Designation: D5465 – 16

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 and health practices and determine the applicability of regulatory limitations prior to use.*

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:*

9215 Heterotrophic Plate Count[3]

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms use in this standard, see Terminologies D1129 and D6161.

3.1.2 *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.

3.1.2.1 *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.

3.1.2.2 *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.

3.1.2.3 *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 online from http://standardmethods.org/store/ProductView.cfm?ProductID=102, or from American Public Health Association (APHA), Standard Methods for the Examination of Water and Wastewater, 800 I Street, NW Washington, DC 20001, http://www.apha.org.

***A Summary of Changes section appears at the end of this standard**

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.

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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:

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

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

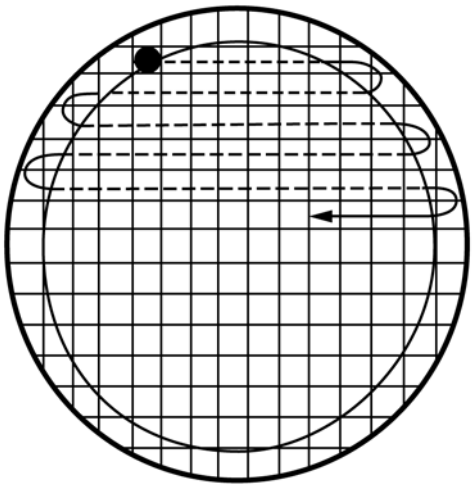

FIG. 1 Colony Counting Pathway (The Inner Circle Indicates the Effective Filtering Area; the Dashed Line Indicates the Pathway)

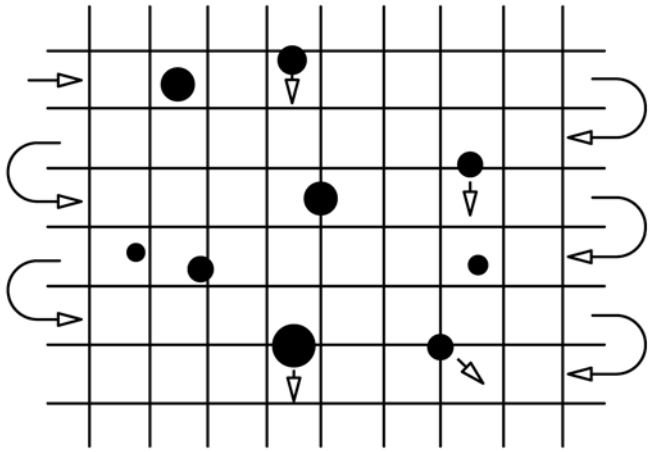

FIG. 2 Enlarged Portion of Grid-Marked Square of Filter (Colonies in Contact with Gridlines are Counted in Squares Indicated by the Arrow)

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:

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

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:

$$\frac{26+36}{2} = 31 \tag{4}$$

Report as 31 CFU/mL.

$$\frac{2600+3600}{2} = 3100 \tag{5}$$

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:

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

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

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

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

$$\frac{750+1\,000}{2} = 875 \tag{7}$$

Report as 880 CFU/mL.

$$\frac{75\,000+100\,000}{2} = 87\,500 \tag{8}$$

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:

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

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:

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

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:

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

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:

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

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:

$$\frac{110}{0.01} \times 1 \left(\text{ or} \times 100 \right) = 11\,000 \left(\text{or } 1\,100\,000 \right) \quad (13)$$

Report as estimated count: 11 000 CFU/mL (or 1 100 000 CFU/100 mL).

6.7.6 If the colonies are too numerous to count, use the upper limit count with the smallest filtration volume. Assume, in the example given in 6.7.5, that volumes 1.0, 0.3, and 0.01 mL all produced too many colonies to count individually, and that the laboratory bench record showed TNTC. For example, with total coliform counts, use 80 CFU (upper limit count for total coliform) as the basis of calculation with the smallest filtration volume; thus:

$$\frac{80}{0.01} \times 100 = 800\,000 \quad (14)$$

Report as >800 000 CFU/100 mL.

6.7.6.1 Alternatively, small sample volumes or sample dilutions can be used to minimize the TNTC problem. Replicates of smaller sample volumes or sample dilutions may be filtered and the results combined. If the MF technique is not applicable, the most probable number (MPN) is useful.

6.7.7 If there is no result because of confluency, laboratory accident, etc., report as no result and specify the reason.

6.8 *Reporting Results*—Report bacterial densities as CFU/mL or CFU/100 mL of sample, as the method requires.

6.9 *Verification*— A verified MF test establishes the validity of organism identification on a differential or selective medium. Obviously, verification is not applied to nonselective media.

6.9.1 A percent verification can be determined for any colony validation test:

$$\frac{\text{colonies meeting verification test}}{\text{colonies subjected to verification}} \times 100 = \text{percent verification} \quad (15)$$

6.9.2 Adjust the original count according to the percent of CFU verified. The verification of all colonies up to at least 10 is recommended. This verification number is required for all positive MF results from potable waters.

6.9.3 Verification is also recommended for establishing quality control in research with new test waters, procedures, or technicians; for identifying atypical colonies; and as support for data used in legal actions. The worker is cautioned not to apply the percent of verification determined for one sample to other samples. The careful worker may also pick non-typical colonies and follow the verification procedure to determine whether false negative responses do occur.

PRACTICE B—COUNTING COLONIES ON POUR PLATES

7. Procedure

7.1 *Manual Counting*—Count the colonies with the aid of magnification under uniform light, using a tally. Alternately, use a Quebec-type colony counter equipped with a guide plate ruled in square centimeters. Do not mistake particles of undissolved medium, sample, or precipitated matter in plates for pinpoint colonies.

7.2 *Automatic Counters*—Do not use plates having scratched surfaces, stippled agar surfaces, or particles or air bubbles in the agar or plates with fingerprints or films on the bottoms of the plates. The colony counters should yield counts within ±10 % of those obtained manually, 90 % of the time.

7.3 Count plates containing between 30 and 300 colonies. Count the number of colonies below 30 if the sample is an exceptionally clean water, such as a drinking water. Count all colonies, including those of pinpoint size, and record the sample volume and dilution used.

7.4 Report the pour plate count as CFU per mL.

PRACTICE C—COUNTING COLONIES ON SPREAD PLATES

8. Procedure

8.1 Count plates containing between 20 and 200 colonies. The maximum number of colonies per spread plate is fewer than that for other plate techniques because surface colonies are larger than subsurface colonies and crowding can result at lower count levels. If the water sample is exceptionally clean, count the actual number of colonies below 20. Report as CFU/mL or CFU/100 mL, depending on the use of the data.

9. Significant Figures

9.1 To prevent false precision in the reporting of counts, the counts must be limited to the digit(s) known definitely plus one digit that is in doubt. These combined digits are termed the significant figures (SFs).

9.1.1 For example, if an analyst reports a plate count of 124 to three SFs, he is indicating that he is certain of the first two digits, 1 and 2, but is uncertain whether the last digit is 3, 4, or 5. If the analyst were reporting that same number to two SFs, he would report the first figure, 1, as certain, the second figure, 2, as uncertain, and the third figure, 4, as unknown. Large counts of 1200, 12 000, and 12 000 000 contain only two significant figures. Of course, zeros can be significant in actual counts of 10, 60, 105, and so forth.

9.2 In plate count and MF methods, the number of significant digits that can be reported is dictated by the test method itself, as follows: within the acceptable counting range of the test method itself, that is, 20 to 60, 20 to 80, 20 to 100, or 30 to 300, the actual number of colonies observed is the best estimate of the true density. The number of SFs is equal to the number of colonies (see Table 2).

9.3 *Rounding Off Counts*—Since plate counts must be limited to the number of SFs obtainable by test method, the non-zero number that is not significant should be treated by the standard scientific convention.

TABLE 2 Number of Significant Figures Reported

Actual Colony Count	Pour Plate/Spread Plate Method	Membrane Filtration Method
1 to 9	1 SF	1 SF
10 to 99	2 SFs	2 SFs
100 to 300	3 SFs	...

9.3.1 If the insignificant digit is below five, replace it with a zero, for example, 3530 becomes 3500.

9.3.2 If the insignificant digit is five, round the preceding significant digit to the nearest even number, for example, with two SFs, 3450 becomes 3400, and 3550 becomes 3600.

9.3.3 If the insignificant digit is greater than five, drop the digit and increase the preceding significant number by one, for example, 3480 becomes 3500.

10. Keywords

10.1 calculating results; counting colonies; membrane filter methods; pour plate methods; reporting results; rounding off; significant figures; spread plate methods; verification of counts

SUMMARY OF CHANGES

Committee D19 has identified the location of selected changes to this standard since the last issue (D6465 – 93 (2012)) that may impact the use of this standard. (Approved June 1, 2016.)

(1) Added new Sections 2 and 3, and renumbered subsequent sections accordingly.