BEFORE THE HON'BLE NATIONAL GREEN TRIBUNAL
PRINCIPAL BENCH, NEW DELHI
ORIGINAL APPLICATION NO. 116 OF 2025
(IA No 366/2025)

IN THE MATTER OF:

Resident Welfare Association A1 Block Janakpuri — Applicant

Versus

Delhi Jal Board and Ors. — Respondents

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Adv. Rajkumar
On behalf of Central Pollution Control Board

Place: Delhi
Date: 28.07.2025

REPORT BY CPCB IN COMPLIANCE TO HON'BLE NGT ORDER DATED 16.07.2025 IN THE MATTER OF OA NO. 116/2025 TITLED; "RESIDENT WELFARE ASSOCIATION A-1 BLOCK JANAKPURI VS DELHI JAL BOARD & ORS."

1.0 BACKGROUND

Hon'ble National Green Tribunal vide order dated **08.04.2025** and **14.05.2025** in Original Application No. 116 of 2025 directed CPCB to collect tap water samples from the individual houses of the affected areas in A-1 Block Janakpuri marked by the applicant in page no.52 of the Original Application. In compliance of the said orders CPCB submitted the report dated **29.05.2025** enclosing the analytical results of the samples of tap water collected on 29.04.2025 and 15.05.2025 that showed presence of Total Coliform and *E.Coli* in 06 samples.

Hon'ble NGT vide its order dated **30.05.2025** further directed CPCB to collect fresh samples of the area where they have failed on **30.06.2025** (i.e of the 06 houses where Total Coliform and *E. Coli* have been detected in the analytical results of the previously collected samples on 29.04.2025 and 15.05.2025). CPCB submitted the report dated **15.07.2025** enclosing the analytical results of the samples of tap water collected on 30.06.2025 that showed the presence of Total Coliform and *E.Coli* in 05 samples. Sample from 6th house could not be collected as owner did not open door for sample collection till time of water supply.

Hon'ble NGT vide its order dated **16.07.2025** directed CPCB as under:

> *Para13: Learned Counsel appearing for the DJB has made an attempt to dispute these reports, but we are of the view that the CPCB being an independent agency with all the facility of testing and having no interest in the matter, their report cannot be doubted. However, in the circumstances of the case, we permit the CPCB to take fresh samples from the taps of the*

households of the area concerned promptly and submit the sample analysis report.

Para 15 The Counsel for the CPCB submits that a video of sample collection will be prepared by the CPCB so that the report is not unnecessarily doubted. Also, CPCB will give reference of analytical method which is universally being followed and the standards provided by the agencies for potable water.

2.0 COMPLIANCE TO THE HON'BLE NGT ORDER DATED 08.04.2025

2.1 Sample Collection

In compliance of the order dated **16.07.2025**, CPCB collected samples of water supply by DJB from **12** houses on 20.07.2025, which included **05** houses where Total Coliform and *E. Coli* have been detected in the analytical results of the previously collected samples on **30.06.2025** and **07** samples were collected from other houses of A-1 Block, Janakpuri. The sample from the house with address, A-1/130, 2nd floor, Janakpuri could not be collected as owner again denied providing access for sampling.

2.2 Analytical Results

The collected samples have been analysed for Bacteriological parameters -Total Coliform (TC), Fecal Coliform (FC) and *E. Coli* and free residual chlorine.

The analytical results of the bacteriological parameters for the samples of tap water supplied by DJB from the **12** houses in A-1 Block Janakpuri are presented in **Table 1**. Out of 12 samples:

- in **06** samples Total Coliform and *E.Coli* have not been detected,
- in **03** samples Total Coliform have been detected but total coliform values close to lowest detect limit and *E.Coli* not detected.
- in **02** samples Total Coliform have been detected but *E.Coli* have not been detected in one and detected in the other but *E.coli* value close to lowest detect limit, and
- in **01** sample both Total Coliform and *E.Coli* have been detected above detection limits.

The Drinking Water Standards of Bureau of Indian Standards-IS 10500:2012 prescribes that Total coliform and *E.Coli* shall not be detected in 100 ml of drinking water samples.

2.3 Analytical Methods

The methodology followed for analyzing the presence and absence of Total Coliform and *E.Coli* in Drinking water is Presence-Absence (P-A) Coliform Test: 9221 D& F, APHA, 24th Edition.

The methodology followed for analyzing the Total coliform and *E.Coli* in water is Standard Total Coliform Fermentation Technique:9221 B,C & G, APHA,24th Edition.

The copy of Presence-Absence (P-A) Coliform Test: 9221 D& F, APHA, 24th Edition and the copy of the Standard Total Coliform Fermentation Technique:9221 B, C & G, APHA,24th Edition is attached as **Annexure-I**.

Nazimuddin
Scientist 'F'
Central Pollution Control Board
28.07.2025

Table No.1 Analytical Results of the bacteriological parameters & free residual chlorine

S. No.	Field Code	Details of the sampling locations at Janakpuri, Delhi	Time of Sampling	Bacteriological Parameters			Free Residual Chlorine (in mg/L)
				Total Coliform (MPN/100 mL)	Fecal Coliform (MPN/100 mL	E. Coli (MPN/100 mL)	
Drinking Water Standards as per IS 10500: 2012				Shall not be detectable in any 100 ml sample	-	Shall not be detectable in any 100 ml sample	0.2 mg/L (Minimum)
Samples collected on 20.07.2025							
1.	J-1	A-1/65, Ground Floor	05:45 AM	11 x 10^2	330	170	N.D
2.	J-2	A-1/63, Ground Floor	06:00 AM	6.8	1.8	BDL	N.D
3.	J-3	A-1/55, 2nd floor	06:10 AM	2.0	2.0	BDL	N.D
4.	J-4	A-1/57,Ground floor	06:20 AM	2.0	2.0	BDL	N.D
5.	J-5	A-1/57, 2nd floor	06:35AM	BDL	BDL	BDL	N.D
6.	J-6	A-1/141,2nd floor	06:45 AM	BDL	BDL	BDL	N.D
7.	J-7	A-1/129,Ground floor	06:50 AM	BDL	BDL	BDL	0.7 mg/l
8.	J-8	A-1/132,Ground floor	07:00 AM	BDL	BDL	BDL	1.4 mg/l
9.	J-9	A-1/148,2nd floor	07:15 AM	BDL	BDL	BDL	N.D
10.	J-10	A-1/46,Ground floor	07:25 AM	BDL	BDL	BDL	N.D
11.	J-11	A-1/36, 1st floor	07:35 AM	2.0	2.0	BDL	0.7 mg/l
12.	J-12	Behind A-1/60	07:40 AM	23	2.0	2.0	0.7 mg/l

BDL stands for Below Detection Limit; N.D stands for Non-Detectable

Detection Limit: TC/FC/E. Coli <1.8 MPN/100 mL

Detection Limit: Free Residual Chlorine: 0.36 mg/L for the Iodometric Method I (4500-Cl B) as per APHA using standard glass burette which was followed in field.

केंद्रीय प्रदूषण नियंत्रण बोर्ड
CENTRAL POLLUTION CONTROL BOARD
HEAD OFFICE - DELHI
Parivesh Bhavan, East Arjun Nagar, Delhi - 110032

जैव-विज्ञान प्रयोगशाला BIO-SCIENCE LABORATORY

Microbiological Analysis Report

1.	रिपोर्ट संख्या और जारी करने की तारीख Report No. & Date of Issue	:	BIO/2526/MB/00101,25/07/2025
2.	रिपोर्ट भेज दी गई है (मांगकर्ता का नाम, मोबाइल नंबर और पता) Report Sent To (Name,Mobile no. & Address of Indentor)	:	DH of WQM-I
3.	नमूने पंजीकरण संख्या और दिनांक Samples Registration No. & Date	:	BIO/MB/2526/SR00137,20/07/2025
4.	विश्लेषण अनुरोध प्रभाग/संगठन Analysis Request Division/Organization	:	WQM-I
5.	एकत्रित किया गया नमूना Sample Collected by	:	Alpana Narula,Yashpa Yadav, Syed Bilal, Gautam Sondhi, Jyoti Singh, Ravi Kumar, Nandan Sarkar, RD Swami
6.	नमूनाकरण योजना प्राथमिकता Sampling Plan Reference	:	
7.	नमूना प्राप्ति की तिथि एवं समय Date & Time of Sample Receipt	:	20/07/2025 11:09 AM
8.	नमूना विश्लेषण अवधि Sample Analysis Period	:	20/07/2025 24/07/2025
9.	नमूना विवरण Sample Details	:	Drinking Water
10.	परियोजना का नाम Name of the Project	:	NGT Monitoring in the matter of OA No.116/2025
11.	रिपोर्ट स्थिति Report Status	:	Final

SR. NO	Field Code	Sample Matrix	Date & Time of Sample Collection	Parameters	Result value	Unit
1	J-1	Fresh Water	20-07-2025 05:45 AM	E.coli	170	MPN/100mL
2	J-1	Fresh Water	20-07-2025 05:45 AM	Faecal Coliform	330	MPN/100mL
3	J-1	Fresh Water	20-07-2025 05:45 AM	Total Coliform	11*10^2	MPN/100mL
4	J-2	Fresh Water	20-07-2025 06:00 AM	E.coli	<1.8	MPN/100mL
5	J-2	Fresh Water	20-07-2025 06:00 AM	Faecal Coliform	1.8	MPN/100mL
6	J-2	Fresh Water	20-07-2025 06:00 AM	Total Coliform	6.8	MPN/100mL
7	J-3	Fresh Water	20-07-2025 06:10 AM	E.coli	<1.8	MPN/100mL
8	J-3	Fresh Water	20-07-2025 06:10 AM	Faecal Coliform	2	MPN/100mL
9	J-3	Fresh Water	20-07-2025 06:10 AM	Total Coliform	2	MPN/100mL
10	J-4	Fresh Water	20-07-2025 06:20 AM	E.coli	<1.8	MPN/100mL
11	J-4	Fresh Water	20-07-2025 06:20 AM	Faecal Coliform	2	MPN/100mL
12	J-4	Fresh Water	20-07-2025 06:20 AM	Total Coliform	2	MPN/100mL
13	J-5	Fresh Water	20-07-2025 06:35 AM	E.coli	<1.8	MPN/100mL
14	J-5	Fresh Water	20-07-2025 06:35 AM	Faecal Coliform	<1.8	MPN/100mL
15	J-5	Fresh Water	20-07-2025 06:35 AM	Total Coliform	<1.8	MPN/100mL
16	J-6	Fresh Water	20-07-2025 06:45 AM	E.coli	<1.8	MPN/100mL
17	J-6	Fresh Water	20-07-2025 06:45 AM	Faecal Coliform	<1.8	MPN/100mL
18	J-6	Fresh Water	20-07-2025 06:45 AM	Total Coliform	<1.8	MPN/100mL
19	J-7	Fresh Water	20-07-2025 06:50 AM	E.coli	<1.8	MPN/100mL
20	J-7	Fresh Water	20-07-2025 06:50 AM	Faecal Coliform	<1.8	MPN/100mL
21	J-7	Fresh Water	20-07-2025 06:50 AM	Total Coliform	<1.8	MPN/100mL
22	J-8	Fresh Water	20-07-2025 07:00 AM	E.coli	<1.8	MPN/100mL

Analyst	Supervisor, Reviewer & Authorized signatory	DH Bioscience Laboratory
Dr. Annu Goel	V. Himajwala	Dr. Yashpal Yadav

Printed on: 25 Jul 2025

केंद्रीय प्रदूषण नियंत्रण बोर्ड
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जैव-विज्ञान प्रयोगशाला BIO-SCIENCE LABORATORY

Microbiological Analysis Report

23	J-8	Fresh Water	20-07-2025 07:00 AM	Faecal Coliform	<1.8	MPN/100mL
24	J-8	Fresh Water	20-07-2025 07:00 AM	Total Coliform	<1.8	MPN/100mL
25	J-9	Fresh Water	20-07-2025 07:15 AM	E.coli	<1.8	MPN/100mL
26	J-9	Fresh Water	20-07-2025 07:15 AM	Faecal Coliform	<1.8	MPN/100mL
27	J-9	Fresh Water	20-07-2025 07:15 AM	Total Coliform	<1.8	MPN/100mL
28	J-10	Fresh Water	20-07-2025 07:25 AM	E.coli	<1.8	MPN/100mL
29	J-10	Fresh Water	20-07-2025 07:25 AM	Faecal Coliform	<1.8	MPN/100mL
30	J-10	Fresh Water	20-07-2025 07:25 AM	Total Coliform	<1.8	MPN/100mL
31	J-11	Fresh Water	20-07-2025 07:35 AM	E.coli	<1.8	MPN/100mL
32	J-11	Fresh Water	20-07-2025 07:35 AM	Faecal Coliform	2	MPN/100mL
33	J-11	Fresh Water	20-07-2025 07:35 AM	Total Coliform	2	MPN/100mL
34	J-12	Fresh Water	20-07-2025 07:40 AM	E.coli	2	MPN/100mL
35	J-12	Fresh Water	20-07-2025 07:40 AM	Faecal Coliform	2	MPN/100mL
36	J-12	Fresh Water	20-07-2025 07:40 AM	Total Coliform	23	MPN/100mL

*** END OF REPORT आख्या समाप्ति ***

Remarks (if any) :

Statement :

1.परिणाम केवल परीक्षण किए गए नमूनों से संबंधित हैं |
The results relate only to the samples tested

2.पूरी रिपोर्ट को छोड़कर दोबारा प्रस्तुत नहीं किया जाएगा। सीपीसीबी के सक्षम प्राधिकारी की लिखित मंजूरी के बिना।
The report shall not be reproduced except in full. without the written approval of the Competent authority of CPCB.

3.इस परीक्षण रिपोर्ट के जारी होने की तारीख से एक सप्ताह तक नमूना रखा जाएगा।
The sample will be retained for one week from the date of issue of this test report.

4. डिटेक्शन लिमिट से नीचे (बीडीएल) <1.8 एमपीएन/100 एमएल इंगित करता है |
Below Detection Limit (BDL) indicates <1.8 MPN/100 mL

Parameter Name	Test Method	Unit	Limit of Detection	Uncertainty of Measurment
E.coli	APHA, 24th Ed., 9221-G	MPN/100mL	<1.8	10-70 MPN/100mL @ 31MPN/100mL
Faecal Coliform	APHA, 24th Ed., 9221-E	MPN/100mL	<1.8	68-700 MPN/100mL @ 230MPN/100mL
Total Coliform	APHA, 24th Ed.,9221-A,B,C	MPN/100mL	<1.8	140-1000 MPN/100mL @ 380MPN/100mL

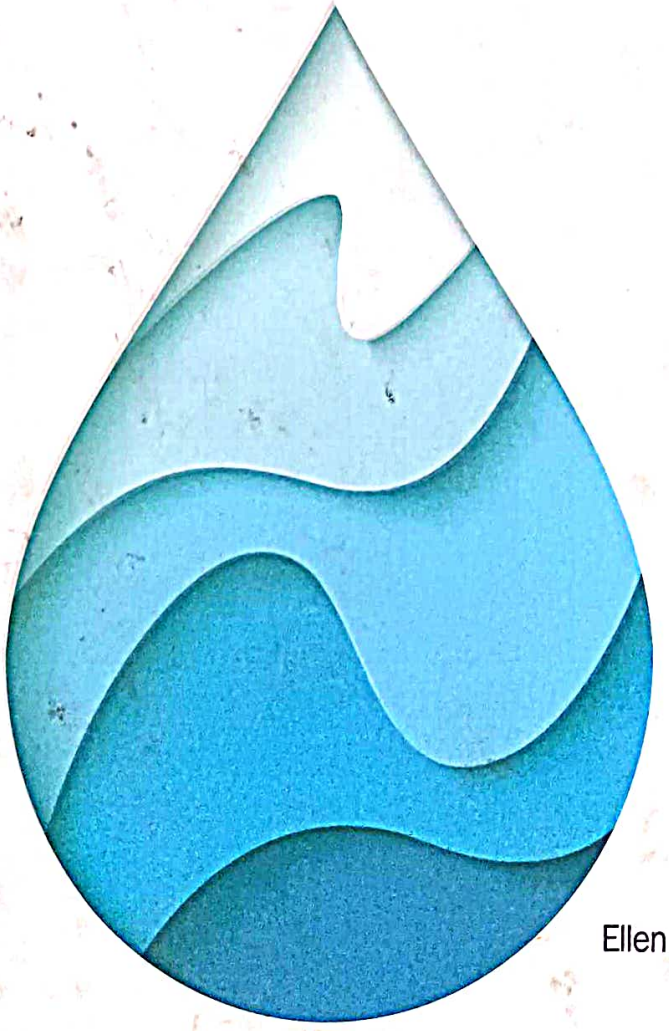
Analyst	Supervisor, Reviewer & Authorized signatory	DH Bioscience Laboratory
Dr. Annu Goel	V. Himajwala	Dr. Yashpal Yadav

Printed on: 25 Jul 2025

Standard Methods

for the Examination of Water and Wastewater

24TH EDITION



Edited by
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American Public Health Association®
American Water Works Association®
Water Environment Federation®

9221

MULTIPLE-TUBE FERMENTATION TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP

Approved by Standard Methods Committee, 2014. Joint Task Group: Ellen B. Braun-Howland (chair), Jennifer Best, Robert J. Blodgett, Laura Boczek, Gil Dichter, Clifford H. Johnson.

9221 A. INTRODUCTION

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

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

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

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

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

1. Potable Water

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

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

2. Nonpotable Water

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

3. Other Samples

The multiple-tube fermentation technique applies to the analysis of salt or brackish waters, as well as muds, sediments, and sludges. Collect samples as directed in Section 9060 A, using sample containers specified in Section 9030 B.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 sample in a sterile blender jar, add 270 mL sterile phosphate buffered or 0.1% peptone dilution water, and blend for 1 to 2 min at high speed (8000 rpm). Prepare the appropriate decimal dilutions of the homogenized slurry as quickly as possible to minimize settling.

9221 B. Standard Total Coliform Fermentation Technique

1. Samples

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

2. Quality Control

All phases of the fermentation technique (9221 B-G) require adherence to the quality assurance/quality control (QA/QC) guidelines presented in Section 9020, including, but not limited to, analytical QC (Section 9020 B.9), instrumentation/equipment (Sections 9020 B.4 and 9030 B), and supplies (Section 9020 B.5). Refer to Table 9020:1 for key QC procedures. Also, note the sections pertaining to appropriate storage and preparation of dehydrated culture media and water quality (Sections 9050 and 9020 B.5*f*).

Use commercial dehydrated media when possible, and ensure that their formulations match those specified here because commercial formulations may vary. Prepared fermentation media can be stored in tightly capped tubes or bottles for up to 3 months in the dark, if temperatures are between 1 and 30 °C and evaporation is less than 10% of the original volume. If the tubes were refrigerated after sterilization, incubate them overnight at room temperature (20 °C) before use and discard those showing growth or bubbles to avoid false-positive results. To demonstrate acceptable medium performance, positive and negative culture controls must be tested before first use and as otherwise specified (see Table 9020:6). Verify and record sterility, volume per tube, and pH. To demonstrate comparability between batches of media, perform a use test (Section 9020 B.5*f*2).

When switching to the multiple-tube fermentation technique, ideally first conduct parallel tests with the previous method to demonstrate applicability and comparability. The results of many coliform performance studies are available in the literature, and the rates of false-positive and -negative results can differ among various media. Carefully select the medium and procedure that best fits the requirements.

3. Presumptive Phase

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

a. Reagents and culture medium:

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. Before sterilization, dispense enough medium into fermentation tubes containing inverted vials (also known as Durham tubes) to cover the inverted vial at least one-half to two-thirds after sterilization. Alternatively, add 0.01 g/L bromocresol purple to lauryl tryptose broth to determine acid production, an indicator of a positive result in this part of the coliform test. Inverted vials are not required if bromocresol purple is added, but their inclusion permits evaluation of both gas and acid production in the sample. Close tubes with metal or heat-resistant plastic caps.

Prepare in accordance with Table 9221:1, making lauryl tryptose broth concentrated enough that adding 100-, 20-, or 10-mL portions of sample to the medium does not reduce ingredient concentrations below those of the standard medium. Autoclave medium at 121 °C for 12 to 15 min. Ensure that inverted vials, if used, are free of air bubbles. The medium pH must be 6.8 ± 0.2 after sterilization.

b. Procedure:

1) Arrange fermentation tubes in rows of 5 or 10 tubes each in a test tube rack. The number of rows and the sample volumes selected depend on the quality and character of the water to be examined. For potable water, 100 mL must be tested. Use five 20-mL portions, ten 10-mL portions, or one 100-mL portion (a single bottle). For nonpotable water, use 5 tubes per dilution (e.g., of 10, 1, 0.1 mL).

When making dilutions and measuring diluted sample volumes, follow the precautions given in Section 9215 B.2. Use Figure 9215:1 as a guide to preparing dilutions. Shake sample and dilutions

Table 9221:1. 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

vigorously 5 s (about 25 times). Inoculate each tube in a set of 5 with replicate sample volumes in increasing decimal dilutions, if decimal quantities of the sample are used. Mix test portions in the medium by gentle agitation.

2) Promptly incubate inoculated tubes or bottles, any culture controls, and sterility blanks at 35 ± 0.5 °C. 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, re-incubate and re-examine at the end of 48 ± 3 h. Record the presence or absence of growth, gas, and acid production. If the inner vial is omitted, growth with acidity (yellow color) signifies a presumptive positive reaction.

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

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 acidic reaction to the confirmed phase (9221 B.4).

4. Confirmed Phase

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

Brilliant green lactose bile broth:

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

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

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

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

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

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

5. Completed Phase

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

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

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

1) *LES Endo agar*—See Section 9222 B.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 must be 7.1 ± 0.2 after sterilization.

3) *Nutrient agar:*

Peptone	5.0 g
Beef extract	3.0g
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. The medium's pH must be 6.8 ± 0.2 after sterilization. After sterilization, immediately place tubes in an inclined position so the agar solidifies with a sloped surface. Tighten screw caps after cooling and store in a protected, cool storage area.

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

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

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

a) Use a sterile 3-mm-diam loop or an inoculating needle slightly curved at the tip;

b) tap and incline the fermentation tube to avoid picking up any membrane or scum on the needle;

c) insert the end of the loop or needle into the liquid in the tube to a depth of approximately 0.5 cm; and

d) streak a plate for isolation with the curved section of the needle in contact with the agar to avoid a scratched or torn surface. Flame the loop between the second and third quadrants to improve colony isolation.

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

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

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

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

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

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

On one slide, prepare separate light emulsions of the test bacterial growth and positive and negative control cultures using drops of reagent-grade 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 the slide in tap water and drain off the excess; apply Lugols solution for 1 min.

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

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

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9221 C. Estimation of Bacterial Density

1. Precision of the Multiple-Tube Fermentation Test

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

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

2. Use of Tables to Determine MPN

Record the coliform concentration as MPN/100 mL. The MPN values for a variety of positive and negative tube combinations are given in Table 9221:2, Table 9221:3 and Table 9221:4. The sample volumes indicated in Tables 9221:2 and 3 are chosen especially for drinking water examinations. Table 9221:4 illustrates MPN values for combinations of positive and negative results when five 10-mL, five 1.0-mL, and five 0.1-mL sample-portion volumes of nonpotable water are tested. If the sample-portion volumes tested are identical to those found in the tables, then report the value corresponding to the appropriate combination of positive and negative results as the MPN/100 mL. However, if the series of decimal dilutions is different, then select the MPN value in Table 9221:4 that corresponds to the combination of positive results and calculate the actual MPN using the following formula:

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

where:

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

If the decimal series[1] includes more than 3 dilutions, use the following guidelines to select the 3 most appropriate dilutions and then use Table 9221:4 and the equation above to calculate the MPN. See Table 9221:5, which provides several examples (A-G) of combinations of positives. First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube. Next, remove the lowest dilution (largest sample volume) if it has all positive tubes and at least one remaining dilution has a positive tube. According to these guidelines, the 3 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 3 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 2 dilutions of the highest dilution with *any* positive tubes, then use the highest dilution with *any* positive tubes and the 2 immediately lower dilutions. In Example C, the highest dilution with all positive tubes is 0.1 mL, which is within 2 dilutions of 0.001 mL, which has 1 positive tube. In Example D, the highest

Table 9221:2. 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 9221:3. 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 9221:4. 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)[a]

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

[a] Results to 2 significant figures.

dilution with all positive tubes is 0.01 mL, which is within 2 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 2 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 2 lowest remaining dilutions corresponding to 1 and 0.1 mL of sample. For the third dilution, add the number of positive tubes in all higher dilutions (0.01 and 0.001 mL of sample), to yield a final combination of 4-4-1.

If no dilution has all positive tubes (Example F), select the lowest 2 dilutions, corresponding to 10 and 1 mL of sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 mL of sample), to yield a final combination of 4-3-2. If the third dilution is assigned more than 5 positive tubes, then the selected combination will not be in Table 9221:4.

If the 3 dilutions selected are not found in Table 9221:4, 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 1 positive tube and the 2 dilutions immediately lower as the 3 selected dilutions. In Example G, the first selection, 4-3-6 (the outcome from the highest 3 dilutions), is not in Table 9221:4 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 9221:4, then use the following formula to calculate the MPN:

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

where:

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

For example, in the series x-x-3-0-0, where the third dilution level (z_s) equals 0.1 mL, $x_s z_s = 0.3$, and $\Sigma n_j z_j = 0.555$. Thus, the calculated MPN = 780/100 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 5 tubes are used per dilution, by using just the last 4 dilutions.

Table 9221:4 shows all but the improbable positive tube combinations for a 3-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 1 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 at 0.1 mL sample/ tube and 1/10 at 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 at 0.1 mL sample/ tube and 0/10 at 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 j^{th} 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 2 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 2 selected dilutions.

When summarizing 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:

Table 9221:5. Examples for Choice of 3 Combinations of Positives from 5 Dilutions

Example	Volume (mL)					Combination of Positives	MPN Index (No./100 mL)
	10	1	0.1	0.01	0.001		
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

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

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

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

1. Samples

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

2. Presumptive Phase

a. Culture medium:

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

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

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

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

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

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

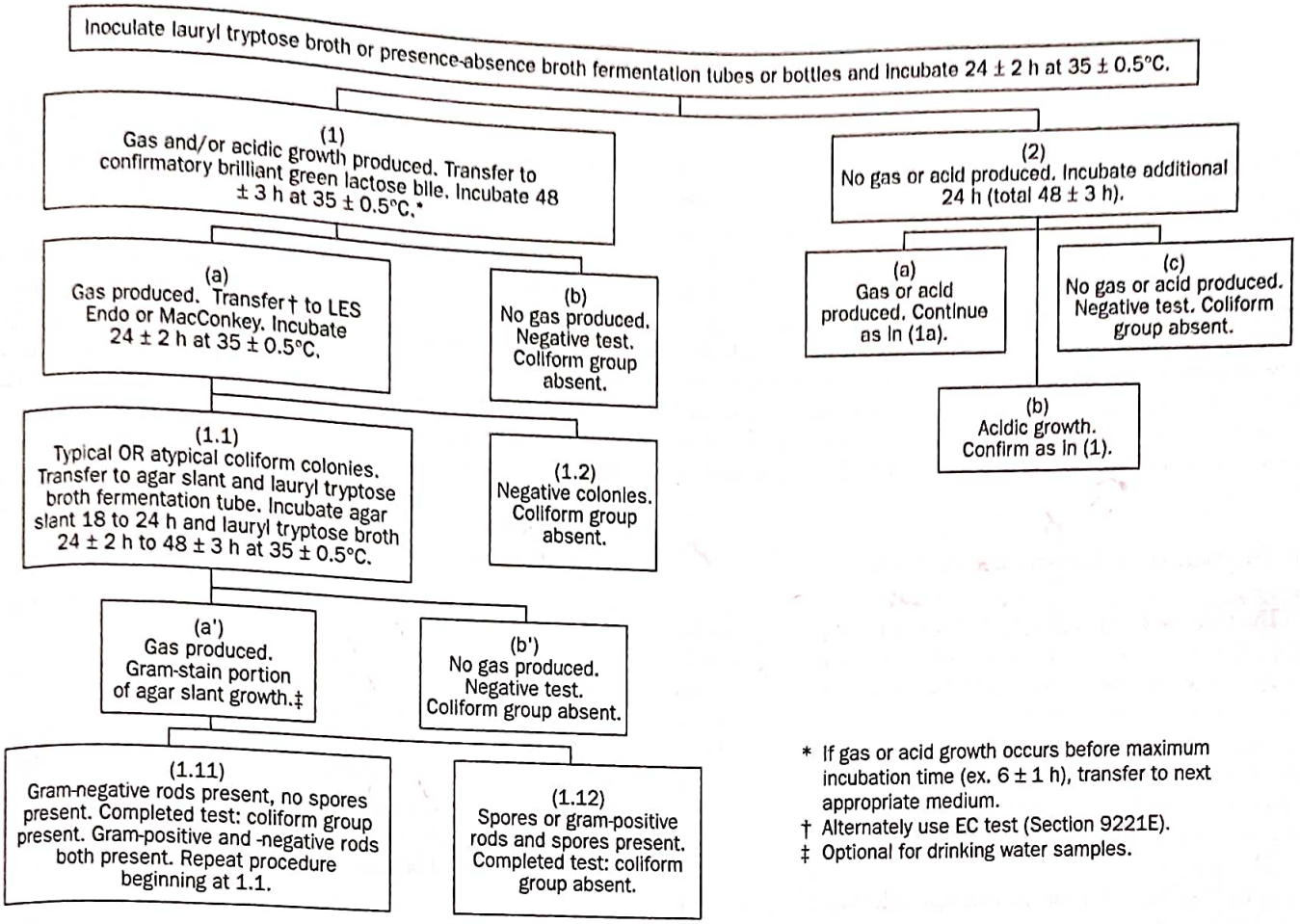

Figure 9221:1. Schematic outline of presumptive, confirmed, and completed phases for total coliform detection.

3. Confirmed Phase

The confirmed phase is outlined in Figure 9221:1.

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

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

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

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

4. Completed Phase

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

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9221 E. THERMOTOLERANT (FECAL) COLIFORM PROCEDURE

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

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

1. Thermotolerant Coliform Test (EC Medium)

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

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

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

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

b. Procedure:

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

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

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

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

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

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

Lactose	5.0 g
Tryptone	20.0 g
Sodium chloride (NaCl)	5.0 g
Salicin	0.5 g
Polyethylene glycol *p*-isooctylphenyl ether (Triton-X)	1.0 mL
Reagent-grade water	1 L

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

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

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

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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, surface, ground, and wastewater. Testing for *E. coli* can be performed using the multiple-tube procedure described here, by the membrane filter method described in Section 9222 I, or by the chromogenic enzyme substrate tests described in Section 9223. Other *E. coli* procedures are presented in 9221 G.

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

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

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

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

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

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

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

b. Procedure:

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

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

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

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

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

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

b. Procedure:

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

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

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

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

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(9221) G. Other *Escherichia coli* Procedures

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

1. *Escherichia coli* Test (GAD Procedure)

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

a. GAD reagent:

L-Glutamic acid	1.0 g
Sodium chloride (NaCl)	90.0 g
Bromocresol green	0.05 g
Polyethylene glycol octylphenyl ether (Triton-X)	3.0 mL
Reagent-grade water	1 L

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

b. Procedure:

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

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

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

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

2. *Escherichia coli* Test (Indole Production)

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

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

1) *Tryptone water:*

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

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

2) *Kovac's reagent:*

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

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

b. Procedure: Gently shake or rotate the 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 the presumptive fermentation tube or bottle to a tube containing 5 mL tryptone water. Incubate the inoculated tryptone water tubes in a water bath or incubator maintained at 44.5 ± 0.2 °C for 24 2 h. After incubation, add 0.2 to 0.3 mL Kovac's reagent to each tube of tryptone water.

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

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9222

Membrane Filter Technique for Members of the Coliform Group

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9222 A. Introduction

The membrane filtration (MF) technique[1] can be used with a variety of different media for the detection and enumeration of many types of bacteria, including coliforms. The medium used depends on the organisms targeted for detection. The MF procedure is reproducible, can be used to test relatively large sample volumes, and usually yields numerical results more rapidly than the multiple-tube fermentation technique.[2] A distinct advantage of the MF technique over using broth media is that it permits colony formation on the surface of the filter. Direct counts of bacterial colonies are useful for various readily filterable sample types including drinking water, some wastewater effluents, natural waters, and others. Also, MF permits the differentiation of the various colony morphologies of bacteria present in the water sample and allows for their subsequent subculture and identification. Additional advantages include the capability of processing larger sample volumes (providing more representative samples) and of analyzing a larger number of samples because the culture dishes require less room in the incubator.[2]

When used for enumerating coliforms, the MF technique is a more precise method than the multiple-tube fermentation procedure, because it uses direct counts as opposed to a statistical estimation (MPN) of results; compare Tables 9221:3 and 9221:4 with Table 9222:3. The data from these tests yield approximately the same qualitative water-quality information, although the numerical results from each method are not interchangeable.[3]

The MF technique has limitations, particularly when testing waters with high turbidity, because the filter can become clogged, resulting in the need for subsamples and multiple membrane filters or difficulties in counting colonies with debris on the membrane filter. In addition, if the sample being filtered contains large numbers of noncoliform (background) bacteria, the morphologies of the coliform colonies can be difficult to discern for counting.[3,4] If noncoliform bacterial interference occurs, the sample result must be invalidated and a new sample collected.

Another disadvantage of the MF technique when compared with broth culture methods is that bacterial numbers are generally