9221 MULTIPLE-TUBE FERMENTATION TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9221 A. Introduction

Coliform bacteria have long been used as water-quality indicators based on the premise that, because these organisms are present in the intestines of warm-blooded animals, their presence in water could indicate that recent fecal contamination has occurred. Historically, this group of organisms has been defined by their ability to ferment lactose, rather than through the tenets of systematic bacteriology, so the group consists of bacteria from several genera belonging to the family Enterobacteriaceae.

The methods described in this section use a lactose-based broth medium to detect the metabolic end products of lactose fermentation. The presence of coliforms must be confirmed in a lactose- and bile salt-containing medium [brilliant green lactose bile (BGLB) broth]. So when the fermentation techniques in this section are used, *coliforms* are defined as all facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid production in the presence of bile salts within 48 h at 35°C.

The standard test for the coliform group may be carried out by the multiple-tube fermentation technique or presence–absence procedure (through the presumptive-confirmed phases or completed test) described herein, the membrane filter (MF) technique (Section 9222), or the enzymatic substrate coliform test (Section 9223). Each technique is applicable within the limitations specified and with due consideration of the purpose of the examination. Production of valid results requires strict adherence to quality control (QC) procedures. QC guidelines are outlined in Section 9020.

The fermentation technique can be used to detect coliforms in drinking water or quantitate coliforms in potable and nonpotable water. When multiple tubes are used, coliform density is estimated via a most probable number (MPN) table. This number, generated using specific probability formulas, is an estimate of the mean density of coliforms in the sample. Coliform testing results, together with other information obtained from engineering or sanitary surveys, provide the best assessment of water-treatment effectiveness and the sanitary quality of source water.

The fermentation test's precision in estimating coliform density depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows acid and/or gas in some or all of the tubes and the smallest sample inoculum shows no acid or gas in any or a majority of the tubes. Bacterial density can be estimated by the formula given or from the table using the number of positive tubes in the multiple dilutions (9221C.2). The number of sample portions selected will be governed by the desired precision of the result. The MPN tables are based on the assumption of a Poisson distribution (random dispersion). However, if the sample is not adequately shaken before aliquots are removed or if bacterial cells clump, the MPN value will be an underestimate of actual bacterial density.

1. Water of Drinking-Water Quality

When analyzing drinking water to determine if its quality meets U.S. Environmental Protection Agency (EPA) standards, a 100-mL sample must be analyzed; use the fermentation technique with 10 replicate tubes each containing 10 mL, 5 replicate tubes each containing 20 mL, or a single bottle containing a 100-mL sample portion. When examining drinking water via the fermentation technique, process all tubes or bottles demonstrating growth—with or without a positive acid or gas reaction—through the confirmed phase (9221B.4). Drinking water samples that are positive for total coliforms also must be tested for thermotolerant (fecal) coliforms (9221E) or *Escherichia coli* (9221F).

For routine examination of public water supplies, the objective of the total coliform test is to determine the efficiency of treatment plant operations and the integrity of the distribution system. The test is also used to screen for the presence of fecal contamination. Some coliform occurrences in a distribution system may be attributed to coliform growth or survival within bacterial biofilms in the mains rather than treatment failure at the plant or well source, or outside contamination of the distribution system. Because it is difficult to distinguish coliforms entering the distribution system and coliforms already present in the pipe biofilm and sediments, assume that all coliforms originate from a source outside the distribution system.

2. Water of Other than Drinking Water Quality

When analyzing nonpotable waters, inoculate a series of tubes with appropriate decimal dilutions of the water (multiples of 10 mL) based on the probable coliform density. Use the presumptive-confirmed phases of the multiple-tube procedure. Use the more labor-intensive completed test (9221B.5) as a QC measure on 10% (or a set percentage) of coliform-positive nonpotable water samples quarterly. Generally, the objective of analyzing nonpotable water is to estimate bacterial density, determine a pollution source, enforce water quality standards, or trace the survival of microorganisms. The multiple-tube fermentation technique may be used to obtain statistically valid MPN estimates of coliform density. Examine a sufficient number of water samples to yield representative results for the sampling station. Generally, the geometric mean or median value of the results of a number of samples will yield a value in which the effect of sample-to-sample variation is minimized.

3. Other Samples

The multiple-tube fermentation technique is applicable to the analysis of salt or brackish waters, as well as muds, sediments, and sludges. Collect samples as directed in Section 9060A, using sample containers specified in Section 9030B.19. Follow the

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precautions given above on portion sizes and numbers of tubes per dilution.

To prepare solid or semisolid samples, weigh the sample and add diluent to make a 10^{-1} dilution. For example, place 30 g sample in a sterile blender jar, add 270 mL sterile phosphate buffered or 0.1% peptone dilution water, and blend for 1 to 2 min at high speed (8000 rpm). Prepare the appropriate decimal dilutions of the homogenized slurry as quickly as possible to minimize settling.

9221 B. Standard Total Coliform Fermentation Technique

1. Samples

Collect samples as directed in Section 9060A, using sample containers specified in Section 9030B.19. Follow the QC guidelines for sample bottles described in Section 9020B.5*d*. Ensure that samples meet laboratory-acceptance criteria upon receipt.

2. Quality Control

All phases of the fermentation technique (9221B–G) require adherence to the quality assurance/quality control (QA/QC) guidelines presented in Section 9020, including, but not limited to, analytical QC (Section 9020B.9), instrumentation/equipment (Sections 9020B.4 and 9030B), and supplies (Section 9020B.5). Refer to Table 9020:I for key QC procedures. Also, note the sections pertaining to appropriate storage and preparation of dehydrated culture media and water quality (Sections 9050 and 9020B.5*f*).

Use commercial dehydrated media when possible, and ensure that their formulations match those specified here because commercial formulations may vary. Prepared fermentation media can be stored in tightly capped tubes or bottles for up to 3 months in the dark, if temperatures are between 1 and 30°C and evaporation is less than 10% of the original volume. If the tubes were refrigerated after sterilization, they should be incubated overnight at room temperature (20°C) before use and those showing growth or bubbles should be discarded to avoid false-positive results. To demonstrate acceptable medium performance, positive and negative culture controls should be tested before first use and as otherwise specified (see Table 9020:VI). Sterility, volume per tube, and pH should also be verified and recorded. To demonstrate comparability between batches of media, perform a use test [Section 9020B.5*f*2)].

If a laboratory is switching to the multiple-tube fermentation technique, analysts ideally should first conduct parallel tests with the previous method to demonstrate applicability and comparability. The results of many coliform performance studies are available in the literature, and the rates of false-positive and -negative results can differ among various media. Users should carefully select the medium and procedure that best fits their needs.

3. Presumptive Phase

Use lauryl tryptose broth in this phase of the multiple-tube test, following the QC guidelines cited in 9221B.2.

a. Reagents and culture medium:

Lauryl tryptose broth:

Tryptose	20.0	g
Lactose	5.0	g

Table 9221:I. Preparation of Lauryl Tryptose Broth

Inoculum *mL*	Amount of Medium in Tube *mL*	Volume of Medium + Inoculum *mL*	Dehydrated Lauryl Tryptose Broth Required *g/L*
1	10 or more	11 or more	35.6
10	10	20	71.2
10	20	30	53.4
20	10	30	106.8
100	50	150	106.8
100	35	135	137.1
100	20	120	213.6

Dipotassium hydrogen phosphate (K_2HPO_4)	2.75	g
Potassium dihydrogen phosphate (KH_2PO_4)	2.75	g
Sodium chloride (NaCl)	5.0	g
Sodium lauryl sulfate	0.1	g
Reagent-grade water	1	L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense enough medium into fermentation tubes containing inverted vials (also known as Durham tubes) to cover the inverted vial at least one-half to two-thirds after sterilization. Alternatively, omit the inverted vial and add 0.01 g/L bromocresol purple to lauryl tryptose broth (to determine acid production, an indicator of a positive result in this part of the coliform test). Close tubes with metal or heat-resistant plastic caps.

Prepare in accordance with Table 9221:I, making lauryl tryptose broth concentrated enough that adding 100-, 20-, or 10-mL portions of sample to the medium will not reduce ingredient concentrations below those of the standard medium. Autoclave medium at 121°C for 12 to 15 min. Ensure that inverted vials, if used, are free of air bubbles. Medium pH should be 6.8 ± 0.2 after sterilization.

b. Procedure:

1) Arrange fermentation tubes in rows of five or ten tubes each in a test tube rack. The number of rows and the sample volumes selected depend on the quality and character of the water to be examined. For potable water, 100 mL must be tested. Use five 20-mL portions, ten 10-mL portions, or one 100-mL portion (a single bottle). For nonpotable water, use five tubes per dilution (of 10, 1, 0.1 mL, etc.).

When making dilutions and measuring diluted sample volumes, follow the precautions given in Section 9215B.2. Use Figure 9215:1 as a guide to preparing dilutions. Shake sample and dilutions vigorously 5 s (about 25 times). Inoculate each tube in a set of five with replicate sample volumes in increasing decimal dilutions, if decimal quantities of the sample are used. Mix test portions in the medium by gentle agitation.

2) Promptly incubate inoculated tubes or bottles, any culture controls, and/or sterility blanks at 35 ± 0.5°C. After 24 ± 2 h, swirl each tube or bottle gently and examine it for growth, gas, and/or acidic reaction (shades of yellow color) and, if no gas or acidic reaction is evident, re-incubate and re-examine at the end of 48 ± 3 h. Record presence or absence of growth, gas, and/or acid production. If the inner vial is omitted, growth with acidity (yellow color) signifies a presumptive-positive reaction.

c. Interpretation: Detection of an acidic reaction (yellow color) and/or gas in the tubes or bottles within 48 ± 3 h constitutes a presumptive-positive reaction. Submit tubes or bottles with a presumptive-positive reaction to the confirmed phase (9221B.4).

The absence of acidic reaction and/or gas formation at the end of 48 ± 3 h of incubation constitutes a negative test. Submit drinking water samples demonstrating growth without a positive gas or acidic reaction to the confirmed phase (9221B.4).

4. Confirmed Phase

a. Culture medium: Use BGLB broth fermentation tubes for the confirmed phase, following QC guidelines cited in 9221B.2.

Brilliant green lactose bile broth:

Peptone	10.0	g
Lactose	10.0	g
Oxgall	20.0	g
Brilliant green	0.0133	g
Reagent-grade water	1	L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense medium into fermentation tubes with an inverted vial, ensuring sufficient volume of medium to cover the inverted vial at least one-half to two-thirds after sterilization. Close tubes with metal or heat-resistant plastic caps. Autoclave medium at 121°C for 12 to 15 min. Ensure that inverted vials are free of air bubbles. Medium pH should be 7.2 ± 0.2 after sterilization.

b. Procedure: Promptly submit all presumptive tubes or bottles showing growth, any amount of gas, or acidic reaction within 24 ± 2 h of incubation to the confirmed phase. If additional presumptive tubes or bottles show active fermentation or acidic reaction at the end of a 48 ± 3 h incubation period, promptly submit these to the confirmed phase. To confirm presumptive coliform colonies growing on a solid medium using fermentation media, see Section 9222B.4*g*.

Gently shake or rotate presumptive tubes or bottles showing gas or acidic growth to resuspend the organisms. With a sterile loop 3.0 to 3.5 mm in diameter, transfer one or more loopfuls of culture to a fermentation tube containing BGLB broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of fermentation tube containing BGLB broth. Remove and discard applicator. Repeat for all other presumptive-positive tubes. Analysts may simultaneously inoculate BGLB broth for total coliforms and EC broth for thermotolerant (fecal) coliforms (see 9221E) or EC-MUG broth for *Escherichia coli* (see 9221F). However, if using the same loop or wooden applicator stick to inoculate a culture into more than one medium, inoculate the most inhibitory medium (BGLB broth) last.

Promptly incubate the inoculated BGLB broth tubes at 35 ± 0.5°C. Any amount of gas formed in the inverted vial of the BGLB broth fermentation tube at any time within 48 ± 3 h constitutes a positive confirmed phase. To estimate the coliform density, calculate the MPN value from the number of positive BGLB tubes as described in 9221C.

c. Alternative procedure: Use this alternative only for polluted water or wastewater known to produce positive results consistently.

If all presumptive tubes are positive in two or more consecutive dilutions within 24 h, then only submit to the confirmed phase the highest-dilution tubes (smallest sample inoculum) in which all tubes are positive, along with any positive tubes in still higher dilutions. Submit to the confirmed phase all tubes in which gas or acidic growth is produced in 24 to 48 h.

5. Completed Phase

The completed test as described here is not required for drinking-water compliance sample analyses. For nonpotable water samples collected under the Clean Water Act, the requirement that 10% of all total-coliform-positive tubes be subjected to the completed test on a seasonal basis no longer exists. The completed test is included here as a QC recommendation and for use when testing results are uncertain. As additional testing for thermotolerant (fecal) coliforms and/or *E. coli* is required of positive coliform tests, further testing using EC and/or EC-MUG broths is considered a completed test. For QC purposes, if no positive drinking-water samples are received within a quarter, then analyze at least one positive source-water sample to confirm that media respond appropriately.

To verify the presence of coliform bacteria and to provide QC data for nonpotable water-sample analysis, use the completed test on at least one positive sample per quarter. If no positive sample occurs within a quarter, perform a QC check using a known positive sample. Analysts may simultaneously inoculate presumptive-positive media into both BGLB broth for confirmation of total coliforms and EC broth for thermotolerant (fecal) coliforms (9221E) or EC MUG broth for *Escherichia coli* (9221F) as long as BGLB broth is inoculated last. Positive results from incubation in EC and/or EC-MUG broths at elevated temperature (44.5 ± 0.2°C) can be considered a completed test. Parallel positive BGLB broth cultures with negative EC or EC-MUG broth cultures indicate the presence of nonfecal coliforms. Parallel positive EC or EC-MUG tubes and negative BGLB broth cultures indicate the presence of thermotolerant (fecal) coliforms or *E. coli*, respectively. Alternatively, the completed test for positive total coliforms may be performed as follows.

a. Culture media and reagents: Follow the QC guidelines cited in 9221B.2.

1) *LES Endo agar*—See Section 9222B.2*a*. Use 100- × 15-mm Petri plates.

2) *MacConkey agar:*

Peptone	17	g
Proteose peptone	3	g
Lactose	10	g

Bile salts	1.5	g
Sodium chloride (NaCl)	5	g
Agar	13.5	g
Neutral red	0.03	g
Crystal violet	0.001	g
Reagent-grade water	1	L

Add ingredients to water, mix thoroughly, and heat to boiling to dissolve. Sterilize by autoclaving for 15 min at 121°C. Temper agar after sterilization and pour into Petri plates (100 × 15 mm). Medium pH should be 7.1 ± 0.2 after sterilization.

3) *Nutrient agar:*

Peptone	5.0	g
Beef extract	3.0	g
Agar	15.0	g
Reagent-grade water	1	L

Add ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense in screw-capped tubes. Autoclave at 121°C for 15 min. Medium pH should be 6.8 ± 0.2 after sterilization. After sterilization, immediately place tubes in an inclined position so the agar will solidify with a sloped surface. Tighten screw caps after cooling and store in a protected, cool storage area.

4) *Gram-stain reagents*—Reagents are commercially available as prepared solutions.

a) *Ammonium oxalate-crystal violet (Hucker's)*—Dissolve 2 g crystal violet (90% dye content) in 20 mL 95% ethyl alcohol. **CAUTION:** ***Flammable.*** Dissolve 0.8 g $(NH_4)_2C_2O_4 \cdot H_2O$ in 80 mL reagent-grade water. Mix the two solutions and age for 24 h before use. Filter through paper into a staining bottle.

b) *Lugol's solution, Gram's modification*—Grind 1 g iodine crystals and 2 g KI in a mortar. Add reagent-grade water, a few milliliters at a time, and grind thoroughly after each addition until solution is complete. Rinse solution into an amber glass bottle with the remaining water, using a total of 300 mL.

c) *Counterstain*—Dissolve 2.5 g safranin dye in 100 mL 95% ethyl alcohol. Add 10 mL to 100 mL reagent-grade water. **CAUTION:** ***Flammable.***

d) *Acetone alcohol*—Mix equal volumes of ethyl alcohol (95%) with acetone. **CAUTION:** ***Flammable.***

b. Procedure:

1) Using aseptic technique, streak one LES Endo agar (Section 9222B.2*a*) or MacConkey agar plate from each presumptive-positive tube of BGLB broth as soon as possible after gas is observed. Streak plates in a manner to ensure the presence of some discrete colonies separated by at least 0.5 cm. To obtain a high proportion of successful isolations if coliform organisms are present, use the following approach:

a) Use a sterile 3-mm-diam loop or an inoculating needle slightly curved at the tip;
b) tap and incline the fermentation tube to avoid picking up any membrane or scum on the needle;
c) insert the end of the loop or needle into the liquid in the tube to a depth of approximately 0.5 cm; and
d) streak a plate for isolation with the curved section of the needle in contact with the agar to avoid a scratched or torn surface. Flame the loop between the second and third quadrants to improve colony isolation.

Incubate plates, inverted, at 35 ± 0.5°C for 24 ± 2 h.

2) The colonies developing on LES Endo agar are defined as *typical* (pink to dark red with a green metallic surface sheen) or *atypical* (pink, red, white, or colorless colonies without sheen) after 24 h incubation. Typical lactose-fermenting colonies developing on MacConkey agar are red and may be surrounded by an opaque zone of precipitated bile. From each plate, pick one or more typical, well-isolated coliform colonies or, if no typical colonies are present, pick two or more colonies considered most likely to be coliforms. Transfer growth from each isolate to a single-strength lauryl tryptose broth fermentation tube and onto a nutrient agar slant.

If needed, use a colony-magnifying device to provide optimum magnification when colonies are picked from the LES Endo or MacConkey agar plates. When transferring colonies, choose well-isolated ones and barely touch the colony surface with a flame-sterilized, air-cooled transfer needle to minimize the danger of transferring a mixed culture.

Incubate secondary broth tubes (lauryl tryptose broth with inverted fermentation vials) at 35 ± 0.5°C for 24 ± 2 h; if gas is not produced within 24 ± 2 h, reincubate and examine again at 48 ± 3 h. Microscopically examine Gram-stained preparations from those 24-h nutrient agar slant cultures corresponding to the secondary tubes that show gas.

3) Gram-stain technique—The Gram stain may be omitted from the completed test for potable-water samples only because Gram-positive bacteria and spore-forming organisms in drinking water rarely survive this selective screening procedure.

Various modifications of the Gram stain technique exist. Use Hucker's modification (as follows) for staining smears of pure cultures; include a Gram-positive and a Gram-negative culture as controls.

On one slide, prepare separate light emulsions of the test bacterial growth and positive and negative control cultures using drops of distilled water on the slide. Air-dry, fix by passing slide through a flame, and stain for 1 min with ammonium oxalate-crystal violet solution. Rinse slide in tap water and drain off excess; apply Lugol's solution for 1 min.

Rinse stained slide in tap water. Decolorize for approximately 15 to 30 s with acetone alcohol by holding the slide between the fingers and letting acetone alcohol flow across the stained smear until the solvent flows colorlessly from the slide. Do not overdecolorize. Counterstain with safranin for 15 s, rinse with tap water, blot dry with absorbent paper or air dry, and examine microscopically. Gram-positive organisms are blue; Gram-negative organisms are red. Results are acceptable only when controls have given proper reactions.

c. Interpretation: Formation of gas in the secondary tube of lauryl tryptose broth within 48 ± 3 h and demonstration of Gram-negative, nonspore-forming, rod-shaped bacteria from the agar culture constitute a positive result for the completed test, demonstrating that a member of the coliform group is present.

6. Bibliography

HUCKER, G.J. & H.J. CONN. 1927. Further Studies on the Methods of Gram Staining; Tech. Bull. No. 128. N.Y. State Agricultural Experiment Station, Geneva, N.Y.

COWLES, P.B. 1939. A modified fermentation tube. *J. Bacteriol.* 38:677.

SKERMAN, V.B.D. 1967. A Guide to the Identification of the Genera of Bacteria. Williams & Wilkins, Baltimore, Md.

EVANS, T.M., C.E. WAARVICK, R.J. SEIDLER & M.W. LECHEVALLIER. 1981. Failure of the most-probable-number technique to detect coliforms in drinking water and raw water supplies. *Appl. Environ. Microbiol.* 41:130.

GERHARDS, P., ed. 1981. Manual of Methods for General Bacteriology. American Soc. Microbiology, Washington, D.C.

SEIDLER, R.J., T.M. EVANS, J.R. KAUFMAN, C.E. WAARVICK & M.W. LECHEVALLIER. 1981. Limitations of standard coliform enumeration techniques. *J. Amer. Water Works Assoc.* 73:538.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2000. National primary drinking water regulations. 40 CFR Parts 141, and 142; *Fed. Reg.* 65(8):1950.

GARRITY, G.M., ed. 2005. Part B: The gammaproteobacteria. *In* Bergey's Manual of Systematic Bacteriology, 2nd ed.; Volume 2: The Proteobacteria. Springer, New York, N.Y.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2005. Manual for the Certification of Laboratories Analyzing Drinking Water; EPA 815-R-05-004. Off. Ground Water and Drinking Water Tech. Support Ctr., Cincinnati, Ohio.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2012. Guidelines for Establishing Test Procedures for the Analysis of Pollutants Under the Clean Water Act; Analysis and Sampling Procedures: Final Rule. 40 CFR Parts 136, 260, *et al.*; *Fed. Reg.* 77(97):29797.

9221 C. Estimation of Bacterial Density

1. Precision of the Multiple-Tube Fermentation Test

The multiple-tube fermentation test is not very precise unless many sample portions are examined, so use caution when interpreting the sanitary significance of any single coliform result. Precision improves greatly when several samples from a given sampling point are estimated separately and their geometric mean is calculated.

Although most probable number (MPN) tables and calculations are described for use in the coliform test, they also can be used to determine the MPN of any organism so long as suitable test media are available. Online MPN calculators are available, but until a calculator's accuracy has been verified, confirm its results using an MPN table in this section.

2. Use of Tables to Determine MPN

Record coliform concentration as MPN/100 mL. The MPN values for a variety of positive and negative tube combinations are given in Tables 9221:II, III, and IV. The sample volumes indicated in Tables 9221:II and III are chosen especially for drinking-water examinations. Table 9221:IV illustrates MPN values for combinations of positive and negative results when five 10-mL, five 1.0-mL, and five 0.1-mL sample-portion volumes of nonpotable water are tested. If the sample-portion volumes tested are identical to those found in the tables, then report the value corresponding to appropriate combination of positive and negative results as the MPN/100 mL. However, if the series of decimal dilutions is different, then select the MPN value in Table 9221:IV that corresponds to the combination of positive results and calculate the actual MPN using the following formula:

$$\text{MPN/100 mL} = (\text{Table MPN/100 mL}) \times 10/V$$

where:

V = volume of sample portion at the lowest selected dilution.

If the decimal series[1] includes more than three dilutions, use the following guidelines to select the three most appropriate dilutions and then use Table 9221:IV and the equation above to calculate the MPN. See Table 9221:V, which provides several examples (A–G) of combinations of positives. First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube. Next, remove the lowest dilution (largest sample volume) if it has all positive tubes and at least one remaining dilution has a positive tube. According to these guidelines, the three dilutions in Example A are selected by removal of the highest (0.001-mL) and the lowest (10-mL) dilutions.

If the lowest dilution does not have all positive tubes, and several of the highest dilutions have all negative tubes, then remove the highest negative dilutions (Example B).

TABLE 9221:II. MPN INDEX AND 95% CONFIDENCE LIMITS FOR ALL COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 20-mL PORTIONS ARE USED

No. of Tubes Giving Positive Reaction Out of 5 (20 mL Each)	MPN Index/ 100 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	–	3.5
1	1.1	0.051	5.4
2	2.6	0.40	8.4
3	4.6	1.0	13
4	8.0	2.1	23
5	>8.0	3.4	–

TABLE 9221:III. MPN INDEX AND 95% CONFIDENCE LIMITS FOR ALL COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN TEN 10-mL PORTIONS ARE USED

No. of Tubes Giving Positive Reaction Out of 10 (10 mL Each)	MPN Index/ 100 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	–	3.4
1	1.1	0.051	5.9
2	2.2	0.37	8.2
3	3.6	0.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.8	34
9	23	8.1	53
10	>23	13	–

Table 9221:IV. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes Are Used per Dilution (10 mL, 1.0 mL, 0.1 mL)*

Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	11	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

* Results to two significant figures.

More than three dilutions may remain after removal of the lowest dilution with all positive tubes and high dilutions with all negative tubes. In this case, if the highest dilution with *all* positive tubes is within two dilutions of the highest dilution with *any* positive tubes, then use the highest dilution with *any* positive tubes and the two immediately lower dilutions. In Example C, the highest dilution with all positive tubes is 0.1 mL, which is within two dilutions of 0.001 mL, which has one positive tube. In Example D, the highest dilution with all positive tubes is 0.01 mL, which is within two decimal dilutions of 0.001 mL, to yield a combination of 4-5-1.

If, after removal of the lowest dilution with all positive tubes, no dilution with all positive reactions remains, then select the lowest

Table 9221:V. Examples for Choice of Three Combinations of Positives from Five Dilutions

Example	Volume *mL* 10	1	0.1	0.01	0.001	Combination of Positives	MPN Index *No./100 mL*
A	5	**5**	**1**	**0**	0	x-5-1-0-x	330
B	**4**	**5**	**1**	0	0	4-5-1-x-x	48
C	5	2	**5**	**2**	**1**	x-x-5-2-1	7000
D	4	5	**4**	**5**	**1**	x-x-4-5-1	4800
E	5	**4**	**4**	**0**	**1**	x-4-4-1-x	400
F	**4**	**3**	**0**	**1**	**1**	4-3-2-x-x	39
G	4	3	**3**	**2**	**1**	x-x-3-2-1	1700

two dilutions and assign the sum of any remaining dilutions to the third dilution. In Example E, the highest dilution with all positive tubes contains 10 mL; this dilution was removed in the second step. Four dilutions, none of which have all positive tubes, remain. Under these circumstances, select the two lowest remaining dilutions corresponding to 1 and 0.1 mL sample. For the third dilution, add the number of positive tubes in all higher dilutions (0.01 and 0.001 mL sample), to yield a final combination of 4-4-1.

If no dilution has all positive tubes (Example F), select the lowest two dilutions, corresponding to 10 and 1 mL sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 mL sample), to yield a final combination of 4-3-2. If the third dilution is assigned more than five positive tubes, then the selected combination will not be in Table 9221:IV.

If the three dilutions selected are not found in Table 9221:IV, then something in the serial dilution was unusual. In this case, the usual methods for calculating the MPN, presented here, may not apply. If a new sample cannot be collected and an MPN value is still desired, use the highest dilution with at least one positive tube and the two dilutions immediately lower as the three selected dilutions. In Example G, the first selection, 4-3-6 (the outcome from the highest three dilutions), is not in Table 9221:IV because 6 is greater than 5. The second selection, according to the above guidelines, would be 3-2-1. If this second set of selected dilutions is not in Table 9221:IV, then use the following formula to calculate the MPN:

$$-\frac{230.3}{z_s}\log_{10}\left(1-\frac{x_s z_s}{\sum_{j=s}^{K} n_j z_j}\right)$$

where:

z_s = the amount of the original sample inoculated into each tube of the sth dilution, and
x_s = the number of positive tubes in the sth dilution,
K = the number of dilutions,
j = a dilution,
s = the highest dilution with at least one positive tube,
n_j = the number of tubes in the jth dilution, and
z_j = the amount of the original sample inoculated into each tube in the jth dilution.

For example, in the series x-x-3-0-0, where the third dilution level (z_s) equals 0.1 mL, $x_s z_s = 0.3$, and $\Sigma\, n_j z_j = 0.555$. Thus, the calculated MPN = 7800/100 mL.

This formula also applies to serial dilutions having all positive tubes in a single dilution, and can serve as an approximation for outcomes like 5-5-5-0-0-0, where five tubes are used per dilution, by using just the last four dilutions.

Table 9221:IV shows all but the improbable positive tube combinations for a three-dilution series. In testing 10 samples, there is a 99% chance of finding all the results among these 95 outcomes. If untabulated combinations occur with a frequency greater than 1%, it indicates that the technique is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled (e.g., growth inhibition at low dilutions).

The MPN for combinations not appearing in the table, or for other combinations of tubes or dilutions, may be *estimated* as follows: First, select the lowest dilution that does not have all positive results. Second, select the highest dilution with at least one positive result. Finally, select all the dilutions between them. For example, from (10/10, 10/10, 4/10, 1/10, 0/10) use only (–, –, 4/10, 1/10, –), corresponding to 4/10 @ 0.1 mL sample/tube and 1/10 @ 0.01 mL sample/tube. Likewise, from (10/10, 10/10, 10/10, 0/10, 0/10), select only (–, –, 10/10, 0/10, –), corresponding to 10/10 @ 0.1 mL sample/tube and 0/10 @ 0.01 mL sample/tube. Use only the selected dilutions in the following formula of Thomas:[1]

$$\text{MPN/100 mL (approx.)} = 100 \times P/(N \times T)^{1/2}$$

where:

P = number of positive results,
N = volume of sample in all the negative portions combined, mL, and
T = total volume of sample in the selected dilutions, mL.

That is, $N = \Sigma(n_j\text{-}x_j)z_j$, $P = \Sigma\, x_j$, and $T = \Sigma\, n_j z_j$, where the summations are over the dilutions selected, and x_j = the number of positive tunes in the jth dilution.

In the first example above,

$$\text{MPN/100 mL (approx.)} = 100 \times 5/(0.69 \times 1.1)^{1/2}$$

$$= 500/0.87 = 570/100 \text{ mL}$$

In the second example above,

$$\text{MPN/100 mL (approx.)} = 100 \times 10/(0.1 \times 1.1)^{1/2}$$

$$= 1000/0.332 = 3000/100 \text{ mL}$$

The two examples compare well with the true MPNs, 590/100 mL and 2400/100 mL, respectively. The second example is a special case for which an exact solution can be calculated directly for the two selected dilutions.

When it is desired to summarize the results from several samples with a single MPN value, use the geometric mean or the median. The geometric mean is calculated by averaging the logarithmic values; for example, the geometric mean of A, B, and C is 10^L where:

$$L = (\log_{10} A + \log_{10} B + \log_{10} C)/3$$

Mean values are reported as the antilog of L.

3. Reference

1. Thomas, H.A., Jr. 1942. Bacterial densities from fermentation tube tests *J. Amer. Water Works Assoc*. 34:572.

4. Bibliography

McCrady, M.H. 1915. The numerical interpretation of fermentation tube results. *J. Infect. Dis*. 12:183.

McCrady, M.H. 1918. Tables for rapid interpretation of fermentation-tube results. *Pub. Health J*. 9:201.

Halvorson, H.O. & N.R. Ziegler. 1933–35. Application of statistics to problems in bacteriology. *J. Bacteriol.* 25:101; 26:331, 559; 29:609.

Hoskins, J.K. 1933. The most probable numbers of *B. coli* in water analysis. *J. Amer. Water Works Assoc*. 25:867.

Hoskins, J.K. 1934. Most probable numbers for evaluation of *coli-aerogenes* tests by fermentation tube method. *Pub. Health Rep*. 49:393.

Eisenhart, C. & P.W. Wilson. 1943. Statistical methods and control in bacteriology. *Bacteriol. Rev.* 7:57.

Cochran, W.G. 1950. Estimation of bacterial densities by means of the "most probable number." *Biometrics* 6:105.

Woodward, R.L. 1957. How probable is the most probable number? *J. Amer. Water Works Assoc*. 49:1060.

DeMan, J.C. 1983. MPN tables, corrected. *Eur. J. Appl. Biotechnol.* 17:301.

Blodgett, R.J. & W.E. Garthright. 1998. Several MPN models for serial dilutions with suppressed growth at low dilutions. *Food Microbiol*. 15:91.

Garthright, W.E. 1998. Appendix 2: Most probable number from serial dilutions. FDA Bacteriological Analytical Manual, 8th ed., Rev. A. AOAC International, Gaithersburg, Md.

Blodgett, R.J. 2002. Measuring improbability of outcomes from a serial dilution test. *Commun. Statist. Theory Meth.* 31:2209.

Garthright, W.E. & R.J. Blodgett. 2003. FDA's Preferred MPN methods for standard, large or unusual tests, with a spreadsheet. *Food Microbiol*. 20:439.

Blodgett, R.J. 2010. Appendix 2: Most probable number from serial dilutions. FDA Bacteriological Analytical Manual. Available at: http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm109656.htm. Accessed November 2016.

9221 D. Presence–Absence (P–A) Coliform Test

The presence–absence (P–A) test for the coliform group is a simple modification of the multiple-tube procedure that is intended for use on routine samples collected from distribution systems or water treatment plants. This simplification using one large test portion (100 mL) in a single culture bottle to determine qualitatively whether coliforms are present or absent is justified on the theory that no coliforms should be present in 100 mL of a drinking water sample. Also, it enables analysts to examine more samples in a given time period compared to quantitative methods. Comparative studies with the membrane-filter procedure indicate that the P–A test may maximize coliform detection in samples containing many organisms that could overgrow coliform colonies and cause detection problems.

The P–A broth contains lactose and a pH indicator to detect the presence of acid production. Analysts observe the culture bottles for gas and/or acid production—the metabolic end products of lactose fermentation. Presumptive-positive coliform results obtained from P–A broth must be confirmed using BGLB broth.

1. Samples

Collect samples as directed in Section 9060, using sample containers specified in Section 9030B.19. Follow the QC guidelines for sample bottles described in Section 9020B.5*d*. Ensure that samples meet laboratory acceptance criteria upon receipt.

2. Presumptive Phase

a. Culture medium:

P-A broth: Follow QC guidelines cited in 9221B.2.

Beef extract	3.0	g
Peptone	5.0	g
Lactose	7.46	g
Tryptose	9.83	g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.35	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.35	g
Sodium chloride (NaCl)	2.46	g
Sodium lauryl sulfate	0.05	g
Bromocresol purple	0.0085	g
Reagent-grade water	1	L

Make this formulation triple strength (3×) when examining 100-mL samples. Dissolve P–A medium in water by stirring (do not use heat). Dispense 50 mL prepared medium into screw-capped 250-mL milk dilution bottles or equivalent containers. A fermentation vial insert is unnecessary. Autoclave for 12 min at 121°C; limit total time in the autoclave to 30 min or less. Medium pH should be 6.8 ± 0.2 after sterilization.

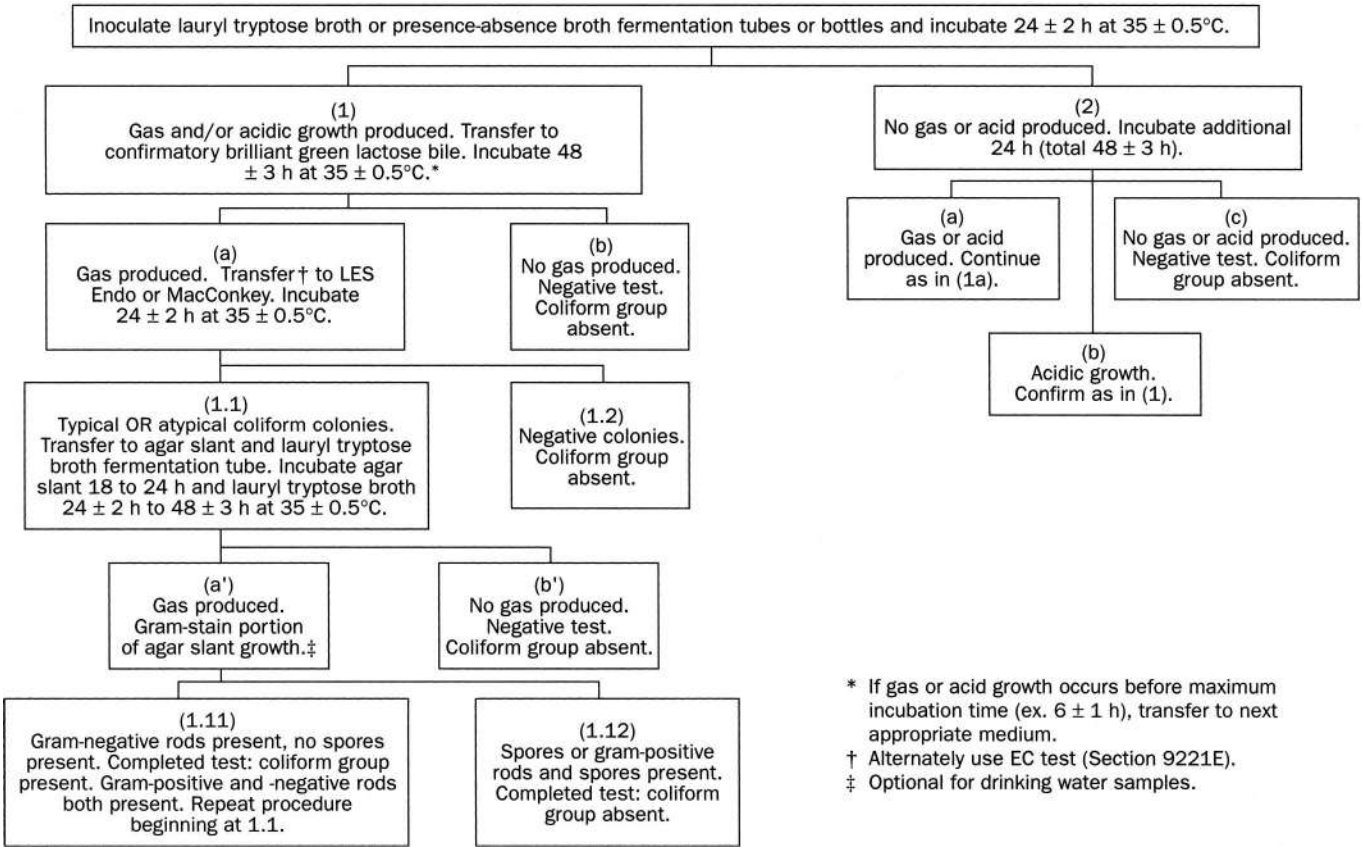

Figure 9221:1. Schematic outline of presumptive, confirmed, and completed phases for total coliform detection.

If sterilized via filtration, a 6× strength P–A medium may be used. Aseptically dispense 20 mL of the 6× medium into a sterile 250-mL dilution bottle or equivalent container.

b. Procedure: Shake sample vigorously for 5 s (approximately 25 times) and inoculate 100 mL into a P–A culture bottle. Mix thoroughly by inverting bottle once or twice to evenly distribute the sample throughout the medium. Incubate at 35 ± 0.5°C and inspect after 24 ± 2 h and 48 ± 3 h for acid reactions.

c. Interpretation: If acidic conditions exist following lactose fermentation, a distinct yellow color will form in the medium. If gas also is being produced, then foaming will occur when the bottle is gently shaken. Any amount of gas and/or acid constitutes a presumptive-positive test that requires confirmation.

3. Confirmed Phase

The confirmed phase is outlined in Figure 9221:1.

a. Culture medium: Use BGLB broth fermentation tubes (see 9221B.4).

b. Procedure: After incubation, promptly use a 3.0- to 3.5-mm-diam sterile loop to transfer one or more loopfuls of culture from a presumptive-positive bottle to a fermentation tube containing BGLB broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing BGLB broth. Remove and discard applicator. Repeat for all other presumptive-positive tubes and inoculate at 35 ± 0.5°C (see 9221B.4).

Loopfuls of culture from presumptive-positive bottles also may be transferred into EC broth [for determination of thermotolerant (fecal) coliforms] and/or EC-MUG broth (for *E. coli* determinations) at the same time, as long as the most inhibitory medium (BGLB broth) is inoculated last.

c. Interpretation: Gas production in the BGLB broth culture within 48 ± 3 h confirms the presence of coliform bacteria. Report result as P–A test positive or negative for total coliforms in 100 mL of sample. Drinking water samples that are positive for total coliforms also must be tested for thermotolerant (fecal) coliforms (9221E) or *E. coli* (9221F).

4. Completed Phase

The completed phase, required for nonpotable water sample analysis, is outlined in 9221B.5 and Figure 9221:1.

5. Bibliography

Weiss, J.E. & C.A. Hunter. 1939. Simplified bacteriological examination of water. *J. Amer. Water Works Assoc.* 31:707.

Clark, J.A. 1969. The detection of various bacteria indicative of water pollution by a presence–absence (P–A) procedure. *Can. J. Microbiol.* 15:771.

CLARK, J.A. & L.T. VLASSOFF. 1973. Relationships among pollution indicator bacteria isolated from raw water and distribution systems by the presence–absence (P–A) test. *Health Lab. Sci.* 10:163.

CLARK, J.A. 1980. The influence of increasing numbers of nonindicator organisms upon the detection of indicator organisms by the membrane filter and presence–absence tests. *Can. J. Microbiol.* 26:827.

CLARK, J.A., C.A. BURGER & L.E. SABATINOS. 1982. Characterization of indicator bacteria in municipal raw water, drinking water and new main water samples. *Can. J. Microbiol.* 28:1002.

JACOBS, N.J., W.L. ZEIGLER, F.C. REED, T.A. STUKEL & E.W. RICE. 1986. Comparison of membrane filter, multiple-fermentation-tube, and presence–absence techniques for detecting total coliforms in small community water systems. *Appl. Environ. Microbiol.* 51:1007.

RICE, E.W., E.E. GELDREICH & E.J. READ. 1989. The presence–absence coliform test for monitoring drinking water quality. *Pub. Health Rep.* 104:54.

9221 E. Thermotolerant (Fecal) Coliform Procedure

Traditionally called *fecal coliforms,* thermotolerant coliforms (those that ferment lactose to produce gas at 44.5°C) have been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. So when looking for evidence of fecal contamination, testing for *E. coli*—a more specific indicator—is recommended. Nevertheless, regulations may require that thermotolerant (fecal) coliforms be identified and enumerated.

To test for thermotolerant coliforms, use one of the multiple-tube procedures described here or the membrane-filter methods described in Sections 9222D and E. In the multiple-tube fermentation technique, thermotolerant coliforms are identified by their ability to ferment lactose to produce gas at 44.5 ± 0.2°C within 24 ± 2 h.

1. Thermotolerant Coliform Test (EC Medium)

The thermotolerant coliform test using EC medium is applicable to investigations of drinking water, stream pollution, unfiltered raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. Do not use EC medium to directly isolate thermotolerant coliforms from water; prior enrichment in a presumptive medium is required for optimum recovery of thermotolerant coliforms. (To test presumptive coliform colonies growing on solid media, refer to Section 9222G.3*c*)

a. EC medium: Prepare EC medium following QC guidelines cited in 9221B.2.

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No. 3	1.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Sodium chloride (NaCl)	5.0 g
Reagent-grade water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense sufficient medium in fermentation tubes with an inverted vial to cover the inverted vial at least one-half to two-thirds after sterilization. Close tubes with metal or heat-resistant plastic caps. Autoclave medium at 121°C for 12 to 15 min. Ensure that inverted vials are free of air bubbles. Medium pH should be 6.9 ± 0.2 after sterilization.

b. Procedure:

1) After incubation, gently shake or rotate fermentation tubes or bottles showing gas, growth, or acidity to resuspend the organisms. Promptly use a sterile 3- to 3.5-mm-diam loop to transfer one or more loopfuls of culture from bottles or tubes showing growth with acid and/or gas production to a fermentation tube containing EC broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing EC broth. Remove and discard applicator. Repeat for all other presumptive-positive tubes and incubate at 44.5 ± 0.2°C.

Simultaneous inoculation into EC broth and/or EC-MUG broth along with BGLB broth is acceptable, if the most inhibitory medium (BGLB broth) is inoculated last.

2) Place all EC tubes into a circulating water bath (preferably with a gabled cover) within 30 min after inoculation. Incubate inoculated EC broth tubes at 44.5 ± 0.2°C for 24 ± 2 h. Maintain a sufficient water depth in the water bath incubator to immerse tubes to the upper level of the medium.

c. Interpretation: Gas production with growth in an EC broth culture within 24 ± 2 h or less is considered a positive thermotolerant (fecal) coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction. If multiple tubes are used, calculate the MPN of thermotolerant coliforms from the number of positive EC broth tubes, as described in 9221C. When using only one tube for subculturing from a single presumptive bottle, report as the presence or absence of thermotolerant coliforms. If heavy growth occurs with no gas production, subject the culture to a thermotolerant coliform or *E. coli* test using a different medium.

2. Thermotolerant (Fecal) Coliform Direct Test (A-1 Medium)

a. A-1 medium: This medium may be used to directly isolate thermotolerant coliforms from unfiltered source water, treated wastewater, and seawater, but not drinking water. Follow guidelines in 9221B.1 for sample collection. Unlike EC medium, A-1 medium does not require prior enrichment in a presumptive medium for optimum recovery of thermotolerant coliforms. Use QC guidelines cited in 9221B.2.

Lactose	5.0 g
Tryptone	20.0 g
Sodium chloride (NaCl)	5.0 g

Salicin	0.5 g
Polyethylene glycol *p*-isooctylphenyl ether*	1.0 mL
Reagent-grade water	1 L

Heat to dissolve solid ingredients, add polyethylene glycol *p*-isooctylphenyl ether, and adjust to pH 6.9 ± 0.1. For 10-mL samples, prepare double-strength medium so the final concentration of ingredients after sample addition is correct. Before sterilization, dispense sufficient medium in fermentation tubes with an inverted vial to cover the inverted vial at least one-half to two-thirds after sterilization. Close with metal or heat-resistant plastic caps. Sterilize by autoclaving at 121°C for 10 min. Ensure that inverted vials are free of air bubbles. Store in the dark at room temperature for not longer than 7 d. Ignore precipitate formed during storage.

b. Procedure: Inoculate tubes of A-1 broth as directed in 9221B.3*b*. Incubate for 3 h at 35 ± 0.5°C. Transfer tubes to a water bath at 44.5 ± 0.2°C and incubate for another 21 ± 2 h.

c. Interpretation: Gas production in any A-1 broth culture within 24 h or less is a positive reaction [i.e., thermotolerant (fecal) coliforms are present]. Calculate the MPN of thermotolerant (fecal) coliforms from the number of positive A-1 broth tubes, as described in 9221C.

* Triton X-100, Sigma-Aldrich, St. Louis, MO, or equivalent.

3. Bibliography

PERRY, C.A. & A.A. HAJNA. 1933. A modified Eijkman medium. *J. Bacteriol.* 26:419.

PERRY, C.A. & A.A. HAJNA. 1944. Further evaluation of EC medium for the isolation of coliform bacteria and *Escherichia coli*. *Amer. J. Pub. Health* 34:735.

GELDREICH, E.E., H.F. CLARK, P.W. KABLER, C.B. HUFF & R.H. BORDNER. 1958. The coliform group. II. Reactions in EC medium at 45°C. *Appl. Microbiol.* 6:347.

GELDREICH, E.E., R.H. BORDNER, C.B. HUFF, H.F. CLARK & P.W. KABLER. 1962. Type distribution of coliform bacteria in the feces of warm-blooded animals. *J. Water Pollut. Control Fed.* 34:295.

GELDREICH, E.E. 1966. Sanitary Significance of Fecal Coliforms in the Environment; FWPCA Pub. WP-20-3. U.S. Dept. Interior, Washington, D.C.

ANDREWS, W.H. & M.W. PRESNELL. 1972. Rapid recovery of *Escherichia coli* from estuarine water. *Appl. Microbiol.* 23:521.

OLSON, B.H. 1978. Enhanced accuracy of coliform testing in seawater by a modification of the most-probable-number method. *Appl. Microbiol.* 36:438.

STANDRIDGE, J.H. & J.J. DELFINO. 1981. A-1 medium: Alternative technique for fecal coliform organism enumeration in chlorinated wastewaters. *Appl. Environ. Microbiol.* 42:918.

9221 F. *Escherichia coli* Procedure Using Fluorogenic Substrate

Escherichia coli is a member of the indigenous fecal flora of warm-blooded animals. The presence of *E. coli* in water is considered a specific indicator of fecal contamination and the possible presence of enteric pathogens. Tests for *E. coli* are applicable to the analysis of drinking, surface, ground, and waste water. Testing for *E. coli* can be performed using the multiple-tube procedure described here, by the membrane filter method described in Section 9222G, or by the chromogenic enzyme substrate tests described in Section 9223. Other *E. coli* procedures are presented in 9221G.

For the *E. coli* test using EC-MUG medium, *E. coli* is defined as the species of coliform bacteria that possesses the enzyme β-glucuronidase, which can cleave the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), thus releasing the fluorogen within 24 ± 2 h or less when grown in EC-MUG medium at 44.5 ± 0.2°C.

1. *Escherichia coli* Test (EC-MUG Medium)

The use of EC-MUG medium to detect *E. coli* is applicable to investigations of drinking water, stream pollution, unfiltered raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. Do not use EC-MUG for the direct isolation of *E. coli*; prior enrichment ina presumptive medium is required for optimum recovery. (To test presumptive coliform colonies growing on solid media, refer to Section 9222G.2.)

Use EC-MUG medium to test for *E. coli* in a total coliform-positive culture, following QC guidelines cited in 9221B.2.

a. EC-MUG medium: Prepare EC-MUG medium following QC guidelines cited in 9221B.2.

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No. 3.	1.5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Sodium chloride (NaCl)	5.0 g
4-Methylumbelliferyl-β-D-glucuronide (MUG)	0.05 g
Reagent-grade water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. Before sterilization, dispense in tubes that do not fluoresce under long-wavelength (365–366 nm) ultraviolet (UV) light. An inverted tube is not necessary. Close tubes with metal or heat-resistant plastic caps. Medium pH should be 6.9 ± 0.2 after sterilization for 15 min at 121°C.

b. Procedure:

1) Gently shake or rotate fermentation tubes or bottles showing growth, gas, or acidity to resuspend the organisms. Using a sterile 3- or 3.5-mm-diam loop, transfer one or more loopfuls of growth from the fermentation tube or bottle to EC-MUG broth. Alternatively, insert a sterile wooden applicator stick at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing EC-MUG broth.

2) Place all EC-MUG tubes in water bath within 30 min after inoculation. Incubate inoculated EC-MUG tubes and negative controls for 24 ± 2 h in a circulating water bath (preferably with a gable cover) maintained at 44.5 ± 0.2°C. Maintain a sufficient

water depth in the water-bath incubator to immerse tubes to the upper level of medium.

c. Interpretation: Examine all tubes exhibiting growth for fluorescence using a 6W, 365–366 nm long-wavelength UV lamp. The presence of bright blue fluorescence is considered a positive result for *E. coli*. Growth in the absence of bright blue fluorescence is considered a negative result. To help interpret results and avoid misidentifying weak autofluorescence of the medium or glass tubes as a positive response, include in the assay a positive control [a known *E. coli* (MUG-positive) culture], a negative control [a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture], and an uninoculated medium control. The distance between the UV lamp and the tubes should be such that the *E. coli* positive control shows distinct fluorescence while the MUG-negative and uninoculated controls do not. If using multiple tubes, calculate the MPN for *E. coli* from the number of positive EC-MUG broth tubes, as described in 9221C. When using only one tube, or subculturing from a single presumptive bottle or colony, report as the presence or absence of *E. coli*.

2. Simultaneous Determination of Thermotolerant Coliforms and *E. coli*

The presence of thermotolerant coliforms and *E. coli* can be determined simultaneously by including an inverted vial (Durham tube) in tubes of EC-MUG broth. Prepare EC-MUG broth according to 9221F.1.

a. Setup: Before sterilization dispense, in fermentation tubes with an inverted vial, sufficient medium to cover the inverted vial at least one-half to two-thirds after sterilization. Close with metal or heat-resistant caps. Medium pH should be 6.9 ± 0.2 after sterilization for 15 min at 121°C.

b. Procedure:

1) Gently shake or rotate fermentation tubes or bottles showing growth, gas, or acidity to resuspend the organisms. Using a sterile 3- or 3.5-mm-diam loop, transfer one or more loopfuls of growth from the fermentation tube or bottle to EC-MUG broth. Alternatively, insert a sterile wooden applicator stick at least 2.5 cm into the culture, promptly remove, and plunge applicator to the bottom of a fermentation tube containing EC-MUG broth.

2) Place all EC-MUG tubes in water bath within 30 min after inoculation. Incubate inoculated EC-MUG tubes, along with positive and negative controls, for 24 ± 2 h in a circulating water bath (preferably with a gable cover) maintained at 44.5 ± 0.2°C. Maintain a sufficient water depth in the water-bath incubator to immerse tubes to the upper level of medium.

c. Interpretation: Examine all tubes exhibiting growth and/or gas for fluorescence using a 6W, 365–366 nm long-wavelength UV lamp. Growth with gas production is considered a positive result for thermotolerant coliforms. The presence of bright blue fluorescence is considered a positive result for *E. coli*. Tubes with growth and/or gas *and* fluorescence are considered positive for both thermotolerant coliforms and *E. coli*. Tubes with growth and/or gas but without bright blue fluorescence are considered positive for thermotolerant coliforms and negative for *E. coli.*

Due to indigenous autofluorescence of media or glass tubes/inserts, use caution in interpreting results. To help interpret results, include in each assay a positive control [a known *E. coli* (MUG-positive) culture], a negative control [a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture], and an uninoculated medium control. The distance between the UV lamp and the tubes should be such that the *E. coli* positive control shows distinct fluorescence while the MUG-negative and uninoculated controls do not. If multiple tubes are used, calculate the MPN for *E. coli* and thermotolerant coliforms from the number of positive EC-MUG broth tubes, as described in 9221C. When using only one tube, or subculturing from a single presumptive bottle or colony, report the presence or absence of *E. coli* and thermotolerant coliforms.

3. Bibliography

FENG, P.C.S. & P.A. HARTMAN. 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli. Appl. Environ. Microbiol.* 43:1320.

HARTMAN, P.A. 1989. The MUG (glucuronidase) test for *E. coli* in food and water. *In* A. Balows, R.C. Tilton & A. Turano, eds. Rapid Methods and Automation in Microbiology and Immunology. Proc. 5th Intl. Symp. on Rapid Methods and Automation in Microbiology & Immunology, Florence, Italy, Nov. 4–6, 1987.

SHADIX, L.C. & E.W. RICE. 1991. Evaluation of β-glucuronidase assay for the detection of *Escherichia coli* from environmental waters. *Can. J. Microbiol.* 37:908.

9221 G. Other *Escherichia coli* Procedures

For the *E. coli* test using the GAD reagent, *E. coli* is defined as the species of coliform bacteria that possesses the enzyme glutamate decarboxylase (GAD), which can produce an alkaline reaction within 4 h in a reagent containing glutamic acid and a lytic agent. This procedure is used to test for *E. coli* after prior enrichment in a medium used to identify coliform bacteria. The procedure is particularly useful for determining the presence of MUG-negative strains of *E. coli,* some of which are pathogenic (see also Section 9260F).

1. *Escherichia coli* Test (GAD Procedure)

Use the GAD procedure to test for *E.* coli in a total coliform-positive culture following the QC guidelines cited in 9221B.2.

a. GAD reagent:

L-Glutamic acid	1.0 g
Sodium chloride (NaCl)	90.0 g
Bromocresol green	0.05 g

Polyethylene glycol octylphenyl ether*	3.0	mL
Reagent-grade water	1	L

Add ingredients to water and mix thoroughly until all ingredients are dissolved. The pH should be 3.4 ± 0.2. The reagent is stable for 2 months when stored at 5°C. It can be filter-sterilized (0.2-μm filter) and treated as a sterile solution.

b. Procedure:

1) Gently shake or rotate presumptive tubes or bottles showing growth, gas, or acidity. Using a graduated pipet, transfer 5 mL broth from the fermentation tube or bottle to 15-mL centrifuge tube.

2) Concentrate the bacterial cells by centrifuging the broth at 2500 to 3000 × *g* for 10 min. Discard supernatant and resuspend cells in 5 mL phosphate buffer. Reconcentrate cells by centrifugation at 2500 to 3000 × *g* for 10 min. Discard supernatant and add 1.0 mL GAD reagent. Vigorously swirl tube to resuspend cells in GAD reagent.

3) Incubate tubes at 35°C and observe after 1 h. Tubes may be incubated for a maximum of 4 h.

c. Interpretation: Examine all tubes for a distinct color change from yellow to blue, which is considered a positive result for *E. coli*. To assist in interpreting results, incorporate in the assay a positive control [a known *E. coli* (GAD-positive) culture], a negative control [a known total coliform organism, such as *Enterobacter cloacae* (GAD-negative)], and an uninoculated GAD reagent control. If multiple tubes are used, calculate the MPN for *E. coli* from the number of positive GAD tubes, as described in 9221C. When using only one tube or presumptive bottle, report as presence or absence of *E. coli*.

2. *Escherichia coli* Test (Indole Production)

For the purposes of this test, *E. coli* is defined as the species of coliform bacteria that can produce indole within 24 ± 2 h when grown in tryptone water at 44.5 ± 0.2°C. There are exceptions: *Klebsiella oxytoca* and some strains of *C. freundii* and *Enterobacter* spp. are also indole positive. Use tryptone water and Kovac's reagent to test for *E. coli* in a total coliform-positive culture.

a. Reagents: Prepare tryptone water and Kovac's reagent following the guidelines cited in 9221B.2.

1) *Tryptone water:*

Tryptone	20	g
Sodium chloride (NaCl)	5	g
Reagent-grade water	1	L

Add ingredients to water and mix thoroughly until dissolved. Adjust pH to 7.5. Dispense 5-mL portions into tubes, cap, and sterilize for 10 min at 121°C.

2) *Kovac's reagent:*

p-Dimethylaminobenzaldehyde	5	g
Amyl alcohol (analytical grade)	75	mL
Hydrochloric acid, conc.	25	mL

Dissolve aldehyde in alcohol. Cautiously add acid to aldehyde-alcohol mixture and swirl to mix. Store in the dark at 4°C. **CAUTION:** ***Reagent is corrosive and flammable.*** This reagent should be pale yellow to light brown in color. Use of low-quality amyl alcohol may produce a dark-colored reagent; do not use such a reagent.

b. Procedure: Gently shake or rotate presumptive tubes or bottles showing growth, gas, or acidity. Using a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from presumptive fermentation tube or bottle to a tube containing 5 mL tryptone water. Incubate inoculated tryptone water tubes in a water bath or incubator maintained at 44.5 ± 0.2°C for 24 ± 2 h. After incubation, add 0.2 to 0.3 mL Kovac's reagent to each tube of tryptone water.

c. Interpretation: Examine all tubes for the appearance of a deep red color in the upper layer, which is considered a positive result for *E. coli*. To assist in interpretation of results, incorporate into the assay a positive control [a known *E. coli* (indole-positive) culture], a negative control [a known total coliform organism, such as *Enterobacter cloacae* (indole-negative)], and an uninoculated reagent control. If multiple tubes are used, calculate the MPN for *E. coli* from the number of indole-positive tubes, as described in 9221C. When using only one tube or presumptive bottle, report as presence or absence of *E. coli*.

3. Bibliography

FIEDLER, J. & J. REISKE. 1990. Glutaminsauredecarboxylase-schnelltest zur identifikation von *Escherichia coli. Z. Ges. Hyg. Grenzgeb*. 36:620.

RICE, E.W., C.H. JOHNSON, M.E. DUNNIGAN & D.J. REASONER. 1993. Rapid glutamate decarboxylase assay for detection of *Escherichia coli*. *Appl. Environ. Microbiol*. 59:4347. Errata. 1995. *Appl. Environ. Microbiol*. 61:847.

STANDING COMMITTEE OF ANALYSTS. 1994. Report on Public Health and Medical Subjects No. 71: Methods for the Examination of Waters and Associated Materials, The Microbiology of Water Part 1—Drinking Water. HMSO Books, London, U.K.

RICE, E.W., C.H. JOHNSON & D.J. REASONER. 1996. Detection of *Escherichia coli* O157:H7 in water from coliform enrichment cultures. *Lett. Appl. Microbiol*. 23:179.

* Triton X-100, Sigma-Aldrich, St. Louis, MO, or equivalent.

9222 MEMBRANE FILTER TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9222 A. Introduction

The membrane filter (MF) technique is reproducible, can be used to test relatively large sample volumes, and usually yields numerical results more rapidly than the multiple-tube fermentation procedure. It is useful in monitoring drinking water and various natural waters. However, the MF technique has limitations, particularly when testing waters with high turbidity or large numbers of noncoliform (background) bacteria. If heterotrophic bacteria interference occurs, for example, sample results may need to be invalidated and new samples collected.

If a laboratory has not used the MF technique before, analysts should conduct parallel tests with the lab's current method to demonstrate applicability and comparability. Many coliform performance studies have been reported in the literature; the rates of false-positive and -negative results can differ among various coliform media, so users should carefully select the medium and procedure that best fit their needs.

1. Terminology

In the MF technique, the *coliform group* is defined as facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that develop colonies with distinctive characteristics on specific media. When purified cultures of coliform bacteria are tested, they produce negative cytochrome oxidase and positive β-galactosidase test reactions. Details of these coliform characteristics are given below for the standard total coliform MF procedure (9222B) (¶ *a* below) and for two procedures for detecting total coliforms and *E. coli* simultaneously (9222J and K) (¶s *b* and *c* below).

a. Endo-type agar medium (Endo agar LES or Endo broth MF): In this procedure, *coliform bacteria* are defined as bacteria that develop red colonies with a metallic (golden-green) sheen within 24 h at 35°C on an Endo-type medium containing lactose. Some members of the total coliform group also produce dark red, mucoid, or nucleated colonies without a metallic sheen; when verified, these are classified as *atypical coliform colonies*. Generally, pink (non-mucoid), blue, white, or colorless colonies lacking sheen on Endo media are considered noncoliforms in this technique.

b. Dual-chromogen m-ColiBlue24 medium: This differential membrane filter medium simultaneously detects and enumerates both total coliforms (TC) and *Escherichia coli* (*E. coli*) in water samples in 24 h based on their specific enzyme activities. Coliform bacteria (other than *E. coli*) are defined as those that produce red colonies within 24 h at 35°C on this medium, which contains lactose and a nonselective dye [2,3,5-triphenyltetrazolium chloride (TTC)]. *E. coli* are distinguished from other coliform bacteria by blue to purple colonies from the action of ß-glucuronidase enzyme on 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (BCIG), also present in the medium.

c. Fluorogen/chromogen MI medium: This differential membrane filter medium simultaneously detects and enumerates both total coliforms (TC) and *Escherichia coli* (*E. coli*) in water samples in 24 h based on their specific enzyme activities. The medium includes two enzyme substrates—the fluorogen 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) and the chromogen indoxyl-β-D-glucuronide (IBDG) to detect the enzymes β-galactosidase and β-glucuronidase produced by TC and *E. coli*, respectively. In this procedure, *coliform bacteria* are defined as bacteria that produce fluorescent colonies within 24 h when exposed to long-wave ultraviolet (UV) light (365–366 nm) at 35°C, while *E. coli* colonies appear blue on this medium.

2. Applications

The MF technique may be used to test drinking, surface, ground, swimming pool, and marine waters. Do not use it to test primary wastewater-treatment effluent unless the sample is diluted, because the high turbidity level may clog the membrane filter before sufficient sample has been filtered. Chlorinated effluents should have low counts and turbidity. Also, do not use the MF technique to test wastewater containing high levels of toxic metals or toxic organic compounds (e.g., phenols) because the filter may concentrate such substances, thereby inhibiting coliform growth. For non-wastewater samples, high turbidity levels may clog the filter and high heterotrophic-bacteria concentrations may interfere with coliform growth on the filter, possibly requiring the use of multiple filters per sample and/or various sample dilutions. To detect stressed total coliforms in treated drinking water and chlorinated secondary or tertiary wastewater-treatment effluents, use a method designed for stressed organism recovery (see Section 9212B.1). A modified MF technique for thermotolerant coliforms in chlorinated wastewater (Section 9212) may be used if 3 months of parallel testing with the multiple-tube fermentation technique shows comparability for each site-specific type of sample.

The standard volume to be filtered is 100 mL for drinking-water samples; this may be distributed among multiple membranes, if necessary. However, for special monitoring purposes (e.g., troubleshooting water-quality problems or identifying coliform breakthrough in low concentrations from treatment barriers), it may be desirable to test 1-L samples. If particulates prevent one filter from processing a 1-L sample, divide sample into four 250-mL portions for analysis. Total the coliform counts on each membrane to report the number of coliforms per liter. Samples other than drinking water should be analyzed using multiple dilution levels. Recommended dilutions for total coliform measurements are presented in Table 9222:I and for thermotolerant coliform and *E. coli* in Table 9222:IV.

Statistical comparisons of results obtained by the multiple-tube method and the MF technique show that MF is more precise

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(compare Tables 9221:III and IV with Table 9222:III). Data from each test yield approximately the same water-quality information, although numerical results are not identical.

3. Bibliography

CLARK, H.F., E.E. GELDREICH, H.L. JETER & P.W. KABLER. 1951. The membrane filter in sanitary bacteriology. *Pub. Health Rep.* 66:951.

KABLER, P.W. 1954. Water examinations by membrane filter and MPN procedures. *Amer. J. Pub. Health* 44:379.

THOMAS, H.A. & R.L. WOODWARD. 1956. Use of molecular filter membranes for water potability control. *J. Amer. Water Works Assoc.* 48:1391.

MCCARTHY, J.A., J.E. DELANEY & R.J. GRASSO. 1961. Measuring coliforms in water. *Water Sewage Works* 108:238.

LIN, S. 1973. Evaluation of coliform test for chlorinated secondary effluents. *J. Water Pollut. Control Fed.* 45:498.

MANDEL, J. & L.F. NANNI. 1978. Measurement evaluation. *In* S.L. Inhorn, ed. Quality Assurance Practices for Health Laboratories, p. 209. American Public Health Assoc., Washington, D.C.

BRENNER, K.P., C.C. RANKIN, Y.R. ROYBAL, G.N. STELMA, JR., P.V. SCARPINO & A.P. DUFOUR. 1993. New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. *Appl. Environ. Microbiol*. 59:3534.

BRENNER, K.P., C.C. RANKIN, M. SIVAGANESAN & P.V. SCARPINO. 1996. Comparison of the recoveries of *Escherichia coli* and total coliforms from drinking water by the MI agar method and the U.S. Environmental Protection Agency-approved membrane filter method. *Appl. Environ. Microbiol*. 62:203.

GRANT, M.A. 1997. A new membrane filtration medium for simultaneous detection and enumeration of *Escherichia coli* and total coliforms. *Appl. Environ. Microbiol*. 63:3526.

FRANCY, D.S. & R.A. DARNER. 2000. Comparison of methods for determining *Escherichia coli* concentrations in recreational waters. *Water Res.* 34:2770.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2002. Method 1604: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium); EPA 821-R-02-024. Off. Water, Washington, D.C.

OLSTADT, J., J.J. SHAUER, J. STANDRIDGE & S. KLUENDER. 2007. A comparison of ten USEPA approved total coliform/*E. coli* tests. *J. Water and Health* 05:267.

9222 B. Standard Total Coliform Membrane Filter Procedure using Endo Media

This method may be used to measure total coliforms in drinking, nonpotable, and other waters using Endo-type media. Typical colonies grown on Endo-type media can be further partitioned to differentiate thermotolerant (fecal) coliforms and *E.coli* using the partitioning methods in 9222G, H, and I. This method may not be appropriate for samples high in particulates that may plug filters or samples with a high proportion of total coliforms relative to *E.coli*. For simultaneous measurement of total coliforms and *E.coli* using membrane filtration, refer to Methods 9222J and K.

1. Laboratory Apparatus

For MF analyses, use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. Sample bottles: See Section 9030B.19.

b. Dilution bottles: See Section 9030B.13.

c. Pipets and graduated cylinders: See Section 9030B.9. Before sterilization, loosely cover opening of graduated cylinders with metal foil or a suitable heavy wrapping-paper substitute. Immediately after sterilization, secure cover to prevent contamination.

d. Containers for culture medium: Use clean borosilicate glass flasks. Any size or shape of flask may be used, but Erlenmeyer flasks with metal caps, metal foil covers, or screw caps provide for adequate mixing of the medium within and are convenient for storage.

e. Culture dishes: Use sterile borosilicate glass or disposable, presterilized plastic Petri dishes, 15- × 60-mm, 9- × 50-mm, or other appropriate size. Wrap convenient numbers of clean, glass culture dishes in metal foil if sterilized via dry heat, or suitable heavy wrapping paper when autoclaved. Incubate loose-lidded glass and disposable plastic culture dishes in tightly closed containers to prevent moisture evaporation with resultant drying of medium and to maintain a humid environment for optimum colony development.

Presterilized disposable plastic dishes with tight-fitting lids that meet the specifications above are available commercially and used widely. Reseal opened packages of disposable dish supplies for storage.

f. Filtration units: The filter-holding assembly (constructed of glass, autoclavable plastic, porcelain, stainless steel, or disposable plastic) consists of a seamless funnel fastened to a base via a locking device or magnetic force. The design should permit the membrane filter to be held securely on the receptacle's porous plate without mechanical damage and allow all fluid to pass through the membrane during filtration. Discard plastic funnels with deep scratches on the inner surface or glass funnels with chipped surfaces. Replace damaged screens on stainless steel units.

Wrap the assembly (as a whole or separate parts) in heavy wrapping paper or aluminum foil, or place in commercial autoclave bags; sterilize via autoclaving; and store until use. Field units may be sanitized by dipping or spraying with alcohol and then igniting or immersing in boiling water for 2 min. Use reagent water to avoid hard-water deposits (see Section 9020B.4*d* for reagent-grade water-quality specifications). After submerging unit in boiling water, cool to room temperature before reuse. Do not ignite plastic parts. Sterile, disposable field units also may be used.

For filtration, mount receptacle of filter-holding assembly on a 1-L filtering flask with a side arm or other suitable device (manifold to hold three to six filter assemblies) such that a pressure differential (34 to 51 kPa) can be exerted on the filter membrane. Connect flask to a vacuum line, an electric vacuum pump, a filter pump operating on water pressure, a hand aspirator, or other means of securing a pressure differential (138 to 207 kPa). Connect a flask of approxi-

mately the same capacity between filtering flask and vacuum source to trap carry-over water.

g. Membrane filters: Use membrane filters with a pore diameter rated to completely retain coliform bacteria (usually 0.45 μm) (for additional specifications, see Section 9020B.5*i*). Only use filter membranes that have been found—through adequate quality control (QC) testing and manufacturer certification—to exhibit the following:

- full retention of the organisms to be cultivated,
- stability in use,
- freedom from chemical extractables that may inhibit bacterial growth and development,
- a satisfactory filtration speed (within 5 min),
- no significant influence on medium pH (beyond ±0.2 units), and
- no increase in the number of confluent colonies or spreaders compared to control membrane filters.

Use membranes grid-marked so bacterial growth is neither inhibited nor stimulated along the grid lines when membranes with entrapped bacteria are incubated on a suitable medium. Preferably use fresh stocks of membrane filters and, if necessary, store them in an environment without temperature and humidity extremes. Obtain no more than a year's supply at any one time.

Preferably use presterilized membrane filters for which the manufacturer has certified that the sterilization technique has neither induced toxicity nor altered the membrane's chemical or physical properties. If membranes are sterilized in the laboratory, autoclave for 10 min at 121–124°C and then let the steam escape rapidly to minimize condensation on filters.

h. Absorbent pads: Use disks of filter paper or other material that the manufacturer has certified, by lot, to be high quality and free of sulfite or other toxic agents at a concentration that could inhibit bacterial growth. Use pads approximately 48 mm in diameter and thick enough to absorb 1.8 to 2.2 mL of medium. Some pads may require 3.0 mL of medium. Sterilize pads in resealable kraft envelopes, or separately in other suitable containers for 15 min in autoclave (dry cycle). Dry pads so they are free of visible moisture before use. See membrane-filter sterilization procedure in ¶ *g* above.

i. Forceps: Use smooth blunt forceps, without corrugations on the inner sides of the tips. Sterilize before use by dipping in 95% ethyl or absolute methyl alcohol and flaming.

j. Incubators: Use incubators to provide a temperature of 35 ± 0.5°C. To avoid excessive drying, maintain a humid environment for the plates during incubation by either using a humidified incubator (between 60 and 90% relative humidity) or placing plates in a sealed container with tight-fitting lid (or sealed bag). The plates should not lose more than 15% of agar weight during incubation.

k. Microscope and light source: To determine colony counts on membrane filters, use 10 to 15× magnification and a cool white fluorescent light source adjusted to give maximum sheen discernment. Optimally, use a binocular wide-field dissecting microscope. Do not use a microscope with an illuminator that concentrates light from an incandescent source when discerning coliform colonies on Endo-type media.

2. Materials and Culture Media

Because test results need to be uniform, use commercial dehydrated media; never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions for rehydration. Store opened supplies of dehydrated media in a desiccator (if necessary). Commercial liquid media (sterile ampule, etc.) may be used if known to give equivalent results. See Section 9020B.5*j*. Test each new medium lot to confirm that performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance. With each new lot of Endo-type medium, verify a minimum 10% of coliform colonies (obtained from natural samples or samples with known additions) to establish the lot's comparative recovery.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls. If commercially prepared medium is unavailable, prepare from individual components as described in ¶s *a* and *b* below.

a. Endo agar LES (Lawrence Experimental Station formulation):

Yeast extract	1.2	g
Casitone or trypticase	3.7	g
Thiopeptone or thiotone	3.7	g
Tryptose	7.5	g
Lactose	9.4	g
Dipotassium hydrogen phosphate (K_2HPO_4)	3.3	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0	g
Sodium chloride (NaCl)	3.7	g
Sodium desoxycholate	0.1	g
Sodium lauryl sulfate	0.05	g
Sodium sulfite (Na_2SO_3)	1.6	g
Basic fuchsin	0.8	g
Agar	15.0	g
Reagent-grade water	1	L

CAUTION: Basic fuchsin is a suspected carcinogen and mutagen. Avoid skin contact, ingestion, and exposure to mucous membrane. Follow manufacturer's and safety data sheet (SDS) instructions.

Rehydrate product according to manufacturer's directions or individual components in 1 L water containing 20 mL 95% ethanol. Do not use denatured ethanol, which reduces background growth and coliform colony size. Bring to near boiling to dissolve agar, promptly remove from heat, and cool to between 45 and 50°C. Do not sterilize by autoclaving. Final pH should be 7.2 ± 0.2. A precipitate is normal in Endo-type media. Dispense in 5- to 7-mL quantities into lower section of 60-mm glass Petri dishes or 4- to 6-mL quantities into lower section of 50-mm plastic Petri dishes and allow to solidify. If dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm. Do not expose poured plates to direct sunlight; refrigerate in the dark, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused medium after 2 weeks [or sooner if there is evidence of moisture loss, medium contamination, medium deterioration (darkening of medium), or surface sheen formation].

*b. m-Endo medium:**

Tryptose or polypeptone	10.0	g
Thiopeptone or thiotone	5.0	g
Casitone or trypticase	5.0	g
Yeast extract	1.5	g
Lactose	12.5	g
Sodium chloride (NaCl)	5.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	4.375	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.375	g
Sodium lauryl sulfate	0.05	g
Sodium desoxycholate	0.10	g
Sodium sulfite (Na_2SO_3)	2.10	g
Basic fuchsin	1.05	g
Agar (optional)	15.0	g
Reagent-grade water	1	L

CAUTION: Basic fuchsin is a suspected carcinogen and mutagen. Avoid skin contact, ingestion, or exposure to mucous membranes. Follow manufacturer's and SDS instructions.

1) Agar preparation—Rehydrate product in 1 L water containing 20 mL 95% ethanol. Do not use denatured ethanol, which reduces background growth and coliform colony size. Do not sterilize by autoclaving. Heat to near boiling to dissolve agar, promptly remove from heat, and cool to between 45 and 50°C. Dispense 5- to 7-mL quantities into 60-mm sterile glass or 4- to 6-mL quantities into 50-mm plastic Petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth. Final pH should be 7.2 ± 0.2. A precipitate is normal in Endo-type media.

Refrigerate finished medium in the dark, and discard unused agar after 2 weeks [or sooner if there is evidence of moisture loss, medium contamination, medium deterioration (darkening of medium), or surface sheen formation].

2) Broth preparation—Prepare as above, omitting agar. Dispense liquid medium (at least 2.0 mL per plate) onto sterile absorbent pads (see 9222B.1*h*) and carefully remove excess medium by decanting plate. The broth may have a precipitate but this does not interfere with medium performance if pads are certified free of sulfite or other toxic agents at concentrations that could inhibit bacterial growth. Refrigerated broth in screw-capped bottles or flasks may be stored for up to 96 h.

c. Buffered dilution rinse water: See Section 9050C.1.

3. Samples

Collect samples as directed in Section 9060A.

4. Procedures

a. Selection of sample size: Sample size will be governed by expected bacterial density, degree of turbidity and, if applicable, regulatory requirements. (See Table 9222:I for suggested sample volumes.)

An ideal sample volume will yield 20 to 80 total coliform colonies and ≤200 colonies of all types (typical, atypical, and noncoliform background colonies) on a membrane-filter surface (Table 9222:II). Analyze drinking waters by filtering 100 mL or replicates of smaller sample volumes (e.g., duplicate 50-mL portions or four replicates of 25-mL portions). Analyze other waters by filtering three different volumes (diluted or undiluted), depending on the expected bacterial density. (See Section 9215B.2 for preparation of dilutions.) When filtering <10 mL of sample (diluted or undiluted), add approximately 10 mL sterile buffered dilution water to the funnel and then add sample followed by another 25 to 50 mL dilution water before filtration or pipet the sample volume into sterile dilution water and then filter the entire contents of dilution bottle. This increase in water volume helps disperse the bacterial suspension uniformly over the entire effective filtering surface.

Table 9222:I. Suggested Sample Volumes for Membrane Filter Total Coliform Test

	Volume (X) To Be Filtered *mL*							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Swimming pools	X							
Wells, springs	X	X	X					
Lakes, reservoirs	X	X	X					
Water supply intake			X	X	X			
Bathing beaches			X	X	X			
River water				X	X	X	X	
Chlorinated sewage				X	X	X		
Raw sewage					X	X	X	X

Table 9222:II. Numbers of Colonies in the Ideal Range for Quantitative Determinations

	Colony Counting Range	
Test	Minimum	Maximum
Total coliform	20	80
Fecal coliform	20	60
Fecal streptococci	20	100
Enterococci	20	60
E. coli	20	80

b. Sterile filtration units and quality control: Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all membrane filter holders in use. (See 9222B.1*f* for sterilization procedures and Sections 9020B.4*l* and *m* for UV cleaning and safety guidelines.)

c. Filtration of sample: Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of the base. Carefully place matched funnel unit over base (lock it in place, if applicable). Thoroughly mix sample or dilution(s) of sample by vigorously shaking (e.g., 25 times up and down in a 1 ft arc in 7 s) to break up clumps of bacteria, which is crucial for a microbial quantitative method. If sample bottle lacks enough headspace for adequate mixing, pour sample into a larger sterile vessel to mix appropriately. Filter sample under partial vacuum (commonly used pressure: 81 kPa, 24 in. Hg, or 79% vacuum).

* Dehydrated Difco m-Endo Broth MF (No. 274920), or equivalent.

With filter still in place, rinse interior surface of funnel by filtering three 20- to 30-mL portions of sterile buffered dilution water from a squeeze bottle (or other appropriate device). This is satisfactory only if squeeze bottle and its contents do not become contaminated during use. Do not reuse partially filled dilution water bottles. Rinsing between samples prevents carryover contamination.

When final rinse and filtration are complete, use aseptic technique to disengage vacuum, unlock and remove funnel, immediately remove membrane filter with sterile forceps, and roll filter onto selected medium to avoid entrapping air. Incorrect filter placement is instantly obvious because patches of unstained membrane indicate entrapped air. If such patches occur, carefully reseat filter on agar surface. Place only one membrane filter per dish. Invert dish, and incubate at 35 ± 0.5°C for 22 to 24 h for m-Endo LES or m-Endo MF. Optionally, to sanitize funnels between samples after filter removal, expose all surfaces of previously cleaned and sterilized assembly to UV radiation for 2 min before reusing units for successive filtrations.

If using broth, aseptically place a sterile pad in the culture dish, saturate it with at least 2.0 to 3.0 mL of medium (depending on pad manufacturer), and carefully remove excess medium by gently decanting it from dish into a disposable terry towel or empty dish. Place sample filter directly on pad, invert dish, and incubate as specified above. If loose-lidded dishes are used, place them in a humid chamber (or humidified incubator).

Differentiation of some colonies may be lost if cultures are incubated >24 h.

For nonpotable water samples, funnels should be rinsed or sanitized with UV after filter removal or after each sample (as described above) because of the high number of coliform bacteria and background flora present in these samples.

d. QC samples: Check for sterility and coliform contamination at the beginning and end of each filtration series, respectively, by filtering 20 to 30 mL of dilution or rinse water through the filter (one funnel per sterilization batch). If controls indicate contamination, reject all data from affected samples and request new samples. Additionally, to check for possible cross-contamination or contaminated rinse water, insert a sterile rinse-water sample (100 mL) after filtration of 10 samples. Incubate these QC samples under the same conditions as the samples being analyzed.

e. Alternative enrichment technique: Use this technique with m-Endo LES media only. Place a sterile absorbent pad in the lid of a sterile culture dish, and pipet at least 2.0 mL lauryl tryptose broth (prepared as directed in Section 9221B.3*a*) to saturate pad. Carefully remove any excess liquid from absorbent pad by decanting plate. Aseptically place filter—through which the sample has been passed—on the pad. Incubate filter, without inverting dish, for 1.5 to 2 h at 35 ± 0.5°C in an atmosphere of at least 60% relative humidity.

Remove enrichment culture from incubator, lift filter from enrichment pad, and roll it onto the m-Endo LES agar surface, which has been allowed to equilibrate to room temperature. Incorrect filter placement is instantly obvious because patches of unstained membrane indicate entrapped air. If such patches occur, carefully reseat filter on agar surface. If broth medium is used, prepare final culture by removing enrichment culture from incubator and separating the dish halves. Place a fresh sterile pad in dish bottom, saturate with at least 2.0 mL of m-Endo medium, and carefully remove excess liquid from absorbent pad by decanting plate. Transfer filter, with same precautions as above, to new pad. Discard used enrichment pad.

With either agar or broth medium and using this two-step procedure, invert dish and incubate for 22 ± 2 h at 35 ± 0.5°C. Proceed to ¶ *f* below.

f. Counting: To count colony-forming units (CFU) on Endo-type membrane filters, use a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device, with a cool white fluorescent light source directed to provide optimal viewing of sheen. The angle of light on the colony affects sheen detection for coliform colonies growing on m-Endo plates. Rocking and turning the Petri plate reflects light at different angles and helps detect sheen on the colony. The typical coliform colony on Endo-type media has a pink to dark-red color with a metallic surface sheen. Count both typical and atypical coliform colonies promptly after incubation. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red, mucoid, or nucleated without sheen. Generally pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms. A high count of noncoliform colonies (>200 CFU) may interfere with the maximum development of coliforms. Refrigerating cultures with high densities of noncoliform colonies (after incubation) for 0.5 to 1 h before counting may aid sheen discernment. Samples of disinfected water or wastewater effluent may include stressed organisms that grow relatively slowly and produce maximum sheen in 22 to 24 h.

g. Coliform verification: Occasionally on Endo-type media, noncoliform organisms may produce typical sheen colonies, and atypical colonies (pink, dark red, or nucleated colonies without sheen) may be coliforms; thus verification of all typical and atypical colony types is recommended. Verify all colonies on Endo media by swabbing the entire membrane or picking at least five typical colonies and five atypical colonies from a given membrane filter culture. (See Section 9020B.9.) Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., for nonpotable samples, verify at least one colony from each typical or atypical colony type from a given membrane filter culture, or verify 10% of positive samples). Adjust counts based on verification results. Verification tests are listed below.

1) Lactose fermentation—Transfer growth from each colony, or swab the entire membrane with a sterile cotton swab (for presence–absence results) and place in single-strength lauryl tryptose broth; incubate broth at 35 ± 0.5°C for up to 48 h. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose broth within 48 h verifies the colony as a coliform. (See Sections 9221B.3 and 4 for media preparation.) Simultaneous inoculation of both media is acceptable (if using same inoculating sterile loop, needle, or wood stick, inoculate lauryl tryptose broth first). Including EC broth inoculation incubated at 44.5 ± 0.2°C for 24 ± 2 h will provide information on the presence of thermotolerant coliforms. Including EC-MUG inoculation incubated at 44.5 ± 0.2°C for 24 h will provide information on presence of *E. coli*. The inoculation order should always be from least to most inhibitory [1) EC or EC-MUG, 2) lauryl tryptose broth, 3) brilliant green lactose broth]. (See 9222G and H for MF partition procedures.)

2) Alternative coliform verifications—Apply this alternative coliform verification procedure to isolated colonies on the Endo membrane filter media. If a mixed culture is suspected or if colony separation is <2 mm, streak the growth to m-Endo medium or MacConkey agar to ensure culture purity or submit the mixed growth to the fermentation tube method.

a) Rapid test—A rapid verification of colonies uses test reactions for cytochrome oxidase (CO) and β-galactosidase. Coliform reactions are CO negative and β-galactosidase positive within 4 h incubation of tube culture or micro (spot) test procedure.

b) Commercial multi-test systems—Verify and/or identify the coliform bacteria by selecting a well-isolated colony, streaking for isolation, and inoculating a pure colony into a multi-test identification system for *Enterobacteriaceae* that includes lactose fermentation and/or β-galactosidase and CO test reactions.

5. Calculation of Coliform Density

Select the membrane(s) with acceptable number of colonies (Table 9222:II) and ≤200 colony-forming units (CFU) of all types per membrane, by the following equation:

$$\text{(Total) coliforms, No./100 mL} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered}} = \text{No. CFU/100 mL}$$

For drinking water samples, if no total coliform colonies are observed, then report the total coliform colonies counted as "<1 CFU/100 mL" or report "total coliform bacteria absent per 100 mL sample."

For nonpotable water samples, if 10.0-, 0.1-, and 0.01-mL portions are examined and all counts are 0, then calculate the number of coliforms per 100 mL that would have been reported if there had been 1 CFU on the filter representing the largest filtration volume. For example, report <10 CFU/100 mL for a 10 mL sample volume with no coliform colonies, i.e,

$$1/10 \times 100 = <10 \text{ CFU/100 mL}$$

For verified coliform counts, adjust the initial count based on the positive verification percentage (both typical and atypical) and report as follows:

$$\text{Verified (total) coliforms, No./100 mL} = \frac{\text{number of verified colonies}}{\text{total number of coliform colonies subjected to verification}} \times \text{total number of presumptive colonies}$$

a. Potable water: In its Total Coliform Rule, the U.S. Environmental Protection Agency (EPA) only requires a record of the presence or absence of total coliforms in 100-mL samples of drinking water; however, quantitative information can indicate the magnitude of a contaminating event and/or remediation progress, especially when comparing sample results from different locations (e.g., repeat samples). Quantitative information only provides a gross estimation of the actual coliform population at collection time due to non-uniform distribution within the matrix.

In good drinking water, coliform occurrence generally will be minimal. Therefore, count all coliform colonies (disregarding the lower limit of 20 cited above) and use the formula given above to obtain coliform density.

If confluent growth occurs—covering either the membrane's entire filtration area or a portion thereof with colonies that are not discrete—report results as "confluent growth with (or without) coliforms." If the total number of bacterial colonies—coliforms plus noncoliforms—is >200 per membrane or if the colonies are not distinct enough to count accurately, then report results as "too numerous to count" (TNTC) or "confluent," respectively. For drinking water samples using Endo-type media, the presence of coliforms in such cultures may be confirmed (see 9222B.4*g*). As an alternative, brush entire filter surface with a sterile loop, applicator stick, or cotton swab and inoculate this growth into 1) a tube of single-strength lauryl tryptose and 2) a tube of brilliant green lactose bile broth. If the brilliant green bile broth tube produces gas within 48 h at 35 ± 0.5°C, coliforms are present. To comply with EPA's Total Coliform Rule, report confluent growth or TNTC with at least one detectable coliform colony (verification only required with Endo-type media) as a "total coliform positive sample." Report confluent growth or TNTC without detectable coliforms as "invalid."

For invalid samples, request a new sample from the same location within 24 h. Select more appropriate volumes to filter per membrane (keeping in mind that the standard drinking-water portion is 100 mL, according to the rule). So instead of filtering 100 mL through one membrane, filter 50-mL portions through two membranes, 25-mL portions through four separate membranes, etc. to reduce interference due to overcrowding. If any membrane contains a verified total coliform colony, report the entire sample as "total coliform positive." If a density determination is desired, total the coliform counts observed on all membranes and report as "[number] per 100 mL." (Alternatively, choose another coliform method that is less subject to heterotrophic bacterial interferences.)

b. Other waters: As with potable water samples, if no filter has a coliform count within the ideal range, total the coliform counts on all filters and report as "[number] per 100 mL." For example, if duplicate 50-mL portions were examined and the two membranes had five and three coliform colonies, respectively, then report the total coliform count as 8 CFU per 100 mL:

$$\frac{[(5 + 3) \times 100]}{(50 + 50)} = 8 \text{ CFU/100 mL}$$

Alternatively, if 50-, 25-, and 10-mL portions were examined and the counts were 15, 6, and <1 coliform colonies, respectively, then calculate based on the most nearly acceptable value and report the total coliform count with a qualifying remark as "estimated 30 CFU/100 mL":

$$\frac{[(15) \times 100]}{(50)} = \text{estimated 30 CFU/100 mL}$$

On the other hand, if 10-, 1.0-, and 0.1-mL portions were examined with counts of 40, 9, and <1 coliform colonies,

Table 9222:III. Confidence Limits for Membrane Filter Coliform Results Using 100-mL Sample

Number of Coliform Colonies Counted	95% Confidence Limits	
	Lower	Upper
0	0.0	3.7
1	0.1	5.6
2	0.2	7.2
3	0.6	8.8
4	1.0	10.2
5	1.6	11.7
6	2.2	13.1
7	2.8	14.4
8	3.4	15.8
9	4.0	17.1
10	4.7	18.4
11	5.4	19.7
12	6.2	21.0
13	6.9	22.3
14	7.7	23.5
15	8.4	24.8
16	9.2	26.0
17	9.9	27.2
18	10.7	28.4
19	11.5	29.6
20	12.2	30.8

respectively, then calculate the coliform density based only on the 10-mL portion because this filter had a coliform count within the acceptable range (see Table 9222:II) and report result as 400 CFU/100 mL:

$$\frac{(40 \times 100)}{10} = 400 \text{ coliforms/100 mL}$$

In this last example, if the membrane with 40 coliform colonies also had a total bacterial colony count >200, then report the coliform count as ≥400 CFU/100 mL or with a qualifying remark as "estimated 400 CFU/100 mL."

If 10.0-, 1.0-, and 0.1-mL portions were examined with counts of TNTC, 150, and 92 coliform colonies, respectively, then calculate based on the most nearly acceptable value and report with a qualifying remark as "estimated 92 000 CFU/100 mL":

$$\frac{(92 \times 100)}{0.1} = \text{estimated } 92\,000 \text{ CFU/100 mL}$$

If 1.0-, 0.3-, 0.1-, and 0.03-mL portions were examined with counts of TNTC, TNTC, 78, and 21 coliform colonies, respectively, then sum the total coliform counts on the latter two countable filters and divide by the sum of their volume to obtain the final reported value of 76 000 CFU/100 mL:

$$\frac{(78 + 21) \times 100}{(0.1 + 0.03)} = 76\,000 \text{ CFU/100 mL}$$

If 1.0-, 0.3-, and 0.01-mL portions were examined with counts of TNTC on all portions, then calculate using the maximum number of colonies acceptable for quantitative determination of that indicator with the smallest filtration volume and report result as >800 000 CFU/100 mL (for total coliform):

$$\frac{80 \times 100}{0.01} = >800\,000 \text{ CFU/100 mL}$$

c. Statistical reliability of membrane filter results: Although MF results are considered more precise than multiple-tube most probable number (MPN) results (5- and 10-tube multiple-tube fermentation formats), membrane counts may underestimate the number of viable coliform bacteria and circumstances may affect this precision (background bacteria and dilution levels, sample types, etc.). Table 9222:III illustrates some 95% confidence limits for MF results. These values are based on the assumption that bacteria are distributed randomly and follow a Poisson distribution.

d. Precision of MF results: Calculate precision of replicate analyses for each type of sample examined and method used if there is enough sample available for replicate analyses (drinking water, ambient water, etc.). (See Section 9020B.9*e*.)

6. Bibliography

Fifield, C.W. & C.P. Schaufus. 1958. Improved membrane filter medium for the detection of coliform organisms. *J. Amer. Water Works Assoc.* 50:193.

McCarthy, J.A. & J.E. Delaney. 1958. Membrane filter media studies. *Water Sewage Works* 105:292.

Rhines, C.E. & W.P. Cheevers. 1965. Decontamination of membrane filter holders by ultraviolet light. *J. Amer. Water Works Assoc.* 57:500.

Geldreich, E.E., H.L. Jeter & J.A. Winter. 1967. Technical considerations in applying the membrane filter procedure. *Health Lab. Sci.* 4:113.

Watling, H.R. & R.J. Watling. 1975. Note on the trace metal content of membrane filters. *Water SA* 1:28.

Geldreich, E.E. 1976. Performance variability of membrane filter procedure. *Pub. Health Lab.* 34:100.

Lin, S.D. 1976. Evaluation of Millipore HA and HC membrane filters for the enumeration of indicator bacteria. *Appl. Environ. Microbiol.* 32:300.

Standridge, J.H. 1976. Comparison of surface pore morphology of two brands of membrane filters. *Appl. Environ. Microbiol.* 31:316.

U.S. Environmental Protection Agency. 1978. Microbiological Methods for Monitoring the Environment Water and Wastes; EPA 600/8-78-017. Off. Res. Develop., Cincinnati, Ohio.

Grabow, W.O.K. & M. Du Preez. 1979. Comparison of m-Endo LES, MacConkey and Teepol media for membrane filtration counting of total coliform bacteria in water. *Appl. Environ. Microbiol.* 38:351.

Dutka, B.D., ed. 1981. Membrane Filtration Applications, Techniques and Problems. Marcel Dekker, Inc., New York, N.Y.

Evans, T.M., R.J. Seidler & M.W. LeChevallier. 1981. Impact of verification media and resuscitation on accuracy of the membrane filter total coliform enumeration technique. *Appl. Environ. Microbiol.* 41:1144.

Franzblau, S.G., B.J. Hinnebusch, T.M. Kelley & N.A. Sinclair. 1984. Effect of noncoliforms on coliform detection in potable groundwater: improved recovery with an anaerobic membrane filter technique. *Appl. Environ. Microbiol.* 48:142.

McFeters, G.A., J.S. Kippin & M.W. LeChevallier. 1986. Injured coliforms in drinking water. *Appl. Environ. Microbiol.* 51:1.

Brenner, K.P., C.C. Rankin, Y.R. Roybal, G.N. Stelma, Jr., P.V. Scarpino & A.P. Dufour. 1993. New medium for the simultaneous

detection of total coliforms and *Escherichia coli* in water. *Appl. Environ. Microbiol*. 59:3534.

BRENNER, K.P., C.C. RANKIN, M. SIVAGANESAN & P.V. SCARPINO. 1996. Comparison of the recoveries of *Escherichia coli* and total coliforms from drinking water by the MI agar method and the U.S. Environmental Protection Agency-approved membrane filter method. *Appl. Environ Microbiol*. 62:203.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2002. Method 1604: Total coliforms and *Escherichia coli* in water by membrane filtration using a simultaneous detection technique (MI medium); EPA 821-R-02-024. Off. Water, Washington, D.C.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2013. National Primary Drinking Water Regulations; Revisions to the Total Coliform Rule; Final Rule. 40 CFR Parts 141 and 142; *Fed. Reg*. 78:10270.

9222 C. Delayed-Incubation Total Coliform Procedure

This modification of the standard MF technique permits membrane shipment or transport after filtration to a distant laboratory for transfer to another substrate, incubation, and completion of the test. The delayed-incubation test may be used when

- conventional procedures are impractical;
- desired sample temperature cannot be maintained during transport;
- the elapsed time between sample collection and analysis would exceed the approved time limit; or
- the sampling location is remote from laboratory services (see Section 9060B).

Independent studies using both fresh and marine water samples have shown consistent results between the delayed-incubation and standard MF tests. Determine the delayed-incubation test's applicability for a specific water source by comparing with results of conventional MF methods.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on transport medium, and ship to laboratory. Complete coliform determination in the laboratory by transferring membrane to standard m-Endo or Endo LES medium, incubating at 35 ± 0.5°C for 20 to 22 h, and counting the typical and atypical coliform colonies that develop. For drinking-water samples collected for compliance with EPA's Total Coliform Rule, report the presence or absence of verified coliforms in 100-mL samples. Verify colonies as outlined previously in 9222B.4*g*.

Transport media are designed to keep coliform organisms viable and generally do not permit visible growth during transit time. Bacteriostatic agents in holding/preservative media suppress growth of microorganisms en route but allow normal coliform growth after transfer to a fresh medium.

The delayed-incubation test follows the methods outlined for the total coliform MF procedure, except as indicated below. Two alternative methods are given: one using the m-Endo preservative medium and the other using the m-ST holding medium. If commercially prepared medium is unavailable, prepare from individual components as described in 9222B.2*a* and 9222C.2*b*.

1. Laboratory Apparatus

a. Culture dishes: Use disposable, sterile, plastic Petri dishes (9 × 50 mm) with tight-fitting lids. Such containers are lightweight and less likely to break in transit. In an emergency or when plastic dishes are unavailable, use sterile glass Petri dishes wrapped in plastic film or similar material. (See 9222B.1*e*.)

b. Field filtration units: See 9222B.1*f*. Ultraviolet light disinfection may be used in the field if an appropriate power source is available (115 V, 60 Hz). Glass or metal filtration units may be sterilized by immersing in boiling water for 2 min. Use reagent water to avoid hard-water deposits. Use a hand aspirator to obtain necessary vacuum.

c. Absorbent pads: See 9222B.1*h*.

d. Forceps: See 9222B.1*i*.

2. Materials and Transport Media

a. m-Endo methods:

1) *m-Endo preservative medium*—Prepare m-Endo medium as described in 9222B.2*b*. After cooling to <45°C, aseptically add 3.84 g sodium benzoate [U.S. Pharmacopeia (USP) grade]/L or 3.2 mL 12% sodium benzoate solution to 100 mL medium. Mix ingredients and refrigerate poured plates. Discard unused medium after 96 h.
2) *Sodium benzoate solution*—Dissolve 12 g $NaC_7H_5O_2$ in sufficient reagent water to make 100 mL. Sterilize by autoclaving or by filtering through a 0.22-μm-pore-size membrane filter. Discard after 6 months.
3) *Cycloheximide**—Optionally, add cycloheximide to m-Endo preservative medium. It may be used for samples that previously have shown overgrowth by fungi, including yeasts. Prepare by aseptically adding 50 mg cycloheximide/100 mL to m-Endo preservative medium. Store cycloheximide solution in refrigerator, and discard after 6 months. **CAUTION: Cycloheximide is a powerful skin irritant. Follow manufacturer's and SDS instructions for proper handling and storage of this chemical.**

b. m-ST method:

m-ST holding medium:

Sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$)	0.1	g
Dipotassium hydrogen phosphate (K_2HPO_4)	3.0	g
Sulfanilamide	1.5	g
Ethanol (95%)	10	mL
Tris (hydroxymethyl) aminomethane	3.0	g
Reagent-grade water	1	L

Dissolve ingredients by rehydrating in water. Sterilize by autoclaving at 121–124°C for 15 min. Final pH should be 8.6 ±

* Actidione®, manufactured by the Upjohn Company, Kalamazoo, MI, or equivalent.

0.2. Dispense at least 2.0 to 3.0 mL (depending on pad manufacturer) to tight-lidded plastic culture dishes containing an absorbent pad, and carefully remove excess liquid from pad by decanting the plate. Store plates in the refrigerator for use within 96 h.

3. Procedure

a. Sample preservation and shipment: Place absorbent pad in bottom of sterile Petri dish and saturate with selected coliform holding medium (see 9222C.2). Remove membrane filter from filtration unit with sterile forceps and roll it, grid side up, onto surface of medium-saturated pad. Protect membrane from moisture loss by tightly closing plastic Petri dish. Seal loose-fitting dishes with an appropriate sealing tape† to prevent membrane dehydration during transit. Place culture dish containing membrane in an appropriate shipping container and send to laboratory for test completion. The sample can be held without visible growth for a maximum of 72 h at ambient temperature on the holding/preservative medium. Visible growth occasionally begins on transport medium when high temperatures are encountered during transit.

† Parafilm, Sigma-Aldrich Co. LLC, or equivalent.

b. Transfer and incubation: At the laboratory, transfer filter from holding medium on which it was shipped to a second sterile Petri dish containing m-Endo or Endo LES medium and incubate at 35 ± 0.5°C for 20 to 22 h.

4. Estimation of Coliform Density

Proceed as described in 9222B.5. Record times of collection, filtration, and laboratory examination, and calculate the elapsed time. Report elapsed time with coliform results.

5. Bibliography

GELDREICH, E.E., P.W. KABLER, H.L. JETER & H.F. CLARK. 1955. A delayed incubation membrane filter test for coliform bacteria in water. *Amer. J. Pub. Health* 45:1462.

PANEZAI, A.K., T.J. MACKLIN & H.G. COLES. 1965. *Coli-aerogenes* and *Escherichia coli* counts on water samples by means of transported membranes. *Proc. Soc. Water Treat. Exam.* 14:179.

BREZENSKI, F.T. & J.A. WINTER. 1969. Use of the delayed incubation membrane filter test for determining coliform bacteria in sea water. *Water Res.* 3:583.

CHEN, M. & P.J. HICKEY. 1986. Elimination of overgrowth in delayed-incubation membrane filter test for total coliforms by M-ST holding medium. *Appl. Environ. Microbiol.* 52:778.

9222 D. Thermotolerant (Fecal) Coliform Membrane Filter Procedure

Thermotolerant (formerly *fecal*) coliform bacterial densities may be determined by either multiple-tube procedure or MF technique. (See Section 9225 for differentiation of *Escherichia coli*.) The thermotolerant coliform MF procedure uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2°C for selectivity. Because incubation temperature is critical, submerge waterproofed (plastic bag enclosures) MF cultures in a water bath for incubation at the elevated temperature or use an appropriate solid heat-sink or other incubator that is documented to hold the temperature at 44.5°C ± 0.2°C throughout the chamber over a 24-h period. The best type of incubator is a gable-covered circulating water bath. In general, this method is applicable under the same circumstances as the multiple-tube thermotolerant coliform procedures (see Section 9221E).

There are limitations to the interpretation of a thermotolerant coliform result from thermal waters (e.g., the tropics) and pulp and paper mill effluent samples where thermotolerant *Klebsiella* have predominated and not been indicative of a sewerage source. As with all coliform results, a sanitary survey should be conducted to identify the most plausible source and public health risk interpretation.

1. Laboratory Apparatus

a. Sample bottles: See Section 9030B.19.
b. Dilution bottles: See Section 9030B.13.
c. Pipets and graduated cylinders: See Section 9030B.9.
d. Containers for culture medium: See 9222B.1*d*.
e. Culture dishes: Tight-fitting plastic dishes (see 9222B.1*e*) are preferred when MF culture plates will be submerged in a water bath during incubation. Place thermotolerant coliform culture plates in plastic bags (remove as much air as possible) or seal individual dishes with waterproof (freezer) tape to prevent leakage during submersion.
f. Filtration units: See 9222B.1*f*.
g. Membrane filters: See 9222B.1*g*.
h. Absorbent pads: See 9222B.1*h*.
i. Forceps: See 9222B.1*i*.
j. Water bath or incubator: The specificity of the thermotolerant coliform test is related directly to incubation temperature. To meet the need for greater temperature control, use a gable-covered water bath, a heat-sink incubator, or any properly designed and constructed incubator that can maintain a temperature tolerance of ±0.2°C. Most circulating water baths equipped with a gable top to reduce water and heat loss can maintain a temperature of 44.5 ± 0.2°C. However, static air incubation may be a problem in some types of incubators because of potential heat layering in the chamber, slower heat transfer from air to the medium, and slow temperature recovery each time the incubator is opened during daily operations.

2. Materials and Culture Medium

mFC medium: The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow the manufacturer's directions for rehydration. Commercial liquid media

(sterile ampule, etc.) may be used if known to give equivalent results. See Section 9020 for QC specifications. If commercially prepared medium is unavailable, prepare from individual components as described below.

mFC medium:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride (NaCl)	5.0 g
Lactose	12.5 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar (optional)	15.0 g
Reagent-grade water	1 L

Rehydrate product or individual components in 1 L water containing 10 mL 1% rosolic acid in 0.2*N* NaOH.* Heat to near boiling, promptly remove from heat, and cool to <50°C. Do not sterilize by autoclaving. If agar is used, dispense 4- to 6-mL quantities to 9- × 50-mm Petri plates (approximately 4 to 5 mm deep) and let solidify. Final pH should be 7.4 ± 0.2. Refrigerate finished medium (preferably in sealed plastic bags or other containers to reduce moisture loss) and discard unused broth after 96 h or unused agar after 2 weeks. NOTE: For samples from sources known to have minimal background growth (e.g., drinking water), 1% rosolic acid addition can be omitted from mFC medium, but it should be used for all unknown sources, stormwaters, and ambient water sources.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls (See Table 9020:VI). Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through filter. If controls indicate contamination, reject all data from affected samples and request new samples. Test each new medium lot to confirm that its performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance.

3. Procedure

a. Select sample size: Select volume of water sample to be examined in accordance with the information in Table 9222:IV. Use sample volumes that will yield counts between 20 and 60 thermotolerant coliform colonies per membrane.

When the sample's bacterial density is unknown, filter several volumes or dilutions to achieve a countable plate. Estimate the volume and/or dilution expected to yield a countable membrane, and select two additional quantities representing one-tenth and ten times (or one third and three times) this volume, respectively.

b. Filter sample: Follow the same procedure and precautions given in 9222B.4*c*.

c. Prepare culture dish: Using aseptic technique, place a sterile absorbent pad in each culture dish and pipet at least 2.0 mL mFC medium (prepared as directed above) to saturate pad. Carefully remove any excess liquid from culture dish by decanting plate. After filtration, aseptically place sample filter on medium-impregnated pad [see 9222B.2*b*2)].

As a substrate substitution for the nutrient-saturated absorbent pad, add 1.5% agar to mFC broth [see 9222B.2*b*1)].

d. Incubate: Place prepared dishes in waterproof plastic bags, remove as much air as possible, seal, invert, and submerge Petri dishes in water bath; incubate for 24 ± 2 h at 44.5 ± 0.2°C. Anchor dishes below water surface; if anchor devices (e.g., "O" rings, bricks, or water bottles) will also be submerged, make sure they are prewarmed before sample use, small enough to maintain critical temperature requirements, and do not interfere with sample incubation. Place all prepared cultures in water bath within 30 min after filtration. Alternatively, use an appropriate, accurate solid heat-sink or equivalent incubator. Do not submerge plates in waterproof hard-sided plastic containers; the extra air space does not allow the plates to reach temperature for many hours.

e. Counting: Colonies produced by thermotolerant coliform bacteria on mFC medium are various shades of blue. Non-thermotolerant coliform colonies are gray to cream-colored. Normally, few non-thermotolerant coliform colonies will be observed on mFC medium because the elevated temperature and addition of rosolic acid salt reagent selects against them. Count colonies with a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device, if needed.

f. Verification: Verify at a frequency established by the laboratory. Verify typical blue colonies and any atypical grey to green colonies (see Section 9020B.10) for thermotolerant coliform analysis. Simultaneous inoculation into single-strength lauryl tryptose and EC broth (or EC-MUG broth) incubated at 35 and 44.5°C, respectively, is acceptable during verification.

TABLE 9222:IV. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER THERMOTOLERANT COLIFORM OR *E. COLI* TEST

	Volume (X) To Be Filtered, *mL*							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Lakes, reservoirs	X	X						
Wells, springs	X	X						
Water supply intake		X	X	X				
Natural bathing waters		X	X	X				
Sewage treatment plant			X	X	X			
Farm ponds, rivers				X	X	X		
Stormwater runoff				X	X	X		
Raw municipal sewage					X	X	X	
Feedlot runoff					X	X	X	
Sewage sludge						X	X	X

4. Calculation of Thermotolerant Coliform Density

a. General: Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 thermotolerant coliform colonies. This colony-density range is more restrictive than the 20 to 80 total coliform range because of the larger colony size on mFC medium. Calculate thermotolerant coliform density as directed in 9222B.5. Record thermotolerant coliform densities as CFU per l00 mL.

* Rosolic acid reagent will decompose if sterilized by autoclaving. Refrigerate stock solution in the dark and discard after 2 weeks, or sooner if its color changes from dark red to muddy brown.

b. Sediment and biosolids samples: For total solids (dry weight basis), see Section 2540G. Calculate thermotolerant coliforms per gram dry weight for biosolids analysis as follows:

$$\text{Thermotolerant coliform, CFU/g dry weight} = \frac{\text{colonies counted}}{(\text{dilution chosen}) \times (\%\ \text{dry solids})}$$

where dilution and % dry solids are expressed in decimal form.

Example 1: Analyst observed 22 colonies on the 1:10 000 dilution plate of a biosolids with 4% dry solids.

$$\frac{22}{(0.0001)\ (0.04)} = 5.5 \times 10^6\ \text{CFU/g dry weight}$$

If no filter has a thermotolerant coliform count falling in the ideal range (20 to 60), total the thermotolerant coliform counts on all countable filters, and report as thermotolerant coliforms per gram dry weight:

Example 2: Analyst observed 18 colonies on the 1:10 000 dilution plate and 2 colonies on the 1:100 000 dilution plate of a biosolids sample with 4% dry solids.

$$\frac{(18 + 2)}{(0.0001 + 0.00001)\ (0.04)} = 4.5 \times 10^6$$

To compute a geometric mean of samples, convert the thermotolerant coliform densities of each sample to $\log_{10}$ values. Determine the geometric mean for the given number of samples† by averaging the $\log_{10}$ values of the thermotolerant coliform densities and taking the antilog of that value.

† Usually seven if collecting for EPA's Pathogen Reduction Rule, 40 CFR Part 503.[1]

5. Reference

1. U.S. Environmental Protection Agency. 1993. Standards for the Use or Disposal of Sewage Sludge: Final Rule. 40 CFR Part 503; *Fed. Reg.* 58:9248.

6. Bibliography

Geldreich, E.E., H.F. Clark, C.B. Huff & L.C. Best. 1965. Fecal-coliform-organism medium for the membrane filter technique. *J. Amer. Water Works Assoc.* 57:208.

Rose, R.E., E.E. Geldreich & W. Litsky. 1975. Improved membrane filter method for fecal coliform analysis. *Appl. Microbiol.* 29:532.

Lin, S.D. 1976. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. *Appl. Environ. Microbiol.* 32:547.

Presswood, W.G. & D.K. Strong. 1978. Modification of MFC medium by eliminating rosolic acid. *Appl. Environ. Microbiol.* 36:90.

Green, B.L., W. Litsky & K.J. Sladek. 1980. Evaluation of membrane filter methods for enumeration of faecal coliforms from marine waters. *Mar. Environ. Res.* 67:267.

Sartory, D.P. 1980. Membrane filtration faecal coliform determinations with unmodified and modified MFC medium. *Water SA* 6:113.

Grabow, W.O.K., C.A. Hilner & P. Coubrough. 1981. Evaluation of standard and modified MFC, MacConkey, and Teepol media for membrane filter counting of fecal coliform in water. *Appl. Environ. Microbiol.* 42:192.

Rychert, R.C. & G.R. Stephenson. 1981. Atypical *Escherichia coli* in streams. *Appl. Environ. Microbiol.* 41:1276.

Pagel, J.E., A.A. Qureshi, D.M. Young & L.T. Vlassoff. 1982. Comparison of four membrane filter methods for fecal coliform enumeration. *Appl. Environ. Microbiol.* 43:787.

U.S. Environmental Protection Agency. 1992. Environmental Regulations and Technology. Control of Pathogens and Vector Attraction in Sewage Sludge; EPA-626/R-92-013. Washington, D.C.

9222 E. Delayed-Incubation Thermotolerant (Fecal) Coliform Procedure

This delayed-incubation procedure is similar to the delayed-incubation total coliform procedure (9222C). Use this test only when the standard immediate thermotolerant coliform test cannot be performed (e.g., if the appropriate field incubator is unavailable or circumstances indicate that a specialized laboratory service is advisable to examine, confirm, or speciate the suspect colonies).

Results obtained by this delayed method have been consistent with results from the standard thermotolerant (fecal) coliform MF test under various laboratory and field use conditions. However, determine test applicability for a specific water source by comparison with the standard MF test, especially for saline waters, chlorinated wastewaters, and waters containing toxic substances.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on m-ST holding medium (see 9222C.2*b*), and ship to the laboratory. Complete thermotolerant coliform test by transferring filter to mFC medium, incubating at 44.5°C for 24 ± 2 h, and counting thermotolerant coliform colonies.

The m-ST medium keeps thermotolerant coliform organisms viable but prevents visible growth during transit. Membrane filters can be held for up to 3 d on m-ST holding medium with little effect on thermotolerant coliform counts.

1. Laboratory Apparatus

a. Culture dishes: See 9222B.1*e*.
b. Field filtration units: See 9222B.1*f*.
c. Absorbent pads: See 9222B.1*h*.
d. Forceps: See 9222B.1*i*.

2. Materials and Transport Medium

a. m-ST medium: Prepare as described in 9222C.2*b*.
b. mFC medium: Prepare as described in 9222D.2.

3. Procedure

a. Membrane filter transport: Using aseptic technique, place an absorbent pad in a plastic Petri dish with a tight lid and saturate with m-ST holding medium. After filtering sample, remove membrane filter from the filtration unit and place it on medium-saturated pad. Use only tightly lidded dishes to prevent moisture loss but avoid excess liquid in the dish. Place culture dish containing the filter in an appropriate shipping container and send to laboratory. Membranes can be held on the transport medium at ambient temperature for a maximum of 72 h with little effect on thermotolerant coliform counts.

b. Transfer: At the laboratory, aseptically remove membrane from holding medium and place it in another dish containing mFC medium.

c. Incubation: After transferring filter to mFC medium, place tight-lidded dishes in waterproof plastic bags, invert, and submerge in a water bath at 44.5 ± 0.2°C for 24 ± 2 h, or use a solid heat-sink or equivalent incubator.

d. Counting: Colonies produced by thermotolerant coliform bacteria are various shades of blue. Non-thermotolerant coliform colonies are gray to cream-colored. Count colonies with a binocular wide-field dissecting microscope at 10 to 15× magnification.

e. Verification: Verify colonies at a frequency established by the laboratory. Verify typical blue colonies and any atypical (grey to green) colonies as described in Section 9020B.10 for thermotolerant coliform analysis.

4. Estimation of Thermotolerant Coliform Density

Count as directed in 9222D.3*e* and compute thermotolerant coliform density as described in 9222D.4. Record time of collection, filtration, and laboratory examination, and calculate and report elapsed time.

5. Bibliography

CHEN, M. & P.J. HICKEY. 1983. Modification of delayed-incubation procedure for detection of fecal coliforms in water. *Appl. Environ. Microbiol.* 46:889.

9222 F. *Klebsiella* Membrane Filter Procedure

Klebsiella bacteria belong to the family Enterobacteriaceae and are included in the total coliform group. *Klebsiella* spp. are excreted in the feces of many healthy humans and animals, and are readily detected in sewage-polluted waters. Approximately 60 to 80% of all *Klebsiella* from feces and from clinical specimens are positive in the thermotolerant coliform test and are *Klebsiella pneumoniae*.

Klebsiella bacteria also are widely distributed in nature, occurring in soil, water, grain, vegetation, etc. Wood pulp, paper mills, textile finishing plants, and sugar-cane processing operations contain large numbers of *Klebsiella* spp. in their effluents (10^4 to 10^6 per 100 mL), and *Klebsiella* spp. are often the predominant coliform in such effluents.

Rapid quantitation may be achieved in the MF procedure by modifying mFC agar base through substitution of inositol for lactose and adding carbenicillin or by using mKleb agar. These methods reduce the necessity for biochemical testing of pure strains. Preliminary verification of differentiated colonies is recommended.

1. Laboratory Apparatus

a. Sample bottles: See Section 9030B.19.
b. Dilution bottles: See Section 9030B.13.
c. Pipets and graduated cylinders: See 9222B.1*c*.
d. Containers for culture medium: See 9222B.1*d*.
e. Culture dishes: See 9222B.1*e*.
f. Filtration units: See 9222B.1*f*.
g. Membrane filters: See 9222B.1*g*.
h. Absorbent pads: See 9222B.1*h*.
i. Forceps: See 9222B.1*i*.
j. Incubators: See 9222B.1*j*.

2. Materials and Culture Media

a. mFCIC agar: This medium may not be available in dehydrated form and may require preparation from the basic ingredients:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride (NaCl)	5.0 g
Inositol	10.0 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar	15.0 g
Reagent-grade water	1 L

Heat medium to boiling, and add 10 mL 1% rosolic acid* dissolved in 0.2 *N* NaOH. Cool to <45°C, and add 50 mg carbenicillin.† Dispense aseptically in 4- to 6-mL quantities into 9- × 50-mm plastic Petri dishes (approximate depth of 4 to 5 mm). Refrigerate until needed. Discard unused agar medium after 2 weeks. Do not sterilize by autoclaving. Final pH should be 7.4 ± 0.2.

b. mKleb agar:

Phenol red agar	31.0 g
Adonitol	5.0 g
Aniline blue	0.1 g
Sodium lauryl sulfate	0.1 g
Reagent-grade water	1 L

* Rosolic acid reagent will decompose if sterilized by autoclaving. Refrigerate stock solution in the dark and discard after 2 weeks, or sooner if its color changes from dark red to muddy brown.
† Available from Geopen, Roerig-Pfizer, Inc., New York, NY.

Sterilize by autoclaving for 15 min at 121–124°C. After autoclaving, cool to 50°C in a water bath; add 20 mL 95% ethyl alcohol (not denatured) and 0.05 g filter-sterilized carbenicillin/L. Shake thoroughly and dispense aseptically into 9- × 50-mm plastic culture plates. The final pH should be 7.4 ± 0.2. Refrigerated medium can be held for 20 d at 4 to 8°C.

3. Procedure

a. Sample size selection and filtration: See 9222B.4 for selection of sample size and filtration procedure. Select sample volumes that will yield counts between 20 and 60 *Klebsiella* colonies per membrane. Place membrane filter on agar surface; incubate for 24 ± 2 h at 35 ± 0.5°C. *Klebsiella* colonies on mFCIC agar are blue or bluish-gray. Most atypical colonies are brown or brownish. Occasional false-positive occurrences are caused by *Enterobacter* species. *Klebsiella* colonies on mKleb agar are deep blue to blue gray; other colonies most often are pink or occasionally pale yellow. Count colonies with a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device.

b. Verification: Verify *Klebsiella* colonies from the first set of samples from ambient waters and effluents, and when *Klebsiella* is suspect in water-supply distribution systems. Verify a minimum of five typical colonies by transferring growth from a colony or pure culture to a commercial multi-test system for Gram-negative speciation. Key tests for *Klebsiella* are citrate (positive), motility (negative), lysine decarboxylase (positive), ornithine decarboxylase (negative), and urease (positive). A *Klebsiella* strain that is indole-positive, liquefies pectin, and demonstrates a negative thermotolerant coliform response is most likely of nonfecal origin.

4. Bibliography

Duncan, D.W. & W.E. Razell. 1972. *Klebsiella* biotypes among coliforms isolated from forest environments and farm produce. *Appl. Microbiol.* 24:933.

Stramer, S.L. 1976. Presumptive identification of *Klebsiella pneumoniae* on M-FC medium. *Can. J. Microbiol.* 22:1774.

Bagley, S.T. & R.J. Seidler. 1977. Significance of fecal coliform-positive *Klebsiella*. *Appl. Environ. Microbiol.* 33:1141.

Knittel, M.D., R.J. Seidler, C. Eby & L.M. Cabe. 1977. Colonization of the botanical environment by *Klebsiella* isolates of pathogenic origin. *Appl. Environ. Microbiol.* 34:557.

Edmonson, A.S., E.M. Cook, A.P.D. Wilcock & R. Shinebaum. 1980. A comparison of the properties of *Klebsiella* isolated from different sources. *J. Med. Microbiol.* (U.K.) 13:541.

Smith, R.B. 1981. A Critical Evaluation of Media for the Selective Identification and Enumeration of *Klebsiella*. M.S. thesis, Dept. Civil & Environmental Engineering, Univ. Cincinnati, Ohio.

Niemela, S.I. & P. Vaatanen. 1982. Survival in lake water of *Klebsiella pneumoniae* discharged by a paper mill. *Appl. Environ. Microbiol.* 44:264.

Geldreich, E.E. & E.W. Rice. 1987. Occurrence, significance, and detection of *Klebsiella* in water systems. *J. Amer. Water Works Assoc.* 79:74.

Duncan, I.B.R. 1988. Waterborne *Klebsiella* and human disease. *Toxicity Assess.* 3:581.

9222 G. Partitioning Thermotolerant Coliforms from MF Total Coliform Using EC Broth

In a drinking water sample, thermotolerant coliform determination can be performed from a total-coliform-positive MF filter within 24 h. This technique may be applicable to other waters if warranted.

1. Laboratory Apparatus

See 9222B.1*a–j*.

2. Materials and Culture Medium

EC broth: See Section 9221E.1*a*.

3. Procedure

a. Selection of sample size and filtration procedure: See 9222B.4.

b. Total coliform verification: Verify total coliforms before using the thermotolerant coliform partition method. Swab surface growth on the total-coliform-positive filter or, if quantification is desired, transfer small portions of each target colony on the filter to the appropriate total coliform verification medium using a sterile needle. See 9222B.4*g* for total coliform verification procedures.

c. Partition method for thermotolerant coliform determination: Using aseptic technique, transfer total-coliform-positive colonies from the membrane filter to a tube containing EC medium by one of the following methods:

- remove membrane containing total coliform colonies from the substrate with sterile forceps and carefully curl and insert membrane into tube of EC medium (do not vortex tube to avoid introducing air bubbles to inverted vial,
- swab the entire membrane filter surface with a sterile cotton swab and transfer the inoculum to EC medium (do not leave cotton swab in the medium), or
- if quantification is desired, inoculate individual total coliform-positive colonies into separate EC tubes. Simultaneous inoculation of both total-coliform verification tests and EC broth is acceptable (order of inoculation should always be EC broth first and then other more inhibitory media).

Incubate tubes in 44.5 ± 0.2°C water-bath incubator within 30 min after inoculation. Maintain a sufficient water depth in incubator to immerse tubes to upper level of medium. Gas production in an EC broth (9221E.1*a*) culture in ≤24 h is considered a positive response for thermotolerant coliform bacteria.

4. Bibliography

MATES, A. & M. SHAFFER. 1989. Membrane filtration differentiation of *E. coli* from coliforms in the examination of water. *J. Appl. Bacteriol.* 67:343.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1989. Drinking Water; National Primary Drinking Water Regulations; Total Coliforms (Including Fecal Coliforms and *E. coli)*; Final Rule. 40 CFR Parts 141 and 142; *Fed. Reg.* 54:27544.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1991. National Primary Drinking Water Regulations; Analytical Techniques; Coliform Bacteria. 40 CFR Part 141; *Fed. Reg.* 56:636.

MATES, A. & M. SHAFFER. 1992. Quantitative determination of *Escherichia coli* from coliforms and fecal coliforms in sea water. *Microbios* 71:27.

SARTORY, D. & L. HOWARD. 1992. A medium detecting beta-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Lett. Appl. Microbiol.* 15:273.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 2013. National Primary Drinking Water Regulations; Revisions to the Total Coliform Rule; Final Rule. 40 CFR Parts 141 and 142; *Fed Reg.* 78:10270.

9222 H. Partitioning *E. coli* from MF Total Coliform using EC-MUG Broth

Escherichia coli is a member of the thermotolerant coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification for *E. coli* may be achieved for a total-coliform- or thermotolerant-coliform-positive MF sample by using media containing 4-methylumbelliferyl-β-D-glucuronide (MUG). In this method, *E. coli* is defined as any coliform that produces the enzyme β-D-glucuronidase and hydrolyzes the MUG substrate to produce a blue fluorescence.

When examining drinking-water samples, use one of the two partition methods to determine the presence of *E. coli* from a total-coliform-positive MF sample on Endo-type media: nutrient agar containing MUG (9222I) or EC containing MUG. When examining wastewater and other nonpotable water samples, use one of the partition methods to determine the presence of *E. coli* from thermotolerant (fecal)-coliform-positive MF samples on mFC media.

1. Laboratory Apparatus

a. See 9222B.1*a–k.*

b. Ultraviolet lamp, long-wave (365–366 nm), 6 W. See Section 9030B.23.

2. Materials and Culture Medium

EC broth with MUG (EC-MUG): See Section 9221F.1.

3. Procedure

a. Selection of sample size and filtration procedure: See 9222B.4.

b. Total coliform verification: Verify total coliforms before using the *E. coli* partition method. Swab surface growth on the total-coliform-positive filter or, if quantification is desired, transfer small portions of each target colony on the filter to the appropriate total coliform verification medium using a sterile needle. See 9222B.4*g* for total coliform verification procedures.

c. Partition method for E. coli *determination:* Use aseptic technique to transfer total coliform-positive colonies on the membrane filter to a tube containing EC-MUG medium by one of the following methods:

- remove membrane containing total coliform colonies from the substrate with sterile forceps and carefully curl and insert membrane into tube of EC-MUG medium,
- swab entire membrane filter surface with a sterile cotton swab and transfer the inoculum to EC-MUG medium (do not leave cotton swab in EC-MUG medium), or
- if quantification is desired, inoculate individual total coliform-positive colonies into separate EC-MUG tubes.

Incubate EC-MUG media at 44.5 $\pm$ 0.2°C for 24 $\pm$ 2 h. Place all EC-MUG tubes in water-bath incubator within 30 min after inoculation. Maintain a sufficient water depth in incubator to immerse tubes to upper level of medium.

Observe EC-MUG tubes using a long-wavelength (365–366-nm) UV light source, preferably containing a 6-W bulb. **CAUTION: UV lamp should never be viewed directly.** Preferably view tubes in a viewing box or hold UV light a few inches in front of tubes (e.g., 3 to 4 in.), facing away from the viewer. Also, using a UV lamp equipped with a specific filter to eliminate most of the visible light interference is desirable and will facilitate fluorescence determination. The presence of a bright blue fluorescence in the tube is a positive response for *E. coli*. Record presence or absence of fluorescence. For nonpotable water samples, this partition method can be used to determine *E. coli* from the thermotolerant coliform MF procedure using mFC medium for initial isolation before transfer to EC-MUG medium. The procedure is the same as the above, except for the total coliform verification process.

A positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret sample results and avoid misidentifying the medium's weak yellow-blue autofluorescence as a positive response. (See Section 9221F.)

4. Bibliography

MATES, A. & M. SHAFFER. 1989. Membrane filtration differentiation of *E. coli* from coliforms in the examination of water. *J. Appl. Bacteriol.* 67:343.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1989. Drinking Water; National Primary Drinking Water Regulations; Total Coliforms (Including Fecal Coliforms and *E. coli)*; Final Rule. 40 CFR Parts 141 and 142; *Fed. Reg.* 54:27544.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1991. National Primary Drinking Water Regulations; Analytical Techniques; Coliform Bacteria. 40 CFR Part 141; *Fed. Reg.* 56:636.

MATES, A. & M. SHAFFER. 1992. Quantitative determination of *Escherichia coli* from coliforms and fecal coliforms in sea water. *Microbios* 71:27.

SARTORY, D. & L. HOWARD. 1992. A medium detecting beta-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Lett. Appl. Microbiol*. 15:273.

9222 I. Partitioning *E. coli* from MF Total Coliforms using NA-MUG Agar

Escherichia coli is a member of the thermotolerant coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification for *E. coli* may be achieved for a total-coliform- or thermotolerant-coliform-positive MF sample by using media containing 4-methylumbelliferyl-β-D-glucuronide (MUG). In this method, *E. coli* is defined as any coliform that produces the enzyme β-glucuronidase and hydrolyzes the MUG substrate to produce a blue fluorescence.

When examining drinking water samples, use one of the two partition methods to determine the presence of *E. coli* from a total-coliform-positive MF sample on Endo-type media; use nutrient agar containing MUG or EC broth containing MUG (9222H). When examining wastewater and other nonpotable water samples, use one of the partition methods to determine the presence of *E. coli* from thermotolerant (fecal)-coliform-positive MF samples on mFC media.

1. Laboratory Apparatus

a. Culture dishes: See 9222B.1*e*.
b. Filtration units: See 9222B.1*f*.
c. Forceps: See 9222B.1*i*.
d. Incubator: See 9222B.1*j*.
e. Ultraviolet lamp, long-wave (365–366 nm), 6 W: See Section 9030B.23.
f. Microscope and light source: See 9222B.1*k*.

2. Materials and Culture Medium

Nutrient agar with MUG (NA-MUG):

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
4-methylumbelliferyl-β-D-glucuronide	0.1 g
Reagent-grade water	1 L

Add dehydrated ingredients to reagent-grade water, mix thoroughly, and heat to dissolve. Sterilize by autoclaving for 15 min at 121–124°C. Dispense 4- to 6-mL quantities aseptically into 50-mm plastic culture plates (approximate depth of 4 to 5 mm) and allow to solidify. Final pH should be 6.8 ± 0.2. Refrigerated prepared medium may be held for 2 weeks.

3. Procedure

a. Selection of sample size and filtration procedure: See 9222B.4.

b. Total coliform verification: For drinking water samples using Endo-type medium, total coliform verification procedures can be performed before or after the partition method. Swab surface growth on the filter or, if quantification is desired, transfer small portions of each target colony on filter to the appropriate total coliform verification medium using a sterile needle. Alternatively, after transferring filter to NA-MUG media, incubating it, and reading the results on this media, either transfer individual colonies, swab surface growth on filter, or place whole filter into appropriate total coliform verification medium. (See 9222B.4*g* for total coliform verification procedures.)

c. Partition method for E. coli determination: Aseptically transfer membrane filter with at least one coliform-positive colony to NA-MUG plate. If quantification is desired, mark each sheen colony (e.g., use a fine-tip pen to mark sheen colony's location on lid and filter/lid orientation) and transfer lid to NA-MUG plate, or use a sterile needle to make a hole in membrane filter next to sheen colony after transferring membrane to NA-MUG medium. Incubate NA-MUG immediately after transfer at 35 ± 0.5°C for 4 h.

Observe individual colonies (on NA-MUG plates) using a long-wavelength (365–366-nm) UV light source, preferably containing a 6-W bulb. **CAUTION: UV lamp should never be viewed directly.** Preferably view plates in a viewing box or hold the UV light a few inches above the plates (e.g., 3 to 4 in.), facing away from the viewer. The presence of a bright blue fluorescence on the periphery (outer edge) of a colony, or observed from the back of the plate is a positive response for *E. coli*. Record presence or absence of fluorescence, or if quantification is desired, count and record the number of target colonies. For nonpotable water samples, this partition method can be used to determine *E. coli* from the thermotolerant coliform MF procedure using mFC medium for initial isolation before transfer to NA-MUG medium. The procedure is the same as the above, except for the total coliform verification process.

4. Bibliography

MATES, A. & M. SHAFFER. 1989. Membrane filtration differentiation of *E. coli* from coliforms in the examination of water. *J. Appl. Bacteriol*. 67:343.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1989. Drinking Water; National Primary Drinking Water Regulations; Total Coliforms (Including Fecal Coliforms and *E. coli)*; Final Rule. 40 CFR Parts 141 and 142; *Fed. Reg.* 54:27544.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1991. National Primary Drinking Water Regulations; Analytical Techniques; Coliform Bacteria. 40 CFR Part 141; *Fed. Reg.* 56:636.

MATES, A. & M. SHAFFER. 1992. Quantitative determination of *Escherichia coli* from coliforms and fecal coliforms in sea water. *Microbios* 71:27.

SARTORY, D. & L. HOWARD. 1992. A medium detecting beta-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Lett. Appl. Microbiol*. 15:273.

SHADIX, L.C., M.E. DUNNIGAN & E.W. RICE. 1993. Detection of *Escherichia coli* by the nutrient agar plus 4-methylumbelliferyl-β-D-glucuronide (MUG) membrane filter method. *Can. J. Microbiol.* 39:1066.

9222 J. Simultaneous Detection of Total Coliform and *E. coli* by Dual-Chromogen Membrane Filter Procedure

1. Laboratory Apparatus

For MF analyses, use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. Sample bottles: See Section 9030B.19.

b. Dilution bottles: See Section 9030B.13.

c. Pipets, sample containers, and graduated cylinders: See Section 9030B.9.

d. Culture dishes: See 9222B.1*e*.

e. Filtration units: See 9222B.1*f*.

f. Membrane filters: See 9222B.1*g*.

g. Absorbent pads: See 9222B.1*h*.

h. Forceps: See 9222B.1*i*.

i. Incubators: See 9222B.1*j*.

2. Materials and Culture Medium

Purchase this medium from a commercial vendor; it cannot be prepared from basic ingredients. See Section 9020B.5*j* for media QC specifications.

Before use, test each lot with positive and negative culture controls (See Table 9020:VI). Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request new samples. Test each new medium lot to confirm that its performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance.

*m-ColiBlue24® Broth**

L-Methionine	0.1	g
Methylene blue	0.016	g
Casitone	8.0	g
Yeast extract	0.5	g
Lactose	0.6	g
Sodium chloride (NaCl)	3.0	g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.75	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.25	g
Triphenyl tetrazolium chloride	0.07	g
Sodium pyruvate	1.0	g
Erythromycin	3.0	g
Octyphenol ethoxylate†	0.5	g
Magnesium sulfate ($MgSO_4$)	0.3	g
5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid	(proprietary)	
Sodium azide	0.02	g
Cyclohexylammonium salt	0.2	g
Reagent-grade water	1	L

CAUTION: Sodium azide is highly toxic and mutagenic. Follow manufacturer's and SDS instructions for proper storage and handling of this medium.

Mix broth gently by inverting ampules two or three times before dispensing. Pour liquid medium (approximately 2 mL per plate) evenly onto sterile absorbent pads and place lid on Petri dish. Final pH should be 7.0 ± 0.2.

3. Procedure

a. Selection of sample size: See 9222B.4*a*.

b. Sterile filtration units: See 9222B.4*b*.

c. Filtration of sample: See 9222B.4*c*, with the following exception: incubate m-ColiBlue24® broth plates at 35 ± 0.5°C for 24 h.

d. Counting: To count colonies on membrane filters, use a low-powered (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device with a cool white fluorescent light source directed to provide optimal viewing. Count all red and blue to purple colonies under normal/ambient light and record as the total coliform result. Count only blue to purple colonies and record as *E. coli* result. Clear or white colonies are considered non-coliform colonies. A high non-coliform count may interfere with the development of coliform colonies.

e. Coliform verification: For drinking water, total coliform colony verification is not required for this medium. For waters other than drinking water, verify at a frequency established by the laboratory (see Section 9020B.10). Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of positive samples) (see Section 9020B.10). Adjust counts based on verification results. Verification tests are listed in 9222B.4*g*.

4. Calculation of Coliform Density

See 9222B.5. Calculate the final values using the formula:

$$\textit{E. coli}/100\text{ mL} = \frac{\text{number of blue-purple colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

$$\text{TC}/100\text{ mL} = \frac{\text{number of red } \textit{and} \text{ blue to purple colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

5. Bibliography

GRANT, M.A. 1997. A new membrane filtration medium for simultaneous detection and enumeration of *Escherichia coli* and total coliforms. *Appl. Environ. Microbiol.* 63:3526.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1999. National Primary and Secondary Drinking Water Regulations: Analytical Methods for Chemical and Microbiological Contaminants and Revisions to Library Certification Requirement Final Rule. 40 CFR Parts 141 and 143; *Fed. Reg.* 64(230):67449.

* m-Coliblue24®, HACH Company, Loveland, CO; EMD Millipore Sigma Corporation, Billerica, MA; or equivalent.
† Triton X-114, or equivalent.

FRANCY, D.S. & R.A. DARNER. 2000. Comparison of methods for determining *Escherichia coli* concentrations in recreational water. *Water Res.* 34:2770.

OLSTADT, J., J.J. SCHAUER, J. STANDRIDGE & S. KLUENDER. 2007. A comparison of the USEPA approved total coliform/*E. coli* tests. *J. Water and Health* 05:267.

9222 K. Simultaneous Detection of Total Coliforms and *E. coli* by Fluorogen/Chromogen Membrane Filter Procedure

1. Laboratory Apparatus

For MF analyses, use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. Sample bottles: See Section 9030B.19.

b. Dilution bottles: See Section 9030B.13.

c. Pipets, sample containers, and graduated cylinders: See Section 9030B.9.

d. Culture dishes: See 9222B.1*e*.

e. Filtration units: See 9222B.1*f*.

f. Membrane filters: See 9222B.1*g*.

g. Absorbent pads: See 9222B.1*h*.

h. Forceps: See 9222B.1*i*.

i. Incubators: See 9222B.1*j*.

2. Materials and Culture Medium

Use commercial dehydrated media whenever possible for uniformity between batches; never prepare media from basic ingredients when suitable dehydrated media are available.

Follow manufacturer's directions for rehydration. Store opened supplies of dehydrated media in a desiccator (if necessary). Commercial liquid media (sterile ampule, etc.) may be used if known to give equivalent results. See Section 9020B.5*j* for media QC specifications.

Before use, test each lot with positive and negative culture controls (See Table 9020:VI). Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request new samples. Test each new medium lot to confirm that its performance is satisfactory (see Section 9020B.5*j*). The use of control charts is helpful to identify trends and ensure long-term consistency in media performance. If commercially prepared medium is not available, prepare as described in ¶s *a*–*c* below.

a. Cefsulodin solution, 1 mg/1 mL: Add 0.02 g of cefsulodin to 20 mL reagent-grade distilled water, sterilize using a 0.22-μm syringe filter, and store in a sterile tube at 4°C until needed. Prepare fresh solution each time MI medium is made. Do not save the unused portion.

*b. MI agar:**

Proteose peptone No. 3	5.0	g
Yeast extract	3.0	g
β-D-Lactose	1.0	g
4-Methylumbelliferyl-β-D-galactopyranoside (MUGal) (final concentration 100 μg/mL)	0.1	g
Indoxyl-β-D-glucuronide (IBDG) (final concentration 320 μg/mL)	0.32	g
NaCl	7.5	g
K_2HPO_4	3.3	g
KH_2PO_4	1.0	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Agar	15.0	g
Reagent-grade distilled water	1	L

Autoclave medium for 15 min at 121–124°C, and add 5 mL of freshly prepared cefsulodin solution (¶ *a* above)(5 μg/mL final concentration) per liter of tempered agar medium. Final pH should be 6.95 ± 0.2. Pipet medium into 9- × 50-mm Petri dishes (5 mL/plate). Store plates at 4°C for up to 2 weeks.

c. MI broth:† Use same ingredients as MI agar, but omit agar. Prepare and sterilize, and add cefsulodin by the methods described for MI agar. Alternately, the broth can be filter-sterilized. Final pH should be 7.1 ± 0.2. Place absorbent pads in 9- × 50-mm Petri dishes and saturate with 2.0 to 3.0 mL MI broth containing 5 μg/mL final concentration of cefsulodin. Store plates in refrigerator, and discard after 96 h. Pour off excess broth before using plates.

3. Procedure

a. Selection of sample size: See 9222B.4*a*.

b. Sterile filtration units: See 9222B.4*b*.

c. Filtration of sample: See 9222B.4*c*.

d. Counting: To count colonies on membrane filters, use a low-powered (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device with a cool white fluorescent light source directed to provide optimal viewing. Count all blue colonies on each MI plate under normal/ambient light and record as *E. coli* results. Positive results that occur in <24 h are valid, but results cannot be recorded as negative until the 24-h incubation period is complete. Expose each MI plate to long-wave UV light (366 nm), and count all fluorescent colonies [blue/green fluorescent *E. coli*, blue/white fluorescent TC other than *E. coli*, and blue/green with fluorescent edges (also *E. coli*)] to obtain the TC count. Record the data. If any blue, non-fluorescent colonies are found on the same plate, add their total to the TC count.

Calculate the final values using the following formula:

$$E.\ coli/100\ \text{mL} = \frac{\text{number of blue colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

* BBL™ MI prepared plates (No. 214986), or equivalent.

† Dehydrated Difco™ MI Broth (No. 214882), or equivalent.

$$\text{TC/100 mL} = \frac{\text{number of fluorescent colonies} + \text{number of blue, non-fluorescent colonies (if any)}}{\text{volume of sample filtered (mL)}} \times 100$$

e. Coliform verification: For drinking water, total coliform colony verification is not required. For waters other than drinking water, verify at a frequency established by the laboratory (see Section 9020B.10). Laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of positive samples) based on need and sample type (see Section 9020B.10). Adjust counts based on verification results. Verification tests are listed in 9222B.4*g*.

4. Calculation of Coliform Density

See 9222B.5.

5. Bibliography

Brenner, K.P., C.C. Rankin, Y.R. Roybal, G.N. Stelma, Jr., P.V. Scarpino & A.P. Dufour. 1993. New medium for the simultaneous detection of total coliforms and *Escherichia coli* in water. *Appl. Environ. Microbiol*. 59:3534.

Brenner, K.P., C.C. Rankin, N. Sivaganesan & P.V. Scarpino. 1996. Comparison of the recoveries of *Escherichia coli* and total coliforms from drinking water by the MI agar method and the U.S. Environmental Protection Agency-approved membrane filter method. *Appl. Environ Microbiol*. 62:204.

U.S. Environmental Protection Agency. 2002. Method 1604: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium); EPA 821-R-02-024. Off. Water, Washington, D.C.