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Standard Test Method for Low Levels of Coliphages in Water[1]

This standard is issued under the fixed designation D6734; 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 an indication of water quality within 6.5 h. This coliphage method can determine coliphages in water down to 1 coliphage per volume of water sampled.

1.2 The test method is applicable to natural fresh water samples and to settled, filtered or finished water samples.

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 and health practices and determine the applicability of regulatory limitations prior to use.*

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

D4201 Test Method for Coliphages in Water (Withdrawn 2005)[3]

3. Terminology

3.1 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 *bacterial lawn, n*—confluent growth of bacteria cultured on an agar plate.

3.2.2 *coliphage, n*—bacterial virus capable of plaquing on the wide-range *E. coli* host strain used in this assay.

3.2.3 *plaque, n*—the circular zone of clearing (lysis) of the visible growth of bacteria on a one or two layer agar plate, caused by the action of one or more bacteriophage.

3.2.4 *plaque forming unit (PFU), n*—the term used to report the number of plaques formed on an agar culture plate previously seeded with a microorganism susceptible to a bacteriophage. Although theoretically, each plaque develops from the action of a single bacteriophage, microbiologists use the term, PFU, to acknowledge that a plaque may have been formed from the action of two or more bacteriophage in close proximity, which is indistinguishable from that formed by a single phage.

4. Summary of Test Method

4.1 A measured water sample is adjusted to pH 6.0 with HCl or NaOH and filtered through a positively-charged filter. The coliphages trapped in the filter are eluted with Trypticase Soy Broth (TSB) at pH 8.5. The total eluate is divided between four Tubes of melted modified nutrient agar (MNA) and *E. coli* C host culture is added to each tube. The contents of each-tube are mixed and poured into a petri plate. The plates are incubated at 35°C for 6 h. The coliphages present infect the host bacteria and form plaques. The total number of plaques on the four plates represents the number of coliphages in the volume of water sample filtered.

5. Significance and Use

5.1 Coliphage organisms may serve as indicators of fecal contamination. The presence of coliphages in water in the absence of a disinfectant indicates the probable presence of fecal contamination. The absolute relationship between the number of coliforms and coliphages in natural waters has not been conclusively demonstrated. Coliphages are generally more resistant than coliforms to chlorination and may have some advantage over coliforms as an indicator of treatment efficiency in disinfected waters. 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 by this concentration procedure in 6.5 h to provide important same-day information on the sanitary quality of water. The lower detection limit of this concentration procedure is 1 coliphage per volume of water sample tested.

[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.

6. Interferences

6.1 High salt concentrations, such as these found in saline or brackish water, interfere with this test method.

6.2 Water sample turbidity in excess of 25 NTU (nepholometric turbidity units using Ratio Turbidimeter) results in decreased plaque formation because bacterial viruses are trapped with the particulate matter in the Zesa Plus filter and are not completely eluted by TSB at pH 8.5.

6.3 Analysis for coliphage can be performed on settled and wastewaters filtered waters, disinfected waters or wastewaters; however, the relationship between coliphage and coliform bacteria will be different from that observed in natural fresh waters. Coliphage are less efficiently removed by settling and filtration than coliforms, and coliphage are generally more resistant than coliforms to chlorination.

7. Apparatus

7.1 *Water Bath,* 46 ± 1°C.

7.2 *Incubator,* 35 ± 0.5°C.

7.3 *Petri Plates,* glass or plastic, sterile, 100 × 15 mm.

7.4 *Pipets,* sterile T.D. bacteriological or Mohr, glass or plastic, 1 and 5 mL.

7.5 *Test Tubes,* with airtight caps or screw caps, 16 × 125 mm and 25 × 150 mm.

7.6 *Platinum Transfer Loop,* 3 mm loop.

7.7 *Sterile Vials,* 12 × 75 mm with crimp or screw caps.

7.8 *Spectrophotometer,* suitable for absorbance measurements at 520 nm.

7.9 *Freezer,* with manual defrost.

7.10 *Filters,* Zeta Plus 60S positively charged 47 mm.[4]

7.11 *Membrane Filtration Units,* (filter base and funnel), reusable glass, plastic or stainless steel units wrapped with aluminum foil or kraft paper and sterilized, or disposable, sterile, plastic units.

7.12 *Vacuum Pump,* capable of creating 15 psi pressure for filtration of wager.

7.13 *Vacuum Flasks,* sterile 1 L.

7.14 *Turbidimeter,* Hach ratio turbidimeter or equivalent.

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 shall conform to the specifications of the committee on Analytical Reagents of the American Chemical Society.[5] 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 D1193, Type II.

8.3 *Host Culture— Escherichia coli* C, ATCC No. 13706.[6]

8.4 *Trytpicase Soy Agar (TSA)*[7]

8.4.1 *Composition per Litre:*

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Final pH 7.3 ± 0.2	

8.4.2 *Preparation*—Add 40 g or the dehydrated medium to 1 L of water and mix well. Heat while stirring on a hot plate. Boil for 1 min or until completely dissolved. Dispense 8-10 mL quantities into screw-cap culture tubes. Autoclave for 15 min at 121°C (15 lbs pressure). Remove from autoclave while still molten and incline tubes at appropriate angle for slants. Let cool to harden.

8.5 *Trypticase (Tryptic) Soy Broth*[8] (TSB) and *Glycerol*

8.5.1 *Composition per Litre:*

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride (NaCl)	5.0 g
Dipotassium Phosphate (K_2PO_4)	2.5 g
Dextrose	2.5 g

8.5.2 *Preparation*—Add 30 g of the dehydrated medium and 100 mL of glycerol to 900 mL of water. Mix well and heat gently to dissolve in a hot water bath. Dispense 5 mL volumes into 16 mm screw-cap test tubes and 50 mL volumes into 125 mL Erlenmyer flasks. Autoclave for 15 min at 121°C. Final pH 7.3 ± 0.2.

8.6 *pH Adjusted Tryptic Soy Broth*

8.6.1 *Preparation*—Add 30 g of dehydrated Tryptic Soy Broth to 1 L of water. Mix well and heat gently in a hot water bath to dissolve. Add in NaOH drop-wise to raise the pH to 8.5. Dispense in 200 mL volumes in 250 mL screw-cap flasks and autoclave for 30 min at 121°C.

8.7 *Modified Nutrient Agar (MNA) :*

8.7.1 *Composition per Litre:*

[4] The sole source of supply of the apparatus known to the committee at this time is Zeta Plus 60S filters available from AMF Cuno, Meriden, CT 06450. 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.

[5] *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 *Annual 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.

[6] The sole source of supply of the material known to the committee at this time is American Type Culture Collection, Rockville, MD 20854. 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.

[7] BBL 11043, Difco 0369, or equivalent.

[8] BBL 11765, Difco 0370, or equivalent.

Nutrient Agar[9]	23.0 g
Nutrient broth	8.0 g
Strontium Nitrate, Sr (NO_3)	0.23 g
Ammonium Nitrate, NH_4NO_3	1.76 g
Sodium Chloride, NaCl	5.0 g

8.7.2 *Preparation*—Add the ingredients to 1 L of water and mix well. Heat in boiling water bath until dissolved completely. Dispense 5.5 mL volumes into 16 × 125 mm screw-cap culture tubes and autoclave for 15 min at 121°C.

9. Sampling

9.1 Collect 1 L water samples in accordance with Practice D3370.

10. Procedure

10.1 *Frozen Host Preparation:*

10.1.1 Inoculate 5 mL sterile TSB in a 16 × 125 mm culture tube with the *E. coli* C host culture from an agar slant or agar plate using a sterile loop. Incubate the inoculated TSB tube 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 + 10 % Glycerol in a 125 mL Erlenmeyer flask. Incubate the culture at 35°C until its absorbance reaches 0.5 as measured at 520 nm with a spectrophotometer previously calibrated with sterile TSB + Glycerol.

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 Seal the vials and store in a freezer at -20°C for no more than 9 weeks.

NOTE 1—Do not use a "frost-free" freezer because the freeze-thaw cycles will kill the bacterial host.

10.2 *Assay Procedure:*

10.2.1 For each water sample, thaw a vial of frozen host culture in a 46°C water bath for 5 min then hold at room temperature until used.

10.2.2 Place a measured volume of water sample (100 mL, 200 mL, 500 mL; analyst selects volume) to be tested in a sterile beaker and adjust the pH to 6.0 with 1 % HCL or 0.1N NaOH.

10.2.3 Place 4 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 46°C water bath and hold for 10 min to stabilize the temperature.

10.2.4 Filter the measured water sample in 10.2.2 through a Zeta Plus 60S positively charged filter contained in a vacuum filter housing.

10.2.5 Remove the filter housing containing the filter with adsorbed coliphages from the vacuum flask and place the filter housing on a sterile vacuum flask.

10.2.6 Elute adsorbed coliphages from the filter by applying 10 mL pH Adjusted TSB and allowing a contact time of 10 min before vacuum is applied. Add second 10 mL aliquot of pH Adjusted TSB to the filter and apply vacuous. Flush the filter with three 1 mL washes of sterile water. (Eluate is the combined filtrates from the pH Adjusted TSB elutions and the water washes).

10.2.7 Plate the total eluate volume from 10.2.6 (20-23 mL). Mix each aliquot of the eluate (ca. 5 mL) with 5.5 mL molten modified Nutrient Agar from 10.2.3.

10.2.8 Add 1.0 mL of thawed host culture from 10.2.1 to each tube containing melted modified Nutrient Agar and eluate.

10.2.9 Gently mix the contents of each tube. Pour the contents of each tube into a separate, labeled petri plate (4 plates per water sample).

10.2.10 Cover the 4 petri plates. Allow the agar to gel at room temperature and incubate the plates at 35°C.

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

11. Calculation

11.1 Count the plaques on each plate. Obtain the number of plaques per total volume of water sample filtered by adding the plaques counted on the four plates.

Example:

Plate No.	1	2	3	4	
No. Plaques	4	2	5	5	Total = 16 PFU

If original water sample filtered was 500 mL, report as 16 PFU (plaque forming units) per 500 mL of water sample.

12. Precision and Bias

12.1 *Single Laboratory Studies:*

12.1.1 Table 1 illustrates coliphage recovery from natural waters using the proposed coliphage method. Recovery of coliphages by the proposed method was compared to actual input as determined by assay of 100 mL of water sample using Test Method D4201.

12.1.2 Fig. 1 presents coliphage/total coliforms data from natural water sources. Creeks, lakes, rivers, and reservoirs in the Washington, D.C. area were. sampled an analyzed for coliphages by the Test Method D4201 and analyzed for total and fecal coliforms by standard membrane filtration techniques. The best fit line was constructed by linear regression analysis of the data.

12.1.3 Fig. 2 presents cotiphage-fecal coliform data from nasural waters. Creeks, lakes, rivers and reservoirs in the Washington, D.C. area were sampled and analyzed for coliphages by the Test Method D4201 and for total and fecal coliforms by the standard membrane filtration technique. The best fit line was constructed by linear regression analysis of the data.

12.1.4 Filtered-chlorinated water samples were collected from water taps in a water treatment plant in the Washington, D.C. area. No chlorination occurred before settling or filtration of the influent river water. Fig. 3 illustrates the relationship between coliphages and total coliforms found in filtered waters using the concentration technique. Statistical analysis of the data by linear regression showed an r value (correlation) of 0.82.

12.1.5 Fig. 4 shows the relationship between coliphages and fecal coliforms found in filtered chlorinated waters using the concentration technique. Statistical analysis of the data by linear regression showed an r value (correlation) of 0.91.

[9] BBL 114472, Difco 0001, or equivalent.