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 room temperature (20°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 or ten tubes each in a test tube rack. The number of 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.

2) Incubate inoculated tubes or bottles at 35 ± 0.5C. After 24 ± 2 h swirl each tube or bottle gently and examine it for growth, gas, and 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 acid production. If the inner vial is omitted, growth with acidity signifies a positive presumptive reaction.

c. Interpretation: Production of an acidic reaction or gas 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 reaction 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.2). 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 presumptive tubes or bottles showing growth, any amount of gas, or acidic reaction within 24 ± 2 h of incubation to the confirmed phase. If active fermentation or acidic reaction appears in the presumptive tube earlier than 24 ± 2 h, transfer to the confirmatory medium; preferably examine tubes at 18 ± 1 h. 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.

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

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 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 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 (e.g., 6 ± 1 h, 24 ± 2 h) 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). Simultaneous inoculation into brilliant green lactose bile broth for total coliforms and EC broth for fecal coliforms (see Section 9221E below) or EC-MUG broth for *Escherichia coli* may be used. Consider positive EC and EC-MUG broths elevated temperature (44.5°C) results as a positive completed test response. Parallel positive brilliant green lactose bile broth cultures with negative EC or EC-MUG broth cultures indicate the presence of nonfecal 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

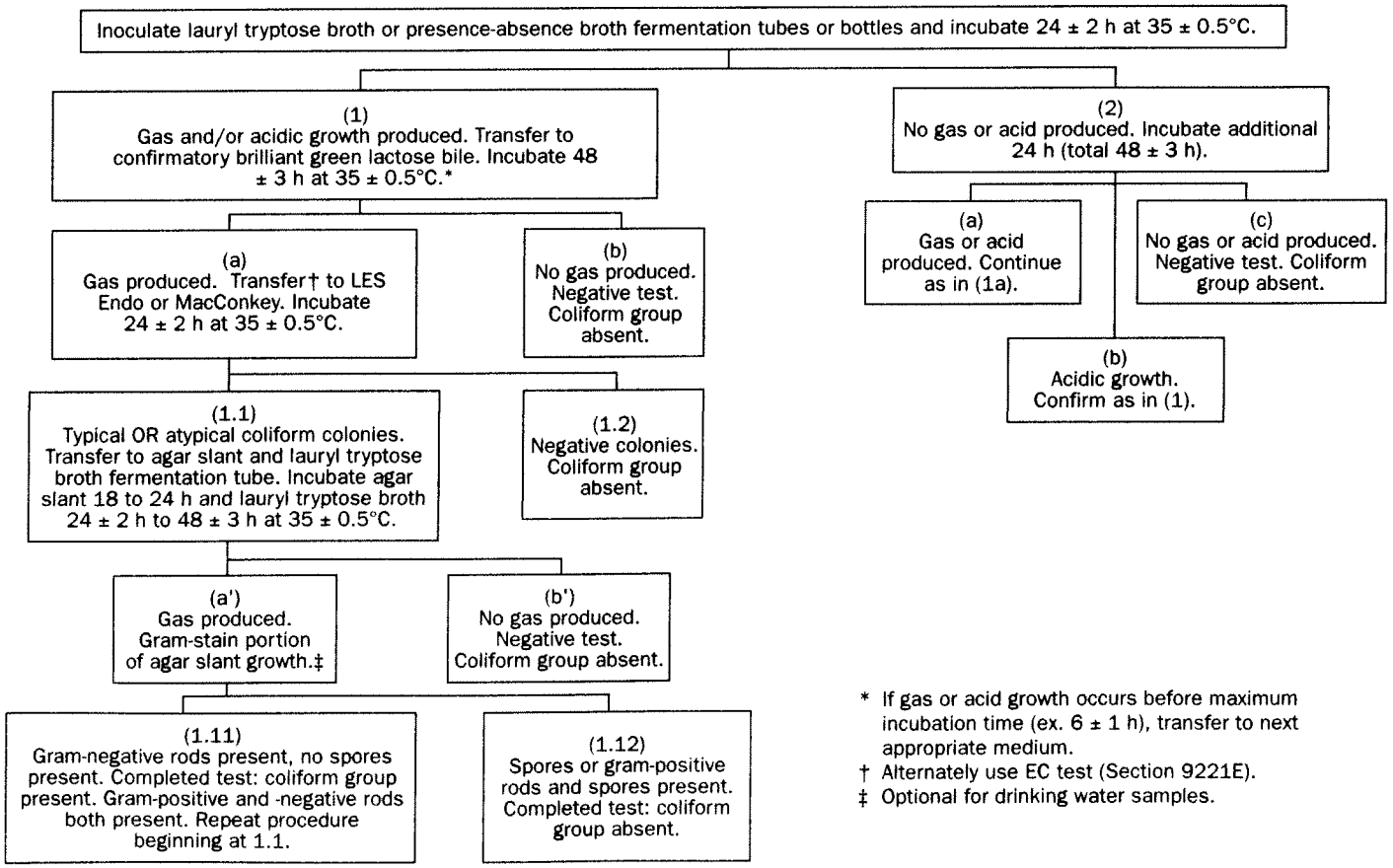

Figure 9221:1. Schematic outline of presumptive, confirmed, and completed phases for total coliform detection.

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

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) 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 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 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.

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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, if only 1 mL is examined in a sample containing 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.

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:

$$\text{MPN value (from table)} \times \frac{10}{\text{largest volume tested in dilution series used for MPN determination}} = \text{MPN/100 mL}$$

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

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

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
0-0-0	<2	—	—	4-2-0	22	9.0	56
0-0-1	2	1.0	10	4-2-1	26	12	65
0-1-0	2	1.0	10	4-3-0	27	12	67
0-2-0	4	1.0	13	4-3-1	33	15	77
				4-4-0	34	16	80
1-0-0	2	1.0	11	5-0-0	23	9.0	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
				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
4-0-0	13	5.0	38	5-5-0	240	100	940
4-0-1	17	7.0	45	5-5-1	300	100	1300
4-1-0	17	7.0	46	5-5-2	500	200	2000
4-1-1	21	9.0	55	5-5-3	900	300	2900
4-1-2	26	12	63	5-5-4	1600	600	5300
				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:

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

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 that 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 qualitative basis. Additional advantages include 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. 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 (3×) 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. When the PA medium is sterilized by 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.

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 (see Section 9221B.2).

c. Interpretation: Gas production in the BGLB broth culture within 48 ± 3 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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