SPIRAL PLATE COUNT METHODS
(Raw Commingled Cow Milk)
IMS #4

[Unless otherwise stated all tolerances are ±5%]

GENERAL REQUIREMENTS

1. Cultural Procedures (CP), items 1-32, as appropriate _____

2. Sample Requirements, see CP item 33 & 34 _____
[For inhibitor testing requirements, refer to Section 6 of the PMO]

3. Comparative Test with SPC _____

a. Analysts certified for SPC _____

b. Test 25 samples in duplicate using the SPC (2400a) and SPLC methods _____

c. Comparisons done by each analyst performing test _____

1. Results must be evaluated by a LEO and shown to be acceptable before official use of test in laboratory _____

2. Copy of comparison and results in QC record (or easily accessible file in laboratory); kept for as long as analyst is certified _____

APPARATUS

4. Spiral Plater _____

a. Model D _____

b. Autoplate® 4000 _____

c. Autoplate® Spiral Plating System _____

d. 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 sec. Close the valve. Allow the detergent to remain in contact with the tubing for 5 min _____

   b. Rinse by lowering the stylus into a container of MS water and opening the valve for 30 sec _____

   c. Repeat with acid cleaner (0.5N 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 _____

3. Autoplate Spiral Plating System _____

   a. Lower the stylus into a solution of 1% detergent and perform a max fill using the manual mode. Allow the detergent to remain in contact with the tubing for 5 min. Press the Syringe Down button until the syringe volume reads zero. _____

   b. Rinse by flushing MS water in manual mode _____

   c. Repeat with acid cleaner (0.5N sulfuric acid) to remove any remaining residual material adhering to the walls of the system _____

   d. Rinse thoroughly with MS water when not in use _____

   e. Sample Volume _____

      1. Model D _____

         a. Dispenses 49.2 µL _____

         b. Checked by 10 consecutive weighings one time per quarter _____

         c. Maintain records _____

      2. Autoplate 4000/Autoplate Spiral Plating System _____

         a. Dispenses 50 µL in default mode _____

         b. Checked quarterly by running validation routine with validation test fixture _____

         c. Maintain records _____

         d. Maintain maintenance log _____

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/Autoplate Spiral Plating System _____

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 (CP item 17) _____

7. Vacuum Source, 50-60 cm Hg with Vacuum Trap (min. 1 L) _____

a. Check annually; maintain records _____

8. Beakers, 5 mL, or Approved Equivalent _____

9. Petri Dishes (100 x 15 mm) _____

10. Standard Methods or Plate Count Agar (CP item 27b) _____

11. Polyethylene Bags, about 30 x 20 x 40 cm _____

12. Sodium Hypochlorite Solution; about 5% for Model D and Autoplate 4000; 1% for Autoplate Spiral Plating System _____

13. Acid Cleaner, 0.5N 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) _____

18. Two Polypropylene 500 mL Capacity Bottles (for Autoplate Spiral Plating System) _____

19. One Polypropylene Waste Container (for Autoplate Spiral Plating System) _____

PLATE PREPARATION

20. Plate Preparation _____

a. Prepare or melt agar quickly in boiling water, flowing steam not under pressure _____

1. Avoid prolonged exposure to high temperatures during and after melting _____

2. Do not melt more than will be poured within 3 hours _____

3. Do not melt agar more than once _____

4. Determine and record pH prior to pouring plates; maintain records _____

5. Pour 15 mL of media tempered to 45±2°C 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 0.0-4.5°C for no longer than 2 weeks _____

Prep. Date: _____ Lab Exp. Date: _____ _____

CALIBRATION

21. Calibration of Counting Grid, Performed Initially and After Maintenance _____

a. Determine and record volume constants for spiral plates _____

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±1°C for 48±3 hours _____

5. Count the SPC plates and compute the SPC/mL for each dilution _____

6. Count the spiral plates over the grid surface using the counting rule of 20 (see item 31.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 SPLC/mL _____

8. Maintain records of calibration check _____

PROCEDURE

22. Work Area _____

a. Plating bench not in direct sunlight _____

b. Sanitize area around instrument before start of plating _____

23. 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 _____

24. 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 _____

25. 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 32°C incubator within 20 min _____

q. After all samples and controls have been plated, repeat step f _____

r. Turn off power and vacuum _____

26. 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, minimum fill (for one plate per sample) or maximum 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 bloom 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) _____

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±1°C for 5 min 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 _____

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 32°C incubator within 20 min _____

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

27. Plating Procedure for Autoplate Spiral Plating System _____

a. Turn on power (ready light on) and ensure that unit is set to 50µL deposition, 100 mm dish size, minimum fill (for one plate per sample) or maximum fill (for multiple replicates)

b. Check stylus alignment daily. Adjust as necessary _____

1. Ensure that the red mark on the upper portion of the stylus support tube is facing forward and is aligned with the slot in the boom (this ensures proper orientation of the Teflon stylus tip). If it is not, loosen the stylus adjustment screw and rotate the stylus support tube so that the mark is correctly aligned _____

2. Check that the boom is approximately level with the turntable surface. If it is not, loosen the stylus adjustment screw and slide the support tube up and down until the boom is level with the turntable surface when the stylus tip rests on the agar _____

3. Place a typical agar plate on the turntable _____

4. Press the Setup button, then Manual button _____

5. Move the stylus one step right, then one step down _____

6. Looking through the agar, check that tip of stylus rests at 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) _____

7. If the stylus is not in the proper position on the plate, lift the stylus and adjust the carriage and stylus using the LEFT/RIGHT arrow buttons on the Stylus Alignment screen until the stylus tip rests on the agar above the section of the radial line and the 13mm circle. Press the check-mark button on the touch panel to save the setting _____

c. Loosen cap and autoclave bottles (item 18) at 120±1°C for 15 min on dry cycle. Let bottles cool with caps loose atop bottles _____

d. Fill bottle labeled "water" with sterile water, and the bottle labeled "disinfectant" with 1% sodium hypochlorite (item 12) without breaking sterility inside bottles and caps. Reattach caps and place into position on the Autoplate Spiral Plating System _____

e. Label plate with sample information and make vertical mark on the side of the plate bottom to indicate the start of sample deposition _____

f. Pour or pipette 3-4 mL of raw milk into a 5mL beaker (item 8) and place into position on the Autoplate _____

g. 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 _____

h. Press the "All" button to initiate a complete cycle of filling, plating and cleaning _____

1. Alternatively, press "Fill", "Plate" and then "Clean" to achieve the same results _____

2. If replicate plates are to be made, such as when comparing to SPC method, press the "Replicates" button on the Home Screen and press the "Up" button until desired number of replicates appears, then press the Checkmark button to save _____

i. After inoculation, when stylus lifts from the agar surface and moves to the starting position, immediately remove the plate and replace lid _____

j. Repeat steps e-i for all samples being tested _____

k. If performing replicate plates, such as when comparing to the SPC method, repeat steps f and g, and press "plate next" for each replicate to be made _____

l. After absorption of liquid, invert plate and place plates into 32°C incubator within 20 min _____

m. After all samples and controls have been plated, press "Clean" to disinfect and rinse the stylus tubing _____

n. Purge the water and disinfectant lines _____

CONTROLS

28. Controls (AM and PM) _____

a. Dye plate control: Prior to beginning plating milk samples, run dye plate. Turn off power 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 with cover completely removed, use timer _____

1. This plate must be placed next to spiral plater and exposed at the start of a run _____

h. Maintain records _____

i. Include control information on work/bench sheet(s) _____

INCUBATION

29. Incubating Plates (see CP item 15) _____

a. Stack plates (upside down) no more than 6 high and incubate within 10 min of agar solidification _____

b. Place stacks to ensure adequate air flow _____

c. Incubate SPLC plates at 32±1°C for 48±3 hours _____

COUNTING COLONIES

30. 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 _____

31. Counting and Recording Spiral Plate Counts _____

a. After incubating plates at 32±1°C for 48±3 hours, promptly count colonies on plates _____

b. Where impossible to count at once, store plates at 0.0-4.5°C for no longer than 24 hours (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 and Autoplate Spiral Plating System, 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 and Autoplate Spiral Plating System: 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 20^{th} 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 and Autoplate Spiral Plating System: If there are not 20 colonies in the 6 segments of the quadrant counted, all the colonies on the whole plate must be counted _____

9. Model D: If the number of colonies in the 2^{nd}, 3^{rd} or 4^{th} segment, which contained the 20^{th} 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 and Autoplate Spiral Plating System: 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 plate exceeds 250 colonies, estimate counts as follows _____

1. Count colonies in portions representative of distribution and estimate the total _____

2. Where there are <10 colonies/sq. cm, count in 12 squares, selecting 6 consecutive squares horizontally across the plate and 6 consecutive squares at right angles _____

3. When there are 10 or more colonies/sq. cm, count 4 random representative squares _____

4. Multiply average number colonies/sq. cm by area of plate in sq. cm _____

g. Record results of sterility and control tests _____

32. Identifying Counting Errors _____

a. Perform monthly counting _____

1. With 3 or more analysts use the RpSm method, (see current SMEDP); maintain records _____

2. With two analysts, comparative counts agree within ≤10% of one another; maintain records _____

3. With only one analyst, replicate counts agree within ≤8% of one another; maintain records _____

4. If using an automated counter compare visual counts to automated counts, with two or more analysts use automated counter as one analyst and use RpSm method; maintain records _____

REPORTING

33. Reporting (SPLC) _____
[When samples are demonstrated to contain inhibitors, no bacteria counts are reported; report as positive for inhibitors or growth inhibitors (GI)]

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 a spreader covers more than half a plate, do not count, report as spreader (SPR) _____

f. If spiral plate contains irregular distributions of colonies, caused by dispensing errors, report as laboratory accident (LA) _____