TOTAL COLIFORM: MULTIPLE TUBE FERMENTATION TECHNIQUE

1.0 SCOPE AND APPLICATION

1.1 This method is used to determine the presence of a member of the coliform group in ground water and surface water.

1.2 The coliform group, as analyzed for in this procedure, is defined as all aerobic and facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas formation within 48 hr at 35°C.

2.0 SUMMARY OF METHOD

2.1 The multiple-tube fermentation technique is a three-stage procedure in which the results are statistically expressed in terms of the Most Probable Number (MPN). These stages -- the presumptive stage, confirmed stage, and completed test -- are briefly summarized below. (For the analysis to be accurate, a five-tube test is required.)

2.1.1 **Presumptive Stage:** A series of lauryl tryptose broth primary fermentation tubes are inoculated with graduated quantities of the sample to be tested. The inoculated tubes are incubated at 35 ± 0.5°C for 24 ± 2 hr, at which time the tubes are examined for gas formation. For the tubes in which no gas is formed, continue incubation and examine for gas formation at the end of 48 ± 3 hr. Formation of gas in any amount within 48 ± 3 hr is a positive presumptive test.

2.1.2 **Confirmed Stage:** The confirmed stage is used on all primary fermentation tubes showing gas formation during the 24-hr and 48-hr periods. Fermentation tubes containing brilliant green lactose bile broth are inoculated with medium from the tubes showing a positive result in the presumptive test. Inoculation should be performed as soon as possible after gas formation occurs. The inoculated tubes are incubated for 48 ± 3 hr at 35 ± 0.5°C. Formation of gas at any time in the tube indicates a positive confirmed test.

2.1.3 **Completed Test:** The completed test is performed on all samples showing a positive result in the confirmed test. It can also be used as a quality control measure on 20% of all samples analyzed. One or more plates of eosin methylene blue are streaked with sample to be analyzed. The streaked plates are incubated for 24 ± 2 hr at 35 ± 0.5°C. After incubation, transfer one or more typical colonies (nucleated, with or without metallic sheen) to a lauryl tryptose broth fermentation tube and a nutrient agar slant. The fermentation tubes and agar slants are incubated at 35 ± 0.5°C for 24 ± 2 hr, or for 48 ± 3 hr if gas is not produced. From the agar slants corresponding to the fermentation tubes in which gas formation occurs, gram-stained samples are examined

microscopically. The formation of gas in the fermentation tube and the presence of gram-negative, non-spore-forming, rod-shaped bacteria in the agar culture may be considered a satisfactorily completed test, demonstrating the positive presence of coliform bacteria in the analyzed sample.

2.2 More detailed treatment of this method is presented in Standard Methods for the Examination of Water and Wastewater and in Microbiological Methods for Monitoring the Environment (see References, Section 10.0).

3.0 INTERFERENCES

3.1 The distribution of bacteria in water is irregular. Thus, a five-tube test is required in this method for adequate statistical accuracy.

3.2 The presence of residual chlorine or other halogens can prevent the continuation of bacterial action. To prevent this occurrence, sodium thiosulfate should be added to the sterile sample container.

3.3 Water samples high in copper, zinc, or other heavy metals can be toxic to bacteria. Chelating agents such as ethylenediaminetetraacetic acid (EDTA) should be added only when heavy metals are suspected of being present.

3.4 It is important to keep in mind that MPN tables are probability calculations and inherently have poor precision. They include a 23% positive bias that generally results in high value. The precision of the MPN can be improved by increasing the number of sample portions examined and the number of samples analyzed from the same sampling point.

4.0 APPARATUS AND MATERIALS

4.1 Incubators:

4.1.1 Incubators must maintain a uniform and constant temperature at all times in all areas, that is, they must not vary more than $\pm$0.5°C in the areas used. Obtain such accuracy by using a water-jacketed or anhydric-type incubator with thermostatically controlled low-temperature electric heating units properly insulated and located in or adjacent to the walls or floor of the chamber and preferably equipped with mechanical means of circulating air. If a hot-air type incubator is used, humidity must be maintained at 75-80%.

4.1.2 Alternatively, use special incubating rooms well insulated and equipped with properly distributed heating units and with forced air circulation, provided that they conform to desired temperature limits and relative humidity. When such rooms are used, record the daily temperature range in areas where plates or tubes are incubated. Provide incubators with open metal wire or sheet shelves so spaced as to assure temperature uniformity throughout the chamber. Leave a 2.5-cm space between walls and stacks of dishes or baskets of tubes.

4.1.3 Maintain an accurate thermometer with the bulb immersed in liquid (glycerine, water, or mineral oil) on each shelf in use within the incubator and record daily temperature readings (preferably morning and afternoon). It is desirable, in addition, to maintain a maximum and minimum registering thermometer within the incubator on the middle shelf to record the gross temperature range over a 24-hr period. At intervals, determine temperature variations within the incubator when filled to maximum capacity. Install a recording thermometer, whenever possible, to maintain a continuous and permanent record of temperature. Mercury thermometers should be graduated in 0.5°C increments and calibrated annually against an NBS certified thermometer. Dial thermometers should be calibrated quarterly.

4.1.4 Keep water depth in the water bath sufficient to immerse tubes to upper level of media.

4.2 Hot-air sterilizing ovens: Use hot-air sterilizing ovens of sufficient size to prevent internal crowding, constructed to give uniform and adequate sterilizing temperatures of 170 ± 10°C and equipped with suitable thermometers. As an alternative, use a temperature-recording instrument.

4.3 Autoclaves:

4.3.1 Use autoclaves of sufficient size to prevent internal crowding, constructed to provide uniform temperatures within the chambers (up to and including the sterilization temperature of 121°C); equipped with an accurate thermometer, the bulb of which is located properly on the exhaust line so as to register minimum temperature within the sterilizing chambers (temperature-recording instrument is optional); equipped with pressure gauge and properly adjusted safety valves connected directly with saturated-steam power lines or directly to a suitable special steam generator (do not use steam from a boiler treated with amines for corrosion control); and capable of reaching the desired temperature within 30 min.

4.3.2 Use of a vertical autoclave or pressure cooker is not recommended because of difficulty in adjusting and maintaining sterilization temperature and the potential hazard. If a pressure cooker is used in emergency or special circumstances, equip it with an efficient pressure gauge and a thermometer, the bulb of which is 2.5 cm above the water level.

4.4 Colony counters: Use Quebec-type colony counter, dark-field model preferred, or one providing equivalent magnification (1.5 diameters) and satisfactory visibility.

4.5 pH Equipment: Use electrometric pH meters, accurate to at least 0.1 pH units, for determining pH values of media. See Method 9040 for standardization of a pH meter.

4.6 Balances: Use balances providing a sensitivity of at least 0.1 g at a load of 150 g, with appropriate weights. Use an analytical balance having a sensitivity of 1 mg under a load of 10 g for weighing small quantities (less than 2 g) of materials. Single-pan rapid-weigh balances are most convenient.

4.7 Media preparation utensils: Use borosilicate glass or other suitable noncorrosive equipment such as stainless steel. Use glassware that is clean and free of residues, dried agar, or other foreign materials that may contaminate media.

4.8 Pipets and graduated cylinders:

4.8.1 Use pipets of any convenient size, provided that they deliver the required volume accurately and quickly. The error of calibration for a given manufacturer's lot must not exceed 2.5%. Use pipets having graduations distinctly marked and with unbroken tips. Bacteriological-transfer pipets or pipets conforming to the APHA standards given in the latest edition of Standard Methods for the Examination of Dairy Products may be used. Optimally, protect the mouth end of all pipets by a cotton plug to eliminate hazards to the worker or possible sample contamination by saliva.

4.8.2 Use graduated cylinders meeting ASTM Standards (D-86 and D-216) and with accuracy limits established by the National Bureau of Standards, where appropriate.

4.9 Pipet containers: Use boxes of aluminum or stainless steel, end measurement 5 to 7.5 cm, cylindrical or rectangular, and length about 40 cm. When these are not available, paper wrappings may be substituted. To avoid excessive charring during sterilization, use best-quality sulfate pulp (Kraft) paper. Do not use copper or copper alloy cans or boxes as pipet containers.

4.10 Dilution bottles or tubes:

4.10.1 Use bottles or tubes of resistant glass, preferably borosilicate glass, closed with glass stoppers or screw caps equipped with liners that do not produce toxic or bacteriostatic compounds on sterilization.

4.10.2 Do not use cotton plugs as closures. Mark gradation levels indelibly on side of dilution bottle or tube. Plastic bottles of nontoxic material and acceptable size may be substituted for glass, provided that they can be sterilized properly.

4.11 Petri dishes: Use glass or plastic Petri dishes about 100 x 15 mm. Use dishes the bottoms of which are free from bubbles and scratches and flat so that the medium will be of uniform thickness throughout the plate. For the membrane-filter technique, use loose-lid glass or plastic dishes, 60 x 15 mm, or tight-lid dishes, 50 x 12 mm. Sterilize Petri dishes and store in metal cans (aluminum or stainless steel, but not copper), or wrap in paper -- preferably best-quality sulfate pulp (Kraft) -- before sterilizing.

4.12 Fermentation tubes and vials: Use only 10-mm x 75-mm fermentation tubes. When tubes are used for a test of gas production, enclose a shell vial, inverted. Use a vial of such size that it will be filled completely with medium and at least partly submerged in the tube.

4.13 Inoculating equipment: Use wire loops made of 22- or 24-gauge nickel alloy (chromel, nichrome, or equivalent) or platinum-iridium for flame sterilization. Single-service transfer loops of aluminum or stainless steel are satisfactory. Use loops at least 3 mm in diameter. Sterilize by dry heat or steam. Single-service hardwood applicators also may be used. Make these 0.2 to 0.3 cm in diameter and at least 2.5 cm longer than the fermentation tube; sterilize by dry heat and store in glass or other nontoxic containers.

5.0 REAGENTS

5.1 ASTM Type II water (ASTM D1193): Water should be monitored for impurities.

5.2 Buffered water:

5.2.1 To prepare **stock phosphate buffer solution**, dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL Type II water, adjust to pH 7.2 $\pm$ 0.5 with 1 N sodium hydroxide (NaOH), and dilute to 1 liter with Type II water.

5.2.2 Add 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride solution (38 g $MgCl_2$/liter Type II water or 81.1 g $MgCl_2 \cdot 6H_2O$/liter Type II water) to 1 liter Type II water. Dispense in amounts that will provide 99 $\pm$ 2.0 mL or 9 $\pm$ 0.2 mL after autoclaving for 15 min.

5.2.3 **Peptone water:** Prepare a 10% solution of peptone in Type II water. Dilute a measured volume to provide a final 0.1% solution. Final pH should be 6.8.

5.2.4 Dispense in amounts to provide 99 $\pm$ 2.0 mL or 9 $\pm$ 0.2 mL after autoclaving for 15 min.

5.2.5 Do not suspend bacteria in any dilution water for more than 30 min at room temperature because death or multiplication may occur, depending on the species.

5.3 Lauryl tryptose broth:

5.3.1 Components of the broth are:

Tryptose	20.0 g
Lactose	5.0 g
Diphosphate 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
Type II water	1 liter

Lauryl tryptose broth is also available in a prepackaged dry powder form.

5.3.2 Make lauryl tryptose broth of such strength that adding 100-mL or 10-mL portions of sample to medium will not reduce ingredient concentrations below those of the standard medium. Prepare in accordance with Table 1.

TABLE 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/liter)
1	10 or more	11 or more	35.6
10	10	20	71.2
10	20	30	53.4
100	50	150	106.8
100	35	135	137.1
100	20	120	213.6

5.3.3 Dispense the broth into fermentation tubes which contain inverted vials. Add an amount sufficient to cover the inverted vial, at least partially, after sterilization has taken place. Sterilize at 121°C for 12 to 15 min. The pH should be 6.8 $\pm$ 0.2 after sterilization.

5.4 Brilliant green lactose bile broth:

5.4.1 Components of the broth are:

Peptone	10.0 g
Lactose	10.0 g
Oxgall	20.0 g
Brillian	0.0133 g
Type II water	1 liter

This broth is also available in a prepackaged dry powder form.

5.4.2 Dispense the broth into fermentation tubes which contain inverted vials. Add an amount sufficient to cover the inverted vial, at least partially, after sterilization has taken place. Sterilize at 121°C for 12 to 15 min. The pH should be 7.2 ± 0.2 after sterilization.

5.5 Ammonium oxalate-crystal violet (Hucker's): Dissolve 2 g crystal violet (90% dye content) in 20 mL 95% ethyl alcohol, dissolve 0.8 g $(NH_4)_2C_2O_4 \cdot H_2O$ in 80 mL Type II water, mix the two solutions, and age for 24 hr before use; filter through paper into a staining bottle.

5.6 Lugol's solution, Gram's modification: Grind 1 g iodine crystals and 2 g KI in a mortar. Add Type II 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).

5.7 Counterstain: Dissolve 2.5 g safranin dye in 100 mL 95% ethyl alcohol. Add 10 mL to 100 mL Type II water.

5.8 Acetone alcohol: Mix equal volumes of ethyl alcohol, 95%, with acetone.

5.9 Gram staining kits: Commercially available kits may be substituted for 5.5, 5.6, 5.7, and 5.8.

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1 All samples must have been collected using a sampling plan that addresses the considerations discussed in U.S. EPA, 1978.

6.2 Clean all glassware thoroughly with a suitable detergent and hot water, rinse with hot water to remove all traces of residual washing compound, and finally rinse with Type II water. If mechanical glassware washers are used, equip them with influent plumbing of stainless steel or other nontoxic material. Do not use copper piping to distribute Type II water. Use stainless steel or other nontoxic material for the rinse-water system.

6.2.1 Sterilize glassware, except when in metal containers, for not less than 60 min at a temperature of 170°C, unless it is known from recording thermometers that oven temperatures are uniform, under which exceptional condition use 160°C. Heat glassware in metal containers to 170°C for not less than 2 hr.

6.2.2 Sterilize sample bottles not made of plastic as above, or in an autoclave at 121°C for 15 min. Perform a sterility check on one bottle per batch.

6.2.3 If water containing residual chlorine and other halogens is to be collected, add sufficient $Na_2S_2O_3$ to clean sample bottle before sterilization to give a concentration of about 100 mg/L in the sample. To a 120-mL bottle add 0.1 mL 10% solution of $Na_2S_2O_3$ (this will neutralize a sample containing about 15 mg/L residual chlorine). Stopper bottle, cap, and sterilize by either dry or moist heat, as directed previously.

6.2.4 Collect water samples high in copper or zinc and wastewater samples high in heavy metals in sample bottles containing a chelating agent that will reduce metal toxicity. This is particularly significant when such samples are in transit for 4 hr or more. Use 372 mg/L of the tetrasodium salt of ethylenediaminetetraacetic acid (EDTA). Adjust EDTA solution to pH 6.5 before use. Add EDTA separately to sample bottle before bottle sterilization (0.3 mL 15% solution in a 120-mL bottle) or combine it with the $Na_2S_2O_3$ solution before addition.

6.3 When the sample is collected, leave ample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, preparatory to examination. Be careful to take samples that will be representative of the water being tested and avoid sample contamination at time of collection or in period before examination.

6.4 Keep sampling bottle closed until the moment it is to be filled. Remove stopper and hood or cap as a unit, taking care to avoid soiling. During sampling, do not handle stopper or cap and neck of bottle and protect them from contamination. Hold bottle near base, fill it without rinsing, replace stopper or cap immediately, and secure hood around neck of bottle.

7.0 PROCEDURE

7.1 Presumptive stage:

7.1.1 Inoculate a series of fermentation tubes ("primary" fermentation tubes) with appropriate graduated quantities (multiples and submultiples of 1 mL) of sample. Be sure that the concentration of nutritive ingredients in the mixture of medium and added sample conforms to the requirements given in Paragraph 5.3. Use a sterile pipet for initial and subsequent transfers from each sample container. If the pipet becomes contaminated before transfers are completed, replace with a sterile pipet. Use a separate sterile pipet for transfers from each different dilution. Do not prepare dilutions in direct sunlight. Use caution when removing sterile pipets from the container; to avoid contamination, do not drag pipet tip across exposed ends of pipets or across lips and necks of dilution bottles. When removing sample, do not insert pipets more than 2.5 cm below the surface of sample or dilution. When discharging sample portions, hold pipet at an angle of about 45°, with tip touching the inside neck of the tube. The portions of sample used for inoculating lauryl-tryptose-broth fermentation tubes will vary in size and number with the character of the water under examination, but

in general use decimal multiples and submultiples of 1 mL. Use Figure 1 as a guide to preparing dilutions. After adding sample, mix thoroughly by shaking the test tube rack. Do not invert the tubes.

7.1.2 Incubate inoculated fermentation tubes at 35 ± 0.5°C. After 24 ± 2 hr shake each tube gently and examine it and, if no gas has formed and been trapped in the inverted vial, reincubate and reexamine at the end of 48 ± 3 hr. Record presence or absence of gas formation, regardless of amount, at each examination of the tubes.

7.1.3 Formation of gas in any amount in the inner fermentation tubes or vials within 48 ± 3 hr constitutes a positive presumptive test. Do not confuse the appearance of an air bubble in a clear tube with actual gas production. If gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be shown by the continued appearance of small bubbles of gas throughout the medium outside the inner vial when the fermentation tube is shaken gently.

7.1.4 The absence of gas formation at the end of 48 ± 3 hr of incubation constitutes a negative test. An arbitrary limit of 48 hr for observation doubtless excludes from consideration occasional members of the coliform group that form gas very slowly and generally are of limited sanitary significance.

7.2 Confirmed stage:

7.2.1 Submit all primary fermentation tubes showing any amount of gas within 24 hr of incubation to the Confirmed Test. If active fermentation appears in the primary fermentation tube earlier than 24 hr, transfer to the confirmatory medium without waiting for the full 24-hr period to elapse. If additional primary fermentation tubes show gas production at the end of 48-hr incubation, submit these to the Confirmed Test.

7.2.2 Gently shake or rotate primary fermentation tube showing gas and do one of two things: (a) with a sterile metal loop, 3 mm in diameter, transfer one loopful of culture to a fermentation tube containing brilliant green lactose bile broth, or (b) insert a sterile wooden applicator at least 2.5 cm long into the culture, remove it promptly, and plunge it to the bottom of fermentation tube containing brilliant green lactose bile broth. Remove and discard applicator.

7.2.3 Incubate the inoculated brilliant green lactose bile broth tube for 48 ± 3 hr at 35 ± 0.5°C. Formation of gas in any amount in the inverted vial of the brilliant green lactose bile broth fermentation tube at any time within 48 ± 3 hr constitutes a positive Confirmed Test.

7.3 Completed test:

7.3.1 Use the Completed Test on positive confirmed tubes to establish definitely the presence of coliform bacteria and provide quality control data for 20% of all samples analyzed.

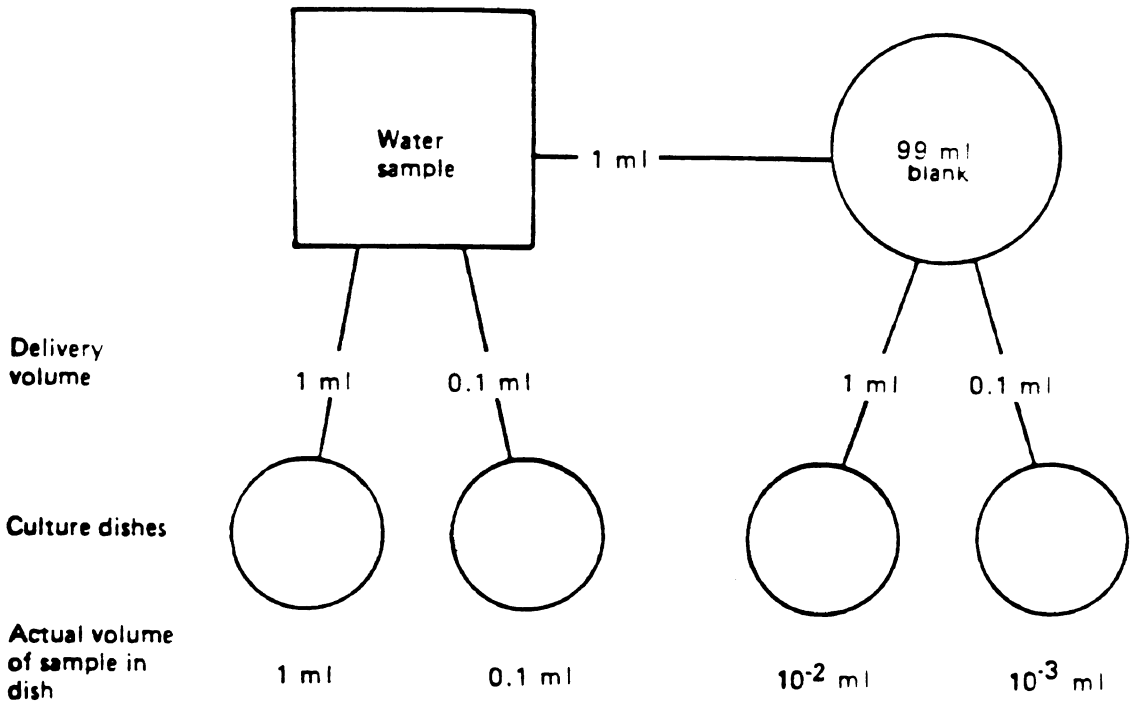

Figure 1. Preparation of dilutions.

7.3.2 Streak one or more eosin methylene blue plates from each tube of brilliant green lactose bile broth showing gas as soon as possible after the appearance of gas. Streak plates to ensure 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 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 needle into the liquid in the tube to a depth of approximately 5.0 mm; and (d) streak plate with curved section of the needle in contact with the agar to avoid a scratched or torn surface.

7.3.3 Incubate plates (inverted) at 35 ± 0.5°C for 24 ± 2 hr.

7.3.4 The colonies developing on eosin methylene blue agar are called: typical (nucleated, with or without metallic sheen); atypical (opaque, unnucleated, mucoid, pink after 24-hr incubation); or negative (all others). From each of these plates, 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 lauryl-tryptose-broth fermentation tube and to a nutrient agar slant.

NOTE: If possible, when transferring colonies, choose well-isolated colonies 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.

7.3.5 Incubate secondary broth tubes at 35 ± 0.5°C for 24 ± 2 hr; if gas is not produced within 24 ± 2 hr, reincubate and examine again at 48 ± 3 hr. Microscopically examine gram-stained preparations (see Paragraph 7.4) from those 24-hr agar slant cultures corresponding to the secondary tubes that show gas.

7.3.6 Formation of gas in the secondary tube of lauryl tryptose broth within 48 ± 3 hr and demonstration of gram-negative, non-spore-forming, rod-shaped bacteria in the agar culture constitute a satisfactory Completed Test, demonstrating the presence of a member of the coliform group.

7.4 Gram-stain procedure:

7.4.1 Prepare a light emulsion of the bacterial growth from an agar slant in a drop of Type II water on a glass slide. Air-dry or fix by passing the slide through a flame and stain for 1 min with the ammonium oxalate-crystal violet solution. Rinse the slide in tap water; apply Lugol's solution for 1 min. (See Paragraphs 5.5-5.8 for reagent.)

7.4.2 Rinse the stained slide in tap water. Decolorize for approximately 15 to 30 sec with acetone alcohol by holding slide between the fingers and letting acetone alcohol flow across the stained smear until no more stain is removed. Do not over-decolorize. Counterstain with safranin (Paragraph 5.7) for 15 sec, then rinse with tap water, blot dry with bibulous paper, and examine microscopically.

7.4.3 Cells that decolorize and accept the safranin stain are pink and defined as gram-negative in reaction. Cells that do not decolorize but retain the crystal violet stain are deep blue and are defined as gram-positive.

7.5 Computing and recording of MPN:

7.5.1 The calculated density of coliform bacteria in a sample can be obtained from the MPN table, based on the number of positive tubes in each dilution of the confirmed or completed test. Table 2 shows MPN indices and 95% confidence limits for potable water testing, and Table 3 describes the MPN indices and 95% confidence limits for general use.

TABLE 2. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 10-mL PORTIONS ARE USED

Number of Tubes Giving Positive Reaction out of 5 of 10 mL each	MPN Index per 100 mL	95% Confidence Limits	
		Lower	Upper
0	<2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16	3.3	52.9
5	>16	8.0	Infinite

7.5.2 Three dilutions are necessary for the determination of the MPN index. For example (see Table 3), if five 10-mL, five 1.0-mL, and five 0.1-mL portions of the samples are used as inocula and four of the 10-mL, two of the 1-mL, and none of the 0.1-mL portions of inocula give positive results, the coded result is 4-2-0 and the MPN index is 22 per 100 mL.

7.5.3 In cases when the serial decimal dilution is other than 10, 1, and 0.1 mL, or when more than three sample volumes are used in the series, refer to the sources cited in Section 10.0, References, for the necessary density determination procedures.

7.5.4 All MPN values for water samples should be reported on the basis of a 100-mL sample.

8.0 QUALITY CONTROL

8.1 Extensive quality control procedures are provided in Part IV of U.S. EPA, 1978 (see Section 10.0, References). These procedures should be adhered to at all times.

TABLE 3. MPN INDEX FOR SERIAL DILUTIONS OF SAMPLE

Number of Tubes Giving Positive Reaction out of			MPN Index per 100 mL	95% Confidence Limits	
5 of 10 mL each	5 of 1 mL each	5 of 0.1 mL each		Lower	Upper
0	0	0	<2		
0	0	1	2	<0.5	7
0	1	0	2	<0.5	7
0	2	0	4	<0.5	11
1	0	0	2	<0.5	7
1	0	1	4	<0.5	11
1	1	0	4	<0.5	11
1	1	1	6	<0.5	15
1	2	0	6	<0.5	15
2	0	0	5	<0.5	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	0	1	17	5	46
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78

Source: U.S. EPA, 1978.

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TABLE 3. MPN INDEX FOR SERIAL DILUTIONS OF SAMPLE
(Continued)

Number of Tubes Giving Positive Reaction out of			MPN Index per 100 mL	95% Confidence Limits	
5 of 10 mL each	5 of 1 mL each	5 of 0.1 mL each		Lower	Upper
4	2	0	22	7	67
4	2	1	26	9	78
4	3	0	27	9	80
4	3	1	33	11	93
4	4	0	34	12	93
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	110
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	150
5	2	0	49	17	130
5	2	1	70	23	170
5	2	2	94	28	220
5	3	0	79	25	190
5	3	1	110	31	250
5	3	2	140	37	340
5	3	3	180	44	500
5	4	0	130	35	300
5	4	1	170	43	490
5	4	2	220	57	700
5	4	3	280	90	850
5	4	4	350	120	1000
5	5	0	240	68	750
5	5	1	350	12	1000
5	5	2	540	180	1400
5	5	3	920	300	3200
5	5	4	1600	640	5800
5	5	5	$\geq$2400		

Source: U.S. EPA, 1978.

8.2 Samples must be maintained as closely as possible to original condition by careful handling and storage. Sample sites and sampling frequency should provide data representative of characteristics and variability of the water quality at that site. Samples should be analyzed immediately. They should be refrigerated at a temperature of 1-4°C and analyzed within 6 hr.

8.3 Quality control of culture media is critical to the validity of microbiological analysis. Some important factors to consider are summarized below:

8.3.1 Order media to last for only 1 yr; always use oldest stock first. Maintain an inventory of all media ordered, including a visual inspection record.

8.3.2 Hold unopened media for no longer than 2 yr. Opened media containers should be discarded after 6 mo.

8.3.3 When preparing media keep containers open as briefly as possible. Prepare media in deionized or distilled (Type II) water of proven quality. Check the pH of the media after solution and sterilization; it should be within 0.2 units of the stated value. Discard and remake if it is not.

8.3.4 Autoclave media for the minimal time specified by the manufacturer because the potential for damage increases with increased exposure to heat. Remove sterile media from the autoclave as soon as pressure is zero. Effectiveness of the sterilization should be checked weekly, using strips or ampuls of Bacillus stearothemophelus.

8.3.5 Agar plates should be kept slightly open for 15 min after pouring or removal from refrigeration to evaporate free moisture. Plates must be free of lumps, uneven surfaces, pock marks, or bubbles, which can prevent good contact between the agar and medium.

8.3.6 Avoid shaking fermentation tubes, which can entrap air in the inner vial and produce a false positive result.

8.3.7 Store fermentation tube media in the dark at room temperature or 4°C. If refrigerated, incubate overnight at room temperature to detect false positive gas bubbles.

8.3.8 Quality control checks of prepared media should include the incubation of 5% of each batch of medium for 2 days at 35°C to inspect for growth and positive/negative checks with pure culture.

8.4 Analytical quality control procedures should include:

8.4.1 Duplicate analytical runs on at least 10% of all known positive samples analyzed.

8.4.2 At least one positive control sample should be run each month for each parameter tested.

8.4.3 At least one negative (sterile) control should be run with each series of samples using buffered water and the medium batch used at the beginning of the test series and following every tenth sample. When sterile controls indicate contamination, new samples should be obtained and analyzed.

8.4.4 The Type II water used should be periodically checked for contamination.

8.4.5 For routine MPN tests, at least 5% of the positive confirmed samples should be tested by the complete test.

9.0 METHOD PERFORMANCE

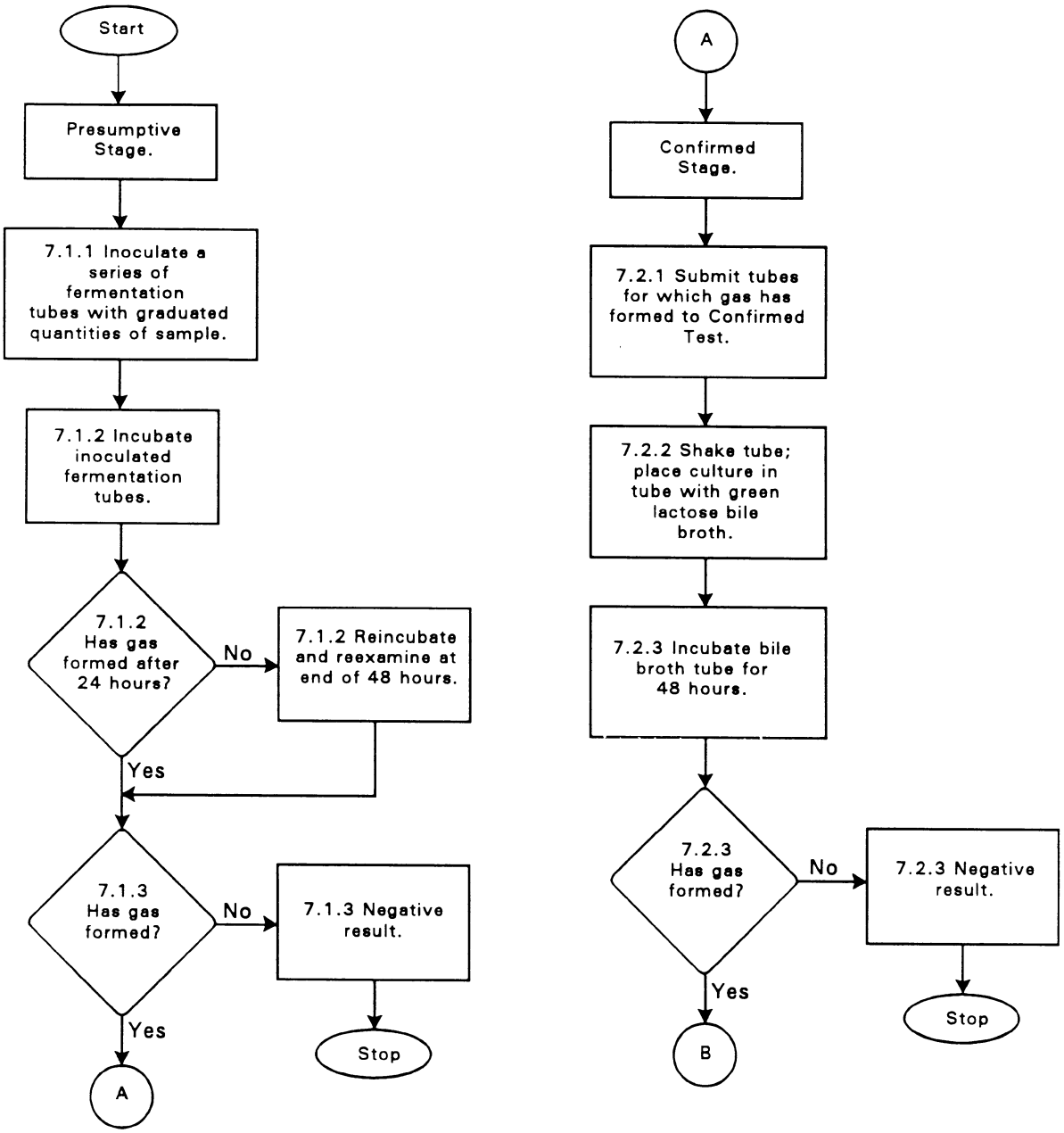
9.1 No data provided.

10.0 REFERENCES

1. Standard Methods for the Examination of Water and Wastewater, 15th ed. (1980).

2. U.S. Environmental Protection Agency, Microbiological Methods for Monitoring the Environment, EPA 600/8-78-017, December 1978.

METHOD 9131
TOTAL COLIFORM, MULTIPLE TUBE FERMENTATION TECHNIQUE



METHOD 9131
TOTAL COLIFORM, MULTIPLE TUBE FERMENTATION TECHNIQUE
(Continued)