

**PLATE LOOP COUNT
AGAR POUR PLATE METHOD
IMS #3**

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

SAMPLES

1. **Laboratory Sample Requirements (see CP items 33 & 34)** _____
[For inhibitor testing requirements, refer to Section 6 of the PMO]

PRE-REQUISITE

2. **Comparative Test with Standard Plate Count (SPC) (SPC – Agar Pour Plate Form 2400a)** _____
- a. Analyst(s) certified for SPC _____
 - b. Comparisons done by each analyst performing test _____
 - 1. Comparison is valid only if done using similar plate count methods, i.e. PCA or SMA agar with pipets (or pipettors) to PCA or SMA agar with the PLC device _____
 - 2. Results must be evaluated by a State/Federal LEO and shown to be acceptable prior to official use of test in laboratory _____
 - 3. Copy of comparison and results in QC record (or easily accessible on file in laboratory); kept for as long as analyst is certified _____

MATERIALS AND APPARATUS

3. **Loop 0.001 mL** _____
- a. True circle, welded I.D. 1.45 ± 0.06 mm, calibrated to contain 0.001 mL, made of appropriate wire _____
 - b. Loop fits over No. 54 but not a No. 53 twist drill bit (lab must have a set), checked monthly; maintain records _____
 - c. Modified by making a 30° bend 3-4 mm from loop, compare to template before use _____
 - d. Opposite end of wire kinked in several places _____
4. **Hypodermic Needle, Luer-Lok™** _____
- a. 13 gauge (sawed off 24-36 mm from the point where the barrel enters the hub) _____

- b. Kinked end of loop wire shank inserted into needle until bend is 12-14 mm from end of barrel; compare to template before use

5. Cornwall™ Continuous Pipetting Outfit (or equivalent)

- a. Consisting of metal holder, Cornwall Luer-Lok syringe and filling outfit
- b. Syringe, 2 mL capacity, adjusted to deliver 1.0 mL
 - 1. Calibrated by checking ten 1 mL discharges (10 mL) using a 10 mL Class A graduated cylinder each day of use; maintain records
- c. With Luer-Lok of needle attached to Luer-Lok fitting of syringe

PREPARATION

6. Media Preparation (reference agar from CP items 14, 27, 28 & 29)

- a. Temperature control (TC) used
 - 1. Contains agar identical to type and volume being used
 - 2. In container identical in size and volume to that being used
 - 3. Undergoes same heat treatment and cooling as test agar
- b. Plate Count Agar or Standard Methods Agar (PCA or SMA)
 - 1. Prepare and sterilize agar for sample series and all controls
 - 2. OR use previously prepared/stored agar; melt agar quickly in boiling water or flowing steam; not under pressure
 - 3. Do not melt agar more than once
 - 4. Promptly place in a circulating water bath to temper and hold melted agar at 45±1°C
 - 5. Record agar temperature with other control information
 - 6. Discard agar that is not used within 3 hours after tempering
 - 7. Avoid prolonged exposure to high temperatures during and after melting; establish lab protocol

7. Heat Treatment of Pipetting Equipment

- a. Sterilize assembled Cornwall Continuous Pipetting Outfit (or equivalent) by wrapping in Kraft paper or in a protective autoclavable container that permits the passage of steam and autoclave at 120±1°C for 15 min

- b. The pipetting outfit cannot be used for more than a 24 hour period of time without re-sterilization

8. Assembly of Complete Apparatus for Use

- a. Carefully place end of rubber supply tube (attached to syringe) in sterile dilution buffer blank and depress syringe plunger several times to pump buffer into syringe. Assure that there are no air bubbles in the syringe
- b. Briefly flame loop and allow to cool 15 sec
- c. Discharge several 1 mL portions to waste, then discharge 1 mL portion of buffer into instrument control plate (performed before testing of first sample)

PROCEDURE

9. Work Area

- a. Level plating bench not in direct sunlight
- b. Sanitize immediately before start of plating

10. Identifying Plates

- a. Select number of samples in any series so that all will be plated within 20 min (pref. ≤ 10 min) after depositing the first sample and pouring the last plate in the series
- b. Label each plate with sample or control identification
- c. Arrange plates in order before testing begins

CONTROLS

11. Controls (AM and PM)

- a. Check sterility of dilution blanks, agar, and Petri dishes used for each group of samples
- b. Expose a poured plate to air with cover completely removed during plating for 15 min; timer used
 - 1. The air control plate must be the first plate poured immediately before samples are shaken and must be located such that it is in the area of the plating activity (not off to the side)
 - 2. After incubation, air plate(s) shall contain ≤ 15 colonies

3. Take and record corrective actions for air control plate(s) with >15 colonies

c. Instrument control, see item 8.c

d. Determine if loop is free rinsing by preparing a rinse control plate after every 20 samples plated

e. After all samples in a series have been tested, discharge a final rinse to a control plate

f. Between sample series on the same day, properly protect the loop from contamination; repeat 8.b & c at the start of the next series

g. Maintain records

h. Include information on bench sheet, work sheet or report sheet(s)

DILUTING SAMPLES

12. Sample Agitation

a. When appropriate, wipe top of unopened containers with sterile, ethyl alcohol-saturated cloth

b. Before removal of any portion, mix raw milk sample(s) by shaking 25 times in 7 sec with a 1 ft movement (containers approx $\frac{3}{4}$ full); use within 3 min

13. Inoculating Plates

a. Dip loop into sample (avoid foam and bubbles) to bend in shank and withdraw vertically from surface three times in 3 sec with uniform movement of 2.5 cm

b. Raise cover of Petri dish (just high enough to insert loop), insert loop and depress plunger causing sterile dilution buffer to flow down the shank, across charged loop washing measured 0.001 mL of sample into dish

c. Do not depress plunger so rapidly that buffer fails to flow across loop

14. Pouring Agar

a. After dispensing test portions, promptly pour 10-12 mL agar into each plate of series, or 12-15 mL where agar weight loss is a problem that cannot be corrected by other actions (Documentation must be kept to indicate that this is a routine practice; amount poured to match agar weight loss test)

1. Lift cover of Petri dish just high enough to pour agar _____
2. After agar is poured, thoroughly and evenly mix agar and test portion in Petri dish(es) _____
 - a. Agar solidification to occur within 10 min _____
 - b. Do not stack plates prior to solidification _____

INCUBATION

15. 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 PLC plates at $32\pm 1^{\circ}\text{C}$ for 48 ± 3 hours _____

COUNTING COLONIES

16. Counting Aids (see CP items 16 & 17) _____

- a. Count colonies with Quebec dark-field model or equivalent with satisfactory grid plate (see CP item 16) _____
- b. Hand tally (see CP item 17) _____

17. Counting, Recording and Computing PLC _____

- a. After incubation count all colonies on plates _____
- b. Where impossible to count at once, store plates at $0.0-4.5^{\circ}\text{C}$ for not longer than 24 hr (avoid as a routine practice) _____
- c. Record results of sterility and control tests _____
- d. Record number of colonies on the plate counted _____
- e. When possible, select spreader free colony plates with 25-250 colonies and count all colonies including those of pinpoint size _____
 1. Use higher magnification if necessary to distinguish colonies from foreign matter _____
 2. Examine edge of Petri dishes for colonies _____

- f. Spreaders _____
 - 1. Count colonies on representative portion only when colonies are well distributed and area covered or repressed does not exceed 25% of plate _____
 - 2. Do not count if repressed growth area > 25% of plate area _____
 - 3. When spreaders must be counted, count each as a single colony _____
 - 4. Count chains/spreaders from separate sources as separate colonies _____
 - 5. If 5% of plates are more than 25% covered by spreaders, take Immediate steps to eliminate and resolve problem _____
- g. 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 colonies 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 _____
- h. If plate yields < 25 colonies, record actual number _____
- i. If the plate shows no colonies, record count as 0 _____
- j. Multiply number of colonies (or estimated number if necessary) by 1,000 _____

18. Identifying Counting Errors _____

- a. Perform monthly counting for PLC _____
 - 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. If only one analyst, replicate counts agree within 8% of one another; maintain records _____

REPORTING

19. Reporting – refer to CP, item 34.b.2.d

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

- a. Report computed count as Plate Loop Count/mL (PLC/mL) when taken from a plate in the 25-250 range
- b. Report PLC plate counts of 0 to 24 as < 25,000 Estimated PLC (EPLC)
- c. When colonies on plate exceed 100/sq. cm, compute count by multiplying 100 x dilution factor x area of plate in sq. cm and report as > computed Count Estimated (EPLC)
- d. If computed count from PLC plate is > 250, report as Estimated PLC (EPLC)
- e. If for any reason, an entire plate is not counted, the computed count is reported as Estimated (EPLC)
- f. 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)
- g. If the plate from a sample has excessive spreader growth, report as spreaders (SPR)
- h. If a laboratory accident renders a plate uncountable, report as laboratory accident (LA)