Designation: D8516 – 23

Standard Test Method for Quantification of Culturable Waterborne Bacteria Using a Defined Culture Medium Coated Plate[1]

This standard is issued under the fixed designation D8516; 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 simple procedure for the quantification of culturable, waterborne bacteria in potable water (drinking water, bottled water, and dental water, for example) and non-potable waters (cooling towers, for example).

1.1.1 The EasyDisc[2,3] plate format is designed to test 1 mL of a water sample on a 47 mm gridded plate containing a growth reagent embedded to the plate's inner surface.

1.1.2 Detection is based on colorimetric technology in which viable, aerobic, heterotrophic, waterborne bacteria grow when present in the water sample, displaying a color reaction which allows for a simplified visualization of colony growth.

1.2 Each plate can accurately detect up to 300 colony forming units per 1 mL (CFU/1 mL) of sample. To increase the quantification range, a sample dilution can be used. Adjust the CFU/mL result to reflect dilutions.

1.3 This test method can be used for potable (for example, drinking, bottled, and dental) waters and non-potable waters such as cooling tower waters. It is the user's responsibility to adhere to all requirements by local regulations and ensure the validity of this test method for waters other than those tested as part of the Interlaboratory Study (ILS).

1.4 The values stated in SI units are to be regarded as the standard. No other units of measurement are included in this standard.

1.5 *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.*

1.6 *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.*

2. Referenced Documents

2.1 *ASTM Standards:*[4]

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

2.2 *ISO Standard:*[5]

ISO 6222 Water quality—Enumeration of culturable micro-organisms—Colony count by inoculation in a nutrient agar culture medium

2.3 *Standard Methods for the Examination of Water and Wastewater:*[6]

9060 Samples

9215 Heterotrophic Plate Count

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this test method, refer to Terminology D1129.

3.1.2 *ambient temperature, n*—temperature of the surroundings, generally assumed to be 20 °C to 25 °C.

3.1.3 *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.

[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] EasyDisc is a registered trademark of IDEXX Laboratories, Inc.

[3] The sole source of supply of the apparatus known to the committee at this time is IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, ME, 04092, USA. 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,[1] which you may attend.

[4] 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.

[5] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[6] Available online from https://www.standardmethods.org/doi.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *confluent growth, n—in this test method,* plated results observed do not contain isolated bacterial colonies but are observed as continuous bacterial growth covering the entire area of the plate's printed grid.

3.2.2 *cooling tower water, n*—water collected from a cooling tower.

3.2.3 *culturable, adj—in this test method,* any aerobic bacteria able to form colonies on solid media.

3.2.4 *dental water, n*—water that comes from dental devices or waterlines of a dental unit providing a water source for dental procedures.

3.2.5 *drinking water, n*—water that meets regulatory requirements or standards for human consumption.

3.2.6 *EasyDisc PCA, n*—method used for the quantification of culturable, waterborne bacteria in drinking water, bottled water, and cooling tower water.[2,3]

3.2.6.1 *Discussion*—The CFU data generated by this test method correlate with the pour plate method using plate count agar (PCA) incubated at 35 °C for 48 h as described in Method 9215 (see 2.3).

3.2.7 *EasyDisc R2A, n*—method used for the quantification of culturable, waterborne bacteria in drinking water, bottled water, and dental water.[2,3]

3.2.7.1 *Discussion*—The CFU data generated by this method correlate with the pour plate method using Reasoners 2 Agar (R2A)[7] incubated at 20 °C to 28 °C for five to seven days as described in Method 9215 (see 2.3).

3.2.8 *EasyDisc YEA, n*—method used for the quantification of culturable, waterborne bacteria in drinking water.[2,3]

3.2.8.1 *Discussion*—The CFU data generated by this method correlate with the pour plate method using yeast extract agar incubated at 22 °C for 68 h and 36 °C for 44 h as described in ISO 6222.

3.2.9 *plate, n—in this test method,* a 47 mm sample plate with a printed grid to aid in counting colonies, which contains a growth reagent for testing a 1 mL ± 0.1 mL specimen.

3.2.10 *specimen, n—in this test method,* a 1 mL portion of a collected water sample.

4. Summary of Test Method

4.1 Bacteria are grown on a defined culture medium coated on a plate.

4.2 From a well-mixed sample, a 1 mL specimen is added to a plate, gently swirled, the white plate lid is reattached, and then the plate is incubated.

NOTE 1—Incubation temperature and interval depends on the plate type selected. Follow local regulatory requirements for plate selection.

4.2.1 EasyDisc PCA plates are incubated, white lid side up, at 35 °C ± 2 °C for 48 h ± 3 h for potable and non-potable water samples.

4.2.2 EasyDisc R2A plates are incubated, white lid side up, at 20 °C to 28 °C for five to seven days for potable and non-potable water samples.

4.2.3 EasyDisc YEA plates are incubated, white lid side up, in a 22 °C ± 2 °C incubator for 68 h ± 4 h or a 36 °C ± 2 °C incubator for 44 h ± 4 h for potable and non-potable water samples.

4.3 Culturable, waterborne bacteria are detected if any colony growth is observed.

4.4 Test results are reported as CFU/1 mL.

NOTE 2—Colonies may be counted with or without the aid of a manual colony counter with magnification under a uniform light.

5. Significance and Use

5.1 This plate format is useful for the routine monitoring of culturable, waterborne bacteria in potable and non-potable waters. The significance of finding these bacteria can help with identifying water quality or water system problems or evaluate compliance with maintenance protocols. This test method uses small volumes of water, or dilutions thereof, and provides an easy and reliable method that eliminates media preparation and reduces laboratory waste.

6. Interferences

6.1 Do not use automated colony counters as the printed grid on the plate can interfere with results from an automatic counter.

6.2 Buffers containing phosphate should be avoided for quality control samples or dilutions as they can interfere with colony visualization.

6.3 Confluent growth can interfere with the colony count on the plates. It is recommended that any plates with confluent growth are reanalyzed with dilutions of the original sample.

7. Apparatus

7.1 *Colony counter,* manual magnification (optional).

7.2 *Equipment for Sample Collection and Transport:*

7.2.1 Sterile 100 mL vessels with or without sodium thiosulfate.

7.2.1.1 *Preparation of Vessels with Sodium Thiosulfate*—Vessels can be purchased containing sodium thiosulfate or 0.1 mL of a 10 % solution of sodium thiosulfate can be added to a 120 mL vessel, to neutralize up to 15 mg/L of residual chlorine as described in Method 9060 (see 2.3).

NOTE 3—Sodium thiosulfate is required for any samples containing an oxidizing agent such as chlorine.

NOTE 4—Dental waters may contain specific disinfectant agents (for example, hydrogen peroxide), therefore the addition of an appropriate neutralizer may be required.

7.2.2 Ice chest.

7.2.3 Ice packs.

7.3 *Incubator,* air microbiological type to maintain a temperature of the selected plate(s) (4.2.1 – 4.2.3).

7.4 *Loop,* inoculating sterile loop, 10 µL capacity or equivalent.

7.5 *Pipettor,* capable of pipetting 1 mL.

[7] Reasoner, D. J. and Geldreich, E. E., "A New Medium for the Enumeration and Subculture of Bacteria from Potable Water," *Applied and Environmental Microbiology*, Vol 49, No. 1, January 1985, pp. 1-7.

7.6 *Pipettor tips,* disposable, 1 mL, sterile.

8. Reagents and Materials

8.1 *Plates*—Plates are commercially available in different premade formats (3.2.6 – 3.2.8) and each plate is suitable for single samples.

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

8.3 *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,[8] where such specifications are available. 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.

8.4 *Quality Control Strains:*

8.4.1 IDEXX-QC HPC/TVC, or

8.4.2 At least one target organism:

8.4.2.1 *Enterococcus faecalis,* American Type Culture Collection (ATCC) 29212/World Data Centre for Microorganisms (WDCM) 00087; or

8.4.2.2 *Escherichia coli,* ATCC 11775/WDCM 00090.

NOTE 5—ATCC target organisms (8.4.2) should be validated by user prior to use.

8.5 Sterile microtubes or sterile tubes for dilutions capable of holding ≥1.5 mL of sample or ≥10.0 mL of sample.

8.6 Water that is oxidant free, nonbuffered, and sterile.

9. Hazards

9.1 The analyst shall 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 or other equipment.

10. Sampling

10.1 Collect samples as described in detail in the USEPA Microbiological Methods Manual[9] and in accordance with Practices D3370.

10.2 *Sample Storage, Temperature, and Handling Conditions*—Keep samples cool but unfrozen (<10 °C) during transit to the laboratory as described in Method 9060 (see 2.3). Use insulated containers to ensure proper maintenance of storage temperatures. Take care that samples containers are not totally immersed in water during transit.

10.3 *Handling Time Limitations*—Adhere to all requirements by local regulations, or it is recommended to examine samples as soon as possible after collection.

11. Quality Control

11.1 Observe and record incubator temperatures at least twice per day when in use. Individual observations shall be ≥4 h apart to ensure temperature is within stated limits.

11.2 Quality control should be conducted at least once on each new lot of plates. Perform the positive control procedure (11.3) and the negative control procedure (11.4).

11.3 *Preparation of Positive Control:*

11.3.1 Use the quality control kit (8.4.1) and perform all steps at ambient temperature.

11.3.2 Remove vial(s) from freezer. Equilibrate at ambient temperature for 15 min.

11.3.3 Open a vial and aseptically transfer the pellet to an appropriately labeled vessel of 100 mL of sterile, nonbuffered, oxidant-free water in a sterile vessel without sodium thiosulfate.

11.3.4 Swirl the sample and allow to stand for 5 min to dissolve the pellet completely.

11.3.5 After the pellet has dissolved, mix by inverting the vessel ten times. Use within 30 min of hydration.

11.3.6 Review the kit's lot activity information on the certificate of analysis to determine if a dilution of hydrated sample is required.[10]

11.3.7 See Section 12 for sample procedure.

11.3.8 Review the quality control kit's lot activity information to determine if results obtained meet criteria as described on the certificate of analysis.[10]

11.4 *Preparation of Negative Control*—Use 1 mL of sterile diluent (8.6) as the test sample.

11.4.1 See Section 12 for sample procedure.

NOTE 6—The negative control test should not contain any colonies.

12. Procedure

12.1 *Sample Preparation:*

12.1.1 The sample should be at ambient temperature and mixed well before adding 1 mL ± 0.1 mL specimen to the plate. Remove the white lid and use a pipettor to add 1 mL ± 0.1 mL of water specimen directly to the plate.

12.1.2 Gently swirl immediately after sample addition to coat the plate entirely and reattach the white lid.

12.1.3 Incubate, undisturbed, at ambient temperature for at least 20 min to allow for the media to set. Transfer the plate to the incubator within 1 h of sample addition.

12.1.4 Follow the respective incubation time and duration for the selected plate(s) as directed in 12.2 – 12.4.

12.2 *EasyDisc PCA Incubation Time and Duration:*

[8] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, 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. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[9] 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.

[10] IDEXX-QC HPC/TVC certificate of analysis is available at idexx.com/water.

12.2.1 Using the prepared plate (see 12.1), incubate the plate, white lid side up, at 35 °C ± 2 °C for 48 h ± 3 h.

12.2.2 See 12.5.

12.3 *EasyDisc R2A Incubation Time and Duration:*

12.3.1 Using the prepared plate (see 12.1), incubate the plate, white lid side up, at 20 °C to 28 °C for five to seven days.

12.3.2 See 12.5.

12.4 *EasyDisc YEA Incubation Time and Duration:*

12.4.1 Using the prepared plate (see 12.1), incubate the plate, white lid side up, at each temperature required in a 22 °C ± 2 °C incubator for 68 h ± 4 h or a 36 °C ± 2 °C incubator for 44 h ± 4 h, or both.

12.4.2 See 12.5.

12.5 *For All Test Samples:*

12.5.1 Examine the plates for colony growth as soon as they are removed from the incubator.

12.5.1.1 Reject any plate with confluent growth.

12.5.2 Sum all colonies for the results. The result is expressed as CFU/mL.

NOTE 7—Most bacteria will produce a blue color on the plates, but some bacteria may produce natural pigments with colors other than blue. All colonies should be summed for the final result.

13. Calculation

13.1 There are no calculations unless dilutions are made.

13.1.1 Adjust the CFU/mL result to reflect dilutions. For example, if a tenfold dilution is prepared by adding 0.1 mL of sample into 0.9 mL of diluent, multiply the result by ten to convert to CFU/1 mL.

14. Report

14.1 Bacteria counts are reported as CFU/1 mL.

14.1.1 If there are more than 300 colonies on the plate inoculated with the highest dilution used, express the results as >300 CFU/1 mL.

14.2 Follow local regulatory requirements for reporting, for example, when to report significant figures or rounding off counts; or it is recommended to report test results to the integer except for dilutions that should report out to the last significant figure.

15. Precision and Bias[11]

15.1 *Precision*—A limited collaborative study was conducted. Ten operators from one laboratory tested EasyDisc PCA and EasyDisc R2A in three different waters and EasyDisc YEA in one water type. All testing was completed at three levels following Practice D2777. The mean count (X), the single operator standard deviation (S_o), the overall standard deviation (S_t), and the percent relative standard deviations (%RSD) are indicated in Tables 1-3.

15.2 *Bias*—The mean values obtained from the samples from the ten operators for the low, mid, and high spiked samples are in Table 4.

15.3 Results of this collaborative study may not be typical of results for matrices other than those studied.

16. Keywords

16.1 bottled water; confluent growth; cooling towers; counting colonies; dental water; drinking water; EasyDisc PCA; EasyDisc R2A; EasyDisc YEA; heterotrophic plate count; plate count agar; pour plate; R2A; Reasoners 2 Agar; total plate count; total viable count; yeast extract agar

[11] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D19-2004. Contact ASTM Customer Service at service@astm.org.

TABLE 1 EasyDisc PCA Mean Count, Single Operator Standard Deviation, Overall Standard Deviation, and Percent Relative Standard Deviations[A]

Matrix	Duration	Temp (°C)	Level	N[B]	X cfu/mL	S_o	% RSD S_o	S_t	% RSD S_t
Bottled water	48 h ± 3 h	35	Low	100	25.8	5.3	20.5	5.6	21.7
			Mid	100	67.0	8.4	12.3	8.4	12.3
			High	100	132.3	12.8	9.7	14.1	10.7
Cooling tower water	48 h ± 3 h	35	Low	90[C]	24.2	5.0	20.7	5.0	20.7
			Mid	100	67.5	7.3	10.8	7.8	11.6
			High	100	126.9	12.6	9.9	14.3	11.3
Drinking water	48 h ± 3 h	35	Low	100	23.9	4.4	18.4	4.8	20.1
			Mid	100	61.1	7.8	12.8	9.5	15.5
			High	100	123.2	11.7	9.5	16.1	13.1

[A] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D19-2004. Contact ASTM Customer Service at service@astm.org.

[B] 10 analysts by 10 replicates/level.

[C] Low level cooling tower water – 9 analysts by 10 replicate/level. Communicating with the analyst, it was identified there was an error with the procedure, therefore the sample was omitted.