U.S. FOOD & DRUG ADMINISTRATION

# Bacteriological Analytical Manual

## Chapter 3: Aerobic Plate Count

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# Authors

Guodong Zhang, Martine Ferguson, Robert J. Blodgett (ret.)

For additional information, contact Guodong Zhang.

# Revision History

March 2025: The suitable colony counting range updated from 25-250 to 15-300 per plate. Mercury thermometers are replaced by non-mercury thermometers, with accuracy checked with a thermometer certified by the National Institute of Standards and Technology (NIST). APC calculation software is added for convenience. More literature has been added to reflect the modifications.

Original Source: Bacteriological Analytical Manual, Edition 8, Revision A, 1998. Chapter 3.

# Introduction

The aerobic plate count (APC) is intended to indicate the level of microorganism in a product. See references (6), (5), (10), (13), (3), (14), and (11) for detailed descriptions and explanations. Detailed procedures for determining the APC of foods using pour plate method have been described by the American Public Health Association (APHA) (1, 2), the Association of Official Analytical Chemists (AOAC) (3), the United States Department of Agriculture (USDA) (14) and the International Organization for Standardization (ISO) (11). Colony counting range (per plate) using pour plate method is 30-300 by USDA (14) and AOAC (3); 25-250 by APHA (1) and APHA (2) and 15-300 by ISO (11). In most scientific publications, scientists presented as low as 0 colony count per plate. Considering ISO is internationally accepted in regulatory affairs and most publications do present results with low colony numbers per plate, in this protocol, the suitable colony counting range is 15-300 per plate. The automated spiral plate count method outlined below conforms to the AOAC Official Methods of Analysis, sec. 977.27 (4).

# Conventional Plate Count Method

## A. Equipment and materials

1. The work bench should be leveled, clean and with ample surface. The laboratory condition should be free of dust and drafts. The microbial density of air in working area, measured in fallout pour plates taken during plating, should not exceed 15 colonies/ plate during 15 min exposure.

2. Storage space, free of dust and insects and adequate for protection of equipment and supplies

3. Petri dishes, glass, or plastic (at least 15 × 90 mm)

4. Pipets with pipet aids (no mouth pipetting) or pipettors, 1, 5, and 10 ml, graduated in 0.1 ml units

5. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps

6. Pipet and petri dish containers, adequate for protection

7. Circulating water bath, for tempering agar, thermostatically controlled to 45 ± 1°C

8. Incubator, 35 ± 1°C; milk, 32 ± 1°C

9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate

10. Tally register

11. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water (R11); milk, 99 ± 2 ml

12. Plate count agar (standard methods) (M124)

13. Refrigerator, to cool and maintain samples at 0-5°C; milk, 0-4.4°C

14. Freezer, to maintain frozen samples from -15 to -20°C

15. Thermometers with appropriate range; accuracy checked with a thermometer certified by the National Institute of Standards and Technology (NIST)

## B. Procedure for analysis of frozen, chilled, precooked, or prepared foods

Using separate sterile pipets, prepare decimal dilutions of 10-2, 10-3, 10-4, and others as appropriate of food homogenate (see Chapter 1 for sample preparation) by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 s. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 s if it stands more than 3 min before it is pipetted into petri dish. Add 12-15 ml plate count agar (cooled to 45 ± 1° C) to each plate within 15 min of original dilution. **For milk samples, pour an agar control, pour a dilution water control and pipet water for a pipet control.**

Add agar to the latter two for each series of samples. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials, e.g., flour and starch. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify. Do not stack plates when pouring agar or when agar is solidifying. Invert solidified petri dishes, and incubate promptly for 48 ± 2 h at 35°C.

# C. Maximum likelihood method background and examples

## 1. Background

The number of colonies is assumed to be Poisson distributed with mean APC density (CFU/ml), $\mu$. Maximum likelihood (ML) is used to estimate the Poisson mean APC (CFU/ml), $\mu$, using the plates for which exact counts are available and the too numerous to count (TNTC) plates. See reference (11) and the Appendix for detailed descriptions and explanations. The likelihood ratio method is used to assess confidence limits (10).

The lower bound is a threshold above which inhibition is suspected for colonies of the target microbe, e.g. 300 colonies.

When there are no TNTC plates, the ML estimate of APC equals a weighted average of the counts as in examples (2.1a, 2.1b, 2.1c, 2.2a), below.

APC = *CFU/ml* = $\sum_{i=1}^{j} w_i \; R_i$ where $w_i = V_i / \sum_{i=1}^{j} V_i$ and $R_i = N_i / V_i$

Where $N_i$ is the colony count (CFU) on plate i

$V_i$ is the volume of original homogenate received by plate i

$R_i$ is the concentration or CFU/ml in the $i^{th}$ plate = $N_i / V_i$

$w_i$ denotes the weight value of plate i (see below)
j is the number of countable plates

The amount of the original homogenate put on a plate, V, equals its dilution ratio times the size of its inoculum. For example: with dilution ratio of 1:100 and an inoculum size of 1 ml,

V=amount of the original homogenate = (1/100)*1ml = 0.01ml.

R, the concentration (CFU/ml), equals the plate colony count (CFU) divided by its homogenate volume (V). For example, if plate i has $N_i$ =150 CFU and $V_i$ =0.01 ml, then $R_i$ =150/0.01=15,000 CFU/ml.

The contribution of each plate to the APC estimate must be weighted (w) by the proportion of the original homogenate that it received. In other words, $w_i$ equals the amount of the original homogenate the $i^{th}$ plate received divided by the total amount of the original homogenate all plates received. For example, for a two-plate experiment, if plate 1 has a homogenate value $V_1$ =0.001 ml and plate 2 has a homogenate volume $V_2$=0.01 ml, and the weight $w_1$ for plate 1 = 0.001/(0.001+0.01)= 0.091

## 2. Examples of APC Calculations

## 2.1 Examples from Common Scenarios

**Example 2.1a: Four plates and two dilutions**

| | Colony Count (N, CFU) | Dilution Ratio | Inoculum Size | Original Homogenate (V, ml) | **Ratio** (R, CFU/ml) |
|---|---|---|---|---|---|
| Plate 1 | 144 CFU | 1:100 | 1 ml | 0.01 ml | **14,400** |
| Plate 2 | 118 CFU | 1:100 | 1 ml | 0.01 ml | **11,800** |
| Plate 3 | 26 CFU | 1:1000 | 1 ml | 0.001 ml | **26,000** |
| Plate 4 | 20 CFU | 1:1000 | 1 ml | 0.001 ml | **20,000** |

The total amount of the original homogenate received by all plates is 2*0.01ml+2*0.001ml = 0.022 ml.
The APC estimate, using MLE, equates to the weighted average of plate R's. The weight for plates 1 and 2 is 0.01/0.022=10/22. The weight for plates 3 and 4 is 0.001/0.022=1/22.

APC = $\Sigma w_i R_i$. = 10/22 (14,400 *CFU/ml)* + 10/22 (11,800 *CFU/ml*)
+ 1/22 (26,000 *CFU/ml*) + 1/22 (20,000 *CFU/ml*) = 308,000/22 *CFU/ml*=14,000 *CFU/ml*.

**Example 2.1b: Two plates with the same dilution**

| | Colony Count | Dilution | Inoculum Size | Original Homogenate | Ratio |
|---|---|---|---|---|---|
| Plate 1 | 27 CFU | 1:100 | 1 ml | 0.01 ml | 2700 |
| Plate 2 | 51 CFU | 1:100 | 1 ml | 0.01 ml | 5100 |

The amount of the original homogenate received by all plates is 0.02 ml. The weight for each plate is 0.01/0.02 = 1/2.

Concentration = 1/2 (2700 *CFU/ml)* + 1/2 (5100 *CFU/ml*)
= 7800/2 *CFU/ml* = *3900* CFU/ml.

**Example 2.1c: Two plates with different dilutions**

| | Colony Count | Dilution | Inoculum Size | Original Homogenate | Ratio |
|---|---|---|---|---|---|
| Plate 1 | 20 CFU | 1:100 | 1 ml | 0.01 ml | 12,000 |
| Plate 2 | 3 CFU | 1:1000 | 1 ml | 0.001 ml | 23,000 |

The amount of the original homogenate received by all plates is 0.011 ml. The weight for plate 1 is 0.01/0.011=10/11. The weight for plate 2 is 0.001/0.011=1/11.

Concentration = 10/11 (12,000 *CFU/ml*) + 1/11 (23,000 *CFU/ml*)
= 143,000/11 *CFU/ml* = 13,000 *CFU/ml.*

## 2.2 Examples from Other Scenarios

**Example 2.2a: One plate**

For one plate the estimated concentration equals its colony count divided by the amount of the original homogenate in its inoculum.

| | Colony Count | Dilution | Inoculum Size | Original Homogenate | Ratio |
|---|---|---|---|---|---|
| Plate 1 | 27 CFU | 1:100 | 1 ml | 0.01 ml | 2700 |

Concentration = (27 CFU)/(0.01 ml) = 2700 *CFU/ml*.

**Example 2.2b: Several plates, one TNTC**

If a count of visible colonies is available, then this is the lower bound. If not, then the lower bound is a threshold above which inhibition is suspected for colonies of the target microbe.

When TNTC plates are present, the ML estimate no longer equals a weighted average of the colony concentrations. Instead, the estimate includes all TNTC plates and must be obtained through software, such as R (see section 4 below and Appendix for details).

| | Colony Count | Dilution | Inoculum Size | Original Homogenate | Ratio |
|---|---|---|---|---|---|
| Plate 1 | 300(TNTC) | 1:100 | 1 ml | 0.01 ml | >30,000 |
| Plate 2 | 127 CFU | 1:1000 | 1 ml | 0.001 ml | 127,000 |
| Plate 3 | 121 CFU | 1:1000 | 1 ml | 0.001 ml | 121,000 |

The estimated concentration is 124,000 CFU/ml (8).

**Example 2.2c: Several TNTC plates**

The estimated concentration changes as more TNTC plates adjust it.

| | Colony Count | Dilution | Inoculum Size | Original Homogenate | Ratio |
|---|---|---|---|---|---|
| Plate 1 | >300(TNTC) | 1:100 | 1 ml | 0.01 ml | >30,000 |
| Plate 2 | >300 (TNTC) | 1:100 | 1 ml | 0.01 ml | >30,000 |
| Plate 3 | 28 CFU | 1:1000 | 1 ml | 0.001 ml | 28,000 |
| Plate 4 | 20 CFU | 1:1000 | 1 ml | 0.001 ml | 20,000 |

The estimate of concentration is 31,000 CFU/ml (8).

## 3. Recording counts

1. Plates with spreading colonies. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth so that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreaders. When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the APC.

2. Plates with no CFU. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 15-300 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident.

3. Rounding of APC result. Report only the first two significant digits. Round off to two significant figures only at the time of conversion to APC. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is odd and round down when the second digit is even. Report APC as estimated results when the colony counts are outside the range of 15-300 per plate.

**Examples**

| Calculated Count | APC |
|---|---|
| 12,700 | 13,000 |
| 12,400 | 12,000 |
| 15,500 | 16,000 |
| 14,500 | 15,000 |

## 4. APC calculations using software

The R package, MPN (8) and its function ‘apc’, calculates the APC (CFU per mL) via maximum likelihood estimation and accompanying confidence interval. Adjustments for TNTC plates are included in the ‘apc’ function. A web application implementing the R package APC function is available at https://cfsan-connect.fda.gov/APC/

# Spiral Plate Method

The spiral plate count (SPLC) method for microorganisms in milk, foods, and cosmetics is an official method of the APHA (2) and the AOAC (3). In this method, a mechanical plater inoculates a rotating agar plate with liquid sample. The sample volume dispensed decreases as the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

## A. Equipment and materials

1. Spiral plater
2. Spiral colony counter with special grid for relating deposited sample volumes to specific portions of petri dishes
3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)
4. Disposable micro beakers, 5 ml
5. Petri dishes, plastic or glass, 150 × 15 mm or 100 × 15 mm
6. Plate count agar (standard methods) (M124)
7. Calculator (optional), inexpensive electronic hand calculator is recommended
8. Polyethylene bags for storing prepared plates
9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)
10. Sterile dilution water
11. Syringe, with Luer tip for obstructions in stylus; capacity not critical
12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.

## B. Preparation of agar plates.

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for pre-pouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70°C into each petri dish. Let agar solidify on level surface with poured plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°C. Bring pre-poured plates to room temperature before inoculation.

## C. Preparation of samples

As described in Chapter 1, select that part of sample with smallest amount of connective tissues and avoid any visible fat layer.

## D. Description of spiral plater

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. Operator with minimum training can inoculate 50 plates per h. Within range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of pre-poured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area. Studies have shown the method to be proficient not only with milk (7) but also with other foods(12, 15).

## E. Plating procedure

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope cover slip against face of stylus tip; if cover slip plane is parallel at about l mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for 48 ± 3 h at 35 ± 1°C.

## F. Sterility controls

Check sterility of spiral plater for each series of samples by plating sterile dilution water. CAUTION: Pre-poured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4°C for longer than one month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.

# G. Counting grid

1. **Description.** Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by 4 arcs labeled 1, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between 2 arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. Spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward center of plate.

2. **Calibration.** The volume of sample represented by various parts of the counting grid is shown in operator's manual that accompanies spiral plater. Grid area constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of $10^6$-$10^3$ cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all Incubate both sets of plates for 48 ± 3 h at 35± 1°C. Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described in H, below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

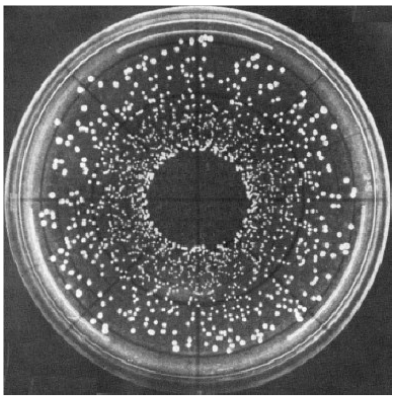
$$\text{Volume (ml) for grid area} = \frac{\text{Spiral Colonies counted in area}}{\text{Baterial count/ml (APC)}}$$

$$\text{Volume (ml)} = \frac{31 + 30 \text{ colonies}}{4.1\times10^4 \text{ bateria/ml}} = 0.0015 \text{ ml}$$

To check total volume dispensed by spiral plater, weigh amount dispensed from stylus tip. Collect in tared 5 ml plastic beaker and weigh on analytical balance (± 0.2 mg).

Figure 1. 10 cm plate, area (3b)

$$\frac{(31+31) \text{ colonies}}{0.0015 \text{ ml}} = 4.1\times10^4$$



## H. Examination and reporting of spiral plate counts.

Counting rule of 20. After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. In this counting procedure, numbers such as 3b, 4c (Fig. I) refer to area segments from outer edge of wedge to designated arc line. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Example of spirally inoculated plate (Fig. I) demonstrates method for determining microbial count. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted. See example under Fig. I.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas. Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described in I-D, above. Report counts as SPLC or estimated SPLC per ml.

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## Appendix:

The likelihood function is

$$L = \left[\prod_{i=1}^{j} \frac{(\bar{\mu}V_i)^{N_i}}{N_i!} exp(-\bar{\mu}V_i)\right] \left\{\prod_{i=j+1}^{k+j} \left[1 - \Gamma\left(N_{L,i} - 1, \bar{\mu}V_i\right)\right]\right\}$$

Countable plates (including plates with 0 counts)

TNTC plates

Where $N_i$ is the count in sample i
$V_i$ is the volume of sample i
$N_{L,i}$ is the lower bound to the uncountable (TNT) range in sample i.
There are j countable plates and k TNTC plates for which only a lower bound is available.

The likelihood function is maximized with respect to $\bar{\mu}$.