Designation: D 4201 – 96

An American National Standard

Standard Test Method for Coliphages in Water[1]

This standard is issued under the fixed designation D 4201; 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 the determination of coliphages infective for *E. coli* C in water. The test method is simple, inexpensive, and yields rapid water quality data. Its sensitivity is limited to 5 coliphages per 100 mL of water sample. This test method is applicable to natural fresh water samples.

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:*

D 1129 Terminology Relating to Water[2]

D 1193 Specification for Reagent Water[2]

D 3370 Practices for Sampling Water from Closed Conduits[2]

3. Terminology

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

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *bacterial lawn*—confluent growth of bacteria.

3.2.2 *coliphage*—as used in this procedure, bacterial virus capable of replication using a specific strain of coliform bacteria (*E. coli* C) as a host.

4. Summary of Test Method

4.1 A measured water sample is added to a tube of melted modified nutrient agar. An *E. coli* C host culture is added to the tube, and the contents of the tube are mixed and poured into a petri dish. The dish is incubated at 35°C. The coliphages present in the water sample lyse the bacteria and form plaques; the total number of plaques represents the number of coliphages in the volume of water sample tested.

5. Significance and Use

5.1 Coliphage organisms may serve as indicators of fecal pollution. The presence of coliphages in water in the absence of a disinfectant indicates the probable presence of fecal contamination, but the absolute relationship between the number of coliforms and coliphages in natural waters has not been demonstrated conclusively. [3, 4, 5]

5.2 The detection of coliphages in a water sample depends upon the use of a sensitive-host strain in the coliphage assay. Coliphages may be detected in 4 to 6 h to provide important same-day information on the sanitary quality of a water. The lower detection limit is 5 coliphages per 100 mL of fresh water sample.

6. Interferences

6.1 High salt concentrations, such as those found in saline or brackish water, interfere in this test method by inhibiting plaque formation.

6.2 Analysis for coliphages can be performed on disinfected waters. However, the quantitative relationship between coliphages and coliform bacteria is different from that observed in natural fresh waters. This difference is due to variations in the survival rates of coliphages and coliform bacteria exposed to disinfectants. For example, coliphages may have been shown to be more resistant to chlorine disinfection than fecal or total coliforms.[6]

7. Apparatus

7.1 *Water Bath*, 44.5± 0.2°C.

7.2 *Incubator*, 35 ± 0.5°C.

7.3 *Balance*.

7.4 *Petri Dishes*, sterile, 100 by 15-mm.

7.5 *Pipets*, plugged, sterile, 1-mL and 5-mL.

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[1] This method is under the jurisdiction of ASTM Committee D-19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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

[3] Kenard, R.P., and Valentine, R.S., "Rapid Determination of the Presence of Enteric Bacteria in Water," *Applied Microbiology*, Vol 27, 1974, p. 484.

[4] Scarpino, P.V., "Bacteriophage Indicators," Berg, G., editor, *Indicators of Viruses in Water and Food*, Ann Arbor Science, Ann Arbor, Mich., 1978, p. 201.

[5] Kott, Y., Ari, B., and Buras, N., "The Fate of Viruses in a Marine Environment," *Proceedings 4th International Conference on Water Pollution Research*, Jenkins, S.H., editor, Pergamon Press, Oxford, 1969, p. 823.

[6] Kott, Y., Roze, N., Sperber, S., and Betzer, N., "Bacteriophages as Viral Pollution Indicators," *Water Research*, Vol 8, p. 165.

7.6 *Test Tubes* with close fitting or airtight caps, 16 by 125-mm and 25 by 150-mm.

7.7 *Platinum Transfer Loop*, sterilized by flaming.

7.8 *Erlenmeyer Flasks*, 125-mL.

7.9 *Sterile Vials*, 12 by 75-mm with caps.

7.10 *Spectrophotometer* set at 520 nm.

7.11 *Refrigerator* with non-frost-free freezer.

8. Reagents

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society.[7] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without decreasing the accuracy of the determination.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type III.

8.3 *Host Culture*[8]—American Type Culture Collection No. 13706, *Escherichia coli* C.

8.4 *Tryptic(ase) Soy Agar*, (sterile) slants or petri dishes for maintaining the *E. coli* C host culture.

8.5 *Tryptic(ase) Soy Broth* (TSB), containing 10 % (v/v) glycerin. Place in a sterile 125-mL Erlenmeyer flask.

8.6 *Modified Nutrient Agar*, 5.5 mL per 16 by 125-mm tube, sterile, formulated as follows:

Nutrient agar, dehydrated	23.0 g
Nutrient broth	8.0 g
$Sr(NO_3)_2$	0.23 g
NH_4NO_3	1.76 g
NaCl	5.0 g
Water	to 1 L

8.7 *Tap Water*, sterile, dechlorinated.

9. Sampling

9.1 Collect the sample in accordance with Practices D 3370.

10. Procedure

10.1 *Frozen Host Preparation*:

10.1.1 Inoculate 5 mL of sterile TSB contained in a 16 by 125-mm test tube with the *E. coli* C host culture from a host culture slant or agar plate using a sterile loop. Incubate the TSB for 18 h at 35°C to allow the host to grow.

10.1.2 Aseptically transfer the 5 mL of host culture from 10.1.1 into 50 mL of sterile TSB (containing 10 % v/v glycerin) in a 125-mL Erlenmeyer flask. Incubate the culture at 35°C until its optical density reaches 0.5 as measured at 520 nm with a spectrophotometer that has been previously calibrated with sterile TSB (containing 10 % v/v glycerin).

10.1.3 Place the Erlenmeyer flask from 10.1.2 in an ice bath for 15 min.

10.1.4 Pipet 5-mL aliquots of the *E. coli* C culture from 10.1.3 into sterile vials.

10.1.5 Cover the vials and place in a freezer at − 20°C. Vials may be stored at − 20°C for ≤9 weeks, for use in the coliphage assay. A“ frost-free” freezer is undesirable because it has freeze-thaw cycles. Viability of the bacteria is lost within 14 days in“ frost-free” freezers.

10.2 *Procedure A—Contamination Less Than 1000 Coliphages per 100* mL *of Sample*:

10.2.1 Thaw a vial(s) of frozen host culture in a 44.5°C water bath for 5 min. One vial of culture is needed for each water sample.

10.2.2 Place approximately 25 mL of the water sample to be tested into a 25 by 150-mm sterile test tube or suitable container. Place the test tube in the 44.5°C water bath for 5 min to allow the temperature to equilibrate.

10.2.3 Place four tubes containing 5.5 mL each of modified nutrient agar in boiling water to melt the agar. Transfer the tubes of melted agar to a 44.5°C water bath and hold for 10 min to stabilize the temperature.

10.2.4 Add 5 mL of the warmed water sample from 10.2.2 to each of the four tubes (10.2.3) containing the melted modified nutrient agar.

10.2.5 Add 1.0 mL of thawed host culture from 10.2.1 to each tube containing melted modified nutrient agar and water sample from 10.2.4.

10.2.6 Gently mix the contents of each tube. Pour the contents of each tube into a separate, labeled petri dish (four dishes per water sample).

10.2.7 Cover the four petri dishes. Allow the agar to gel and then incubate the plates, inverted at 35°C.

10.2.8 Count the plaques after 6 h (±0.5 h) of incubation.

10.3 *Procedure B—Contamination Greater Than 1000 Coliphages per 100* mL *of Sample*—Dilute 1 volume of water sample with 4 or more volumes of sterile dechlorinated tap water and proceed as in 10.2 (Procedure A).

11. Report

11.1 *Procedure A*—Count the plaques on each plate. Obtain the number of plaques per 100 mL of water sample by adding the plaques on the four plates (total plaque forming unit in 20 mL) and multiplying the total by five.

Example:

Plate No.	1	2	3	4	
Plaques	4	5	9	6	Total = 24 plaques

24 x 5 = 120 plaques per 100 mL.

11.2 *Procedure B*—Count the plaques on each plate. Obtain the number of plaques per 100 mL of water sample by adding the plaques on the four plates and multiplying by five and by the reciprocal of the dilution.

Example:

Plate No.	1	2	3	4	
Plaques	4	5	9	6	Total = 24 plaques

Dilution = 1/10: Reciprocal of the dilution is 10/1
Therefore, $24 \times 5 \times 10 = 1200$ plaques per 100 mL.

[7] *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. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[8] Available from Atlantic Research Corp., 5390 Cherokee Ave., Alexandria, VA., or from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD.

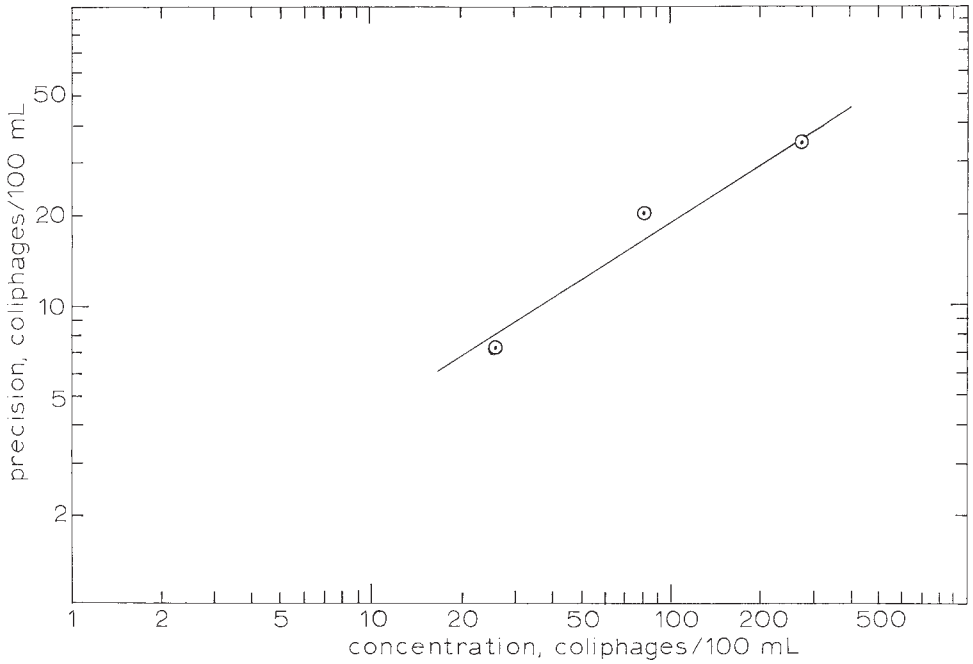

FIG. 1 Single-Operator Precision versus Coliphage Concentration

12. Precision and Bias [9]

12.1 Two operators in each of three laboratories tested three bacteriophage concentrations in triplicate in the ranges from 0 to 50, 50 to 150, and 150 to 500 coliphages per 100 mL of water using Procedure A. The pooled single-operator precision (S_0) versus coliphage concentration is shown in Fig. 1. The equation of the line is:

$$\log S_0 = 0.176 + 0.578 \log \text{coliphages per 100 mL}$$

Because of the instability of microbiological samples, identical samples could not be analyzed by each laboratory. Therefore, S_T(total standard deviation) could not be calculated. However, a pooled multiple-operator value was generated. The equation of the line is:

$$\log (\text{pooled multiple}-\text{operators value}) = 0.179 + 0.571 \log (\text{coliphages per 100 mL}).$$

[9] Round-robin test data are on file at ASTM Headquarters as RR:D19-1086.