



Designation: D5916 – 96 (Reapproved 2002)

Standard Test Method for Detection and Enumeration of *Clostridium perfringens* from Water and Extracted Sediments by Membrane Filtration (MF)¹

This standard is issued under the fixed designation D5916; 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 This test method can enumerate *Clostridium perfringens* spores and vegetative cells from marine water, sediment, wastewater, ambient water, and drinking water. Since *C. perfringens* spores are present in large numbers in human and animal wastes and are resistant to wastewater treatment practices, extremes in temperature, and environmental stress, they are an indicator of present fecal contamination as well as a conservative tracer of past fecal contamination. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

1.2 *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

D1193 Specification for Reagent Water

D1888 Methods of Test for Particulate and Dissolved Matter in Water³

D2777 Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water

D3370 Practices for Sampling Water from Closed Conduits

D3863 Test Method for Retention Characteristics of 0.40 to 0.45- μ m Membrane Filters Used in Routine Filtration Procedures for the Evaluation of Microbiological Water Quality

D3870 Practice for Establishing Performance Characteris-

tics for Colony Counting Methods in Microbiology (Withdrawn 2000)⁴

D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E200 Practice for Preparation, Standardization, and Storage of Standard and Reagent Solutions for Chemical Analysis

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *Clostridium perfringens*—in this test method, *C. perfringens* is defined as an obligate anaerobic gram-positive, spore forming, nonmotile bacillus, 0.9–1.3 by 3.0–9.0 μ m in size that ferments sucrose, ferments lactose with stormy gas production, does not ferment cellobiose, and produces acid phosphatase. *Clostridium perfringens* also produces toxins which cause gas gangrene and gastroenteritis.

3.2.2 *spores*—*C. perfringens* produces single oval subterminal spores less than 1.0 μ m in diameter during adverse conditions. Sporulation can also occur in the intestinal tract. The endospore that develops is a highly refractile body formed within the cell. Spores are notably resistant to heat, drying, and chemical disinfectants which would kill the vegetative forms of *C. perfringens*. This resistance to unfavorable conditions preserves the organisms for long periods of time.

4. Summary of Test Method

4.1 Appropriate volumes of water are passed through membrane filters (MF) that retain the bacteria present in the sample. The MFs are placed on mCP agar modified by Armon and Payment (1)⁵ from the medium of Bisson and Cabelli (2) and are incubated anaerobically at 44.5°C for 24 h. The yellow, straw-colored *C. perfringens* colonies which turn dark pink to magenta on exposure to ammonium hydroxide are counted and

¹ This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology. Current edition approved Feb. 10, 1996. Published April 1996. DOI: 10.1520/D5916-96R02.

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 Withdrawn.

⁴ Withdrawn. The last approved version of this historical standard is referenced on www.astm.org.

⁵ The boldface numbers in parentheses refer to the list of references at the end of this test method.

reported *C. perfringens* colony forming units (CFU) per 100 mL. Because of the selectivity of the mCP medium, presumptive counts are normally reported for routine monitoring purposes. If verification is desired, colonies are confirmed by anaerobic growth in thioglycollate, a positive gram stain reaction and stormy fermentation of iron milk, and mCP counts adjusted based on the percent confirmation.

4.2 For sediment analyses, 1 to 10 g of wet sediment is weighed, water added, and mixed by vortex and sonication. After settling, the water layer is analyzed as described in 4.1.

4.3 Verification of counts is not required. However, if verification is desired, colonies can be confirmed by anaerobic growth in thioglycollate, a positive gram stain reaction and stormy fermentation of iron milk. The mCP counts may be adjusted based on the percent confirmation.

5. Significance and Use

5.1 *Clostridium perfringens* is a strict obligate anaerobe that is found in fecal material. Under moderately adverse conditions these organisms produce endospores that can withstand extreme environmental conditions and are conservative tracers of past and present pollution in fresh and marine waters and sediments.

6. Interferences

6.1 Waters containing sediment, large quantities of colloidal or suspended materials such as iron, manganese, alum floc, or algae can clog the filter pores and prevent filtrations or cause the development of spreading bacterial colonies which may mask target colonies and prevent accurate counting.

6.2 When bacterial densities are high, a smaller sample volume or sample dilution can be filtered to minimize the interference of turbidity or high background (nontarget) bacterial densities. Replicates of smaller sample volumes or dilutions of sample may be filtered and the results combined. However, the membrane filter techniques may not be applicable to highly turbid waters with low *Clostridium* densities.

6.3 Toxic materials such as metals, phenols, acids, caustics, chloramines, and other disinfection by-products may also adversely affect recovery of *Clostridium* vegetative cells on the MF. The most probable number (MPN) method should be considered as an alternative procedure for these samples (3). Use with discretion.

7. Apparatus

7.1 *Microscope*, stereoscopic, wide-field type, with magnification of 10 to 15 $\times$.

7.2 *Microscope Lamp*, that produces diffuse light from a cool white fluorescent or tungsten lamp adjusted to give maximum visibility.

7.3 *Counting Device*, hand tally or electronic.

7.4 *Pipet Container*, stainless steel or aluminum, for sterilization and storage of glass pipets.

7.5 *Pipets*, sterile T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 *Graduated Cylinders*, 100 to 1000 mL, covered with aluminum foil or kraft paper and sterilized.

7.7 *Membrane Filtration Units* (filter base and funnel), glass, plastic, or stainless steel, wrapped with aluminum foil or kraft paper and sterilized.

7.8 *Ultraviolet Unit*, for disinfecting the filter funnel between filtrations (optional).

7.9 *Line Vacuum*, electric vacuum pump or aspirator as a vacuum source. In an emergency or in the field, a hand pump or a syringe can be used if equipped with a check valve to prevent the return flow of air.

7.10 *Flask, Vacuum*, usually 1 L, with appropriate tubing. Filter manifolds to hold a number of filter bases are optional.

7.11 *Flask, Safety Trap*, placed between the filter flask and the vacuum source.

7.12 *Forceps*, straight or curved, with smooth tips to permit handling of filters without damage.

7.13 *Thermometers*, 0 to 50 $\pm$ 0.2°C and 0 to 100 $\pm$ 0.5°C, which have been checked against a National Institute of Standards and Technology (NIST) certified thermometer or against one traceable to NIST.

7.14 *Petri Dishes*, sterile, plastic or glass, 50 by 9 mm with tight-fitting lids, or 60 by 15 mm with loose-fitting lids.

7.15 *Bottles*, milk dilution, borosilicate glass or plastic, screw-cap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.

7.16 *Test Tubes*, 150 by 20 mm, borosilicate glass or disposable plastic.

7.17 *Caps*, aluminum or autoclavable plastic, for 150 by 20 mm test tubes.

7.18 *Centrifuge Tubes*, 50 mL, polycarbonate.

7.19 *Inoculation Loops*, (3 mm diameter) and needles, nichrome or platinum wire, 26 B & S gage, in suitable holders. Disposable applicator sticks or plastic loops are acceptable alternatives to inoculation loops.

7.20 *Waterbaths*, that maintain 46 to 48°C for tempering agar and 60 $\pm$ 0.5°C for heat shock.

7.21 *Sonication Unit*, to break up soil and sediment particles prior to analyses.⁶

7.22 *Anaerobic System* (anaerobic jar, reaction chamber, hydrogen/carbon dioxide disposable generator and anaerobic indicator), or any other system capable of producing the appropriate anaerobic conditions to support the growth of the organisms.⁷

7.23 *Incubator*, with temperature capability of 44.5 $\pm$ 0.2°C, large enough to hold the anaerobic system.

7.24 *Filter Paper*, circular, 11 cm, Whatman 40 or 110, or equivalent, for separation of mCP agar during anaerobic incubation.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on

⁶ Bronson Sonifier, 500 W, or Tekmar Sonic Disrupter, 500 W with 3 mm tip set at 18 W, or equivalent.

⁷ BBL 60460 or BBL 60466 GasPak Anaerobic System with BBL 70308 Disposable Hydrogen and Carbon Dioxide Generator Envelopes, BBL Microbiological Systems, Cockeysville, MD 21030, or equivalent.

Analytical Reagents of the American Chemical Society where such specifications are available.⁸ Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. Use microbiological grade agar in preparation of culture media. Whenever possible, use commercial culture media as a means of quality control.

8.2 Purity of Water—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type II of Specification **D1193**.

8.3 Ethanol 95 %, Pure, for preparation of acetone alcohol and for flame-sterilization of forceps.

8.4 Membrane Filters, sterile, white, grid marked, 47 mm diameter, with $0.45 \pm 0.02 \mu\text{m}$ pore size or other pore sizes for which the manufacturer provides data demonstrating equivalency.

8.5 Buffered Dilution and Rinse Water:

8.5.1 Stock Phosphate Buffer Solution—Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of water. Adjust pH to 7.2 with 1 N NaOH and bring to 1000 mL with water. Dispense aseptically into screw-cap bottles and autoclave at 121°C for 15 min. Alternatively, sterilize by filtration through a $0.2 \mu\text{m}$ pore membrane filter and dispense aseptically in sterile screw-cap bottles. Store in refrigerator and handle aseptically. If cloudiness, a marked change in pH, or other evidence of contamination appears, discard the stock. Confirm that pH is 7.2 ± 0.5 before use.

8.5.2 Magnesium Chloride Solution—Dissolve 81.4 g of hexahydrate magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) in 1000 mL of water. Mix well and sterilize by filtration or autoclave at 121°C for 15 min. Store in refrigerator and handle aseptically. If cloudiness or other evidence of contamination occurs, discard the stock solution.

8.5.3 Phosphate Buffered Dilution Water—Add 1.25 mL of stock phosphate buffer solution and 5 mL of magnesium chloride solution to 1000 mL of water in a volumetric flask and mix well. Dispense dilution water in amounts which will provide 99 ± 2 mL after sterilization in screw-cap dilution bottles, or in larger volume containers for use as rinse water if desired. Autoclave immediately at 121°C for 15 min. Autoclave larger volumes for longer periods as appropriate. Alternatively, sterilize by filtration through a $0.2 \mu\text{m}$ pore membrane filter and dispense aseptically into sterile screw-cap bottles.

8.6 Ferric Chloride Solution—Weigh out 4.5 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and dissolve in 100 mL of water. Filter, sterilize, and store in refrigerator.

8.7 Phenolphthalein Diphosphate Solution—Weigh out 0.5 g of phenolphthalein diphosphate and dissolve in 100 mL of water. Filter, sterilize, and store in refrigerator.

8.8 Indoxyl β -D Glucoside Solution—Weigh out 0.06 g of indoxyl β -D glucoside and dissolve in 80 mL of water

(0.075 % solution). Sonicator in **7.21** can be used to speed dissolution. Filter, sterilize, and use in **8.9.2**.

8.9 mCP Agar; (modified) (1).

8.9.1 Composition/Litre:

Tryptose	30.0 g
Yeast extract	20.0 g
Sucrose	5.0 g
L-cysteine	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g
Bromocresol purple	0.04 g
Agar	15.0 g

8.9.2 Preparation—In a 1000 mL Erlenmeyer flask add medium ingredients from **8.9.1** to 900 mL water, stir, and heat to dissolve. Bring the pH to 7.6 with 1 N NaOH. Autoclave for 15 min at 121°C (15 lb pressure). Cool to 50°C . Add the following reagents aseptically and mix well:

D-cycloserine	0.4 g
Polymyxin B sulfate	0.025 g
4.5 % $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution	2.0 mL
0.5 % Phenolphthalein diphosphate solution	20.0 mL
0.075 % Indoxyl- β -D-glucoside solution	80.0 mL

8.9.3 Dispense 4 to 4.5 mL into each 50 by 9 mm petri plate using a sterile Cornwall syringe or Brewer pipette and allow to harden. Store agar inverted in a sealed plastic bag in a refrigerator at 4°C for no more than one month. It is recommended that the agar plates be stored in an anaerobic chamber in the refrigerator if possible.

8.10 Iron Milk Medium.⁹

8.10.1 Composition/Litre:

Fresh whole milk	1 L
Ferrous sulfate- $7\text{H}_2\text{O}$	1 g

8.10.2 Preparation—Dissolve ferrous sulfate in 50 mL water. Add slowly to 1 L milk and mix with magnetic stirrer. Dispense 11 mL medium into culture tubes. Autoclave at 118°C for 12 min.

8.11 Fluid Thioglycollate Medium.¹⁰

8.11.1 Composition/Litre:

L-cysteine	0.5 g
Agar (granulated)	0.75 g
NaCl	2.5 g
Dextrose (anhydrous)	5.0 g
Yeast extract	5.0 g
Tryptone	15.0 g
Sodium thioglycollate	0.5 g
Resazurin	0.001 g

8.11.2 Preparation—Suspend 29.5 g of medium in 1 L of water. Mix thoroughly and heat to boil for 1 to 2 min or until solution is complete. Final pH is 7.1 ± 0.1 . Dispense 15 mL portions into culture tubes. Cap and autoclave at 121°C for 15 min. Store tubes in the dark at room temperature. Do not refrigerate. If medium becomes oxidized (if more than 30 % of medium is pink), reheat once only in boiling water bath and cool before use.

⁸ Reagent Chemicals, American Chemical Society Specifications, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

⁹ FDA Bacteriological Analytical Manual, 7th Ed., AOAC International, Arlington, VA, 1992, pp. 476–477, iron milk medium (modified).

¹⁰ Fluid Thioglycollate Medium (BBL 11259), Benton-Dickinson Microbiology Systems, Cockeysville, MD; (Difco 0432-02-6), Difco Laboratories, Detroit, MI, or equivalent.