Designation: D5465 – 16 (Reapproved 2020)

Standard Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods[1]

This standard is issued under the fixed designation D5465; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 These practices cover recommended procedures for counting colonies and reporting colony-forming units (CFU) on membrane filters (MF) and standard pour and spread plates.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D5259 Test Method for Isolation and Enumeration of Enterococci from Water by the Membrane Filter Procedure

D5392 Test Method for Isolation and Enumeration of ***Escherichia coli*** in Water by the Two-Step Membrane Filter Procedure

D6161 Terminology Used for Microfiltration, Ultrafiltration, Nanofiltration, and Reverse Osmosis Membrane Processes

D6974 Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels—Filtration and Culture Procedures

E2563 Practice for Enumeration of Non-Tuberculosis ***Mycobacteria*** in Aqueous Metalworking Fluids by Plate Count Method

2.2 *Other Standards:*

APHA 9215 Heterotrophic Plate Count[3]

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminologies D1129 and D6161.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *colony forming unit (CFU), n—in microbiology,* a visible mass of cells (algae, bacteria, or fungi) originating from either an individual cell or cluster of cells that have been placed onto or dispersed into a solid or semi-solid nutrient medium and subsequently incubated under prescribed conditions.

3.2.1.1 *Discussion*—Prescribed growth conditions can include, but are not limited to: growth medium pH and nutrient composition, incubation temperature, incubation environment (for example: gas mixture, pressure and relative humidity), and incubation interval. Any given set of growth conditions will select for the culture recovery of a fraction of a sample's microbiome and against the culture recovery of the balance of that microbiome.

3.2.1.2 *Discussion*—Recognizing that all culture test methods are selective, CFU data invariably underestimate the population densities of viable microbes in samples tested by those methods. Moreover, incomplete disaggregation of masses of microbial cells during sample preparation contributes to decreasing the ratio of CFU to total viable microbes in the sample.

3.2.1.3 *Discussion*—Colonies normally become visible to the naked eye only after approximately 1 billion cells have amassed. Assuming that the colony derived from a single cell, it requires approximately 30 generations for a single cell to proliferate to a mass of 1 billion cells. Consequently, the time lapse between inoculation and detection of a CFU is directly dependent on the generation time(s) of taxa present in sample. Moreover, because colony diameter increases as the cells

[1] These practices are under the jurisdiction of ASTM Committee D19 on Water and are the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American Public Health Association (APHA), 800 I St., NW, Washington, DC 20001, http://www.apha.org.

within the colony continue proliferate, in samples containing microbes with different generation times, colonies of microbes with longer generation times are likely to be eclipsed (and therefore undetected) by colonies of microbes with shorter generation times. This phenomenon further contributes to CFU data underestimating total viable cell numbers.

4. Significance and Use

4.1 Numerous ASTM test methods and practices (for example: Test Methods D5259 and D5392, and Practices D6974 and E2563) report colony counts as their measured parameter.

4.2 These practices provide a uniform set of counting, calculating, and reporting procedures for ASTM test methods in microbiology.

	Section
A—Counting Colonies on Membrane Filters	6
B—Counting Colonies on Pour Plates	7
C—Counting Colonies on Spread Plates	8

4.3 The counting rules provide a best attainable estimate of microorganisms in the sample, since the samples cannot be held and reanalyzed at a later date.

5. Hazards

5.1 The analyst/technician must know and observe the normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials.

PRACTICE A—COUNTING COLONIES ON MEMBRANE FILTERS

6. Procedure

6.1 The grid lines help in counting the colonies. Count them for the organism of interest following a preset plan such as that shown in Fig. 1. Some colonies will be in contact with the grid lines. A suggested procedure for reducing error in counting is shown in Fig. 2. Count the colonies in the squares indicated by the arrows.

6.2 The fluorescent lamp tube should be nearly parallel with and directly over the membrane filter. Ideally, the lamp is attached to and surrounds the objective nosepiece of the stereoscopic microscope. Count the colonies individually, even if they are in contact with each other. The technician must learn to recognize the difference between two or more colonies that have grown into contact with each other and the single, irregularly shaped colonies that sometimes develop on membrane filters. The latter colonies are usually associated with a fiber or particulate material and conform to the shape and size of the fiber or particulates. Colonies that have grown together almost invariably show a very fine line of contact.

6.3 Count the colonies with a stereoscopic (dissecting) microscope that provides a magnification of at least 10 to 15×.

6.4 See Table 1 for guidance on acceptable counting limits.

6.5 *Calculation of Results*—Select the membrane with the number of CFU in the acceptable range and calculate the count/reporting volume according to the following general formula:

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{volume of sample filtered in mL}} \times 1 \quad (1)$$

$$\text{CFU/100 mL} = \frac{\text{colonies counted}}{\text{volume of sample filtered in mL}} \times 100 \quad (2)$$

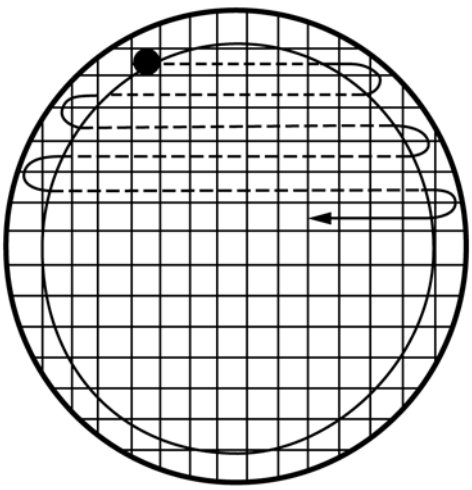

FIG. 1 Colony Counting Pathway (The Inner Circle Indicates the Effective Filtering Area; the Dashed Line Indicates the Pathway)

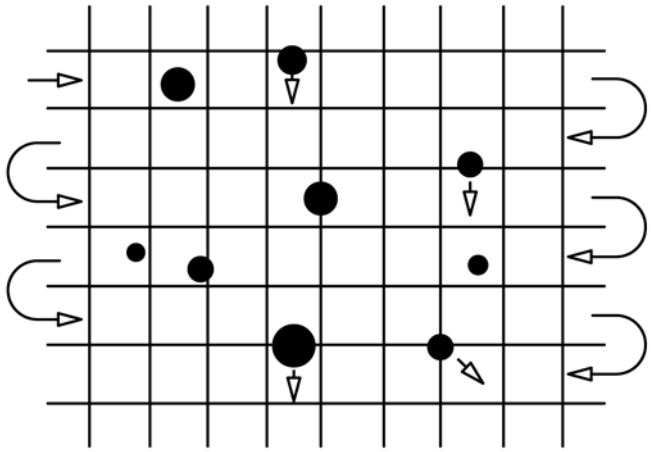

FIG. 2 Enlarged Portion of Grid-Marked Square of Filter (Colonies in Contact with Gridlines are Counted in Squares Indicated by the Arrow)

TABLE 1 Recommended Counting Range for High-Density Samples[A]

Microorganism	Colony Count	Remarks
Total coliform bacteria, MF, 47 mm	20 to 60	Upper limit, 200 colonies of all types
Fecal coliform bacteria, MF, 47 mm	20 to 60	
Fecal streptococci, MF, 47 mm	20 to 100	
Heterotrophic spread plate count	20 to 200	
Heterotrophic pour plate count	30 to 300	Upper limit, 300

[A] Colony counts below or exceeding the limits cited above must be identified as outside of this range.

6.6 *Counts Within the Acceptable Limits:*

6.6.1 The acceptable range of counts on a membrane for samples that are diluted is a function of the organism/test combination as given in Table 1.

6.6.2 Assume that the filtration of volumes of 80, 20, 5, and 1 mL produced counts of 250, 60, 15, and 4, respectively. Do not count the colonies on all filters. Select the MF(s) within the acceptable counting range and then limit the actual counting to such membranes. After selecting the best MF for counting, in this case that with a 60-CFU count, the analyst counts CFU according to the procedures shown in Fig. 1 and Fig. 2 and applies the general formula as follows:

$$\frac{60}{20} \times 1 \text{ (or} \times 100) = 3 \text{ (or } 300) \quad (3)$$

Report as 3 CFU/mL or 300 CFU/100 mL.

6.6.3 If there are acceptable counts on replicate plates, carry the counts independently to final reporting units, and then calculate the arithmetic mean of these counts to obtain the final reported value. For example, 1 mL volumes produced counts of 26 and 36 CFU/mL or counts of 2600 and 3600 CFU/100 mL:

$$\frac{26+36}{2} = 31 \quad (4)$$

Report as 31 CFU/mL.

$$\frac{2600+3600}{2} = 3100 \quad (5)$$

Report as 3100 CFU/100 mL.

6.6.4 If more than one dilution produced acceptable counts, count the colonies for each dilution, carry the counts independently to final reporting units, and then average for the final reported value. For example, assume that volumes of 0.3, 0.1, 0.03, and 0.01 mL produced colony counts of too numerous to count (TNTC), 75, 30, and 8, respectively. In this example, two volumes, 0.1 and 0.03, produce colonies in an acceptable counting range. Carry each MF count independently to a count/mL or count/100 mL:

$$\frac{75}{0.1} \times 1 \text{ (or} \times 100) = 750 \text{ CFU/mL (or } 75\,000 \text{ CFU/100 mL)} \quad (6)$$

$$\frac{30}{0.03} \times 1 \text{ (or} \times 100)$$

$$= 1\,000 \text{ CFU/mL (or}100\,000 \text{ CFU/100 mL)}$$

Then calculate the arithmetic mean of these counts to obtain the final reported value:

$$\frac{750+1\,000}{2} = 875 \quad (7)$$

Report as 880 CFU/mL.

$$\frac{75\,000+100\,000}{2} = 87\,500 \quad (8)$$

Report as 88 000 CFU/100 mL.

6.6.5 For finished drinking water samples only, countable membranes may contain from one colony to the upper limit of the test (see Table 1). Count the target colonies/volume filtered. Calculate and report the number of CFU/100 mL.

6.7 *Counts Outside Acceptable Limits:*

6.7.1 Zero counts recorded as < values/volume filtered are acceptable for sample volumes of 100 mL or more.

6.7.2 If full-volume samples are filtered, such as 25, 50, or 100 mL, and the resulting count is 1 to 19 colonies, these values are acceptable although <20. The count is adjusted to 1 or 100 mL for reporting. For example, a count of 1 colony/25 mL is adjusted:

$$\frac{1}{25} \times 1 \text{ (or} \times 100) = <1 \text{ (or } 4) \quad (9)$$

Report as <1 CFU/mL (or 4 CFU/100 mL).

6.7.3 If all MF counts are <20/volume filtered, select the most nearly acceptable count (for non-drinking waters). For example, assume a count in which sample volumes of 1, 0.3, and 0.01 mL produced CFU counts of 14, 3, and 0, respectively. No CFU count falls within recommended limits here. Calculate on the basis of the most nearly acceptable plate count, 14, and report with a qualifying remark:

$$\frac{14}{1.0} \times 1 \text{ (or} \times 100) = 14 \text{ (or } 1400) \quad (10)$$

Report as estimated count: 14 CFU/mL (or 1400 CFU/100 mL).

6.7.4 If counts from all membranes are zero, calculate using the count from largest filtration volume. For example, sample volumes of 25, 10, and 2 mL produced CFU counts of 0, 0, and 0, respectively, and no actual calculation is possible, even as an estimated report. Calculate the number of CFU per 100 mL that would have been reported if there had been one CFU on the filter representing the largest filtration volume; thus:

$$\frac{<1}{25} \times 1 \text{ (or} \times 100) = <0.04 \text{ (or}<4) \quad (11)$$

Report as <0.04 CFU/1 mL (or <4 CFU/100 mL).

6.7.4.1 If 100 mL is sampled and the results are zero:

$$\frac{<1}{100} \times 1 \text{ (or} \times 100) = <0.01 \text{ (or}<1) \quad (12)$$

Report as <1 CFU/1 mL (or <1/100 mL).

6.7.5 If all membrane counts are above the upper limit for the method (see Table 1), calculate the count with the smallest volume filtered. For example, assume that volumes 1, 0.3, and 0.01 mL produced CFU counts of TNTC, 150, and 110 CFU. Since all of the CFU counts are above the recommended limit, use the CFU count from the smallest sample volume filtered and estimate as follows: