STANDARD METHODS 9221 B. STANDARD TOTAL COLIFORM FERMENTATION TECHNIQUE

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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. NaC1	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 l0-mL portions of sample to medium will not reduce ingredient concentrations below those of the standard medium. Prepare in accordance with Table 9221:1.

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).

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

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 ....1.0 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.

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- x 15-mm petri plates.

2) *MacConkey agar:*

Peptone	17.0 g
Proteose peptone	3.0 g
Lactose	10.0 g
Bile salts	1.5 g
Sodium chloride. NaC1	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Reagent-grade water	1.0 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 X 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	1.0 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 $(NH_4)_2C_2O_4H_2O$ 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 K1 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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STANDARD METHODS 9221 C. ESTIMATION OF BACTERIAL DENSITY

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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, Table 9221:IV shows that the 95% confidence limits are often one-third times and three times the estimate. 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

2. Table Reading and Recording of MPN

Record coliform concentration as the Most Probable Number (MPN)/100 mL. The MPN values, for a variety of positive and negative 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 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 sample portion volumes 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 the MPN/100 mL, or, when appropriate, as presence or absence. 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 results and calculate according to the following formulas:

$$\text{MPN/100 mL} = \text{MPN value (from table)} \times 10/V$$

where:

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

When more than three dilutions are used in a decimal series of dilutions, select the three most appropriate dilutions and refer to Table 9221:IV. Several examples are shown in the following table.

	Volume mL						
Example	**10 mL**	**1 mL**	**0.1 mL**	**0.01 mL**	**0.001 mL**	**Combination of positives**	**MPN Index No./100 mL**
a	5	5	1	0	0	5-1-0	330
b	4	5	1	0	0	4-5-1	48
c	0	0	1	0	0	0-0-1	1.8
d	5	4	4	1	0	4-4-1	400
e	5	4	4	0	1	4-4-1	400
f	5	5	5	5	2	5-5-2	54,000

Select 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 (Example a). If the lowest dilution tested has less than five portions with positive results, select it and the two next succeeding higher dilutions (Examples b, c).

When a positive result occurs in a dilution higher than the three selected according to the fore-going rules, change the selection to the lowest dilution that has less than five positive results and the next two higher dilutions (Example d). When all the foregoing selection rules have left unselected any higher dilutions with positive results, add those higher-dilution positive results to the tested to select three dilutions, then select the next lower dilution (Example f).

When it is desired to summarize with a single MPN value the results from several samples, use the geometric mean or the median. The geometric mean is computed by averaging the logarithmic values; e.g., 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.

Table 9221:IV shows all but the very improbable positive tube combinations for a three-dilution series. In testing 10 different samples, there is a 99% chance of finding all the results among these 95 outcomes. If untabulated combinations occur with a frequency greater than 0.1%, 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 (5/5, 10/10, 4/10, 1/10, 0/5) use only (-, -, 4/10, 1/10, -), 4/10 @ 0.1 mL sample/tube and 1/10 @ 0.01 mL sample/tube; from (5/5, 10/10, 10/10, 0/10, 0/5) select only (-,-,10/10, 0/10, -), 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.

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.

Although MPN tables and calculations are described for use in the coliform test, they also determine the MPN of any other organisms provided that suitable test media are available.

When all the results at the lower dilutions are positive and all the results at higher dilutions are negative, it is possible to calculate an exact MPN for two selected dilutions as follows: When V is the volume of each individual sample portion at the highest dilution with all positive portions,

$$\text{MPN/100 mL} = (1/V)\ [230.3 \log_{10} (T/N)]$$

where T and N are defined as for Thomas's formula. The last example discussed above was (5/5, 10/10, 10/10, 0/10, 0/5), with portions 10, 1, 0.1, 0.01, and 0.001. The third dilution is the highest with positive portions, so V = 0.1. The MPN for the third and fourth dilution would be exactly

$$\text{MPN/100 mL} = (1/0.1) \times [230.3 \log_{10} (1.1/0.1)]$$

$$= 2400/100 \text{ mL}$$

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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 (20mLEach)	MPN Index/ I00 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	—	3.5
I	1.1	0.051	5.4
2	2.6	0.4	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 Various 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

STANDARD METHODS 9221 E. FECAL COLIFORM PROCEDURE

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Elevated-temperature tests for distinguishing organisms of the total coliform group that also belong to the fecal coliform group are described herein. Modifications in technical procedures. standardization of methods, and detailed studies of the fecal coliform group have established the value of this procedure. 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 A-l broth is a single-step method.

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. Prior enrichment in presumptive media is required for optimum recovery of fecal coliforms when using EC medium. 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 is used to distinguish those total coliform organisms that are fecal coliforms. 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_2HPO_4 ....4.0 g
Potassium dihydrogen phosphate. KH_2PO_4 ....1.5 g
Sodium chloride. NaCl ....5.0 g
Reagent-grade water ....1.0 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, growth, or acidity within 48 h of incubation to the fecal coliform test.

1) Gently shake or rotate presumptive 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 fermentation tube or bottle to EC broth (see Section 9221B.2).

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 ± 2 h or less is considered a positive fecal coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction. 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 be used for the direct isolation of fecal coliforms from water. Prior enrichment in a presumptive medium is not required.

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 ....1.0 L

*Triton X-l00. Rohm and Haas Co., or equivalent.

Heat to dissolve solid ingredients, add polyethylene glycol *p*-isooctyiphenyl 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 l0-mL sample portions to medium will not reduce ingredient concentrations below those of the standard medium. For l0-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.

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

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STANDARD METHODS 9221 F. *ESCHERICHIA COLI* PROCEDURE

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Escherichia coli is a member of the fecal coliform group of bacteria. This organism in water indicates fecal contamination. Enzymatic assays have been developed that allow for the identification of this organism. Assays for $- glucuronidase or glutamate decarboxylase may be used to determine the presence of *E. coli.* In method 9221F.1, *E. coli* are defined as coliform bacteria that possess the enzyme $-glucuronidase and are capable of cleaving the fluorogenic substrate 4-methylumbel-liferyl-$-D-glucuronide (MUG) with the corresponding release of the fluorogen when grown in EC-MUG medium at 44.5/C within 24 ± 2 h or less. In method 9221F.2, *E. coli* are defined as coliform bacteria that possess the enzyme glutamate decarboxylase and are capable of producing an alkaline reaction within 4 h in a reagent containing a lytic agent and glutamic acid. The procedure is used as a confirmatory test after prior enrichment in a presumptive medium for total coliform bacteria. These tests are performed by the tube procedure described here or by the membrane filter method as described in Section 9222. The chromogenic substrate procedure (Section 9223) can be used for direct detection of *E. coli*.

Tests for *E. coli* are applicable for the analysis of drinking water, surface and ground water, and wastewater. *E. coli* is a member of the indigenous fecal flora of warm-blooded animals. The occurrence of *E. coli* is considered a specific indicator of fecal contamination and the possible presence of enteric pathogens.

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

Use EC-MUG medium for the confirmation of *E. coli*.

a. EC-MUG medium:

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No.	31.5 g
Dipotassium hydrogen phosphate. K_2HPO_4	4.0 g
Potassium dihydrogen phosphate, KH_2PO_4	1.5 g
Sodium chloride, NaC1	5.0 g
4-methylumbelliferyl-$-D-glucuronide (MUG)	0.05 g
Reagent-grade water	1.0 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 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.

b. Procedure: Submit all presumptive fermentation tubes or bottles showing growth, gas, or acidity within 48 ± 3 h of incubation to the *E. coli* test.

1) Gently shake or rotate presumptive 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 presumptive fermentation tube or bottle to EC-MUG broth.

2) Incubate inoculated EC-MUG tubes in a water bath or incubator maintained at 44.5 ± 0.2/C for 24 ± 2 h. Place all EC-MUG tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in the water-bath incubator to immerse tubes to upper level of medium.

c. *Interpretation:* Examine all tubes exhibiting growth for fluorescence using a long-wavelength UV lamp (preferably 6 W). The presence of bright blue fluorescence is considered a positive response for *E. coli*. A positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret the results and to avoid confusion of weak auto-fluorescence of the medium as a positive response. If multiple tubes are used, calculate MPN 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, report as presence or absence of *E. coli*.

2. *Escherichia coli* Test (GAD Procedure)

The GAD test procedure is not approved for use in LT2 monitoring.

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