



Designation: D 5392 – 93 (Reapproved 2000)

Standard Test Method for Isolation and Enumeration of *Escherichia Coli* in Water by the Two-Step Membrane Filter Procedure¹

This standard is issued under the fixed designation D 5392; 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 describes a membrane filter (MF) procedure for the detection and enumeration of *Escherichia coli*, a bacterium found exclusively in the feces of humans and other warm-blooded animals. The presence of these microorganisms in water is an indication of fecal pollution and the possible presence of enteric pathogens. These bacteria are found in water and wastewater in a wide range of densities. The detection limit of this procedure is one colony forming unit (CFU) per volume filtered.

1.2 This test method has been used successfully with temperate fresh and marine ambient waters, and wastewaters. It is the user's responsibility to ensure the validity of this test method for waters of other types.

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.* For specific hazard statements, see Section 9.

2. Referenced Documents

2.1 ASTM Standards:

- D 1129 Terminology Relating to Water²
- D 1193 Specification for Reagent Water²
- D 3370 Practices for Sampling Water from Closed Conduits²
- D 3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology³
- D 5465 Practice for Determining Microbial Counts from Waters Analyzed by Plating Methods³

3. Terminology

3.1 *Definitions*—For definitions of terms used in this test method, refer to Terminology D 1129.

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 April 15, 1993. Published September 1993.

² *Annual Book of ASTM Standards*, Vol 11.01.

³ *Annual Book of ASTM Standards*, Vol 11.02.

3.2 Definitions of Terms Specific to This Standard:

3.2.1 *Escherichia coli* (*E. coli*)—a species of bacteria that is a member of the total coliform group and known to originate in the feces of warm-blooded animals.

3.3 Performance Characteristics (Practice D 3870)

3.3.1 *precision*—the degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95 % confidence limits of the mean computed from the results of a series of controlled determinations.

3.3.2 *bias*—the persistent positive or negative deviation of the average value of the test method from the assumed or accepted true value.

3.3.3 *specificity*—the ability of a test method to select or distinguish, or both, the target bacteria in the same water sample; the specificity characteristic of the method is usually reported as the percent of false positive and false negative results.

3.3.4 *upper counting limit (UCL)*—that colony count above which there is an unacceptable counting error; the error may be due to overcrowding or antibiosis.

3.3.5 *accuracy*—the proportion of the observed count to the true density of a sample.

4. Summary of Test Method

4.1 This two-step test method⁴ provides a direct count of bacterial colonies developing on the surface of the filter when placed on a selective nutrient medium. The water sample is passed through a membrane filter that retains the bacteria. After filtration, the membrane filter containing the bacterial cells is placed on a selective, differential medium, mTEC. The membrane on the medium is first incubated at 35°C for 2 h so that injured or stressed bacteria can be resuscitated and then the medium is incubated at 44.5°C for 22 h. Following incubation the filter is transferred to a filter pad saturated with urea substrate. After 15 min all yellow or yellow-brown colonies are counted with the aid of 10 to 15 \times magnifier and a fluorescent lamp.

⁴ Dufour, A., Strickland, E., and Cabelli, V., "Membrane Filter Method for Enumerating *Escherichia coli*," *Appl. and Environ. Microbiol.* 41:1152–1158, 1981.

5. Significance and Use

5.1 This test method is useful for measuring recreational water quality and chlorinated wastewaters, although it can be used for any water suspected of contamination by fecal wastes of warm-blooded animals. The significance of finding *E. coli* in recreational water samples, especially samples obtained from fresh recreational waters, is that there is a risk of gastrointestinal illness, directly related to the *E. coli* density, associated with swimming.⁵

5.2 Since small or large volumes of water or dilutions thereof can be analyzed by the MF technique, a wider range of levels of *E. coli* in water can be detected and enumerated than with other methods.

6. Interferences

6.1 Water with high levels of colloidal or suspended materials can clog the membrane filter pores and prevent filtration. Also, suspended materials cause spreading colonies that could interfere with target colonies and thereby prevent accurate counting.

6.2 Smaller sample size or sample dilution can be used to minimize the interference of turbidity or high background (nontarget) bacterial densities. Replicates of sample volumes or dilutions of sample may be filtered and the results combined. However, the membrane filter techniques may not be applicable to high turbid waters with low bacterial densities.

6.3 In some samples, chemicals may have toxic effects on the target organism.

7. Apparatus

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

7.2 *Microscope Lamp*, producing diffuse light from a cool, white fluorescent lamp adjusted to give maximum visibility.

7.3 *Counting Device*, hand tally or electronic.

7.4 *Pipet Container*, stainless steel, aluminum, or borosilicate glass, for 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 sterile.

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

7.8 *Ultraviolet Unit*, for sterilizing the filtration unit (optional).

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

7.10 *Flask*, filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 *Forceps*, straight or curved, with smooth tips to handle filters without damage.

7.12 *Thermometer*, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one traceable to a NIST thermometer.

7.13 *Petri Dishes*, sterile, plastic, 50 by 12 mm, with tight-fitting lids.

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

7.15 *Inoculation Loops*, at least 3-mm diameter, and needles, nichrome or platinum wire, 26 B & S gage, in suitable holders.

7.16 *Incubator*, air, maintained at $35 \pm 0.5^\circ\text{C}$.

7.17 *Incubator, Waterbath*, maintained at 44 to 46 $^\circ\text{C}$.

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

7.19 *Test Tubes*, 75 by 10 mm, borosilicate glass.

7.20 *Caps*, aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.21 *Test Tubes*, screw-cap, borosilicate glass, 125 by 16 mm or other appropriate size.

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 Analytical Reagents of the American Chemical Society where such specifications are available.⁶ 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. The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media and reagents as 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 III of Specification **D 1193**.

8.3 *Ethanol, Methanol, or Isopropanol*, denatured, in a small, wide-mouth container, for flame-sterilization or pipets.

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 Water/Buffered Rinse Water:*

8.5.1 *Composition Per Litre:*

Sodium Dihydrogen Phosphate (NaH_2PO_4)	0.58 g
Sodium Monohydrogen Phosphate (Na_2HPO_4)	2.50 g
Sodium Chloride	8.50 g

8.5.2 *Preparation*— Dissolve the ingredients in 1 L of water in a flask and dispense in appropriate amounts for dilutions in

⁵ Cabelli, V. J., Dufour, A. P., Levin, M. A., McCabe, L. J., and Haberman, P. W., "Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches," *American Journal of Public Health*, 69:690–696, 1979.

⁶ *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.