



Designation: D6503 – 24

Standard Test Method for Enterococci in Water Using Enterolert^{1,2}

This standard is issued under the fixed designation D6503; 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 simple procedure for the detection of enterococci in water and wastewater. It is based on IDEXX's patented Defined Substrate Technology (DST).² This product, Enterolert, utilizes a nutrient indicator that fluoresces when metabolized. It can detect these bacteria at one most probable number (MPN)/100 mL within 24 h. The presence of this microorganism in water is an indication of fecal contamination and the possible presence of enteric pathogens.

1.2 This test method can be used successfully with drinking water, source water, recreational (fresh and marine) water, wastewater, and bottled water. It is the user's responsibility to ensure the validity of this test method for waters of untested matrices.

1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this 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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *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.*

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 1, 2024. Published April 2024. Originally approved in 1999. Last previous edition approved in 2019 as D6503 – 19. DOI: 10.1520/D6503-24.

² Enterolert and Quanti-Tray are trademarks of IDEXX Laboratories, Inc., in Westbrook, ME. IDEXX Laboratories, Inc. is the sole source of supply of Enterolert and Quanti-Tray. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

2. Referenced Documents

2.1 *ASTM Standards:*³

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

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

D3370 Practices for Sampling Water from Flowing Process Streams

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 *enterococci, n*—a gram positive bacteria possessing the enzyme β -D-glucosidase, which cleaves the nutrient indicator and produces fluorescence under a long wave length (365 nm to 366 nm) ultraviolet (UV) light.

3.2.2 *most probable number (MPN), n*—a statistical method for determining bacterial density based on the Poisson distribution.

3.2.3 *presence-absence, n*—a term used to indicate if enterococci are present or absent in a water sample.

3.2.3.1 *Discussion*—It is a qualitative value, “yes” or “no” for reporting results.

3.2.4 *Quanti-Tray, n*—a system for the quantification of enterococci.²

3.2.4.1 *Discussion*—It consists of a sealer and trays which have multi-wells and can enumerate up to 2419 MPN/100 mL without dilution using the Quanti-Tray 2000 tray or it can detect up to 200.5 MPN/100 mL using the 51-well Quanti-Tray.

3.2.5 *snap pack, n*—a package containing Enterolert reagent for testing 100 mL sample either in the P/A format or quantitatively.

³ 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.

4. Summary of Test Method

4.1 This test method is used for the detection of enterococci, such as *E. faecium*, *E. faecalis* in drinking water, source water, recreational waters (marine water and fresh), wastewaters, and bottled water. When the reagent is added to the sample and incubated at $41\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ for 24 h and up to 28 h, Enterolert can detect these bacteria at 1 MPN/100 mL. Fluorescence is produced when enterococci metabolizes the nutrient indicator. Enterolert can be used as a presence-absence test or for quantification (5-tube, 10-tube MPN, 15-tube serial dilution or the Quanti-Tray² system).

5. Significance and Use

5.1 This test provides an easy and reliable method for the detection of enterococci in water within 24 h. For recreational water (fresh and marine) testing is performed to insure areas are safe for swimming. Enterolert also can be used for testing bottled water, wastewater, ground water, and drinking water.

6. Interferences

6.1 The presence of *Bacillus* spp. can interfere with the testing of marine water samples. To eliminate interference, a 1:10 dilution is required with sterile water (deionized or distilled).

7. Apparatus

7.1 *Ultraviolet Lamp*, 6 watt long wavelength or equivalent (365 nm to 366 nm).

7.2 $41\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$ *Incubator*, air or water bath.

7.3 *Vessels*, sterile, non-fluorescent.

7.4 *Quanti-Tray Sealer*.

7.5 *Quanti-Tray 51-well or Quanti-Tray 2000*.

8. Reagents and Materials

8.1 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification **D1193**, Type IV. Sterilize the water by either autoclaving or by sterile filtration (0.22 micron-filtered water).

8.2 *Enterolert Test Kit*.

9. Precautions

9.1 The analyst must observe the normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents and materials and while operating sterilization equipment and other equipment.

10. Sampling

10.1 Collect the sample as described in detail in the USEPA microbiological methods manual⁴ and in accordance with Practices **D3370**.

10.2 *Sample Storage Temperature and Handling Conditions*—Ice or refrigerate water samples at a temperature of $2\text{ }^{\circ}\text{C}$ to $8\text{ }^{\circ}\text{C}$ during transit to the laboratory. Use insulated containers to ensure proper maintenance of storage temperatures. Take care that sample bottles are not totally immersed in water during transit or storage.

10.3 *Holding Time Limitations*—Examine samples, as soon as possible, after collection. Do not hold samples longer than 8 h between collection and incubation of samples.

11. Quality Control Check

11.1 Check and record temperatures in incubators twice daily (separated by at least 4 h) when in use to ensure temperature is within stated limits.

11.2 Quality control should be conducted on each new lot of Enterolert. See package insert for the recommended quality control procedure, using American Type Culture Collection (ATCC) bacteria strains or equivalent. The following protocol is an example using the ATCC bacterial strains:

11.2.1 For each type of the ATCC bacterial strain listed below, streak the culture onto labeled TSA or blood agar plates and incubate at $35\text{ }^{\circ}\text{C}$ for 18 h to 24 h.

11.2.2 For each bacterial strain, touch a $1\text{ }\mu\text{L}$ loop to a colony and use it to inoculate a labeled test tube containing 5 mL of sterile deionized water. Close cap and shake thoroughly.

11.2.3 For each bacterial strain, take a $1\text{ }\mu\text{L}$ loop from the test tube (11.2.2) and use it to inoculate a labeled vessel containing 100 mL of sterile deionized water.

11.2.4 Follow the Enterolert presence/absence or MPN steps listed in Section 12, Procedure, to test these controls. Compare the test results to the following expected results:

Control	ATCC No.	Expected Result
<i>Enterococcus faecium</i>	335667	Fluorescence
<i>Serratia marcescens</i> (g, -)	43862	No fluorescence
<i>Aerococcus viridians</i> (g, +)	10400	No fluorescence

12. Procedure

NOTE 1—A 1:10 dilution is required for marine water samples or samples containing the presence of salt.

12.1 *Presence/Absence*—See package insert.

12.1.1 Samples should be brought to room temperature ($18\text{ }^{\circ}\text{C}$ to $30\text{ }^{\circ}\text{C}$).

12.1.2 Carefully separate one snap pack from the strip.

12.1.3 Tap the snap pack to insure that all of the powder is towards the bottom of the pack.

12.1.4 Open the pack by snapping back the top of the score line. Do not touch the opening of pack.

12.1.5 Add the reagent to a 100 mL water sample, which is in a sterile, transparent, non-fluorescent vessel.

12.1.6 Aseptically cap and seal the vessel.

12.1.7 Shake until dissolved.

12.1.8 Incubate Enterolert for 24 h and up to 28 h at $41\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$,

12.1.9 Read results at 24 h and up to 28 h. If the sample is inadvertently incubated over 28 h without observation, the following guidelines apply: Lack of fluorescence after 28 h is a valid negative test. Fluorescence after 28 h is an invalid result.

⁴ Bordner, R.H., Winter, J.A., and Scarpino, P.V., Eds., *Microbiological Methods for Monitoring the Environment, Water, and Wastes*, EPA-600/8-78-017.

12.1.10 Check for fluorescence by placing a 6 watt or equivalent 365 nm to 366 nm UV light within 5 in. of the sample in a dark environment. Be sure the light is facing away from your eyes and towards the vessel. If fluorescence is observed, the presence of enterococci is confirmed.

12.2 *MPN*—Quanti-Tray enumeration test procedure for 100 mL sample (see package insert).

12.2.1 Follow steps 12.1.1 – 12.1.7.

12.2.2 Pour the reagent sample into the Quanti-Tray avoiding contact with the foil tab and seal the tray according to the Quanti-Tray package insert.

12.2.3 Incubate for 24 h and up to 28 h at 41 °C ± 0.5 °C.

12.2.4 Follow the same interpretation instructions from 12.1.9 through 12.1.10, and count the number of positive wells.

Refer to the MPN table (see Table 1) provided with the Quanti-Tray to determine the MPN/100 mL.

12.3 *MPN*—5-tube × 20 mL, 10-tube × 10 mL and 15-tube serial dilution.

12.3.1 Follow 12.1.1 – 12.1.7.

12.3.2 Use sterile nonfluorescent tubes or transfer 20 mL of the reagent sample into five sterile non-fluorescent tubes or transfer 10 mL of the reagent sample into five sterile non-fluorescent tubes.

12.3.3 For 15 tube serial dilution.

12.3.4 Incubate for 24 h and up to 28 h at 41 °C ± 0.5 °C.

12.3.5 Follow 12.1.9 and 12.1.10 for interpretation.

12.3.6 Refer to the MPN tables (see Tables 2-5) to determine the MPN/100 mL.

TABLE 1 51-Well Quanti-Tray MPN Table

No. of Wells Giving Positive Reaction	MPN/100-mL Sample	95 % Confidence Limits	
		Lower	Upper
0	<1	0.0	3.7
1	1.0	0.3	5.6
2	2.0	0.6	7.3
3	3.1	1.1	9.0
4	4.2	1.7	10.7
5	5.3	2.3	12.3
6	6.4	3.0	13.9
7	7.5	3.7	15.5
8	8.7	4.5	17.1
9	9.9	5.3	18.8
10	11.1	6.1	20.5
11	12.4	7.0	22.1
12	13.7	7.9	23.9
13	15.0	8.8	25.7
14	16.4	9.8	27.5
15	17.8	10.8	29.4
16	19.2	11.9	31.3
17	20.7	13.0	33.3
18	22.2	14.1	35.2
19	23.8	15.3	37.3
20	25.4	16.5	39.4
21	27.1	17.7	41.6
22	28.8	19.0	43.9
23	30.6	20.4	46.3
24	32.4	21.8	48.7
25	34.4	23.3	51.2
26	36.4	24.7	53.9
27	38.4	26.4	56.6
28	40.6	28.0	59.5
29	42.9	29.7	62.5
30	45.3	31.5	65.6
31	47.8	33.4	69.0
32	50.4	35.4	72.5
33	53.1	37.5	76.2
34	56.0	39.7	80.1
35	59.1	42.0	84.4
36	62.4	44.6	88.8
37	65.9	47.2	93.7
38	69.7	50.0	99.0
39	73.8	53.1	104.8
40	78.2	56.4	111.2
41	83.1	59.9	118.3
42	88.5	63.9	126.2
43	94.5	68.2	135.4
44	101.3	73.1	146.0
45	109.1	78.6	158.7
46	118.4	85.0	174.5
47	129.8	92.7	195.0
48	144.5	102.3	224.1
49	165.2	115.2	272.2
50	200.5	135.8	387.6
51	>200.5	146.1	infinite