Designation: D5259 – 14

Standard Test Method for Isolation and Enumeration of Enterococci from Water by the Membrane Filter Procedure[1]

This standard is issued under the fixed designation D5259; 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 covers a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. The enterococci, which include *Enterococcus faecalis* (*E. faecalis*), *E. faecium*, and their varieties are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, enterococci in water are 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 is one colony forming unit (CFU)/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 untested types.

1.3 The values stated in SI units are to be regarded as the standard.

1.4 *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:*[2]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D3370 Practices for Sampling Water from Closed Conduits

D3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology (Withdrawn 2000)[3]

3. Terminology

3.1 *Definitions:*

3.1.1 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 *Enterococcus*—in this test method, *Enterococcus* species are those bacteria that produce red to maroon colonies with black or reddish-brown precipitate on underside, after incubation on mE agar and subsequent transfer to EIA medium.

3.2.1.1 *Discussion*—Enterococci include *E. faecalis, E. faecium, E. avium*, and their variants.

4. Summary of Test Method

4.1 The procedure given in this test method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter.[4] A water sample is filtered through the membrane that retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective, medium, mE agar, and incubated for 48 h at 41°C, then transferred to EIA agar and held at 41°C for 20 min. Enterococci develop as red to maroon colonies with black or reddish-brown precipitate on the underside of the filter.

5. Significance and Use

5.1 The enterococci are indicators of the bacteriological quality for potable water, shellfish growing waters, ambient, and recreational waters. A direct relationship between swimming, associated gastroenteritis, and enterococci has been established through epidemiological studies and marine and fresh water bathing beaches. These studies have led to the development of criteria that can be used to establish bathing water standards based on established health-water quality relationships.

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[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.

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

[4] 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* , Vol 69, 1979, pp. 690–696.

5.2 Since small or large volumes of water or dilutions thereof, can be analyzed by the membrane filter technique, a wide range of levels of enterococci in water can be enumerated and detected.

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 (non-target) bacterial densities. Replicates of smaller sample volumes or dilutions of sample may be filtered and the results combined. If the membrane filter technique is not applicable, the most probable number (MPN) method for fecal streptococci is recommended, with verification.

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 15X.

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 tip delivery 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 disinfecting 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 or 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 an 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 1:10 dilutions.

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

7.16 *Incubator* maintained at 41 ± 0.5°C.

7.17 *Waterbath* maintained at 44 to 46°C for tempering agar.

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

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

7.20 *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.[5] 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.

8.1.1 The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control.

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

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

8.2 *Membrane Filters,* sterile, white, grid marked, 47 mm diameter, with 0.45 ± 0.02 µm pore size or other pore sizes for which the manufacturer provides data demonstrating equivalency.

8.3 *Buffered Dilution Water/Buffered Rinse Water:*

8.3.1 *Composition/Litre:*

Sodium dihydrogen phosphate (NaH_2PO_4)	0.58 g
Sodium monohydrogen phosphate (Na_2HPO_4)	2.50 g
Sodium chloride	8.50 g

8.3.2 *Preparation*—Dissolve the ingredients in 1 L of water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes or into containers for use as rinse water, or both. Autoclave after preparation at 121°C (15 lb pressure at sea level) for 15 min. The final pH should be 7.4 ± 0.2.

8.4 *mE Agar:*

[5] *Reagent Chemicals, American Chemical Society Specifications,* American Chemical Society, Washington, DC, www.chemistry.org. 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. Pharmacopeial Convention, Inc. (USPC), Rockville, MD, http://www.usp.org.