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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 aerobic and facultative anaerobic, gram-negative, nonspore-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 either by the multiple-tube fermentation technique (through the presumptive-confirmed phases or completed test) described herein, by the membrane filter (MF) technique (Section 9222) or by the proposed chromogenic 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, results of the examination of replicate tubes and dilutions are reported in terms of the Most Probable Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of the mean density of coliforms in the sample. Coliform density, together with other information obtained by engineering or sanitary surveys, provides the best assessment of water treatment effectiveness and the sanitary quality of untreated water.

The precision of each test depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows gas in some or all of the tubes and the smallest sample inoculum shows no gas in all or a majority of the tubes. Bacterial density (9221C.2) can be estimated by the formula given or from the table using the number of positive tubes in the multiple dilutions. The number of sample portions selected will be governed by the desired precision of the result. 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 clumping of bacterial cells occurs, the MPN value will be an underestimate of the actual bacterial density.

1. Water of Drinking Water Quality

When drinking water is analyzed to determine if the quality meets the standards of the U.S. Environmental Protection Agency (EPA), 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 all drinking water by the fermentation technique, use the confirmed phase (9221B.2). Apply the completed test (9221B.3) to selected samples on a seasonal basis and at least quarterly for quality control.

For the routine examination of most public water supplies, particularly those that are disinfected, the object of the coliform test is to determine compliance with EPA standards as a measure of the efficiency of treatment plant operation or water/effluent quality. A high proportion of coliform occurrences in a distribution system may be attributed not to treatment failure at the plant or the well source, but to bacterial regrowth in the mains. Because it is difficult to distinguish between coliform regrowth and new contamination, assume all coliform occurrences to be new contamination unless otherwise demonstrated.

2. Water of Other than Drinking Water Quality

In the examination of nonpotable waters inoculate a series of tubes with appropriate decimal dilutions of the water (multiples and submultiples of 10 mL), based on the probable coliform density. Use the presumptive-confirmed phase of the multiple-tube procedure. Use the more labor-intensive completed test (9221B.3) as a quality control measure on at least 10% of coliform-positive nonpotable water samples on a seasonal basis. The object of the examination of nonpotable water generally is to estimate the density of bacterial contamination, determine a source of pollution, enforce water quality standards, or trace the survival of microorganisms. Each objective requires a numerical value for reporting results. The multiple-tube fermentation technique may be used to obtain statistically valid MPN estimates of coliform density. Examine a sufficient number of 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. The direct-count, membrane filter technique may prove the better procedure to accomplish this objective.

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. 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 50 g sample in sterile blender jar, add 450 mL sterile phosphate buffer or 0.1% peptone dilution water, and blend for 1 to 2 min at low speed (8000 rpm). Prepare the appropriate decimal dilutions of the homogenized slurry as quickly as possible to minimize settling.

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* Approved by Standard Methods Committee, 1991.

9221 B. Standard Total Coliform Fermentation Technique

1. 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 35°C before use. Discard tubes showing growth and/or bubbles.

a. Reagents and culture medium:

1) *Lauryl tryptose broth:*

Tryptose	20.0	g
Lactose	5.0	g
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. pH should be 6.8 ± 0.2 after sterilization. Before sterilization, dispense sufficient medium, in fermentation tubes with an inverted vial, to cover inverted vial at least one-half to two-thirds after sterilization. Alternatively, omit inverted vial and add 0.01 g/L bromcresol purple to presumptive medium to determine acid production, the 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 medium will not reduce ingredient concentrations below those of the standard medium. Prepare in accordance with Table 9221:I.

b. Procedure:

1) Arrange fermentation tubes in rows of five tubes each in a test tube rack. The number of five-tube rows and the sample volumes selected depend upon 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.).

In 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.

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

2) Incubate inoculated tubes or bottles at 35 ± 0.5C. After 24 ± 2 h swirl each tube or bottle gently and examine it for heavy growth, gas, and acidic reaction (shades of yellow color) and, if no gas or acidic growth has formed, reincubate and reexamine at the end of 48 ± 3 h. Record presence or absence of heavy growth, gas, and acid production. If the inner vial is omitted, growth with acidity signifies a positive presumptive reaction.

c. Interpretation: Production of gas or acidic growth in the tubes or bottles within 48 ± 3 h constitutes a positive presumptive reaction. Submit tubes with a positive presumptive reaction to the confirmed phase (9221B.2).

The absence of acidic growth or gas formation at the end of 48 ± 3 h of incubation constitutes a negative test. An arbitrary 48-h limit for observation doubtless excludes occasional members of the coliform group that grow very slowly (see Section 9212).

2. Confirmed Phase

a. Culture medium: Use brilliant green lactose bile broth fermentation tubes for the confirmed phase.

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. pH should be 7.2 ± 0.2 after sterilization. Before sterilization, dispense, in fermentation tubes with an inverted vial, sufficient medium to cover inverted vial at least one-half to two-thirds after sterilization. Close tubes with metal or heat-resistant plastic caps.

b. Procedure: Submit all primary tubes or bottles showing heavy growth, any amount of gas, or acidic growth within 24 h of incubation to the confirmed phase. If active fermentation or acidic growth appears in the primary tube earlier than 24 h, transfer to the confirmatory medium, preferably without waiting for the full 24-h period to elapse. If additional primary tubes or bottles show active fermentation or acidic growth at the end of a 48-h incubation period, submit these to the confirmed phase.

Gently shake or rotate primary tubes or bottles showing gas or acidic growth to resuspend the organisms. With a sterile metal loop 3 mm in diameter, transfer one loopful of culture to a fermentation tube containing brilliant green lactose bile broth or 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 tube for 48 ± 3 h 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. Calculate the MPN value from the number of positive brilliant green lactose bile tubes as described in Section 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 only after 48 h.

3. Completed Phase

To establish the presence of coliform bacteria and to provide quality control data, use the completed test on at least 10% of positive confirmed tubes (see Figure 9221:1). Double confirmation into brilliant green lactose bile broth for total coliforms and EC broth for fecal coliforms (see Section 9221E below) may be used. Consider positive EC broth elevated temperature (44.5°C) results as a positive completed test response. Parallel positive brilliant green lactose bile broth cultures with negative EC broth cultures indicate the presence of nonfecal coliforms and must be submitted to the completed test procedure to validate the presence of coliforms.

a. Culture media and reagents:

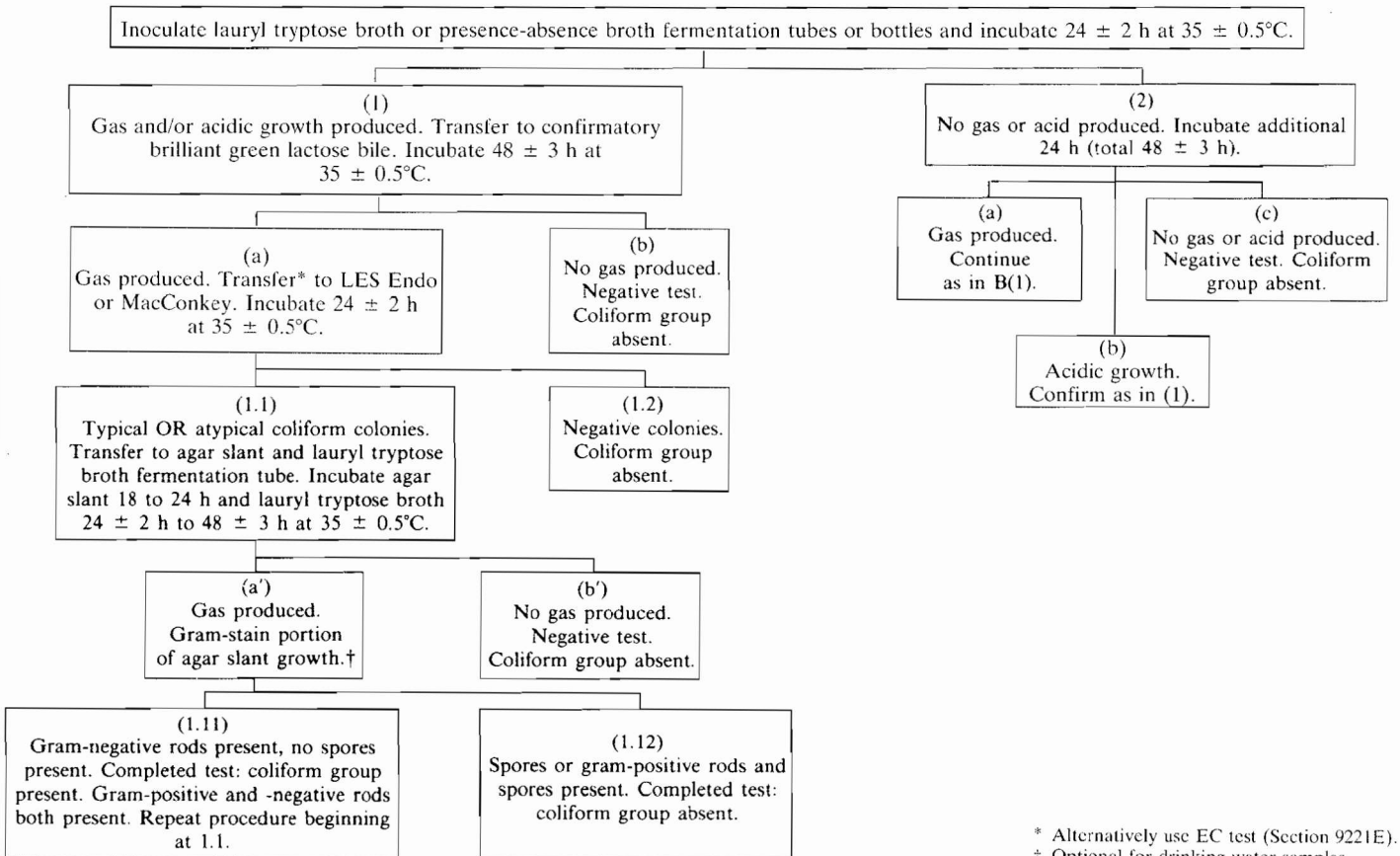1) *LES Endo agar:* See Section 9222B. Use 100- × 15-mm petri plates.

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). 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

Inoculate lauryl tryptose broth or presence-absence broth fermentation tubes or bottles and incubate 24 ± 2 h at 35 ± 0.5°C.

(1) Gas and/or acidic growth produced. Transfer to confirmatory brilliant green lactose bile. Incubate 48 ± 3 h at 35 ± 0.5°C.

(2) No gas or acid produced. Incubate additional 24 h (total 48 ± 3 h).

(a) Gas produced. Transfer* to LES Endo or MacConkey. Incubate 24 ± 2 h at 35 ± 0.5°C.

(b) No gas produced. Negative test. Coliform group absent.

(a) Gas produced. Continue as in B(1).

(c) No gas or acid produced. Negative test. Coliform group absent.

(b) Acidic growth. Confirm as in (1).

(1.1) Typical OR atypical coliform colonies. Transfer to agar slant and lauryl tryptose broth fermentation tube. Incubate agar slant 18 to 24 h and lauryl tryptose broth 24 ± 2 h to 48 ± 3 h at 35 ± 0.5°C.

(1.2) Negative colonies. Coliform group absent.

(a') Gas produced. Gram-stain portion of agar slant growth.†

(b') No gas produced. Negative test. Coliform group absent.

(1.11) Gram-negative rods present, no spores present. Completed test: coliform group present. Gram-positive and -negative rods both present. Repeat procedure beginning at 1.1.

(1.12) Spores or gram-positive rods and spores present. Completed test: coliform group absent.

* Alternatively use EC test (Section 9221E).
† Optional for drinking water samples.

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

Add ingredients to water, mix thoroughly, and heat to dissolve. pH should be 6.8 ± 0.2 after sterilization. Before sterilization, dispense in screw-capped tubes. After sterilization, immediately place tubes in an inclined position so that 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:

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.

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

b. *Procedure:*

1) Using aseptic technique, streak one LES Endo agar (Section 9222B.2) 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 insure presence of some discrete colonies separated by at least 0.5 cm. Observe the following precautions when streaking plates to obtain a high proportion of successful isolations if coliform organisms are present: (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 end of loop or needle into the liquid in the tube to a depth of approximately 0.5 cm; and (d) streak plate for isolation with curved section of the needle in contact with the agar to avoid a scratched or torn surface. Flame loop between 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); *atypical* (pink, red, white, or colorless colonies without sheen) after 24 h incubation; or *negative* (all others). 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 consist of organisms of the coliform group, and transfer growth from each isolate to a single-strength lauryl tryptose broth fermentation tube and onto a nutrient agar slant. (The latter is unnecessary for drinking water samples.)

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 inserted) 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 culture; 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 and 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 over-decolorize. 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. If no gas is produced in the secondary tube of lauryl tryptose broth within 48 ± 3 h, adjust original MPN result calculated from the confirmed test accordingly (Section 9221C).

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9221 C. Estimation of Bacterial Density

1. Precision of Fermentation Tube Test

Unless a large number of sample portions is examined, the precision of the fermentation tube test is rather low. For example, even when the sample contains 1 coliform organism/mL, about 37% of 1-mL tubes may be expected to yield negative results because of random distribution of the bacteria in the sample. When five tubes, each with 1 mL sample, are used under these conditions, a completely negative result may be expected less than 1% of the time.

Even when five fermentation tubes are used, the precision of the results obtained is not of a high order. Consequently, exercise great caution when interpreting the sanitary significance of coliform results obtained from the use of a few tubes with each sample dilution, especially when the number of samples from a given sampling point is limited.

2. Computing and Recording of MPN

To calculate coliform density, compute in terms of the Most Probable Number (MPN). The MPN values, for a variety of planting series and results, are given in Tables 9221:II, III, and IV. Included in these tables are the 95% confidence limits for each MPN value determined. If the sample volumes used are those found in the tables, report the value corresponding to the number of positive and negative results in the series as the MPN/100 mL or report as total or fecal coliform presence or absence.

The sample volumes indicated in Tables 9221:II and III relate more specifically to finished 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 volumes of samples are tested. When the series of decimal dilutions is different from that in the table, select the MPN value from Table 9221:IV for the combination of positive tubes and calculate according to the following formula:

$$\text{MPN value (from table)} \times \frac{10}{\text{largest volume tested}} = \text{MPN/100 mL}$$

When more than three dilutions are used in a decimal series of dilutions, use the results from only three of these in computing the MPN. To select the three dilutions to be used in determining the MPN index, choose the highest dilution that gives positive results in all five portions tested (no lower dilution giving any negative results) and the two next succeeding higher dilutions. Use the results at these three volumes in computing the MPN index. In the examples given below, the significant dilution results are shown in boldface. The number in the numerator rep-

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

No. of Tubes Giving Positive Reaction Out of 5 of 20 mL Each	MPN Index/ 100 mL	95% Confidence Limits (Approximate)	
		Lower	Upper
0	<1.1	0	3.0
1	1.1	0.05	6.3
2	2.6	0.3	9.6
3	4.6	0.8	14.7
4	8.0	1.7	26.4
5	>8.0	4.0	Infinite

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

No. of Tubes Giving Positive Reaction Out of 10 of 10 mL Each	MPN Index/ 100 mL	95% Confidence Limits (Approximate)	
		Lower	Upper
0	< 1.1	0	3.0
1	1.1	0.03	5.9
2	2.2	0.26	8.1
3	3.6	0.69	10.6
4	5.1	1.3	13.4
5	6.9	2.1	16.8
6	9.2	3.1	21.1
7	12.0	4.3	27.1
8	16.1	5.9	36.8
9	23.0	8.1	59.5
10	>23.0	13.5	Infinite

resents positive tubes; that in the denominator, the total tubes planted; the combination of positives simply represents the total number of positive tubes per dilution:

Example	1 mL	0.1 mL	0.01 mL	0.001 mL	Combination of positives	MPN Index /100 mL
a	5/5	5/5	2/5	0/5	5-2-0	5000
b	5/5	4/5	2/5	0/5	5-4-2	2200
c	0/5	1/5	0/5	0/5	0-1-0	20

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	95% Confidence Limits		Combination of Positives	MPN Index/ 100 mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
				4-2-0	22	9.0	56
0-0-0	< 2	—	—	4-2-1	26	12	65
0-0-1	2	1.0	10	4-3-0	27	12	67
0-1-0	2	1.0	10	4-3-1	33	15	77
0-2-0	4	1.0	13	4-4-0	34	16	80
				5-0-0	23	9.0	86
1-0-0	2	1.0	11	5-0-1	30	10	110
1-0-1	4	1.0	15	5-0-2	40	20	140
1-1-0	4	1.0	15	5-1-0	30	10	120
1-1-1	6	2.0	18	5-1-1	50	20	150
1-2-0	6	2.0	18	5-1-2	60	30	180
2-0-0	4	1.0	17	5-2-0	50	20	170
2-0-1	7	2.0	20	5-2-1	70	30	210
2-1-0	7	2.0	21	5-2-2	90	40	250
2-1-1	9	3.0	24	5-3-0	80	30	250
2-2-0	9	3.0	25	5-3-1	110	40	300
2-3-0	12	5.0	29	5-3-2	140	60	360
3-0-0	8	3.0	24	5-3-3	170	80	410
3-0-1	11	4.0	29	5-4-0	130	50	390
3-1-0	11	4.0	29	5-4-1	170	70	480
3-1-1	14	6.0	35	5-4-2	220	100	580
3-2-0	14	6.0	35	5-4-3	280	120	690
3-2-1	17	7.0	40	5-4-4	350	160	820
				5-5-0	240	100	940
4-0-0	13	5.0	38	5-5-1	300	100	1300
4-0-1	17	7.0	45	5-5-2	500	200	2000
4-1-0	17	7.0	46	5-5-3	900	300	2900
4-1-1	21	9.0	55	5-5-4	1600	600	5300
4-1-2	26	12	63	5-5-5	≥1600	—	—

In *c*, select the first three dilutions so as to include the positive result in the middle dilution.

When a case such as that shown below in line *d* arises, where a positive occurs in a dilution higher than the three chosen according to the rule, incorporate it in the result for the highest chosen dilution, as in *e*:

Example	1 mL	0.1 mL	0.01 mL	0.001 mL	Combination of positives	MPN Index /100 mL
d	5/5	3/5	1/5	1/5	5-3-2	1400
e	5/5	3/5	2/5	0/5	5-3-2	1400

When it is desired to summarize with a single MPN value the results from a series of samples, use the geometric mean or the median.

Table 9221:IV shows the most likely positive tube combinations. If unlikely combinations occur with a frequency greater than 1% it is an indication that the technique is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled. The MPN for combinations not appearing in the table, or for other combinations of tubes or dilutions, may be estimated by Thomas' simple formula:

$$\text{MPN/100 mL} = \frac{\text{no. of positive tubes} \times 100}{\sqrt{\left(\begin{array}{c}\text{mL sample in}\\ \text{negative tubes}\end{array} \times \begin{array}{c}\text{mL sample in}\\ \text{all tubes}\end{array}\right)}}$$

While the MPN tables and calculations are described for use in the coliform test, they are equally applicable to determining the MPN of any other organisms provided suitable test media are available.

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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. The P-A test also provides the optional opportunity for further screening of the culture to isolate other indicators (fecal coliform, *Aeromonas*, *Staphylococcus*, *Pseudomonas*, fecal streptococcus, and *Clostridium*) on the same qualititative basis. Additional advantages include the possibility of examining a larger number of samples per unit of time and 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 sample submissions collected from distribution systems or water treatment plants. When sample locations produce a positive P-A result for coliforms, it may be advisable to determine coliform densities in repeat samples. Quantitative information may indicate the magnitude of a contaminating event.

1. Presumptive Phase

a. Culture media:

1) *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 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 a screw-cap 250-mL milk dilution bottle. A fermentation tube insert is not necessary. Autoclave for 12 min at 121°C with the total time in the autoclave limited to 30 min or less. pH should be 6.8 ± 0.2 after sterilization.

2) *Lauryl tryptose broth:* See Section 9221B.1.

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 triple-strength medium throughout the sample. Incubate at 35 ± 0.5°C and inspect after 24 and 48 h for acid reactions.

c. Interpretation: A distinct yellow color forms in the medium when acid 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.

2. Confirmed Phase

The confirmed phase is outlined in Figure 9221:1.

a. Culture medium: Use brilliant green lactose bile fermentation tubes (see 9221B.2).

b. Procedure: Transfer all cultures that show acid reaction or acid and gas reaction to brilliant green lactose bile (BGLB) broth for incubation at 35 ± 0.5°C.

c. Interpretation: Gas production in the BGLB broth culture within 48 h confirms the presence of coliform bacteria. Report result as presence-absence test positive or negative for total coliforms in 100 mL of sample.

3. Completed Phase

The completed phase is outlined in Section 9221B.3 and Figure 9221:1.

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9221 E.   Fecal Coliform Procedure

Elevated-temperature tests for the separation of organisms of the coliform group into those of fecal origin and those derived from nonfecal sources are described herein. Modifications in technical procedures, standardization of methods, and detailed studies of members of the coliform group found in the feces of various warm-blooded animals compared with those from other environmental sources have established the value of a fecal coliform determination. The test can be performed by one of the multiple-tube procedures described here or by membrane filter methods as described in Section 9222. The procedure using EC medium yields adequate information about the source of the coliform group (fecal or nonfecal) when used in confirmation. Do not use it for direct isolation of coliforms from water because prior enrichment is required in a presumptive medium for optimum recovery of fecal coliforms. The procedure using A-1 broth is a single-step method not requiring confirmation.

The fecal 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. The procedure is not recommended as a substitute for the total coliform test in the examination of potable waters, because no coliform bacteria of any kind should be tolerated in a treated water. The test using A-1 medium is applicable to source water, seawater, and treated wastewater.

1. Fecal Coliform Test (EC Medium)

The fecal coliform test differentiates between coliforms of fecal origin (intestines of warm-blooded animals) and coliforms from other sources. Use EC medium or, for a more rapid test of the quality of shellfish waters, treated wastewaters, or source waters use A-1 medium in a direct test.

a. EC 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$_2$HPO$_4$	4.0	g
Potassium dihydrogen phosphate, KH$_2$PO$_4$	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. pH should be 6.9 ± 0.2 after sterilization. Before sterilization, dispense in fermentation tubes, each with an inverted vial, sufficient medium to cover the inverted vial at least partially after sterilization. Close tubes with metal or heat-resistant plastic caps.

b. Procedure: Submit all presumptive fermentation tubes or bottles showing any amount of gas, heavy growth, or acidity within 48 h of incubation to the confirmed test.

1) Gently shake or rotate presumptive fermentation tubes or bottles showing gas, heavy growth, or acidity. With a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from each presumptive fermentation tube or bottle to EC broth.

2) Incubate inoculated EC broth tubes in a water bath at 44.5 ± 0.2°C for 24 ± 2 h.

Place all EC tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in water bath incubator to immerse tubes to upper level of the medium.

c. Interpretation: Gas production with growth in an EC broth culture within 24 h or less is considered a positive fecal coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction indicating a source other than the intestinal tract of warm-blooded animals. If multiple tubes are used, calculate MPN from the number of positive EC broth tubes as described in Section 9221C. When using only one tube for subculturing from a single presumptive bottle, report as presence or absence of fecal coliforms.

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

a. A-1 broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

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. Before sterilization dispense in fermentation tubes with an inverted vial sufficient medium to cover the inverted vial at least partially after sterilization. Close with metal or heat-resistant plastic caps. Sterilize by autoclaving at 121°C for 10 min. Store in dark at room temperature for not longer than 7 d. Ignore formation of precipitate.

Make A-1 broth of such strength that adding 10-mL sample portions to medium will not reduce ingredient concentrations below those of the standard medium. For 10-mL samples prepare double-strength medium.

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

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

c. Interpretation: Gas production in any A-1 broth culture within 24 h or less is a positive reaction indicating coliforms of fecal origin. Calculate MPN from the number of positive A-1 broth tubes as described in Section 9221C.

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