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

MILK LABORATORY EVALUATION FORM

LABORATORY

LOCATION | LAB #

DATE

X = DEVIATION | U = UNDETERMINED
O = NOT USED | NA = NOT APPLICABLE

SPIRAL PLATE COUNT METHODS

[Unless otherwise stated all tolerances are ±5%]

GENERAL REQUIREMENTS

1. **Cultural Procedures, items 1 - 32, as appropriate** ..... _____
2. **Sample Requirements, see CP item 33 & 34** ..... _____
   a. Raw milk tested only ..... _____
3. **Comparative Test with SPC** ..... _____
   a. Test 25 samples in duplicate using the SPC and SPLC methods ..... _____
   b. Comparisons done by each analyst performing test ..... _____
      1. Results must be shown to be acceptable before official tests may be performed by the analyst ..... _____
   c. Copy of comparison and results in QC record (or easily accessible file in laboratory) ..... _____
   d. Analysts certified for Standard Plate Count ..... _____

APPARATUS

4. **Spiral Plater** ..... _____
   a. Model D ..... _____
   b. Autoplate® 4000 ..... _____
   c. Rinse and clean apparatus weekly ..... _____
      1. Model D ..... _____
         a. Remove the valve from syringe, insert hand held syringe (item 15) containing water and apply pressure
         b. Repeat with alcohol or acid detergent to remove any remaining residual material adhering to walls of the system ..... _____
         c. Rinse with water before reassembling ..... _____
      2. Autoplate® 4000 ..... _____
         a. Lower the stylus into a solution of 5% detergent and open the valve for 5 seconds. Close the valve. Allow the detergent to remain in contact with the tubing for 5 minutes ..... _____
         b. Rinse by lowering the stylus into a container of MS water and opening the valve for 30 seconds ..... _____
         c. Repeat with acid cleaner (0.5 N sulfuric acid) to remove any remaining residual material adhering to the walls of the system ..... _____
         d. Rinse thoroughly with MS water and leave the system full of water when not in use ..... _____
   d. Sample volume ..... _____
      1. Model D ..... _____
         a. Dispenses 49.2 µL ..... _____
         b. Checked by 10 consecutive weighings one time per quarter ..... _____
         c. Records maintained ..... _____
      2. Autoplate® 4000 ..... _____
         a. Dispenses 50 µL in default mode ..... _____
         b. Checked quarterly by running validation routine with validation test fixture ..... _____
         c. Records maintained ..... _____
   e. Maintenance log maintained ..... _____
5. **Spiral Plate colony viewer with appropriate grid** ..... _____
   a. Model D ..... _____
      1. Counting grid divided into 8 equal wedges ..... _____
      2. Each wedge divided into 6 arcs (segments) labeled 3a, 3b, 3c, 4a, 4b and 4c from the outside edge ..... _____
   b. Autoplate® 4000 ..... _____
      1. Spiral counting grid divided into 4 quadrants ..... _____
      2. Each quadrant is divided into 6 arcs (segments) labeled 8, 9, 10, 11, 12 and 13 ..... _____
      3. For high count plates, the grid is divided into 8 circumferential sectors labeled a, b, c, d, e, f, g and h ..... _____
6. **Hand tally** ..... _____
7. **Vacuum source, 50 - 60 cm Hg with vacuum trap (min. 1 L)** ..... _____
   a. Checked annually, records maintained ..... _____
8. **Beakers, 5 mL, or approved equivalent** ..... _____
9. **Petri dishes (100 x 15 mm)** ..... _____
10. **Standard Methods Agar** ..... _____
11. **Polyethylene bags, about 30 x 20 x 40 cm** ..... _____
12. **Sodium hypochlorite solution, about 5% (equivalent to full strength commercial bleach solution), or equivalent** ..... _____
13. **Acid cleaner, 0.5 N sulfuric acid** ..... _____
14. **Sterile water** ..... _____
15. **Syringe, with Luer-Lok tip, 10 - 20 cc (for Model D)** ..... _____
16. **Dye solution, crystal violet, 0.7% solution** ..... _____
17. **Three polypropylene 75 mL capacity reservoirs (for 4000)** ..... _____

PLATE PREPARATION

18. **Plate Preparation** ..... _____
   a. Prepare or melt agar quickly in boiling water, flowing steam not under pressure, or microwave oven (use extreme care) ..... _____
      1. Avoid prolonged exposure to high temperatures during and after melting ..... _____
      2. Do not melt more than will be used within 3 hr ..... _____
      3. Do not melt agar more than once ..... _____
      4. Determine and record pH prior to pouring plates ..... _____
      5. Pour 15 mL of media tempered to 60 - 70C into each plate ..... _____
      6. Allow to solidify on a sanitized, level surface ..... _____
      7. Optionally, use automated dispenser ..... _____
   b. After solidification examine plates for uniformity of agar depth (no more than 2 mm difference), invert plates and allow to cool to room temperature ..... _____
      1. Plates used immediately ..... _____
      2. Or, stored inverted in sealed plastic bags (item 11) at 0 - 4.4C for no longer than 2 weeks ..... _____

   Date prep. _____ ..... _____
19. **Calibration of Counting Grid, performed initially and after maintenance** ..... _____
   a. Determine and record volume constants for spiral plates ..... _____

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SPIRAL PLATE COUNT METHODS

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1. Make a series of consecutive 1:2 dilutions of a bacterial suspension (no spreaders) .....
2. Prepare 11 bacterial concentrations in the range of 10^3 to 10^6 cell/mL .....
3. Plate all dilutions in duplicate by both the SPC and SPLC methods .....
4. Incubate both sets of plates at 32±1C for 48±3 hr .....
5. Count and calculate the SPC/mL for each dilution .....
6. Count the spiral plates over the grid surface using the counting rule of 20 (see item 28.c.) to record the number of colonies counted and the grid area over which they were counted .....
7. For each of the SPLC colony counts for a particular grid area, divide by the SPC/mL for the corresponding bacterial concentration used .....

$$\frac{(\text{SPLC/area})}{\text{SPC/mL}} = \text{volume (mL) for grid area}$$

8. Maintain records of calibration check .....

PROCEDURE

20. Work Area .....
- a. Plating bench not in direct sunlight .....
- b. Sanitize area around instrument before start of plating .....

21. Preliminary Set up and Examination of Plates .....
- a. Allow plates to reach room temperature prior to use .....
  1. Allow refrigerated plates to dry at room temperature for 12 to 24 hours prior to use .....
- b. Examine plates for uniform agar depth and smooth surface .....
  1. If agar depth too low or high and/or water, defects or contamination are detected, do not use .....
- c. Place plates for easy access near instrument .....

22. Sample Agitation .....
- a. When appropriate, wipe top of unopened containers with sterile, ethyl alcohol-saturated cloth .....
- b. Before removing test portion, thoroughly mix contents of each container (approx ¾ full) by shaking 25 times in 7 sec with 1 ft movement .....
- c. Remove test portion and plate within 3 min of sample agitation .....

23. Plating Procedure for Model D .....
- a. Turn on vacuum .....
- b. Turn on power, ready light on, and set unit to automatic .....
- c. Check stylus tip angle daily and adjust as necessary .....
  1. Tip of stylus touches back of arc marking the starting point on the turntable, tip OK .....
  2. Tip of stylus does not touch back of arc marking the starting point on the turntable, adjust tip and check using steps a and b .....
     - a. Use vacuum to hold a microscope cover slip, or equivalent, against the face of the stylus .....
     - b. Hold stylus/cover slip about 1 mm above platform surface, if parallel using level gauge proceed, if not adjust and recheck .....
  3. Run dye solution (item 16) as in steps g - n to assure spiral plater is dispensing liquid uniformly over plate surface .....
- d. CAM follower arm bearing touches flag on stationary CAM, adjust as necessary .....
- e. Fill one 5 mL beaker (or approved equivalent) with sterile water and another with 5% Sodium hypochlorite solution (or approved equivalent) .....
- f. Clean stylus tip by rinsing for 1 second in sodium hypochlorite solution (item 12) 3x and then in sterile water 3x prior to introducing **EACH** sample .....
- g. Label plate with sample information and make a vertical mark on the side of the plate bottom to indicate the start of sample deposition .....
- h. Insert tip into agitated sample in rigid container, or poured into sterile 5 mL beaker, or approved equivalent, avoiding foam .....
- i. Open vacuum filling valve .....
- j. Draw up sample through sight glass and close valve .....
  1. Assure that there is a solid column of sample in the sight glass, i.e. no bubbles .....
- k. Lift stylus out of sample and touch off excess sample onto dry area of sample container .....
- l. Place agar plate on platform and remove cover .....
- m. Place stylus tip on agar surface and start motor .....
- n. After inoculation, when stylus lifts from agar surface and moves to starting position immediately remove plate and replace lid .....
- o. Repeat f - n for each sample to be tested .....
- p. After absorption of liquid, invert plate and place in 32C incubator within 20 minutes .....
- q. After all samples and controls have been plated, repeat step f
- r. Turn off power and vacuum .....

24. Plating Procedure for Autoplate® 4000 .....
- a. Turn on vacuum .....
- b. Turn on power, ready light on and ensure that unit is set to 50 µL deposition, 100 mm dish size, min. fill (for one plate per sample) or max. fill (for multiple replicates) .....
- c. Check stylus alignment daily and adjust as necessary .....
  1. Place a typical agar plate on the turntable, press test .....
  2. Check that the boom is parallel to the turntable surface .....
  3. If boom is not parallel to the turntable surface, loosen the stylus adjustment screw and slide the support tube up or down until the boom is in the correct location parallel to the turntable surface .....
  4. Check that the scribed line on the stylus support tube faces forward .....
  5. If the scribed line on the stylus support tube does not face forward, loosen the stylus adjustment screw and rotate the tube until the scribed line faces forward .....
  6. Looking through agar, check that tip of stylus rests at the intersection of the 13 mm diameter circle (±0.5 mm left to right) and the 9 o'clock radial line (±3.0 mm front to back) .....

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7. If tip of stylus does not rest as described above adjust tip by loosening the boom adjustment screw and move the boom until the stylus tip rests at the correct position ..... _____
8. Run dye solution (item 16) as in steps g - n below to assure spiral plater is dispensing liquid uniformly over the plate surface ..... _____

d. Wrap and autoclave reservoirs (item 17) at 120±1C for 5 minutes on dry cycle ..... _____
e. Fill reservoirs labeled "water 1" and "water 2" with sterile water to the top of their black tolerance bands and place in position on the Autoplate® 4000 ..... _____
f. Fill the reservoir labeled "disinfectant" with 5% sodium hypochlorite (item 12) to the top of the black tolerance band and place in position on the Autoplate® 4000 ..... _____
g. Label plate with sample information and make a vertical mark on the side of the plate bottom to indicate the start of sample deposition ..... _____
h. Pour or pipet 3 - 4 mL of raw milk into a 5 mL beaker (item 8) and place in position on the Autoplate® 4000 ..... _____
i. Remove the agar plate cover and place the plate on the turntable so that the vertical mark aligns with the radial scribed line on the turntable ..... _____
j. Press "All" to initiate a complete cycle of cleaning, filling and plating ..... _____
   1. Alternatively, press "Clean", "Fill" and then "Plate" to achieve the same results ..... _____
   2. If replicate plates are to be made, such as when comparing to SPC method, select "Max" as the fill option, otherwise set "Min" as the fill option ..... _____
k. After inoculation, when stylus lifts from the agar surface and moves to the starting position, immediately remove plate and replace lid ..... _____
l. Repeat steps g - k for all samples being tested ..... _____
m. If performing replicate plates, such as when comparing to the SPC method, repeat steps h and i, and press "Plate" for each replicate to be made ..... _____
n. After absorption of liquid, invert plate and place plates into 32C incubator within 20 minutes ..... _____
o. After all samples and controls have been plated, press "Clean" to disinfect and rinse the stylus tubing ..... _____
p. Remove and rinse reservoirs ..... _____
q. Turn off power and vacuum ..... _____

CONTROLS

25. Controls ..... _____
a. Dye plate control: Prior to beginning plating milk samples, run dye plate as in appropriate procedure section ..... _____
   1. Examine for good distribution of liquid over surface ..... _____
   2. If distribution is not even do not proceed until corrected ..... _____
b. Initial rinse control with sterile dilution buffer, for Autoplater® run "All" cycle to intake and plate sterile buffer ..... _____
c. Determine if spiral plater is rinsing free by preparing a rinse control plate after every 20 samples plated ..... _____
d. Determine if sanitizing solution is rinsing free between samples by running a known (spiked) sample after last sample and before final rinse control ..... _____
e. After all samples have been run discharge a final rinse to a control plate ..... _____
f. Check sterility of rinse buffer and medium for each group of samples ..... _____
g. Expose a plate to air for 15 min during plating, AM and PM ..... _____
   1. This plate must be placed next to spiral plater and exposed at the start of a run ..... _____
h. Records maintained ..... _____
i. Include control information on work/bench sheet(s) ..... _____

INCUBATION

26. Incubation (32±1C) ..... _____
a. Plates must reach incubation temperature within 2 hr ..... _____
b. Stack plates no more than 6 high ..... _____
c. Arrange stacks so each is at least 2.5 cm from adjacent stacks and from incubator surfaces ..... _____
d. Place stacks directly over each other on successive shelves ..... _____

COUNTING COLONIES

27. Counting Aids ..... _____
a. Count colonies with aid of magnification under uniform and properly controlled artificial illumination with a hand tally ..... _____
b. Or approved automated plate counter ..... _____

28. Counting and Recording Spiral Plate Counts ..... _____
a. After incubating plates at 32±1C for 48±3 hr, promptly count colonies on plates ..... _____
b. Where impossible to count at once, store plates at 0 - 4.4C for not longer than 24 hr (avoid as a routine practice) ..... _____
c. Count SPLC plates using the "Counting Rule of 20" ..... _____
   1. Center the plate over the grid. For Autoplate® 4000 position vertical mark on side of plate at 12 o'clock on grid ..... _____
   2. Model D: Choose any wedge and count the colonies from the outer edge of the first segment toward the center until 20 colonies have been counted ..... _____
   3. Autoplate® 4000: Choose any of the 4 quadrants and count the colonies beginning in the outer segment #8 toward the center until 20 colonies have been counted ..... _____
   4. Complete the count by counting the remainder of the colonies observed in the segment in which the 20th colony occurred ..... _____
   5. Count segment in opposite wedge to original one counted
   6. Record counts and wedges/segments counted ..... _____
   7. Model D: If there are not 20 colonies in the 4 segments of the wedge counted, all the colonies on the whole plate must be counted ..... _____
   8. Autoplate® 4000: If there are not 20 colonies in the 6 segments of the quadrant counted, all the colonies on the whole plate must be counted ..... _____

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9. Model D: If the number of colonies in the 2nd, 3rd or 4th seg-ment, which contained the 20th colony exceeds 75, recount plate by counting the circumferentially adjacent segments in all 8 wedges (minimum of 50 colonies must be counted) ..... _____
10. Autoplate® 4000: If the number of colonies in segment #8 in one quadrant exceeds 75, recount plate by counting the circumferentially adjacent sectors (single spirals) in 1/8th increments (marked a - h on the grid) until at least 50 colonies have been counted, record count and last sector _____
    counted ..... _____
11. If spreader covers no more than half a plate count well distributed colonies in the spreader free portion of the plate ..... _____
12. Estimate the number of bacteria by dividing the count obtained by the volume contained in all the segments or sectors counted ..... _____

$$\frac{X + X}{\text{volume}} = \text{count/mL}$$

d. Record total number of colonies on each plate counted ..... _____
e. If plates show no colonies, record plate count as 0 ..... _____
f. If plates show excessive colonies and can not be counted record as TNTC for largest dilution factor ..... _____
g. Record results of sterility and control tests ..... _____

29. Personal Errors ..... _____

a. Avoid inaccurate counting due to carelessness, fatigue, or impaired vision ..... _____
b. Discover cause and correct if unable to duplicate your own counts on the same plate ..... _____
c. Perform monthly counting ..... _____
   1. If 3 or more analysts use the RpSm method, see current SMEDP, records maintained ..... _____
   2. If less than three analysts, comparative counts agree ≤8% for the same analyst and ≤10% between two analysts, records maintained ..... _____
   3. If using an automated counter compare visual counts to automated counts, with two or more analysts use the automated counter as one analyst and compare counts using the RpSm method, records maintained ..... _____

REPORTS

30. Reporting Spiral Plate Counts (SPLC) ..... _____

a. Report calculated count as SPLC/mL ..... _____
b. If fewer than 20 colonies are counted on a total plate, report as <400 ESPLC/mL ..... _____
c. If plate is recorded as being TNTC, report as >400,000 ESPLC/ mL ..... _____
d. Report only first two left-hand digits ..... _____
   1. If the third digit is 5 round the second number using the following rules ..... _____
      a. When the second digit is odd round up (odd up, 235 to 240) ..... _____
      b. When the second digit is even round down (even down, 225 to 220) ..... _____
e. If spiral plate contains irregular distributions of colonies, caused by dispensing errors, report as laboratory accident (LA) ..... _____
f. If a spreader covers more than half a plate, do count, report as spreader (SPR) ..... _____
g. If presence of growth inhibitor is detected colony count can not be reported, report as growth inhibitor (GI) ..... _____