

**STANDARD PLATE AND COLIFORM COUNT
AGAR POUR PLATE METHODS
IMS #2 (SPC), IMS #21 (CPC)**

[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]

MEDIA PREPARATION

2. **Media Preparation (reference agars/broth from CP items 14, 27, 28 & 29)** _____

a. Temperature Control (TC) used for each test agar type _____

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, hold melted agar at $45 \pm 1^\circ\text{C}$ _____

5. Record agar temperature with other control information _____

6. Agar should be discarded if not used within 3 hours after tempering _____

7. Avoid prolonged exposure to high temperatures during and after melting; establish lab protocol _____

c. Violet Red Bile Agar (VRB) _____

1. Boil for at least 1 min, but no more than 2 min. Do not autoclave. _____

2. Promptly place in a circulating water bath to temper; hold melted agar at $45 \pm 1^\circ\text{C}$ _____

3. Record agar temperature with other control information _____

- 4. Agar should be discarded if not used within 3 hours after tempering _____
- d. Brilliant Green Lactose Bile Broth (BGB) _____
 - 1. Examine Durham/fermentation tubes for presence of air bubbles _____
 - 2. If air bubbles cannot be removed from tubes; DO NOT USE _____

PROCEDURE

3. Work Area _____

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

4. Selecting Dilutions _____

- a. Standard Plate Count (SPC) _____
 - 1. Plate two decimal dilutions per sample _____
 - 2. Select dilutions that would be expected to yield one plate with 25-250 colonies _____
 - a. Raw milk is normally diluted to 1:100 and 1:1000 _____
 - b. Finished products are normally diluted to 1:10 and 1:100 _____
 - 3. SPC not performed on cultured or acidified products _____
- b. Coliform Plate Count (CPC) _____
 - 1. For pasteurized fluid milk samples, 1 mL direct and/or decimal dilutions as appropriate _____
 - 2. For samples other than milk (item 11) and dry milk products (item 12) distribute 10 mL of a 1:10 dilution among three plates _____

5. Identifying Plates _____

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

CONTROLS

6. Controls (AM and PM)

- a. Check sterility of dilution blanks, agar, Petri dishes, and pipets/tips 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. Maintain records
- d. Include information on bench sheet, work sheet or report sheet(s)

DILUTING SAMPLES

7. Sample Agitation

- a. When appropriate, wipe top of unopened containers with sterile, ethyl alcohol-saturated cloth
- b. Before removal of any portion or sub-samples, thoroughly mix contents of each container
 - 1. Mix raw milk sample(s) by shaking 25 times in 7 sec with a 1 ft movement (containers approx $\frac{3}{4}$ full)
 - 2. Mix retail milk samples by inverting containers top to bottom, then bottom to top (a complete half circle or 180 degrees) without pausing, 25 times
- c. Remove test portion within 3 min of sample agitation

8. Dilution Agitation

- a. Before removal of any portion, shake each dilution bottle 25 times in 7 sec with a 1 ft movement
- b. Remove test portion within 3 min of dilution agitation

- c. Mechanical shakers may be used only if a laboratory provides validation data on a specific unit. Data must pass validation criteria _____

PLATING

9. Sample & Dilution Measurements, Pipets _____

- a. Use separate sterile pipets for the initial transfers from each container, adjusting pipets in pipet container without touching the pipets _____
- b. Do not drag pipet tip over exposed exterior of pipets in pipet container _____
- c. Do not drag pipet across lip or neck of sample container or dilution blank _____
- d. Insert pipet not more than 2.5 cm (1") below sample surface or dilution surface (avoid foam and bubbles) _____
- e. Using pipet aid, draw test portion above pipet graduation mark and remove pipet from liquid (mouth pipetting not permitted) _____
- f. Adjust test volume to mark with lower side of pipet:
 - 1. In contact with inside of sample container (above the sample surface) _____
 - 2. Or, in contact with inside of dilution blank neck or area above buffer on straight-walled container _____
 - 3. Ensure excess liquid does not adhere when pipet is removed from the sample container or dilution blank _____
- g. For dilutions, dispense test portion to dilution blank (with lower side of pipet in contact with neck of dilution blank, or area above buffer on straight-walled containers) with column drain of 2-4 sec _____
- h. Gently lift cover of Petri dish just high enough to insert pipet _____
- i. Hold pipet at approximately a 45° angle with tip touching dish _____
- j. Release sample or dilution portion to Petri dish (with lower side of pipet in contact with plate) with column drain of 2-4 sec _____
 - 1. Using pipet aid, blow out the last drop of undiluted sample, away from main part of sample _____
 - 2. On diluted samples, touch pipet tip once against dry spot on dish bottom _____
 - a. When depositing 0.1mL, do not re-touch to dry area _____

- k. Discard pipets into disinfectant **OR** dispose into biohazard bags or containers to be sterilized (using this method of disposal does not require placing into disinfectant first)

10. Sample & Dilution Measurements, Pipettors [for electronic pipettors, follow manufacturer instructions] Mechanical _____ Electronic _____

- a. Each day before use, vigorously depress plunger 10x to redistribute lubrication and assure smooth operation (mechanical pipettors)
- b. Before each use, examine pipettor to assure that no liquid is expelled from the pipettor nose-cone (contaminated), if fouling is detected do not use until cleaned as per manufacturer recommendation
- c. Use separate sterile tip for the initial transfers from each container
- d. Depress plunger to first stop (mechanical pipettors)
- e. Do not drag tip/barrel across lip or neck of sample container or dilution blank, and do not allow pipettor barrel within sample container
- f. Insert tip approximately 0.5-1.0 cm below sample or dilution surface (avoid foam and bubbles)
- g. With pipettor vertical, slowly and completely release plunger on mechanical pipettor; do not lay pipettor down once sample is drawn up, use vertical rack or charging stand if necessary
- h. Touch off lower side of tip:
 - 1. To inside of sample container above the sample surface, excess liquid not adhering to tip
 - 2. Or to the inside of dilution blank neck or area above buffer on straight-walled containers, excess liquid not adhering to tip
 - a. For dilutions, hold pipettor nearly vertical with lower side of tip touching neck of dilution blank (or area above buffer on straight-walled containers), dispense test portion to blank by slowly depressing plunger to stop (mechanical pipettor)
 - 3. For two (2) stop pipettors, depress plunger to second stop with tip remaining in contact with dilution blank
- i. Gently lift cover of Petri dish just high enough to insert tip with pipettor approximately vertical to dish
 - 1. Release sample or dilution portion onto plate with tip slightly above but not in contact with the plate by slowly depressing plunger completely

- a. For two (2) stop pipettors, depress plunger to second stop _____
 - b. Do not touch off pipettor tip(s) to Petri dish _____
 - c. Optionally, deposit samples with pipettor capable of making a 1:10 dilution in the tip _____
- j. Discard tips into disinfectant **OR** dispose into biohazard bags or containers to be sterilized, (using this method of disposal does not require placing into disinfectant first) _____

11. Samples Other than Milk _____

- a. Weigh 11 g aseptically into a 99 mL dilution blank heated to 40-45°C _____

12. Dry Milk Product Samples _____

- a. Weigh 11 g aseptically into a 99 mL dilution blank heated to 40-45°C _____
 - 1. Use standard dilution blank _____
 - 2. Or, 2.0 % sodium citrate blank (pH<8.0) for relatively insoluble sample (e.g. whey) _____
- b. Wet sample completely with gentle inversions _____
- c. Let soak a minimum of 2 min; shake 25 times in 7 sec with a 1 ft movement; use within 3 min of agitation _____

13. Pouring Agar _____

- a. After dispensing test portions, promptly pour 10-12 mL of agar into each plate of series, or 12-15 mL for > 1 mL portion/plate or 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 _____
- b. For dry milk product sample(s), overlay plate with 3-5 mL PCA or SMA _____
- c. For coliform count, overlay plate with 3-4 mL VRB _____

INCUBATION

14. 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 SPC plates at $32\pm 1^{\circ}\text{C}$ for 48 ± 3 hours (dry milk products for 72 ± 3 hours)
- d. Incubate Coliform plates at $32\pm 1^{\circ}\text{C}$ for 24 ± 2 hours

COUNTING COLONIES

15. Counting Aids (see CP items 16 and 17)

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

16. Counting, Recording and Computing SPC

- a. After incubation, count all colonies on selected 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 dilutions used and number of colonies on each plate counted
- e. When possible, select spreader free 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. If consecutive plates yield 25-250 colonies, count all colonies on plates from both dilutions
- g. 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 _____
- h. If there is no plate yielding 25-250 colonies, use plate having nearest to 250 colonies _____
- i. If plates from all dilutions exceed 250 colonies, estimate counts as follows _____
1. Count colonies in portions representative of distribution and estimate total _____
 2. Where there are < 10 colonies/sq. cm, count colonies in 12 squares, selecting 6 consecutive squares horizontally across the plate and six 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 _____
- j. If plates from all dilutions yield < 25 colonies each, record actual number in lowest dilution _____
- k. If all plates from a sample show no colonies, record count as 0 _____
- l. Multiply number of colonies (or estimated number if necessary) by the reciprocal of the dilution _____
1. If consecutive dilutions yield 25-250 colonies, compute count using formula below _____

$$N = \Sigma C / [(1 \times n_1) + (0.1 \times n_2)]d$$

- Where,
- N = number of colonies per milliliter or gram
 - ΣC = sum of all colonies on all plates counted
 - n1 = number of plates in lower dilution counted
 - n2 = number of plates in next highest dilution counted
 - d = dilution from which the first counts were obtained

Example: 1:100 = 244 colonies

1:1,000 = 28 colonies

$$\begin{aligned} N &= (244 + 28) / [(1 \times 1) + (0.1 \times 1)]0.01 \\ &= 272 / [1.1]0.01 \\ &= 272 / 0.011 \\ &= 24,727 [25,000 \text{ (reported)}] \end{aligned}$$

Note: In the NCIMS Program the denominator will always be 0.11 for 1:10 dilutions and 0.011 for 1:100 dilutions

17. Counting, Recording and Computing CPC

- a. After incubation, count all colonies on selected plates
- b. Where impossible to count at once, store plates at 0.0-4.5°C for not longer than 24 hr (avoid as a routine practice)
- c. Confirmation of colonies
 - 1. Pick 10% up to 10 representative colonies per plate with relative percentages of each colony type and inoculate into BGB; incubate for 48±3 hours at 32±1°C
 - 2. Gas production at any time during the incubation is considered a confirmed test
 - 3. Record the number of picked colonies and the number of colonies that produced gas (if necessary calculate % confirmed and multiply by total number of colonies)
- d. If no colonies appear on plate(s), record count as 0
- e. If there are 1-154 colonies on a plate, record number counted
- f. If >154 colonies develop on the highest dilution plate, record number as >150
- g. When multiple plates of a dilution are used, sum counts of plates
- h. Multiply number of colonies (or estimated number if necessary) by the reciprocal of the dilution

18. Identifying Counting Errors

- a. Perform monthly counting for SPC
 - 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 _____

REPORTING

19. Reporting (see 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. SPC _____

1. Report computed count as Standard Plate Count/mL or /g (SPC/mL or SPC/g) when taken from plate(s) in the 25-250 range _____
2. Report SPC plate counts of 0 to 24 as < 25 times the reciprocal of the dilution and report as Estimated SPC (ESPC) _____
3. When colonies on SPC plates 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 (ESPC) _____
4. If computed counts from SPC plates are >250 , report as Estimated SPC (ESPC) _____

b. CPC _____

1. Report count as Coliform Plate Count (confirmed)/mL or /g when taken from plate(s) in the 1-154 range (CPC/mL) _____
2. If no colonies appear on coliform plates, report as < 1 times the reciprocal of the dilution and report as Estimated (ECPC) _____
3. Counts from coliform plates > 154 are reported as > 150 Estimated Coliform Count (ECPC) _____
4. If for any reason, an entire plate is not counted, the computed count is reported as Estimated (ECPC) _____

c. 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) _____

d. If all plates from a sample have excessive spreader growth, report as spreaders (SPR) _____

e. If a laboratory accident renders a plate uncountable, report as laboratory accident (LA) _____