUG
(SM 9222B/SM 9222G)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

mEndo incubation start temperature (°C):	mEndo incubation start date/time:
mEndo incubation end temperature (°C):	mEndo incubation end date/time:
NA-MUG incubation start temperature (°C):	NA-MUG incubation start date/time:
NA-MUG incubation end temperature (°C):	NA-MUG incubation end date/time:

Replicate number 1

		Total coliforms		*E. coli*	
Analyst initials	Sample volume filtered	No. colonies per filter	Total Coliforms per 100 mL	No. colonies per filter	*E. coli* per 100 mL
	100 mL				
	10.0 mL				
	1.0 mL				
	0.1 mL				

Replicate number 2

		Total coliforms		*E. coli*	
Analyst initials	Sample volume filtered	No. colonies per filter	Total Coliforms per 100 mL	No. colonies per filter	*E. coli* per 100 mL
	100 mL				
	10.0 mL				
	1.0 mL				
	0.1 mL				

Replicate number 3

		Total coliforms		*E. coli*	
Analyst initials	Sample volume filtered	No. colonies per filter	Total Coliforms per 100 mL	No. colonies per filter	*E. coli* per 100 mL
	100 mL				
	10.0 mL				
	1.0 mL				
	0.1 mL				

Membrane Filtration: mFC/NA-MUG
(SM 9222D/SM 9222G)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

mFC incubation start temperature (°C):	mFC incubation start date/time:
mFC incubation end temperature (°C):	mFC incubation end date/time:
NA-MUG incubation start temperature (°C):	NA-MUG incubation start date/time:
NA-MUG incubation end temperature (°C):	NA-MUG incubation end date/time:

Replicate number 1

		Fecal coliforms		*E. coli*	
Analyst initials	Sample volume filtered	No. colonies per filter	Fecal coliforms per 100 mL	No. colonies per filter	*E. coli* per 100 mL
	100 mL				
	10.0 mL				
	1.0 mL				
	0.1 mL				

Replicate number 2

		Fecal coliforms		*E. coli*	
Analyst initials	Sample volume filtered	No. colonies per filter	Fecal coliforms per 100 mL	No. colonies per filter	*E. coli* per 100 mL
	100 mL				
	10.0 mL				
	1.0 mL				
	0.1 mL				

Replicate number 3

		Fecal coliforms		*E. coli*	
Analyst initials	Sample volume filtered	No. colonies per filter	Fecal coliforms per 100 mL	No. colonies per filter	*E. coli* per 100 mL
	100 mL				
	10.0 mL				
	1.0 mL				
	0.1 mL				

E. coli Membrane Filtration: mTEC
(EPA 1103.1, SM 9213D)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

mTEC incubation start temperature (°C):	mTEC incubation start date/time:
mTEC incubation end temperature (°C):	mTEC incubation end date/time:
Urease substrate incubation start date/time:	Urease substrate incubation end date/time:

Replicate number 1

		E. coli	
Analyst initials	Sample volume filtered	No. colonies per filter	*E. coli* per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 2

		E. coli	
Analyst initials	Sample volume filtered	No. colonies per filter	*E. coli* per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 3

		E. coli	
Analyst initials	Sample volume filtered	No. colonies per filter	*E. coli* per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

E. coli Membrane Filtration: Modified mTEC (EPA 1603)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

modified mTEC incubation start temperature (°C):	modified mTEC incubation start date/time:
modified mTEC incubation end temperature (°C):	modified mTEC incubation end date/time:

Replicate number 1

		E. coli	
Analyst initials	Sample Volume filtered	No. colonies per filter	*E.coli* per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 2

		E. coli	
Analyst initials	Sample Volume filtered	No. colonies per filter	*E.coli* per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 3

		E. coli	
Analyst initials	Sample Volume filtered	No. colonies per filter	*E.coli* per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Multiple-Tube Fermentation: Fecal streptococcus
(SM 9230B)

Laboratory: _____ **Sample Collection time:** _____

Sample Collection Date: _____ **Sampling point:** _____

Replicate Number 1

Azide Dextrose Broth (ADB)							BEA Plates							
	Analyst Initials	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL		Analyst Initials	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr ADB read							24 hr BEA read from 24 hr ADB						
48 hr ADB read							24 hr BEA read from 48 hr ADB						
Comments:							Final tube combination:		Fecal streptococcus/100 mL:				

Replicate Number 2

Azide Dextrose Broth (ADB)							BEA Plates							
	Analyst Initials	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL		Analyst Initials	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr ADB read							24 hr BEA read from 24 hr ADB						
48 hr ADB read							24 hr BEA read from 48 hr ADB						
Comments:							Final tube combination:		Fecal streptococcus/100 mL:				

Replicate Number 3

Azide Dextrose Broth (ADB)							BEA Plates							
	Analyst Initials	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL		Analyst Initials	10 mL	1.0 mL	0.1 mL	0.01 mL	0.001 mL
24 hr ADB read							24 hr BEA read from 24 hr ADB						
48 hr ADB read							24 hr BEA read from 48 hr ADB						
Comments:							Final tube combination:		Fecal streptococcus/100 mL:				

ADB incubation start date/time:

Start temp:

ADB incubation end date/time:

End temp:

BEA incubation start date/time:
(From 24 hr ADB)

Start temp:

BEA incubation end date/time:
(From 24 hr ADB)

End temp:

BEA incubation start date/time:
(From 48 hr ADB)

Start temp:

BEA incubation end date/time:
(From 48 hr ADB)

End temp:

Membrane Filtration: Fecal Streptococcus
(SM 9230C)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

mEnterococcus incubation start temperature (^{o}C):	mEnterococcus incubation start date/time:
mEnterococcus incubation end temperature (^{o}C):	mEnterococcus incubation end date/time:

Replicate number 1

		Fecal streptococcus	
Analyst initials	Sample volume filtered	No. colonies per filter	Fecal streptococcus per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 2

		Fecal streptococcus	
Analyst initials	Sample volume filtered	No. colonies per filter	Fecal streptococcus per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 3

		Fecal streptococcus	
Analyst initials	Sample volume filtered	No. colonies per filter	Fecal streptococcus per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Membrane Filtration: Enterococcus (mE-EIA)
(EPA 1106.1, SM 9230C)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

mE incubation start temperature (°C):	mE incubation start date/time:
mE incubation end temperature (°C):	mE incubation end date/time:
EIA incubation start temperature (°C):	EIA incubation start date/time:
EIA incubation end temperature (°C):	EIA incubation end date/time:

Replicate number 1

		Enterococcus	
Analyst initials	Volume filtered	No. colonies per filter	Enterococci per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 2

		Enterococcus	
Analyst initials	Volume filtered	No. colonies per filter	Enterococci per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 3

		Enterococcus	
Analyst initials	Volume filtered	No. colonies per filter	Enterococci per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Membrane Filtration: Enterococcus (mEI)
(EPA 1600)

Laboratory: _____ **Sample collection time:** _____

Sample collection date: _____ **Sampling point:** _____

mEI incubation start temperature (°C):	mEI incubation start date/time:
mEI incubation end temperature (°C):	mEI incubation end date/time:

Replicate number 1

		Enterococcus	
Analyst initials	Volume filtered	No. colonies per filter	Enterococcus per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 2

		Enterococcus	
Analyst initials	Volume filtered	No. colonies per filter	Enterococcus per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Replicate number 3

		Enterococcus	
Analyst initials	Volume filtered	No. colonies per filter	Enterococcus per 100 mL
	100 mL		
	10.0 mL		
	1.0 mL		
	0.1 mL		

Batch-specific Cover Sheet:
Method 1605 (Aeromonas) - ADA with Vancomycin

Note: *Please complete one sheet per week of analysis.*

Laboratory name: _____

Section 1. Media Preparation Information

1	Kovac's expiration date	
2	Oxidase dry slides expiration date	
3	Date of nutrient agar plate preparation	
4	Date of nutrient agar slant preparation	
5	Date nutrient agar slants for positive controls and matrix spikes were inoculated	
6	Time nutrient agar slants for positive controls and matrix spikes were inoculated	

Section 2: Sample Processing Information

7	Dilution preparation date (for IDC, ODC, and MS/MSD samples)	
8	Dilution preparation time (for IDC, ODC, and MS/MSD samples)	
9	Analyst preparing dilutions for IDC, ODC, and MS/MSD samples)	
10	Sample spiking date	
11	Sample spiking time	
12	Analyst spiking samples	
13	Analyst performing filtration	
14	Funnel decontamination method	

Section 3: Sample Analysis Information

15	Incubator temperature at start date	°C	
16	Incubator temperature at read date	°C	
16	ADA-V incubation start date / time		
17	ADA-V read date / time		
18	ADA-V read analyst		
19	Nutrient agar plate incubation start date / time		
20	Nutrient agar plate read date / time		
21	Nutrient agar plate read analyst		
22	Oxidase confirmation date / time		
23	Oxidase read analyst		
24	Trehalose incubation start date / time		
25	Trehalose read date / time		
26	Trehalose read analyst		
27	Tryptone (indole) incubation start date / time		
28	Tryptone (indole) read date / time		
29	Tryptone (indole) read analyst		

QC Checklist:
Method 1605 (Aeromonas) - ADA with Vanomycin

Note: *Please complete one sheet per week of analysis. Please circle the appropriate response and provide supporting information as necessary.*

Laboratory name: _____

Batch identification: _____

1	Did all method blanks (dilution/rinse water) exhibit the appropriate response?	Yes	No
	If no, please list contaminated method blank(s) and associated samples.		
2	Did ADA-V media sterility check exhibit the appropriate response?	Yes	No
	If no, please explain.		
3	Did nutrient agar **plate** media sterility check exhibit the appropriate response?	Yes	No
	If no, please explain.		
4	Did nutrient agar **slant** media sterility check exhibit the appropriate response?	Yes	No
	If no, please explain.		
5	Did trehalose media sterility check exhibit the appropriate response?	Yes	No
	If no, please explain.		
6	Did tryptone media sterility check exhibit the appropriate response?	Yes	No
	If no, please explain.		
7	Did the unspiked reagent water sample exhibit the appropriate response?	Yes	No
	If no, please explain.		
8	Did negative controls for oxidase, trehalose, and indoleexhibit the appropriate responses?	Yes	No
	If no, please explain.		

Sample-specific Data Report Form:
Method 1605 (Aeromonas) - ADA With Vanomycin

Laboratory name: _____

Section 1: Sample information

1	Sample number:	5	Volume (mL) of spike (QC samples only):
2	Utility:	6	Volume filtered (mL):
3	Sampling point:	7	Dilution bottle (for laboratory-prepared QC samples only, plea D D2
4	QC Analysis or Matrix filtered (please circle one): sterility check, direct streak, streak with filter reagent, finished, dilution/rinse	8	If this report form is for a method blank, please indicate samples that are associated with this method blank:

Please note: It is important to record the number of colonies for each presumptively positive morphological type so that the final density of *Aeromonas* per sample can be reported based on percent confirmation of each colony type.

Section 2: Sample results

	ADA-V colony description **(this section is optional)**			No. of presumptive positive colonies for this colony type	No. of presumptive colonies submitted to confirmation for this colony type	Nutrient agar colony description **(this section is optional)**			No. oxidase positive per colony type	No. trehalose positive per colony type	No. Indole positive per colony type
	Color	Morphology*	size (mm)			Color	Morphology*	size (mm)			
1	pale yellow	1358	2-4 mm	60	2	off-white	1358	2-4 mm	2 of 2	2 of 2	1 of 2
2	dark yellow	1358	1-3 mm	5	1	off-white	1358	1-2 mm	1 of 1	1 of 1	1 of 1
3											
4											
5											
6											
7											
8											
9											
10											

*Morphology choices ***(list all that apply)***: (1) Round, (2) oval, (3) symmetric, (4) asymmetric, (5) shiny, (6) dull, (7) translucent, (8) opaque, (9) grainy, (10) fuzzy, (11) other

Section 3: Calculations (Use one row for each presumptive positive colony color and morphology. If more than five colony types, please attach another sheet.)

A No. presumptive positive colonies for each colony type	**B** No. of each colony type submitted to confirmation	**C** How many submitted colonies per colony type confirmed (oxidase positive, ferments trehalose, and produces indole)?	**A * (C/B) = D** Calculated no. of confirmed *Aeromonas* per colony type	**D1 + D2 + D3 + D4 + D5** Total confirmed Aeromonas **per sample**
60	2	1	D1= 30	30 + 5 = 35
5	1	1	D2= 5	
			D3=	
			D4=	
			D5=	

Section 5: Comments

Sample-specific Data Report Form:
Method 1605 (Aeromonas) - ADA With Vanomycin

Laboratory name: _____

Section 1: Sample information

1	Sample number:	5	Volume (mL) of spike (QC samples only):
2	Utility:	6	Volume filtered (mL):
3	Sampling point:	7	Dilution bottle (for laboratory-prepared QC samples only, please circle one): D D2
4	QC Analysis or Matrix filtered (please circle one): sterility check, direct streak, streak with filter reagent, finished, dilution/rinse	8	If this report form is for a method blank, please indicate samples that are associated with this method blank:

Please note: It is important to record the number of colonies for each presumptively positive morphological type so that the final density of *Aeromonas* per sample can be reported based on percent confirmation of each colony type.

Section 2: Sample results

	ADA-V colony description **(this section is optional)**			No. of presumptive positive colonies for this colony type	No. of presumptive colonies submitted to confirmation for this colony type	Nutrient agar colony description **(this section is optional)**			No. oxidase positive per colony type	No. trehalose positive per colony type	No. Indole positive per colony type
	Color	Morphology*	size (mm)			Color	Morphology*	size (mm)			
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											

*Morphology choices ***(list all that apply)***: (1) Round, (2) oval, (3) symmetric, (4) asymmetric, (5) shiny, (6) dull, (7) translucent, (8) opaque, (9) grainy, (10) fuzzy, (11) other

Section 3: Calculations (Use one row for each presumptive positive colony color and morphology. If more than five colony types, please attach another sheet.)

A No. presumptive positive colonies for each colony type	**B** No. of each colony type submitted to confirmation	**C** How many submitted colonies per colony type confirmed (oxidase positive, ferments trehalose, and produces indole)?	**A * (C/B) = D** Calculated no. of confirmed *Aeromonas* per colony type	**D1 + D2 + D3 + D4 + D5** Total confirmed Aeromonas **per sample**
			D1=	
			D2=	
			D3=	
			D4=	
			D5=	

Section 5: Comments

Laboratory name:	Laboratory ID:

Method 1622/23 Bench Sheet

1. Client sample number		
2. Internal laboratory sample ID (if applicable)		
3. Date and time of sample receipt		
4. Received by		
5. Temperature of sample and condition of sample upon arrival		
6. Storage location and storage temperature		
7. Sample turbidity, in NTU		
8. Sample type (IPR, method blank, field sample, OPR, MS, PT sample)		
9. Spiking suspension number (for IPR, OPR, MS, and PT samples only)		
10. Estimated number of oocysts/cysts spiked (for IPR, OPR, MS, and PT samples only)	*Crypto*	*Giardia*
11. Spiking date and time		
12. Sample volume spiked, in L		
13. Sample filtration start date and time		
14. Type of filter used (Envirochek, Envirochek HV, FiltaMax, CrypTest, other [specify]) and lot number:		
15. Name of analyst performing filtration		
16. Sample volume filtered, to nearest ¼ L (*do not include rinse volume*)		
17. Did filter clog?		
18. Elution date and time *(must be performed within 96 hours of sample collection/filtration)*		
19. Elution procedure: 9 wrist shaker 9 FiltaMax wash station 9 stomacher 9 backflush/sonication		
20. Name of analyst performing elution		
21. Elution buffer:_____ Elution buffer lot number and expiration date:		
22. Concentration procedure (centrifugation, FiltaMax concentrator, other [specify])		
23. Name of analyst performing concentration		
24. Pellet volume after concentration, in mL		
25. (a) Total volume of resuspended concentrate; (b) volume transferred to IMS (in mL)	(a)	(b)
26. Number of subsamples processed independently through the remainder of the method		
27. IMS system used (Dynal anti-*Cryptosporidium*, Dynal GC-Combo, other [specify]) and lot number		
27. Name of analyst performing IMS procedure		
28. Slide(s) used (Meridian, Dynal, other [specify]) and lot number		
29. Date and time sample applied to slide(s) to dry *(must be completed same working day as Row 18)*		
30. Detection kit used (Merifluor, AquaGlo, Crypt-a-Glo, Giardi-a-Glo, other [specify]) and lot number		
31. Analyst performing staining procedure		
32. Staining completion date and time *(must be complete within 72 hours of Row 29)*		
33. Total number of oocysts and cysts counted in sample (sum of counts in subsamples, if applicable)	*Crypto*	*Giardia*
Comments:		

These steps must be completed in one working day

HV=high volume
NTU=nephelometric turbidity unit
IPR=initial precision and recovery
OPR=ongoing precision and recover
MS=matrix spike
PT=performance test

Laboratory name:	Laboratory ID:

Method 1622/1623 *Cryptosporidium* Report Form

Client sample number:	Internal laboratory sample ID (if applicable):
10-mL subsample ID (if packed pellet > 0.5 mL):	Volume examined (in L) on this slide:
Analyst:	Positive staining control acceptable 9 YES 9 NO Negative staining control acceptable 9 YES 9 NO

Object located by FA No.	Shape (oval or round)	Size L x W (Fm)	DAPI -	DAPI +		D.I.C.		
			Light blue internal staining, no distinct nuclei, green rim (A)	Intense blue internal staining (B)	Number of nuclei stained sky blue (C)	Empty oocysts (D)	Oocysts with amorphous structure (E)	Oocysts with internal structure (F) Number of sporozoites
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								

Total FA number from this slide:	Examination completion date:
	Examination completion time (must be complete within 7 days of staining):
DAPI -: Total number (A):	D.I.C. - Total number of empty oocysts (D):
DAPI +: Total number (B):	D.I.C. - Total number of oocysts with amorphous structure (E):
DAPI +: Total number (C):	D.I.C. - Total number of oocysts with internal structure (F):
Total count DAPI + (C) that show structure by D.I.C. (F):	

Laboratory name:	Laboratory ID (if applicable):

Method 1623 *Giardia* Report Form

Client sample number:	Internal laboratory sample ID (if applicable):
10-mL subsample ID (if packed pellet > 0.5 mL):	Volume examined (in L) on this slide:
Analyst:	Pos. staining control acceptable 9 YES 9 NO Neg. staining control acceptable 9 YES 9 NO

Object located by FA No.	Shape (oval or round)	Size L x W (Fm)	DAPI -	DAPI +		D.I.C.				
			Light blue internal staining, no distinct nuclei, green rim (A)	Intense blue internal staining (B)	Number of nuclei stained sky blue (C)	Empty cysts (D)	Cysts with amorphous structure (E)	Cysts with internal structure (F)		
								Number of nuclei	Median body	Axonemes
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										

Total FA number from this slide:	Examination completion date:
	Examination completion time (must be complete within 7 days of staining):
DAPI-: Total number (A):	D.I.C.: Total number of empty cysts (D):
DAPI+: Total number (B):	D.I.C.: Total number of cysts with amorphous structure (E):
DAPI+: Total number (C):	D.I.C.: Total number of cysts with one internal structure (F):
Total number DAPI + (C) that show structure by D.I.C. (F):	D.I.C.: Total number of cysts with >one internal structure (F):

APPENDIX E
EPA HEADQUARTERS AND REGIONAL ATP CONTACTS

Headquarters
William Telliard
Director, Analytical Methods
Attn: ATP Program Coordinator
Mail Code 4303T
U.S. EPA Office of Water, Office of Science and Technology
1200 Pennsylvania Avenue, NW
Washington, DC 20460

Region 1
Arthur Clark
QA Chemist
USEPA Region 1
EQA
60 Westview Street
Lexington, MA 02173

Region 2
Carol Lynes
ATP Program Coordinator
USEPA Region 2
Division of Science and Monitoring
2890 Woodbridge Avenue (MS-220)
Building 10
Edison, NJ 08837-3679

Region 3
Charles Jones
Regional QA Officer
USEPA Region 3
Environmental Assessment and Protection Division
1650 Arch Street, 3ES-10
Philadelphia, PA 19103-2029

Region 4
Wayne Turnbull
Chemist/ATP Program Coordinator
USEPA Region 4
Room: SESD
960 College Station Road
Athens, GA 30605-2720

Region 5
Kenneth Gunter
ATP Program Coordinator
USEPA Region 5
77 W. Jackson Blvd., WT-15J
Chicago, IL 60604

Region 6
David Stockton
USEPA Region 6 Laboratory
Houston Branch
10625 Fallstone Road (6MD-HI)
Houston, TX 77099

Region 7
Doug Brune
ATP Program Coordinator
USEPA Region 7
726 Minnesota Avenue, ENSV/QA
Kansas City, KS 66101

Region 8
Rick Edmonds
Regional Quality Assurance Officer
USEPA Region 8
999 18th Street - Suite 500 (8TMS-L)
Denver, CO 80202-2466

Region 9
Roseanne Sakamoto
ATP Program Coordinator
USEPA Region 9
75 Hawthorne Street, PMD-3
San Francisco, CA 94105

Region 10
Bruce Woods
QAO
USEPA Region 10
200 Sixth Avenue, OEA-095
Seattle, WA 98101