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

9221 A. Introduction

The coliform group consists of several genera of bacteria belonging to the family Enterobacteriaceae. The historical definition of this group has been based on the method used for detection, lactose fermentation, rather than on the tenets of systematic bacteriology. Accordingly, when the fermentation technique is used, this group is defined as all facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation 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 procedures. Quality control guidelines are outlined in Section 9020.

When multiple tubes are used in the fermentation technique, coliform density can be estimated by using a most probable number (MPN) table. This number, based on certain probability formulas, is an estimate of the mean density of coliforms in the sample. Results of coliform testing, 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 precision of the fermentation test 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 and/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.3). 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 the portions 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 the quality meets U.S. Environmental Protection Agency (EPA) standards, 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, to the confirmed phase (9221B.3). Drinking water samples that are positive for total coliforms also must be tested for thermotolerant (fecal) coliforms (9221E) or *Escherichia coli* (9221F).

For routine examinations 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. It is also used as a screen for the presence of fecal contamination. Some coliform occurrences in a distribution system may be attributed to growth or survival of coliforms in 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 between 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 examining 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.4) as a quality control measure on at least 10% of coliform-positive nonpotable water samples on a seasonal basis. The objective of the examination of nonpotable water, generally, is to estimate the bacterial density, determine a source of pollution, 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 Sections 9060A and B, using sample containers specified in Section 9030B.19. Follow the 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

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sample in 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 Sections 9060A and B, using sample containers specified in Section 9030B.19.

2. Presumptive Phase

Use lauryl tryptose broth in the presumptive portion of the multiple-tube test. If the medium has been refrigerated after sterilization, incubate overnight at room temperature (20°C) before use. Discard tubes showing growth and/or bubbles.

a. Reagents and culture medium: If possible, use a commercially available medium.

Lauryl tryptose broth:

Tryptose	20.0	g
Lactose	5.0	g
Dipotassium hydrogen phosphate, K_2HPO4	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—in fermentation tubes with an inverted vial (Durham tube)—sufficient medium 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 bromcresol 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.

Make lauryl tryptose broth of such strength that adding 100-mL, 20-mL, or 10-mL portions of sample to the medium will not reduce ingredient concentrations below those of the standard medium. Prepare in accordance with Table 9221:I. 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.

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

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, use five 20-mL portions, ten 10-mL portions, or a single bottle of 100-mL portion; 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 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) Incubate inoculated tubes or bottles 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, reincubate and reexamine 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 positive presumptive reaction.

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

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 acid reaction to the confirmed phase (9221B.3). An arbitrary 48-h limit for observation doubtless excludes occasional members of the coliform group that grow very slowly (see Section 9212).

3. Confirmed Phase

a. Culture medium: Use brilliant green lactose bile broth fermentation tubes for the confirmed phase. If possible, use a commercially available medium.

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—in fermentation tubes with an inverted vial—sufficient medium to cover the inverted vial at least one-half to two-thirds after sterilization. Close tubes

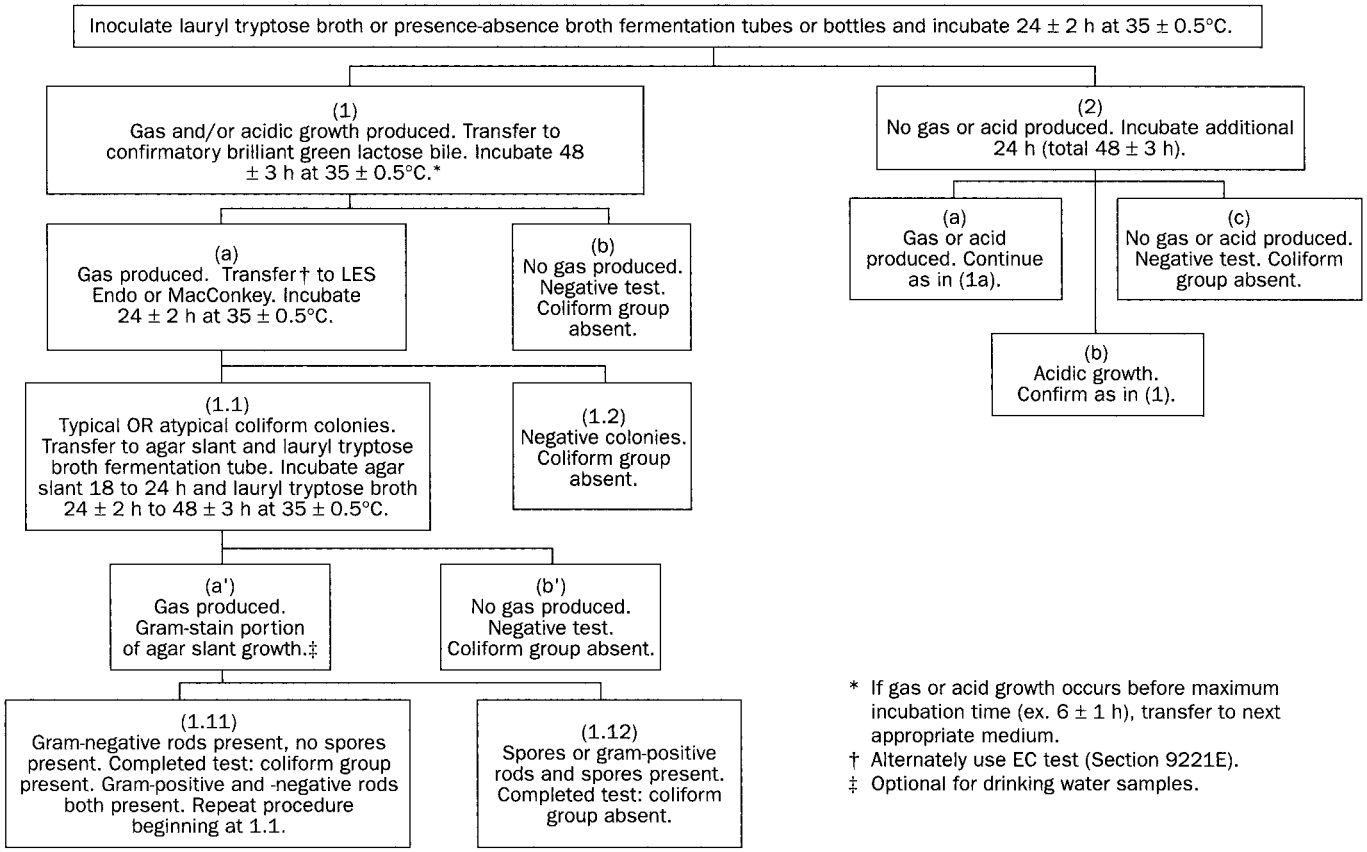


Figure 9221:1. Schematic outline of presumptive, confirmed, and completed phases for total coliform detection.

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: 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, submit these to the confirmed phase. Simultaneous inoculation into brilliant green lactose bile broth for total coliforms and EC broth for thermotolerant (fecal) coliforms (see 9221E) or EC-MUG broth for *Escherichia coli* (see 9221F) may be used. To confirm presumptive coliform colonies growing on a solid medium using fermentation media, see Section 9222B.5.*f.*

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 brilliant green lactose bile broth. Alternatively, insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to bottom of fermentation tube containing brilliant green lactose bile broth. Remove and discard applicator. Repeat for all other positive presumptive tubes.

Incubate the inoculated brilliant green lactose bile broth tubes at 35 ± 0.5°C. Formation of gas in any amount in the inverted vial of the brilliant green lactose bile 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 brilliant green lactose bile 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, submit to the confirmed phase only the tubes of the highest dilution (smallest sample inoculum) in which all tubes are positive and 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.

4. Completed Phase

To verify the presence of coliform bacteria and to provide quality control data for nonpotable water sample analysis, use the completed test on 10% (or a set percentage) of positive confirmed tubes (see Figure 9221:1). Use the completed test on at least one positive sample per quarter. If no positive sample occurs within a quarter, a QC check must be performed using a known positive sample. Simultaneous inoculation into brilliant green lactose bile broth for total coliforms and EC broth for thermotolerant (fecal) coliforms (9221E) or EC MUG broth for *Escherichia coli* (9221F) may be used. Positive results from incubation in EC and/or EC-MUG broths at elevated temperature (44.5 ± 0.2°C) can be considered as a completed test. Parallel

positive brilliant green lactose bile 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 brilliant green lactose bile broth cultures are recorded as positive for fecal coliforms or *E. coli*, respectively. Alternatively, the completed test for positive total coliforms may be performed as follows.

a. Culture media and reagents: If possible, use a dehydrated, commercially available medium.

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. 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 tube of brilliant green lactose bile broth showing gas, as soon as possible after the observation of gas. 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, the following approach may be used: (*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 organisms of the coliform group. 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 surface of the colony 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 the occurrences of gram-positive bacteria and spore-forming organisms surviving this selective screening procedure are infrequent in drinking water.

Various modifications of the Gram stain technique exist. Use the following modification by Hucker for staining smears of pure cultures; include a gram-positive and a gram-negative culture as controls.

Prepare separate light emulsions of the test bacterial growth and positive and negative control cultures on the same slide, 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 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 the presence of a member of the coliform group.

5. Bibliography

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9221 C. Estimation of Bacterial Density

1. Precision of the Multiple-Tube Fermentation Test

Unless many sample portions are examined, the precision of the Multiple-Tube Fermentation Test is rather low. Consequently, use caution when interpreting the sanitary significance of any single coliform result. When several samples from a given sampling point are estimated separately and the results combined in their geometric mean, the precision is greatly improved. 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 other organisms, provided that suitable test media are available.

2. Table Reading and Recording of Most Probable Number (MPN)

Record coliform concentration as MPN/100 mL. 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 examining drinking waters. 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 used are those found in the tables, report the value corresponding to the number of positive and negative results in the series as MPN/100 mL. When the series of decimal dilutions is different from that in Table 9221:IV, select the MPN value from Table 9221:IV for the combination of positive results and calculate according to the following formula:

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

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	10	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.

where:

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

When more than three dilutions are used in a decimal series[1] of dilutions, use the following guidelines to select the three most appropriate dilutions and refer to Table 9221:IV. Several illustrative examples (A through G) of combinations of positives are shown in Table 9221:V. 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. Accord-

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

ing 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).

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 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:

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

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, $N = \Sigma(n_j - x_j)z_j$, $P = \Sigma x_j$, and $T = \Sigma n_j z_j$, where the summations are over the dilutions selected, and x_j = the number of positive tubes 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.

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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. Simplification—by use of one large test portion (100 mL) in a single culture bottle to obtain qualitative information on the presence or absence of coliforms—is justified on the theory that no coliforms should be present in 100 mL of a drinking water sample. Another advantage includes the possibility of examining a larger number of samples per unit of time. 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 problems in detection.

The P–A test is intended for use on routine samples collected from distribution systems or water treatment plants.

1. Samples

Collect samples as directed in Sections 9060A and B, using sample containers specified in Section 9030B.19.

2. Presumptive Phase

a. Culture medium:

P–A broth: This medium is commercially available in dehydrated and in sterile concentrated form.

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
Bromcresol purple	0.0085	g
Reagent-grade water	1	L

Make this formulation triple strength (3×) when examining 100-mL samples. Dissolve the P–A broth medium in water without heating, using a stirring device. Dispense 50 mL prepared medium into screw-capped 250-mL milk dilution bottles

or equivalent containers. A fermentation tube insert is not necessary. Autoclave for 12 min at 121°C, with the total time in the autoclave limited to 45 min or less. Medium pH should be 6.8 ± 0.2 after sterilization. When the P–A medium is sterilized via filtration, a 6× strength 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 achieve even distribution of the medium throughout the sample. Incubate at 35 ± 0.5°C and inspect after 24 ± 2 and 48 ± 3 h for acid reactions.

c. Interpretation: A distinct yellow color forms in the medium when acidic conditions exist following lactose fermentation. If gas also is being produced, gently shaking the bottle will result in a foaming reaction. Any amount of gas and/or acid constitutes a positive presumptive test requiring confirmation. Simultaneous inoculation of EC broth or EC MUG and then brilliant green lactose bile broth is permitted.

3. Confirmed Phase

The confirmed phase is outlined in Figure 9221:1.

a. Culture medium: Use brilliant green lactose bile (BGLB) fermentation tubes (see 9221B.3).

b. Procedure: Use a sterile wire loop or wooden applicator to transfer all cultures that show acid reaction or acid and gas reaction to BGLB broth for incubation at 35 ± 0.5°C (see 9221B.3).

c. Interpretation: Gas production in 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.4 and Figure 9221:1.

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9221 E. Fecal Coliform Procedure

Thermotolerant coliforms (those that ferment lactose to produce gas at 44.5°C) were traditionally called *fecal coliforms,* but they also have been documented in organically rich waters or tropical climates in the absence of recent fecal contamination. So, testing for *E. coli*—a specific indicator of fecal contamination—is recommended.

Nevertheless, current regulations may require that thermotolerant (formerly fecal) coliforms be identified and enumerated. In the multiple-tube fermentation technique, this group of organisms is identified by their ability to ferment lactose to produce gas at 44.5°C.

A test for thermotolerant coliforms can be performed using one of the multiple-tube procedures described here or the membrane filter methods described in Sections 9222D and E.

1. Thermotolerant Coliform Test (EC Medium)

The thermotolerant coliform test using EC medium is applicable to investigations of drinking water, stream pollution, raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. Do not use EC medium for the direct isolation of thermotolerant coliforms from water. Prior enrichment in a presumptive medium is required for optimum recovery of thermotolerant coliforms. For testing of presumptive coliform colonies growing on solid media, refer to Section 9222G.2.

a. EC medium: Preferably use a dehydrated, commercially available medium.

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) Gently shake or rotate fermentation tubes or bottles showing gas, growth, or acidity. Using a sterile 3- or 3.5-mm-diam loop or sterile wooden applicator stick, transfer growth from each presumptive or confirmed fermentation tube or bottle to EC broth (see Section 9221B.3).

2) Place all EC tubes in a water bath within 30 min after inoculation. Incubate inoculated EC broth tubes in a water bath 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 and growth in an EC broth culture within 24 ± 2 h or less is considered a positive thermotolerant 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 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 Coliform Direct Test (A-1 Medium)

a. A-1 medium: This medium may be used for the direct isolation of thermotolerant coliforms from source water, treated wastewater and seawater, but not drinking water. Unlike EC medium, A-1 medium does not require prior enrichment in a presumptive medium for optimum recovery of thermotolerant (fecal) coliforms. Preferably use a dehydrated, commercially available medium.

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

* Triton X-100, Rohm and Haas Co., or equivalent.

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, 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 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 formation of precipitate during storage.

b. Procedure: Inoculate tubes of A-1 broth as directed in Section 9221B.2.*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 indicating the presence of thermotolerant coliforms. Calculate the MPN of thermotolerant coliforms from the number of positive A-1 broth tubes (as described in 9221C).

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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 water, surface water, groundwater, and wastewater. 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 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 possessing the enzyme β-glucuronidase and capable of cleaving the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) with the corresponding release of the fluorogen within 24 ± 2 h or less when grown in EC-MUG medium at 44.5°C.

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

Use EC-MUG medium to test for *E. coli* in a total coliform-positive culture. The procedure also can be used to confirm

presumptive total coliform colonies growing on solid media, described in Section 9222B.5*.f.*

a. EC-MUG medium:

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 (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. Using a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from the fermentation tube or bottle to EC-MUG broth.

2) Place all EC-MUG tubes in water bath within 30 min after inoculation. Incubate inoculated EC-MUG tubes for 24 ± 2 h in a water bath 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 aid in the interpretation of results and to avoid confusion of weak autofluorescence of the medium or glass tubes as a positive response, include in the assay 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. If multiple tubes are used, calculate the MPN for *E. coli* from the number of positive EC-MUG broth tubes as described in Section 9221C. When using only one tube, or subculturing from a single presumptive bottle or colony, report as presence or absence of *E. coli*.

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9221 G. Other *Escherichia coli* Procedures (PROPOSED)

For the *E. coli* test using the GAD reagent, *E. coli* is defined as the species of coliform bacteria possessing the enzyme glutamate decarboxylase (GAD), and capable of producing an alkaline reaction within 4 h in a reagent containing glutamic acid and a lytic agent. The procedure is used to test for *E. coli* after prior enrichment in a medium used for the identification of 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

a. GAD reagent:

L-Glutamic acid	1.0	g
Sodium chloride, NaCl	90.0	g
Bromcresol 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. 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 from the broth by centrifugation at 2500 to 3000 × *g* for 10 min. Discard supernatant and resuspend cells in 5 mL phosphate buffer. Reconcentrate cells by centrifugation (2500 to 3000 × *g,* 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. The presence of a blue color is considered a positive result for *E. coli*. To assist in interpretation of results, incorporate in the assay a positive control consisting of a known *E. coli* (GAD-positive) culture, a negative control consisting of a known total coliform organism [e.g., *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 Section 9221C. When using only one tube or a single presumptive bottle, report as presence or absence of *E. coli*.

* Triton X-100, Union Carbide Co., or equivalent.

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. However, it should be noted that *Klebsiella oxytoca* is indole positive. Use tryptone water and Kovacs' reagent to test for *E. coli* in a total coliform-positive culture.

a. Reagents:

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 115°C.

2) *Kovacs' 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°C for 24 ± 2 h. After incubation, add 0.2 to 0.3 mL Kovacs' reagent to each tube of tryptone water.

c. Interpretation: Examine all tubes for the appearance of a deep red color in the upper layer. The presence of a red color is considered a positive result for *E. coli*. To assist in interpretation of results, incorporate into the assay a positive control consisting of a known *E. coli* (indole-positive) culture, a negative control consisting of a known total coliform organism [e.g., *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 Section 9221C. When using only one tube or a single presumptive bottle, report as presence or absence of *E. coli*.

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