DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION

MILK LABORATORY EVALUATION FORM

LABORATORY		
LOCATION		LAB #
DATE	X = DEVIATION O = NOT USED	U = UNDETERMINED NA = NOT APPLICABLE

PHOSPHATASE TEST – SCHARER RAPID METHOD

[Unless otherwise stated all tolerances are ±5%]

SAMPLES

1. Laboratory Requirements (See CP, item 33 & 34) .....
   a. Store phenolic compounds in laboratory so that cross contamination does not occur .....

APPARATUS

2. See Cultural Procedures, items 1-32 (as necessary) .....
3. Water Baths, circulating .....
   a. Thermostatically controlled, 40±1C .....
   b. Thermostatically controlled, 34±1C .....
4. Pipets/pipettors (_____) .....
   a. 1.1 mL milk pipets .....
   b. 1 mL, 5 mL and/or 10 mL as appropriate .....
   c. Graduations distinctly marked with contrasting pigment .....
   d. Discard those with broken tips or other damage .....
   e. Or, use fixed volume pipettors (see CP, item 6e) .....
5. Test Tubes (Brand _____) .....
   a. Matched set, marked at 5, 5.5 and 8.5 mL .....
   b. Tubes purchased from same manufacturer as reagents .....
6. Test Tube Stoppers or screw caps .....
   a. Phenol-free and fit tubes .....
7. Test Tube Racks, tubes do not tilt when racks on side .....
8. Glassware and Other Apparatus (Phenol-free) .....
9. Amber Glass or Light-Proof Storage Bottle for CQC/Indo-Phax .....
10. Light Source and Filter .....
   a. Dental X-ray film viewer with daylight-type fluorescent bulb and suitable filter .....
   b. Or equivalent lighted source .....
11. Centrifuge (optional) .....
   a. Suitable for centrifuging assay tubes .....

REAGENTS

12. Buffer .....
   a. Dissolve 100g sodium sesquicarbonate dihydrate ($NaHCO_3NA_2CO_3\times2H_20$) in MS water and make up to 1 L .....
   b. Optionally, dissolve 46.8g sodium carbonate and 37.17g sodium bicarbonate in MS water and make up to 1 liter .....
   c. Store in glass stoppered bottle in refrigerator .....
13. Buffer Control/Buf-fax .....
   a. Dilute 25 mL buffer (item 12) to 500 mL with MS water .....
   b. Optionally, dissolve 1 Buf-Fax tablet in 50 mL MS water .....
      Brand _____ .....
      Exp. Date _____ Open Date _____ .....
   c. For cultured products, prepare double strength buffer control by diluting 25 mL buffer (item 12) to 250 mL with MS water or dissolve 1 Buf-Fax tablet in 25 mL MS water .....
14. Buffer Substrate/Phos-Phax .....
   a. Purify Reagent .....
      1. Dissolve 0.5g phenol-free disodium phenyl phosphate crystals in 10 mL MS water, add 25 mL buffer (item 12) and 0.1 mL CQC reagent (items 15 a&b or 0.1 mL Indo-Phax (item 15d) .....
      2. Mix and let stand 5 min .....
      3. Add 5 mL butyl alcohol, mix, let stand .....
      4. Remove alcohol layer and discard .....
      5. Repeat steps 3 and 4 until all blue color has been removed .....
   b. Dilute purified reagent to 500 mL with MS water .....
   c. For cultured products, prepare double strength buffer substrate by adding 50 mL buffer .....
   d. Optionally, prepare reagent with Phos-Phax tablet .....
      Brand _____ .....
      Exp. Date _____ Open Date _____ .....
      1. Purify by dissolving 1 tablet in 5 mL MS water .....
      2. Add 0.1 mL CQC reagent or 0.1 mL Indo-Phax (item 15), mix, let stand 5 min .....
      3. Add 2 mL butyl alcohol, mix, let stand .....
      4. Remove alcohol layer and discard .....
      5. Repeat steps 3 and 4 until all blue color has been removed .....
      6. Dilute aqueous solution to 50 mL with MS water .....
      7. For cultured products, prepare double strength by diluting to 25 mL with MS water .....
15. CQC Reagent/Indo-Phax .....
   a. Dissolve 30 mg crystalline 2,6 dichloroquinone-chlorimide (special for phosphatase work) in 10 mL methyl or ethyl alcohol (CQC) .....
   b. Dissolve 200 mg copper sulfate ($CuS0_4\times5H_20$) in 100 mL MS water (catalyst) .....
   c. Do not prepare in large quantities .....
   d. Optionally, prepare solution by dissolving 1 Indo-Phax tablet in 5 mL methyl alcohol (Indo-Phax contains $CuS0_4$ catalyst) .....
      Brand _____ .....
      Exp. Date _____ Open Date _____ .....
   e. Store CQC/Indo-Phax reagent in amber bottle under refrigeration .....
   f. Discard CQC/Indo-Phax reagent after 1 week or if it turns brown .....
16. N-Butyl Alcohol (neutralized) .....
   a. Add 0.1 N NaOH to alcohol until small portion tested with bromthymol blue gives green or light blue color .....
   b. Optionally, add 50 mL of diluted buffer [10 mL of buffer (item 12) and 40 mL of MS water] per gallon of alcohol .....
   c. Or, dissolve 4 Buf-Fax tablets in 50 mL of MS water and add to each gallon of alcohol .....
   d. To test, shake alcohol with an equal quantity of neutral MS water, allow them to separate, and add a few drops of indicator solution to the water layer .....

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e. Prior to use re-check alcohol for neutrality .....

17. Color Standards .....

a. Commercial Standards, purchased from same company as assay tubes, i.e., standards matched to tubes .....

Brand _____ Date Rcd. _____ .....

1. 1, 2 and 5 unit standards .....
2. Stored in dark .....
3. Replaced as necessary .....

b. Phenol Standards .....

1. Weigh 1.000g of reagent grade anhydrous phenol, transfer to 1 liter volumetric flask and make up to 1 liter with 0.1 N HCl and mix. Stock solution contains 1 mg phenol/mL .....
2. Stock solution is stable for several months under refrigeration stored in glass stoppered amber bottle .....
3. Prepare fresh daily a working solution of phenol containing 10 µg phenol/mL by diluting 5 mL of the stock solution to 500 mL with MS water (Solution I) .....
4. Dilute 10 mL of Solution I to 100 mL with MS water (Solution II) .....
5. Prepare color standards containing 1.0, 2.0, and 5.0 µg phenol/5 mL in a series of test tubes by diluting 0.5, l.0, 2.5 mL, respectively of the Solution II with 4.5, 4.0 and 2.5 mL of phenol-free MS water .....
6. Add 0.5 mL buffer (item 12) and mix; pH must be 9.5-10.0 .....
7. Add 0.1 mL of CQC and 0.1 mL catalyst, or 0.1 mL Indo-Phax (see item 15) .....
8. Mix immediately, incubate for 5 min. at 40C in a water bath .....
9. Extract each standard with 3 mL butyl alcohol (item 16) following procedure in item 25i-l .....
10. Withdraw butyl alcohol layer and transfer to appropriate tubes .....

PREPARATION

18. Preparation of Glassware and Other Apparatus .....

a. Preferably, rinse all glassware immediately after use in warm water .....

b. Thoroughly wash in warm water with suitable detergent which does not contain phenolic compounds .....

c. Rinse thoroughly in clean tap and MS water (glassware and stoppers should be clean and free from soaps and detergents) .....

d. Test representative pieces to determine if phenol-free using test procedure in item 25 with no milk .....

19. Preparation of Materials .....

a. All aqueous solutions made with phenol-free MS water .....

b. Where suitability of reagents is doubtful, test each separately to determine if it causes false results .....

c. Since reagents may decompose with age, prepare them shortly before use, or store under refrigeration .....

d. Store in well-stoppered containers with pouring lips protected from dust contamination .....

e. Do not use reagents that give false results. Use only reagents that are free from phenolic or other contamination .....

20. Negative Control .....

a. Heat raw milk to 95±1C and hold for 1 min while stirring (temperature control [TC] required) .....

b. Cool rapidly in an ice bath .....

c. When tested, this control must show no color .....

d. If desired, distribute 1 mL quantities in small tubes, seal and freeze in a non-frost-free freezer, or place in a styrofoam container and place in the center of a frost-free freezer for no more than 2 months .....

21. Positive Control .....

a. To a portion of negative control, add mixed-herd raw milk (approximately 0.2 mL to 100 mL heated milk, use more or less raw milk as needed to get the correct positive control color), checked before official use .....

b. This control must show approx. 1-2 µg phenol equivalent/mL when compared to standards in item 17 .....

c. If desired, distribute 1 mL quantities in small tubes, seal and freeze in a non-frost-free freezer, or place in a styrofoam container and place in the center of a frost-free freezer for no more than two months .....

22. Interfering Substance Control .....

a. Substitute buffer control (item 13) for buffered substrate; any blue color is attributable to interference substance and that amount is subtracted from original test .....

SCREENING PROCEDURE

23. Tube Identification .....

a. Before transferring sample to test tubes, arrange tubes in order .....

b. Identify each tube with sample number .....

24. Sample Mixing .....

a. Thoroughly mix milk or cream by inversion 25 times before transferring test portions to tubes .....

b. Remove test sample within 3 minutes of agitation .....

c. Samples must be kept cool (0-4.4C) before testing .....

25. Procedure .....

a. Use clean pipet for each sample .....

b. Add 0.5 mL of sample or control to each appropriately labeled tube .....

c. Add 5.0 mL of buffered substrate to each appropriately labeled tube .....

1. For cultured or acidified products, use 5.0 mL of double strength buffer substrate (item 14c or 14d7); test pH after addition of double strength buffer substrate and adjusted if necessary to pH 9.5-10 with solid buffer crystals (item 12a) .....
2. If interfering substance is known or suspected, add 5.0 mL of buffer control (item 13) as indicated in item 22 above .....

d. Stopper/cap tubes and mix by inverting several times; place in water bath .....

e. Warm mixture to 40±1C and then incubate for 15 min (TC used, milk and reagent mixture) .....

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f. Remove from water bath, add 0.1 mL of CQC (item 15a) and 0.1 mL catalyst (item 15b) or 0.1 mL Indo-Phax (item 15c) and mix well ..... _____
g. Re-incubate for 5 min (start timer immediately) ..... _____
h. Remove tubes, cool in ice water bath (to below 10C), and add 3 mL neutralized cold (-20C recommended) butyl alcohol (item 16) ..... _____
i. Re-stopper and extract indo-phenol blue by gently inverting tubes through four (4) half-circles ..... _____
j. Take 1 sec to invert, pause 1 sec to allow bubbles to break and alcohol to separate, taken another sec to return tube(s) to upright position, pause 1 sec and repeat ..... _____
k. Immediately lay tubes on flat surface for 2 min to permit separation of butyl alcohol ..... _____
l. Repeat extraction and separation steps i - k ..... _____
m. Extraction of indo-phenol blue complete ..... _____
n. Without emulsification of all butyl alcohol ..... _____
   1. Emulsification interference can be clarified sufficiently by cooling tubes in ice water for 5 min and then centrifuging tubes at 3,000 rpm for 5 min ..... _____
o. Stand tubes erect, compare colors to 1-, 2-, and 5-unit standards (item 17) using a standard uniform light and suitable filter ..... _____
p. Record results in µg phenol/mL (<1, 1, >1<2, 2, >2<5, 5 or >5 µg/mL based on the comparison to the standards (item 17) ..... _____
q. A value of 1 µg or more must be confirmed ..... _____

CONFIRMATION PROCEDURE

CONTROLS

26. Negative Controls ..... _____
a. Prepare separate controls for each suspect positive sample tested ..... _____
b. Prepare by heating for 1-2 min after the thermometer registers 95±1C (using TC) , stirring or mixing as necessary ..... _____
c. Cool rapidly using an ice bath ..... _____
d. This control must show no blue color ..... _____
27. Positive Control (See item 21) ..... _____
28. Interfering Substance Control (See item 22) ..... _____
29. Microbial Phosphatase Control ..... _____
a. To determine presence of microbial phosphatase, heat 5 mL of suspect milk at 63±1C for 30 min, stirring or mixing as necessary (if fat content is >10%, heat at 66±1C) ..... _____
b. Test heated portion, unheated portion and negative control ..... _____
c. Interpretation ..... _____
   1. If heated and unheated portions have equal activity, the sample is regarded negative for residual phosphatase, the activity originally measured is microbial ..... _____
   2. If the heated portion has no activity, the sample contains milk phosphatase activity either residual or reactivated ..... _____

30. Reactivated Phosphatase Control ..... _____
a. Magnesium acetate solution ..... _____
   1. Dissolve 35.4 g of $Mg(C_2H_3O_2)_2 \times 4H_2O$ in 25 mL MS water warming slightly to aid solution. Pour solution into 100 mL volumetric flask, rinse original container several times and add rinses to flask. After cooling, make up to 100 mL (stable for 1 year at 0-4.4C) Date prep. _____ ..... _____
b. Procedure ..... _____
   1. Place 10 mL of each milk or milk product sample to be tested in a boiling water bath and hold 1 min after temperature sample has reached 95±1C (TC used) ..... _____
   2. Cool samples and use a portion of each for dilution as required and for a negative control ..... _____
   3. Place a 5 mL aliquot of unheated sample to be tested in a screw-cap (phenol-free) test tube and add 0.1 mL MS water ..... _____
   4. Place a second 5 mL aliquot in an identical tube, add 0.1 mL Mg acetate solution ..... _____
   5. Incubate both aliquots for 1 hr at 34±1C (with TC) ..... _____
   6. Remove samples from water bath, cool, and dilute 1 mL of sample containing magnesium with 5 mL of corresponding boiled milk or milk product control ..... _____
   7. Test undiluted sample containing no magnesium and 1:6 dilution of sample containing magnesium for phosphatase activity ..... _____
c. Interpretation ..... _____
   1. If the 1:6 dilution of the aliquot containing magnesium has equal or greater phosphatase activity than the undiluted aliquot containing no magnesium, the sample is regarded negative for residual phosphatase, and the phosphatase originally measured is of **reactivated** origin ..... _____
   2. If the diluted aliquot contains less activity than the undiluted aliquot, the sample is considered positive for **residual phosphatase** ..... _____
   3. A false-positive for residual phosphatase may also be obtained if a reactivatable sample has been allowed to stand at elevated temperatures (20C) for periods of 1 hr or more before testing (SPC <20,000/mL) ..... _____

REPORT

31. Confirmatory Interpretation ..... _____
a. Report as residual phosphatase if ≥1 µg/mL and interfering substances, microbial phosphatase, and reactivatable phosphatase are not present ..... _____
b. Report as <1 µg/mL for residual phosphatase if: ..... _____
   1. Interfering substance present (buffer and substrate tubes have equal color) ..... _____
   2. Or, if microbial phosphatase present ..... _____
   3. Or, if reactivatable phosphatase present ..... _____
   4. Or, if product was treated such that reactivatable phosphatase may be present (must document) ..... _____