

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

[Unless otherwise stated all tolerances are  $\pm 5\%$ ]

**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  $\mu$ L \_\_\_\_\_
      - b. Checked by 10 consecutive weighings one time per quarter \_\_\_\_\_
      - c. Maintain records \_\_\_\_\_
    - 2. Autoplate 4000/Autoplate Spiral Plating System \_\_\_\_\_
      - a. Dispenses 50  $\mu$ L 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  $45\pm 2^{\circ}\text{C}$  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  $0.0\text{-}4.5^{\circ}\text{C}$  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  $32\pm 1^{\circ}\text{C}$  for  $48\pm 3$  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  $\mu$ L 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 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 ( $\pm 0.5$  mm left to right) and the 9 o'clock radial line ( $\pm 3.0$  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  $120 \pm 1^\circ\text{C}$  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 ( $\pm 0.5$  mm left to right) and the 9 o'clock radial line ( $\pm 3.0$  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  $120 \pm 1^\circ\text{C}$  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  $32^\circ\text{C}$  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  $32\pm 1^{\circ}\text{C}$  for  $48\pm 3$  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  $32\pm 1^{\circ}\text{C}$  for  $48\pm 3$  hours, promptly count colonies on plates \_\_\_\_\_

- b. Where impossible to count at once, store plates at  $0.0\text{-}4.5^{\circ}\text{C}$  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<sup>th</sup> 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<sup>nd</sup>, 3<sup>rd</sup> or 4<sup>th</sup> segment, which contained the 20<sup>th</sup> 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  $\leq 10\%$  of one another; maintain records \_\_\_\_\_
  3. With only one analyst, replicate counts agree within  $\leq 8\%$  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) \_\_\_\_\_