9222 MEMBRANE FILTER TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9222 A. Introduction

The membrane filter (MF) technique is highly reproducible, can be used to test relatively large sample volumes, and yields numerical results more rapidly than the multiple-tube procedure. The MF technique is extremely useful in monitoring drinking water and a variety of natural waters. However, the MF technique has limitations, particularly when testing waters with high turbidity or noncoliform (background) bacteria. For waters with high levels of noncoliform bacteria, the chromogenic substrate coliform method is recommended although densities above 10^4 can interfere. When the MF technique has not been used previously, it is desirable to conduct parallel tests with the multiple-tube fermentation technique (Section 9221) to demonstrate applicability and comparability.

1. Definition

As related to the MF technique, the coliform group is defined as comprising many facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that develop red colonies with a metallic (golden) sheen within 24 h at 35°C on an Endo-type medium containing lactose. Some members of the total coliform group may produce dark red or nucleated colonies without a metallic sheen. When verified these are classified as atypical coliform colonies. When purified cultures of coliform bacteria are tested they produce negative cytochrome oxidase and positive β-galactosidase test reactions.† Generally, all red, pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms by this technique.

2. Applications

Turbidity caused by the presence of algae, particulates, or other interfering material may not permit testing of a sample volume sufficient to yield significant results. Low coliform estimates may be caused by the presence of high numbers of noncoliforms or of toxic substances. The MF technique is applicable to the examination of saline waters, but not wastewaters that have received only primary treatment followed by chlorination because of turbidity in high volume samples or wastewaters containing toxic metals or toxic organic compounds such as phenols. For the detection of stressed total coliforms in treated drinking water and chlorinated secondary or tertiary wastewater effluents use a method designed for stressed organism recovery (see Section 9212B.1). A modified MF technique for fecal coliforms (Section 9212) in chlorinated wastewater may be used if parallel testing over a 3-month period with the multiple-tube fermentation technique shows comparability for each site-specific type of sample.

The standard volume to be filtered for drinking water samples is 100 mL. This may be distributed among multiple membranes if necessary. Because treated drinking water may contain low densities of total coliforms that may not be detectable in 100-mL sample portions, water plant laboratories should consider testing 1-L samples of finished water, provided that particulates are not present to interfere with filtration or development of discrete colonies. The purpose of using large samples is to discover small leakages of coliforms through treatment barriers. If particulates prevent filtering a 1-L sample, divide the sample into four portions of 250 mL for analysis and total any coliforms on each membrane into a single report on 1 L examined. Smaller samples may be necessary for source or recreational waters and wastewater effluents.

Statistical comparisons of results obtained by the multiple-tube method and the MF technique show that the MF is more precise (compare Tables 9221:I, II, and III with Table 9222:II). Data from each test yield approximately the same water quality information, although numerical results are not identical (see Section 9010B for drinking water). For raw water sources, 80% of the MF test results would be expected to fall within the 95% confidence limits of the multiple-tube completed test results. Results from the multiple-tube test would be expected to be higher than MF results because of a built-in positive statistical bias of approximately 23%.

3. Bibliography

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* Approved by Standard Methods Committee, 1994.

† ONPG is a substrate for the β-galactosidase test.

9222 B. Standard Total Coliform Membrane Filter Procedure

1. Laboratory Apparatus

For MF analyses use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

Sterilize glassware as described in Washing and Sterilization, Section 9040.

a. Sample bottles: See Section 9030B.18.

b. Dilution bottles: See Section 9030B.13.

c. Pipets and graduated cylinders: See Section 9030B.9. Before sterilization, loosely cover opening of graduated cylinders with metal foil or a suitable heavy wrapping-paper substitute. Immediately after sterilization secure cover to prevent contamination.

d. Containers for culture medium: Use clean borosilicate glass flasks presterilized to reduce bacterial contamination. Any size or shape of flask may be used, but erlenmeyer flasks with metal caps, metal foil covers, or screw caps provide for adequate mixing of the medium contained and are convenient for storage.

e. Culture dishes: Use sterile borosilicate glass or disposable, presterilized plastic petri dishes, 60 × 15 mm, 50 × 9 mm, or other appropriate size. Wrap convenient numbers of clean, glass culture dishes in metal foil if sterilized by dry heat, or suitable heavy wrapping paper when autoclaved. Incubation of loose-lidded glass and disposable plastic culture dishes in tightly closed containers with wet paper or cloth may be necessary to prevent moisture evaporation with resultant drying of medium and to maintain a humid environment for optimum colony development.

Disposable plastic dishes that are tight-fitting and meet the specifications noted above are available commercially and are used widely. Reseal opened packages of disposable dish supplies for storage.

f. Filtration units: The filter-holding assembly (constructed of glass, autoclavable plastic, porcelain, or stainless steel) consists of a seamless funnel fastened to a base by a locking device or held in place by magnetic force. The design should permit the membrane filter to be held securely on the porous plate of the receptacle without mechanical damage and allow all fluid to pass through the membrane during filtration. Discard plastic funnels with deep scratches on inner surface or glass funnels with chipped surfaces.

Separately wrap the two parts of the assembly in heavy wrapping paper, sterilize by autoclaving, and store until use. Alternatively treat previously cleaned unwrapped parts by ultraviolet radiation (2 min exposure) for the initial sanitization before use in the test procedure, or before reusing units during a filtration series. Field units may be sanitized by igniting alcohol or immersing in boiling water for 2 min. After submerging unit in boiling water, cool it to room temperature before reuse. Do not ignite plastic parts. Sterile, disposable field units may be used.

For filtration, mount receptacle of filter-holding assembly in a 1-L filtering flask with a side tube or other suitable device (manifold to hold three to six filter assemblies) such that a pressure differential (34 to 51 kPa) can be exerted on the filter membrane. Connect flask to an electric vacuum pump, a filter pump operating on water pressure, a hand aspirator, or other means of securing a pressure differential (138 to 207 kPa). Connect a flask of approximately the same capacity between filtering flask and vacuum source to trap carry-over water.

g. Membrane filter: Use membrane filters (for additional specifications, see Section 9020) with a rated pore diameter such that there is complete retention of coliform bacteria. Use only those filter membranes that have been found, through adequate quality control testing and *certification by the manufacturer*, to exhibit: full retention of the organisms to be cultivated, stability in use, freedom from chemical extractables that may inhibit bacterial growth and development, a satisfactory speed of filtration (within 5 min), no significant influence on medium pH (beyond ± 0.2 units), and no increase in number of confluent colonies or spreaders compared to control membrane filters. Use membranes grid-marked in such a manner that bacterial growth is neither inhibited nor stimulated along the grid lines when the membranes with entrapped bacteria are incubated on a suitable medium. Preferably use fresh stocks of membrane filters and if necessary store them in an environment without extremes of temperature and humidity. Obtain no more than a year's supply at any one time.

Preferably use presterilized membrane filters for which the manufacturer has certified that the sterilization technique has neither induced toxicity nor altered the chemical or physical properties of the membrane. If membranes are sterilized in the laboratory, autoclave for 10 min at 121°C. At the end of the sterilization period, let the steam escape rapidly to minimize accumulation of water of condensation on filters.

h. Absorbent pads consist of disks of filter paper or other material certified for each lot by the manufacturer to be of high quality and free of sulfites or other substances of a concentration that could inhibit bacterial growth. Use pads approximately 48 mm in diameter and of sufficient thickness to absorb 1.8 to 2.2 mL of medium. Presterilized absorbent pads or pads subsequently sterilized in the laboratory should release less than 1 mg total acidity (calculated as $CaCO_3$) when titrated to the phenolphthalein end point, pH 8.3, using 0.02*N* NaOH and produce pH levels of 7 ± 0.2. Sterilize pads simultaneously with membrane filters available in resealable kraft envelopes, or separately in other suitable containers. Dry pads so they are free of visible moisture before use. See sterilization procedure described for membrane filters above and Section 9020 for additional specifications on absorbent pads.

i. Forceps: Smooth-tipped, without corrugations on the inner sides of the tips. Sterilize before use by dipping in 95% ethyl or absolute methyl alcohol and flaming.

j. Incubators: Use incubators to provide a temperature of 35 ± 0.5°C and to maintain a humid environment (60% relative humidity).

k. Microscope and light source: To determine colony counts on membrane filters, use a magnification of 10 to 15 diameters and a cool white fluorescent light source adjusted to give maximum sheen discernment. Optimally use a binocular wide-field dissecting microscope. Do not use a microscope illuminator with optical system for light concentration from an incandescent light source for discerning coliform colonies on Endo-type media.

2. Materials and Culture Media

The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions

for rehydration. Store opened supplies of dehydrated media in a desiccator. Commercially prepared media in liquid form (sterile ampule or other) also may be used if known to give equivalent results. See Section 9020 for media quality control specifications.

Test each new medium lot against a previously acceptable lot for satisfactory performance as described in Section 9020B. With each new lot of Endo-type medium, verify a minimum 10% of coliform colonies, obtained from natural samples, to establish the differential accuracy of the medium lot.

*a. LES Endo agar:**

Yeast extract	1.2 g
Casitone or trypticase	3.7 g
Thiopeptone or thiotone	3.7 g
Tryptose	7.5 g
Lactose	9.4 g
Dipotassium hydrogen phosphate, K_2HPO_4	3.3 g
Potassium dihydrogen phosphate, KH_2PO_4	1.0 g
Sodium chloride, NaCl	3.7 g
Sodium desoxycholate	0.1 g
Sodium lauryl sulfate	0.05 g
Sodium sulfite, Na_2SO_3	1.6 g
Basic fuchsin	0.8 g
Agar	15.0 g
Reagent-grade water	1 L

Rehydrate in 1 L water containing 20 mL 95% ethanol. Do not use denatured ethanol, which reduces background growth and coliform colony size. Bring to a near boil to dissolve agar, then promptly remove from heat and cool to 45 to 50°C. Do not sterilize by autoclaving. Final pH 7.2 ± 0.2. Dispense in 5- to 7-mL quantities into lower section of 60-mm glass or plastic petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth. Do not expose plates to direct sunlight; store in the dark at 4 to 8°C, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused medium after 3 weeks or sooner if there is evidence of moisture loss or medium contamination (darkening of the medium).

b. M-Endo medium:†

Tryptose or polypeptone	10.0 g
Thiopeptone or thiotone	5.0 g
Casitone or trypticase	5.0 g
Yeast extract	1.5 g
Lactose	12.5 g
Sodium chloride, NaCl	5.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	4.375 g
Potassium dihydrogen phosphate, KH_2PO_4	1.375 g
Sodium lauryl sulfate	0.05 g
Sodium desoxycholate	0.10 g
Sodium sulfite, Na_2SO_3	2.10 g
Basic fuchsin	1.05 g
Agar (optional)	15.0 g
Reagent-grade water	1 L

1) Agar preparation—Rehydrate in 1 L water containing 20 mL 95% ethanol. Heat to near boiling to dissolve agar, then promptly remove from heat and cool to between 45 and 50°C. Dispense 5- to 7-mL quantities into 60-mm sterile glass or plastic petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth. Do not sterilize by autoclaving. Final pH should be 7.2 ± 0.2. A precipitate is normal in Endo-type media.

Store finished medium in the dark at 4 to 8°C and discard unused agar after 2 weeks.

2) Broth preparation—Prepare as above, omitting agar. Dispense liquid medium (1.8 to 2.2 mL per plate) onto absorbent pads (see absorbent pad specifications, Section 9222B.1). The broth may have a precipitate but this does not interfere with medium performance if pads are certified free of sulfite or other toxic agents at a concentration that could inhibit bacterial growth. Broth may be stored at 4°C for up to 4 d.

c. Buffered dilution rinse water: See Section 9050C.1.

3. Samples

Collect samples as directed in Sections 9060A and B.

4. Coliform Definition

All bacteria that produce a red colony with a metallic (golden) sheen within 24 h incubation at 35°C on an Endo-type medium are considered members of the coliform group. The sheen may cover the entire colony or may appear only in a central area or on the periphery. The coliform group thus defined is based on the production of aldehydes from fermentation of lactose. While this biochemical characteristic is part of the metabolic pathway of gas production in the multiple-tube test, some variations in degree of metallic sheen development may be observed among coliform strains. However, this slight difference in indicator definition is not considered critical to change its public health significance, particularly if suitable studies have been conducted to establish the relationship between results obtained by the MF and those obtained by the standard multiple-tube fermentation procedure.

Verify typical and atypical colonies by lactose fermentation or reaction to CO and ONPG to avoid false-positive and false-negative results.

Preferably verify all typical and atypical colonies but when there are more than 50 typical/atypical colonies present from a wastewater sample, verify at least 10%.

A rapid (4 h) verification using CO and ONPG is desirable to determine followup procedures by a water supply utility. For coliform counts of more than 5/100 mL from drinking water, verify all colonies or at least 10, if there are 10 or more, included in the direct count. See Section 9222B.5*f* for specific verification procedure. The continued presence of confluent growth or TNTC non-sheen colonies may be an early signal of water quality problems; do not disregard.

5. Procedures

Generally, an enrichment procedure may improve the assessment of drinking water quality. However, this step may be eliminated in the routine examination of drinking water where repeated determinations have shown that adequate results are obtained by a single-step MF technique. Enrichment usually is not necessary in the examination of nonpotable water or wastewater.

* Dehydrated Difco M-Endo Agar LES (No. 0736), dehydrated BBL M-Endo Agar LES (No. 11203), or equivalent.

† Dehydrated Difco M-Endo Broth MF (No. 0749), dehydrated BBL *m*-Coliform Broth (No. 11119), or equivalent may be used if absorbent pads are used.

a. Selection of sample size: Size of sample will be governed by expected bacterial density. In drinking water analyses, sample size will be limited only by the degree of turbidity or by the noncoliform growth on the medium (Table 9222:I). For regulation purposes, 100 mL is the official sample size.

An ideal sample volume will yield 20 to 80 coliform colonies and not more than 200 colonies of all types on a membrane-filter surface. Analyze drinking waters by filtering 100 to 1000 mL, or by filtering replicate smaller sample volumes such as duplicate 50-mL or four replicates of 25-mL portions. Analyze other waters by filtering three different volumes (diluted or undiluted), depending on the expected bacterial density. See Section 9215B.2 for preparation of dilutions. When less than 10 mL of sample (diluted or undiluted) is to be filtered, add approximately 10 mL sterile dilution water to the funnel before filtration or pipet the sample volume into a sterile dilution flask, then filter the entire dilution. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

b. Sterile filtration units: Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all membrane filter holders in use. Decontaminate this equipment between successive filtrations by using an ultraviolet (UV) sterilizer for 2 min, flowing steam, or boiling water for 2 min. Do not expose membrane-filter culture preparations to random UV radiation leaks that might emanate from the sterilization cabinet. Eye protection is recommended; either safety glasses or prescription-ground glasses afford adequate eye protection against stray radiation from a UV sterilization cabinet that is not light-tight during the exposure interval. Clean UV tube regularly and check it periodically for effectiveness to insure that it will produce a 99.9% bacterial kill in a 2-min exposure. See also Section 9020.

c. Filtration of sample: Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of receptacle. Carefully place matched funnel unit over receptacle and lock it in place. Filter sample under partial vacuum. With filter still in place, rinse the interior surface of the funnel by filtering three 20- to 30-mL portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle. This is satisfactory only if the squeeze bottle and its contents do not become contaminated during use. Rinsing between samples prevents carryover contamination. Upon completion of final rinse and the filtration process disengage vacuum, unlock and remove funnel, immediately remove membrane filter with sterile forceps, and place it on selected medium with a rolling motion to avoid entrapment of air. Insert a sterile rinse water sample (100 mL) after filtration of a series of 10 samples to check for possible cross-contamination or contaminated rinse water. Incubate the rinse water control membrane culture under the same conditions as the sample.

d. Enrichment technique: Place a sterile absorbent pad in the lid of a sterile culture dish and pipet 1.8 to 2.2 mL lauryl tryptose broth, prepared as directed in 9221B.1.*a*1), to saturate pad. Carefully remove any excess liquid from absorbent pad. Aseptically place filter through which the sample has been passed on pad. Incubate filter, without inverting dish, for 1.5 to 2 h at 35 ± 0.5°C in an atmosphere of at least 60% relative humidity.

If the agar-based Endo-type medium is used, remove enrichment culture from incubator, lift filter from enrichment pad, and roll it onto the agar surface. Incorrect filter placement is at once obvious, because patches of unstained membrane indicate entrapment of air. Where such patches occur, carefully reseat filter on agar surface. If the liquid medium is used, prepare final culture by removing enrichment culture from incubator and separating the dish halves. Place a fresh sterile pad in bottom half of dish and saturate it with 1.8 to 2.0 mL of M-Endo medium. Transfer filter, with same precautions as above, to new pad. Discard used enrichment pad.

With either the agar or the liquid medium, invert dish and incubate for 20 to 22 h at 35 ± 0.5°C. Proceed to ¶ *f* below.

e. Alternative single-step direct technique: If the agar-based medium is used, place prepared filter directly on agar as described in preceding section, invert dish, and incubate for 22 to 24 h at 35 ± 0.5°C.

If liquid medium is used, place a pad in the culture dish and saturate with 1.8 to 2.0 mL M-Endo medium. Place prepared filter directly on pad, invert dish, and incubate for 22 to 24 h at 35 ± 0.5°C.

Differentiation of some colonies from either agar or liquid medium substrates may be lost if cultures are incubated beyond 24 h.

f. Counting: To determine colony counts on membrane filters, use a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device, with a cool white fluorescent light source directed to provide optimal viewing of sheen. The typical coliform colony has a pink to dark-red color

TABLE 9222:I. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER TOTAL COLIFORM TEST

	Volume (X) To Be Filtered *mL*							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Swimming pools	X							
Wells, springs	X	X	X					
Lakes, reservoirs	X	X	X					
Water supply intake			X	X	X			
Bathing beaches			X	X	X			
River water				X	X	X	X	
Chlorinated sewage				X	X	X		
Raw sewage					X	X	X	X

with a metallic surface sheen. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red or nucleated without sheen. Colonies that lack sheen may be pink, red, white, or colorless and are considered to be noncoliforms. The total count of colonies (coliform and noncoliform) on Endo-type medium has no consistent relationship to the total number of bacteria present in the original sample. A high count of noncoliform colonies may interfere with the maximum development of coliforms. Refrigerating cultures (after 22 h incubation) with high densities of noncoliform colonies for 0.5 to 1 h before counting may deter spread of confluence while aiding sheen discernment.

Samples of disinfected water or wastewater effluent may include stressed organisms that grow relatively slowly and produce maximum sheen in 22 to 24 h. Organisms from undisinfected sources may produce sheen at 16 to 18 h, and the sheen subsequently may fade after 24 to 30 h.

g. Coliform verification: Typical sheen colonies may be produced occasionally by noncoliform organisms. Atypical colonies (dark red or nucleated colonies without sheen) occasionally may be coliforms. Verification of both colony types is advisable. Verify by a test for lactose fermentation or by using alternative procedures involving either a rapid (4 h) test for cytochrome oxidase (CO) and β-galactosidase or a multi-test system for speciation. See Section 9020B.4*d*. For drinking water, verify all suspect colonies or at least 10 typical colonies from a given membrane filter culture.

1) Lactose fermentation—Verify all typical and atypical coliform colonies included in the direct count or a minimum of ten such colonies from drinking water samples by transferring growth from each colony to lauryl tryptose broth; incubate at 35 ± 0.5°C for 48 h. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose broth (Section 9221B.2 for medium preparation) within 48 h verifies the colony as a coliform. Simultaneous inoculation of both media for gas production is acceptable. Inclusion of EC broth inoculation for 44.5°C incubation will provide information on the presence of fecal coliforms. Use of EC-MUG with incubation at 35°C for 24 h will provide information on presence of *E. coli*.

2) Alternative coliform verifications—Apply this alternative coliform verification procedure to isolated colonies on the membrane filter culture. If a mixed culture is suspected or if colony separation is less than 2 mm, streak the growth to M-Endo medium or MacConkey agar to assure culture purity or submit the mixed growth to the fermentation tube method.

a) Rapid test—A rapid verification of colonies utilizes test reactions for cytochrome oxidase (CO) and β-galactosidase. Coliform reactions are CO negative and β-galactosidase positive within 4 h incubation of tube culture or micro (spot) test procedure.

b) Commercial multi-test systems—Verify the colony by streaking it for purification, selecting a well-isolated colony, and inoculating into a multi-test identification system for Enterobacteriaceae that includes lactose fermentation and/or β-galactosidase and CO test reactions.

6. Calculation of Coliform Density

Compute the count, using membrane filters with 20 to 80 coliform colonies and not more than 200 colonies of all types per membrane, by the following equation:

$$\text{(Total) coliform colonies/100 mL} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered}}$$

For verified coliform counts, adjust the initial count based upon the positive verification percentage and report as "verified coliform count per 100 mL."

$$\text{Percentage verified coliforms} = \frac{\text{number of verified colonies}}{\text{total number of coliform colonies subjected to verification}} \times 100$$

a. Water of drinking water quality: While the EPA Total Coliform Rule for public water supply samples requires only a record of coliform presence or absence in 100-mL samples, it may be advisable to determine coliform densities in repeat sampling situations. This is of particular importance when a coliform biofilm problem is suspected in the distribution system. Quantitative information may provide an indication of the magnitude of a contaminating event. This information cannot be determined by presence/absence methods unless they are configured in a multiple-tube, multiple-dilution arrangement.

With water of good quality, the occurrence of coliforms generally will be minimal. Therefore, count all coliform colonies (disregarding the lower limit of 20 cited above) and use the formula given above to obtain coliform density.

If confluent growth occurs, that is, growth covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth with (or without) coliforms" and request a new sample from the same location. If the total number of bacterial colonies, coliforms plus noncoliforms, exceeds 200 per membrane, or if the colonies are not distinct enough for accurate counting, report results as "too numerous to count" (TNTC). The presence of coliforms in such cultures showing no sheen may be indicated by placing the entire membrane filter culture into a sterile tube of brilliant green lactose bile broth. As an alternative, brush the entire filter surface with a sterile loop, applicator stick, or cotton swab and inoculate this growth to the tube of brilliant green lactose bile broth. If gas is produced from this culture within 48 h at 35 ± 0.5°C, conclude that coliforms are present. For compliance with the EPA Total Coliform Rule, report confluent growth or TNTC with at least one detectable coliform colony (which is verified) as a total coliform positive sample. Report confluent growth or TNTC without detectable coliforms as invalid. Request a new sample from the same location within 24 h and select more appropriate volumes to be filtered per membrane, observing the requirement that the standard drinking water portion is 100 mL, or choose another coliform method that is less subject to heterotrophic bacterial interferences. Thus, to reduce interference from overcrowding, instead of filtering 100 mL per membrane, filter 50-mL portions through each of two membranes, 25-mL portions through each of four membranes, etc. Total the coliform counts observed on all membranes and report as number per 100 mL.

b. Water of other than drinking water quality: As with potable water samples, if no filter has a coliform count falling in the ideal range, total the coliform counts on all filters and report as number per 100 mL. For example, if duplicate 50-mL portions were examined and the two membranes had five and three coliform

colonies, respectively, report the count as eight coliform colonies per 100 mL, i.e.,

$$\frac{[(5 + 3) \times 100]}{(50 + 50)}$$

Similarly, if 50-, 25-, and 10-mL portions were examined and the counts were 15, 6, and <1 coliform colonies, respectively, report the count as 25/100 mL, i.e.,

$$\frac{[(15 + 6 + 0) \times 100]}{(50 + 25 + 10)}$$

On the other hand, if 10-, 1.0-, and 0.1-mL portions were examined with counts of 40, 9, and <1 coliform colonies, respectively, select the 10-mL portion only for calculating the coliform density because this filter had a coliform count falling in the ideal range. The result is 400/100 mL, i.e.,

$$\frac{(40 \times 100)}{10}$$

In this last example, if the membrane with 40 coliform colonies also had a total bacterial colony count greater than 200, report the coliform count as ≥400/100 mL.

TABLE 9222:II. 95% CONFIDENCE LIMITS FOR MEMBRANE FILTER COLIFORM RESULTS USING 100-mL SAMPLE

Number of Coliform Colonies Counted	95% Confidence Limits	
	Lower	Upper
0	0.0	3.7
1	0.1	5.6
2	0.2	7.2
3	0.6	8.8
4	1.0	10.2
5	1.6	11.7
6	2.2	13.1
7	2.8	14.4
8	3.4	15.8
9	4.0	17.1
10	4.7	18.4
11	5.4	19.7
12	6.2	21.0
13	6.9	22.3
14	7.7	23.5
15	8.4	24.8
16	9.4	26.0
17	9.9	27.2
18	10.7	28.4
19	11.5	29.6
20	12.2	30.8

Report confluent growth or membranes with colonies too numerous to count as described in *a* above. Request a new sample and select more appropriate volumes for filtration or utilize the multiple-tube fermentation technique.

c. Statistical reliability of membrane filter results: Although the statistical reliability of the MF technique is greater than that of the MPN procedure, membrane counts may underestimate the number of viable coliform bacteria. Table 9222:II illustrates some 95% confidence limits. These values are based on the assumption that bacteria are distributed randomly and follow a Poisson distribution. For results with counts, c, greater than 20 organisms, calculate the approximate 95% confidence limits using the following normal distribution equations:

$$\text{Upper limit} = c + 2\sqrt{c} \qquad \text{Lower limit} = c - 2\sqrt{c}$$

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9222 C. Delayed-Incubation Total Coliform Procedure

Modification of the standard MF technique permits membrane shipment or transport after filtration to a distant laboratory for transfer to another substrate, incubation, and completion of the test. This delayed-incubation test may be used where it is impractical to apply conventional procedures. It also may be used: (*a*) where it is not possible to maintain the desired sample temperature during transport; (*b*) when the elapsed time between sample collection and analysis would exceed the approved time limit; (*c*) where the sampling location is remote from laboratory services; (*d*) when it is necessary to monitor streams for water

quality or pollution control activities by a standardized procedure; or (*e*) for other reasons that prevent analysis of the sample at or near the sample site.

Independent studies using both fresh- and salt-water samples have shown consistent results between the delayed incubation and standard direct test. Determine the applicability of the delayed-incubation test for a specific water source by comparing with results of test procedures using conventional methods.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on the transport medium, and ship to the laboratory. Complete the coliform determination in the laboratory by transferring the membrane to standard M-Endo or LES Endo medium, incubating at 35 ± 0.5°C for 20 to 22 h, and counting typical and atypical coliform colonies that develop. For drinking water samples collected for compliance with the EPA Total Coliform Rule, report the presence or absence of verified coliforms in 100-mL samples. Verify colonies as outlined previously in Section 9222B.5*g*.

Transport media are designed to keep coliform organisms viable and generally do not permit visible growth during transit time. Bacteriostatic agents in holding/preservative media suppress growth of microorganisms en route but allow normal coliform growth after transfer to a fresh medium.

The delayed-incubation test follows the methods outlined for the total coliform MF procedure, except as indicated below. Two alternative methods are given, one using the M-Endo preservative medium and the other the M-ST holding medium.

1. Apparatus

a. Culture dishes: Use disposable, sterile, moisture-tight plastic petri dishes (50 × 12 mm). Such containers are light in weight and are less likely to break in transit. In an emergency or when plastic dishes are unavailable, use sterile glass petri dishes wrapped in plastic film or similar material. See Section 9222B.1*e* for specifications.

b. Field filtration units: See Section 9222B.1*f* for specifications. Disinfect by adding methyl alcohol to the filtering chamber, igniting the alcohol, and covering unit to produce formaldehyde. Ultraviolet light disinfection also may be used in the field if an appropriate power source is available (115 V, 60 Hz). Glass or metal filtration units may be sterilized by immersing in boiling water for 2 min. Use a hand aspirator to obtain necessary vacuum.

2. Materials and Transport Media

a. M-Endo methods:

1) *M-Endo preservative medium:* Prepare as described in Section 9222B.2*b*. After cooling to below 45°C, aseptically add 3.84 g sodium benzoate (USP grade)/L or 3.2 mL 12% sodium benzoate solution to 100 mL medium. Mix ingredients and dispense in 5- to 7-mL quantities to 50- × 9-mm petri plates. Store poured plates at 2 to 10°C. Discard unused medium after 96 h.

2) *Sodium benzoate solution:* Dissolve 12 g $NaC_7H_5O_2$ in sufficient distilled water to make 100 mL. Sterilize by autoclaving or by filtering through a 0.22-µm pore size membrane filter. Discard after 6 months.

3) *Cycloheximide:** Optionally add cycloheximide to M-Endo preservative medium. It may be used for samples that previously have shown overgrowth by fungi, including yeasts. Prepare modification by aseptically adding 50 mg cycloheximide/100 mL M-Endo preservative medium. Store cycloheximide solution at 5 to 10°C and discard after 6 months. Cycloheximide is a powerful skin irritant; handle with caution according to the manufacturer's directions.

b. M-ST method:

M-ST holding medium:

Sodium phosphate, monobasic, $NaH_2PO_4 \cdot H_2O$	0.1 g
Dipotassium hydrogen phosphate, KH_2PO_4	3.0 g
Sulfanilamide	1.5 g
Ethanol (95%)	10 mL
Tris (hydroxymethyl) aminomethane	3.0 g
Reagent-grade water	1 L

Rehydrate in water. Sterilize by autoclaving at 121°C for 15 min. Final pH should be 8.6 ± 0.2. Dispense 1.8 to 2.2 mL to tight-lidded plastic culture dishes containing an absorbent pad. Store in refrigerator for use within 96 h.

3. Procedure

a. Sample preservation and shipment: Place absorbent pad in bottom of sterile petri dish and saturate with selected coliform holding medium (see Section 9222C.2 above). Remove membrane filter from filtration unit with sterile forceps and roll it, grid side up, onto surface of medium-saturated pad. Protect membrane from moisture loss by tightly closing plastic petri dish. Seal loose-fitting dishes with an appropriate sealing tape. Prevent membrane dehydration during transit. Place culture dish containing membrane in an appropriate shipping container and send to the laboratory for test completion. The sample can be held without visible growth for a maximum of 72 h on the holding/preservative medium. This usually allows use of the mail or a common carrier. Visible growth occasionally begins on the transport medium when high temperatures are encountered during transit.

b. Transfer and incubation: At the laboratory, transfer membrane from holding medium in which it was shipped to a second sterile petri dish containing M-Endo or LES Endo medium and incubate at 35 ± 0.5°C for 20 to 22 h.

4. Estimation of Coliform Density

Proceed as in Section 9222B.6 above. Record times of collection, filtration, and laboratory examination, and calculate the elapsed time. Report elapsed time with coliform results.

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* Actidione®, manufactured by the Upjohn Company, Kalamazoo, Mich., or equivalent.

9222 D. Fecal Coliform Membrane Filter Procedure

Fecal coliform bacterial densities may be determined either by the multiple-tube procedure or by the MF technique. See Section 9225 for differentiation of *Escherichia coli*, the predominant fecal coliform. If the MF procedure is used for chlorinated effluents, demonstrate that it gives comparable information to that obtainable by the multiple-tube test before accepting it as an alternative. The fecal coliform MF procedure uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2°C for selectivity and gives 93% accuracy in differentiating between coliforms found in the feces of warm-blooded animals and those from other environmental sources. Because incubation temperature is critical, submerge waterproofed (plastic bag enclosures) MF cultures in a water bath for incubation at the elevated temperature or use an appropriate, accurate solid heat sink incubator. Alternatively, use an equivalent incubator that will hold the 44.5°C temperature within 0.2°C (throughout the chamber) over a 24-h period. Areas of application for the fecal coliform method in general are stated in the introduction to the multiple-tube fecal coliform procedures, Section 9221C.

1. Materials and Culture Medium

a. M-FC medium: The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions for rehydration. Commercially prepared media in liquid form (sterile ampule or other) also may be used if known to give equivalent results. See Section 9020 for quality control specifications.

M-FC medium:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride, NaCl	5.0 g
Lactose	12.5 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar (optional)	15.0 g
Reagent-grade water	1 L

Rehydrate dehydrated medium in 1 L water containing 10 mL 1% rosolic acid in 0.2*N* NaOH.* Heat to near boiling, promptly remove from heat, and cool to below 50°C. Do not sterilize by autoclaving. If agar is used, dispense 5- to 7-mL quantities to 50- × 12-mm petri plates and let solidify. Final pH should be 7.4 ± 0.2. Store finished medium at 4 to 8°C, preferably in sealed plastic bags or other containers to reduce moisture loss, and discard unused broth after 96 h or unused agar after 2 weeks.

Test each medium lot against a previously acceptable lot for satisfactory performance as described in Section 9020B, by making dilutions of a culture of *E. coli* (Section 9020) and filtering appropriate volumes to give 20 to 60 colonies per filter. With each new lot of medium verify 10 or more colonies obtained from several natural samples, to establish the absence of false positives. For most samples M-FC medium may be used without the 1% rosolic acid addition, provided there is no interference with background growth. Such interference may be expected in stormwater samples collected during the first runoff (initial flushing) after a long dry period.

b. Culture dishes: Tight-fitting plastic dishes are preferred because the membrane filter cultures are submerged in a water bath during incubation. Place fecal coliform cultures in plastic bags or seal individual dishes with waterproof (freezer) tape to prevent leakage during submersion. Specifications for plastic culture dishes are given in Section 9222B.1*e*.

c. Incubator: The specificity of the fecal coliform test is related directly to the incubation temperature. Static air incubation may be a problem in some types of incubators because of potential heat layering within the chamber and the slow recovery of temperature each time the incubator is opened during daily operations. To meet the need for greater temperature control use a water bath, a heat-sink incubator, or a properly designed and constructed incubator giving equivalent results. A temperature tolerance of 44.5 ± 0.2°C can be obtained with most types of water baths that also are equipped with a gable top for the reduction of water and heat losses. A circulating water bath is excellent but may not be essential to this test if the maximum permissible variation of 0.2°C in temperature can be maintained with other equipment.

2. Procedure

a. Selection of sample size: Select volume of water sample to be examined in accordance with the information in Table 9222:III. Use sample volumes that will yield counts between 20 and 60 fecal coliform colonies per membrane.

When the bacterial density of the sample is unknown, filter several volumes or dilutions to achieve a countable density. Estimate volume and/or dilution expected to yield a countable membrane and select two additional quantities representing one-tenth and ten times this volume, respectively.

b. Filtration of sample: Follow the same procedure and precautions as prescribed under Section 9222B.5*b* above.

c. Preparation of culture dish: Place a sterile absorbent pad in each culture dish and pipet 1.8 to 2.0 mL M-FC medium, prepared as directed above, to saturate pad. Carefully remove any excess liquid from culture dish. Aseptically, place prepared filter on medium-impregnated pad as described in Section 9222B above.

As a substrate substitution for the nutrient-saturated absorbent pad, add 1.5% agar to M-FC broth as described in Section 9222B above.

d. Incubation: Place prepared cultures in waterproof plastic bags or seal, invert, and submerge petri dishes in water bath, and incubate for 24 ± 2 h at 44.5 ± 0.2°C. Anchor dishes below water surface to maintain critical temperature requirements. Place all prepared cultures in the water bath within 30 min after filtration. Alternatively, use an appropriate, accurate solid heat sink or equivalent incubator.

e. Counting: Colonies produced by fecal coliform bacteria on M-FC medium are various shades of blue. Nonfecal coliform colonies are gray to cream-colored. Normally, few nonfecal col-

* Rosolic acid reagent will decompose if sterilized by autoclaving. Store stock solution in the dark at 2 to 10°C and discard after 2 weeks or sooner if its color changes from dark red to muddy brown.

TABLE 9222:III. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER FECAL COLIFORM TEST

Water Source	Volume (X) To Be Filtered, *mL*						
	100	50	10	1	0.1	0.01	0.001
Lakes, reservoirs	X	X					
Wells, springs	X	X					
Water supply intake		X	X	X			
Natural bathing waters		X	X	X			
Sewage treatment plant, secondary effluent			X	X	X		
Farm ponds, rivers				X	X	X	
Stormwater runoff				X	X	X	
Raw municipal sewage					X	X	X
Feedlot runoff					X	X	X

iform colonies will be observed on M-FC medium because of selective action of the elevated temperature and addition of rosolic acid salt reagent. Elevating the temperature to 45.0 ± 0.2°C may be useful in eliminating environmental *Klebsiella* strains from the fecal coliform population. Count colonies with a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device.

f. Verification: Verify typical blue colonies and any pale-yellow atypical colonies as described in Section 9020 for fecal coliform analysis. Simultaneous inoculation at both temperatures is acceptable.

3. Calculation of Fecal Coliform Density

Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 fecal coliform colonies. This colony density range is more restrictive than the 20 to 80 total coliform range because of larger colony size on M-FC medium. Calculate fecal coliform density as directed in Section 9222B.6 above. Record densities as fecal coliforms per 100 mL.

4. Bibliography

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9222 E. Delayed-Incubation Fecal Coliform Procedure

This delayed-incubation procedure is comparable to the delayed-incubation total coliform procedure (Section 9222C). It may be used where the appropriate field incubator is not available, or where, under certain circumstances, a specialized laboratory service is advisable to examine, confirm, or speciate the suspect colonies.

Results obtained by this delayed method have been consistent with results from the standard fecal coliform MF test under various laboratory and field use conditions. However, determine test applicability for a specific water source by comparison with the standard MF test, especially for saline waters, chlorinated wastewaters, and waters containing toxic substances. Use the delayed incubation test only when the standard immediate fecal coliform test cannot be performed.

To conduct the delayed-incubation test filter sample in the field immediately after collection, place filter on M-ST holding medium (see Section 9222C.2*b* below), and ship to the laboratory. Complete fecal coliform test by transferring filter to M-FC medium, incubating at 44.5°C for 24 ± 2 h, and counting fecal coliform colonies.

The M-ST medium keeps fecal coliform organisms viable but prevents visible growth during transit. Membrane filters can be held for up to 3 d on M-ST holding medium with little effect on the fecal coliform counts.

1. Apparatus

a. Culture dishes: See Section 9222C.1*a* for specifications.
b. Field filtration units: See Section 9222C.1*b*.

2. Materials and Transport Medium

a. M-ST medium: Prepare as described in Section 9222C.2*b*.
b. M-FC medium: Prepare as described in Section 9222D.1*a*.

3. Procedure

a. Membrane filter transport: Place an absorbent pad in a tight-lid plastic petri dish and saturate with M-ST holding medium. After filtering sample remove membrane filter from filtration unit and place it on medium-saturated pad. Use only tight-lid dishes to prevent moisture loss; however, avoid having excess liquid in the dish. Place culture dish containing membrane in an appropriate shipping container and send to laboratory. Membranes can be held on the transport medium at ambient temperature for a maximum of 72 h with little effect on fecal coliform counts.

b. Transfer: At the laboratory remove membrane from holding medium and place it in another dish containing M-FC medium.

c. Incubation: After transfer of filter to M-FC medium, place tight-lid dishes in waterproof plastic bags, invert, and submerge in a water bath at 44.5°C ± 0.2°C for 24 ± 2 h or use a solid heat sink or equivalent incubator.

d. Counting: Colonies produced by fecal coliform bacteria are various shades of blue. Nonfecal coliform colonies are gray to cream-colored. Count colonies with a binocular wide-field dissecting microscope at 10 to 15 magnifications.

e. Verification: Verify typical blue colonies and any atypical (pale-blue or pale-yellow) colonies as described in Section 9020 for fecal coliform analysis.

4. Estimation of Fecal Coliform Density

Count as directed in Section 9222D.2*e* above and compute fecal coliform density as described in Section 9222D.3. Record time of collection, filtration, and laboratory examination, and calculate and report elapsed time.

5. Bibliography

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9222 F. *Klebsiella* Membrane Filter Procedure

Klebsiella bacteria belong to the family Enterobacteriaceae and are included in the total coliform group. The outermost layer of *Klebsiella* bacteria consists of a large polysaccharide capsule, a characteristic that distinguishes this genus from most other bacteria in this family; this capsule provides some measure of protection from disinfectants. *Klebsiella* bacteria are commonly associated with coliform regrowth in large water supply distribution systems.

Klebsiellae may be opportunistic pathogens that can give rise to bacteremia, pneumonia, urinary tract, and several other types of human infection. Approximately 60 to 80% of all *Klebsiella* from feces and from clinical specimens are positive in the fecal coliform test and are *Klebsiella pneumoniae*.

Klebsiella bacteria also are widely distributed in nature, occurring in soil, water, grain, vegetation, etc. Wood pulp, paper mills, textile finishing plants, and sugar-cane processing operations contain large numbers of klebsiellae in their effluents (10^4 to 10^6), and *Klebsiella* sp. are often the predominant coliform in such effluents.

Rapid quantitation may be achieved in the MF procedure by modifying M-FC agar base through substitution of inositol for lactose and adding carbenicillin or by using M-Kleb agar. These methods reduce the necessity for biochemical testing of pure strains. Preliminary verification of differentiated colonies is recommended.

1. Apparatus

a. Culture dishes: See Section 9222B.1*e* for specifications.

b. Filtration units: See Section 9222B.1*f*.

2. Materials and Culture Medium

a. Modified M-FC agar (M-FCIC agar): This medium may not be available in dehydrated form and may require preparation from the basic ingredients:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride, NaCl	5.0 g
Inositol	10.0 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar	15.0 g
Reagent-grade water	1 L

Heat medium to boiling and add 10 mL 1% rosolic acid dissolved in 0.2*N* NaOH. Cool to below 45°C and add 50 mg carbenicillin.* Dispense aseptically in 5- to 7-mL quantities into 50- × 9-mm plastic petri dishes. Store at 4 to 8°C until needed. Discard unused agar medium after 2 weeks. Do not sterilize by autoclaving. Final pH should be 7.4 ± 0.2.

b. M-Kleb agar:

Phenol red agar	31.0 g
Adonitol	5.0 g
Aniline blue	0.1 g
Sodium lauryl sulfate	0.1 g
Reagent-grade water	1 L

Sterilize by autoclaving for 15 min at 121°C. After autoclaving, cool to 50°C in a water bath; add 20 mL 95% ethyl alcohol (not denatured) and 0.05 g filter sterilized carbenicillin/L. Shake thoroughly and dispense aseptically into 50- × 9-mm plastic culture plates. The final pH should be 7.4 ± 0.2. Prepared medium can be held for 20 d at 4 to 8°C.

* Available from Geopen, Roerig-Pfizer, Inc. New York, N.Y.

3. Procedure

a. See Section 9222B.5 for selection of sample size and filtration procedure. Select sample volumes that will yield counts between 20 and 60 *Klebsiella* colonies per membrane. Place membrane filter on agar surface; incubate for 24 ± 2 h at 35 ± 0.5°C. *Klebsiella* colonies on M-FCIC agar are blue or bluish-gray. Most atypical colonies are brown or brownish. Occasional false positive occurrences are caused by *Enterobacter* species. *Klebsiella* colonies on M-Kleb agar are deep blue to blue gray, whereas other colonies most often are pink or occasionally pale yellow. Count colonies with a low-power (10 to 15 magnifications) binocular wide field dissecting microscope or other optical device.

b. Verification: Verify *Klebsiella* colonies from the first set of samples from ambient waters and effluents and when *Klebsiella* is suspect in water supply distribution systems. Verify a minimum of five typical colonies by transferring growth from a colony or pure culture to a commercial multi-test system for gram-negative speciation. Key tests for *Klebsiella* are citrate (positive), indole (negative), motility (negative), lysine decarboxylase (positive), ornithine decarboxylase (negative), and urease (positive). A *Klebsiella* strain that is indole-positive, liquefies pectin, and demonstrates a negative fecal coliform response is most likely of nonfecal origin.

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9222 G. MF Partition Procedures (PROPOSED)

1. *Escherichia coli* Partition Methods

a. Applications: Escherichia coli is a member of the fecal coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification may be achieved with the MF procedure by transferring the membrane from a total-coliform- or fecal-coliform-positive sample to a nutrient agar substrate containing 4-methylumbelliferyl-β-D-glucuronide (MUG). In this method *E. coli* is defined as any coliform that produces the enzyme β-glucuronidase and hydrolyzes the MUG substrate to produce a blue fluorescence around the periphery of the colony.

Limit use of this method to verify the presence of *E. coli* from a total-coliform-positive MF on Endo-type media to drinking water samples. In the examination of wastewater and other nonpotable water samples, use this procedure to verify positive filters from mFC medium used in the fecal coliform MF procedure.

b. Apparatus:

1) *Culture dishes:* See Section 9222B.1*e*.
2) *Filtration units:* See Section 9222B.1*f*.
3) *Forceps:* See Section 9222B.1*i*.
4) *Incubator:* See Section 9222B.1*j*.
5) *Ultraviolet lamp,* long wave (366 nm), preferably 6 W.
6) *Microscope* and *light source:* See Section 9222B.1*k*.

c. Materials and culture medium:

1) *Nutrient agar with MUG (NA-MUG):*

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
4-methylumbelliferyl-β-D-glucuronide	0.1 g
Reagent-grade water	1 L

Add dehydrated ingredients to reagent-grade water, mix thoroughly, and heat to dissolve. Sterilize by autoclaving for 15 min at 121°C. Dispense aseptically into 50-mm plastic culture plates. The final pH should be 6.8 ± 0.2. Prepared medium may be held for 2 weeks at 4 to 8°C.

2) *EC broth with MUG (EC-MUG):*

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	1.4 g
Sodium chloride, NaCl	5.0 g
4-methylumbelliferyl-β-D-glucuronide	0.1 g
Reagent-grade water	1 L

Add dehydrated ingredients to reagent-grade water, mix thoroughly and heat to dissolve. pH should be 6.9 ± 0.2 after sterilization. Before sterilization, dispense into culture tubes and cap with metal or heat-resistant plastic caps.

d. Procedure:

See Section 9222B.5 for selection of sample size and filtration procedure. For drinking water samples using Endo-type medium, count and record the metallic golden sheen colonies. Before transfer of the membrane, transfer a small portion of each target colony to the appropriate total coliform verification medium, using a sterile needle.

Alternatively, after transfer and incubation on NA-MUG, swab the surface growth on the MF and transfer to the appropriate medium. Aseptically transfer the membrane from the Endo-type medium to NA-MUG or EC-MUG medium. If quantification of the total coliforms is desired using NA-MUG medium, mark

each sheen colony with a fine-tipped marker or by puncturing a hole in the membrane adjacent to the colony with a sterile needle. Incubate NA-MUG at 35 ± 0.5°C for 4 h or EC-MUG at 44.5 ± 0.2 for 24 h. Observe individual colonies or tubes using a long-wave-length (366-nm) ultraviolet light source, preferably containing a 6-W bulb. The presence of a blue fluorescence in the tube, on the periphery (outer edge) of a colony, or observed from the back of the plate is considered a positive response for *E. coli*. Count and record the number of target colonies, if quantification is desired, or just record presence or absence of fluorescence.

For nonpotable water samples, use mFC medium for initial isolation before transfer to NA-MUG or EC-MUG medium. The procedure is the same as the above, with the exception of the total coliform verification process. Use a MUG-positive *E. coli* culture as a positive control.

Verify at least 10% of colonies exhibiting characteristic fluorescence. Aseptically transfer growth from target colonies and make a streak plate on a non-selective medium such as plate count agar. Streak plates in such a manner to ensure presence of discrete colonies separated by a least 0.5 cm. Transfer growth from colonies for species identification using either a commercially available multi-test system for *Enterobacteriaceae* or by conventional biochemical tests. See Section 9225.

2. Fecal Coliform Partition Method

a. Applications: Further partitioning of total coliforms from the original MF coliform-positive culture in a presence/absence search for fecal coliform in a drinking water sample may be achieved within 24 h. This procedure provides additional information from the original sample that might otherwise be lost in the request for a new sample for verification of laboratory findings.

b. Materials and culture medium: EC broth. See Section 9221E.1*a*.

c. Procedure: See Section 9222B.5 for selection of sample size and filtration procedure. For drinking water samples using Endo-type media, count and record the metallic (golden) sheen colonies. Before transfer of membrane or swabbing of plate, transfer a small portion of each target colony to the appropriate total coliform verification media using a sterile needle (see Section 9222B.5*g*). Use a sterile cotton swab to collect bacteria from the membrane surface, or pick discrete colonies with a 3-mm loop or sterile applicator stick, or transfer the entire membrane to inoculate a tube of EC medium. Incubate inoculated EC broth in a water bath at 44.5 ± 0.2°C for 24 ± 2 h. Place all EC tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in water bath incubator to immerse tubes to upper level of the medium. Gas production in an EC broth culture in 24 h or less is considered a positive response for fecal coliform bacteria.

3. Bibliography

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9223 CHROMOGENIC SUBSTRATE COLIFORM TEST*

9223 A. Introduction

The chromogenic substrate test utilizes hydrolyzable substrates for the simultaneous detection of enzymes of total coliform bacteria and *Escherichia coli*. When the chromogenic technique is used the total coliform group is defined as all bacteria possessing the enzyme β-D-galactosidase, which cleaves the chromogenic substrate, resulting in release of the chromogen. *Escherichia coli* are defined as bacteria giving a positive total coliform response and possessing the enzyme β-glucuronidase, which cleaves a fluorogenic substrate, resulting in the release of the fluorogen. The test can be used in either a multiple-tube or a presence-absence (single 100-mL sample) format.

1. Principle

a. Total coliform bacteria: Chromogenic substrates, such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG), are used to detect the enzyme β-D-galactosidase, which is produced by total coliform bacteria. The β-D-galactosidase enzyme hydrolyzes the substrate and produces a color change, which indicates and substantiates a positive test for total coliforms within 24 to 28 h without additional procedures. Noncoliform bacteria, such as species of the genera *Aeromonas* and *Pseudomonas*, that produce small amounts of the enzyme β-D-galactosidase, are suppressed and generally will not produce a positive response within 28 h unless more than 10^4 colony-forming units (CFU)/mL (10^6 CFU/100 mL) are present.

* Approved by Standard Methods Committee, 1994.

b. Escherichia coli: A substrate such as the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) is used to detect the enzyme β-glucuronidase, which is produced by *E. coli*. The β-glucuronidase enzyme hydrolyzes the substrate and produces a fluorescent product when viewed under long-wavelength (366-nm) ultraviolet (UV) light. The presence of fluorescence indicates a positive test for *E. coli*. Some strains of *Shigella* spp. also may produce a positive fluorescence response. Because *Shigella* spp. are overt human pathogens this is not considered a detriment for testing the sanitary quality of water.

2. Applications

The chromogenic substrate coliform test is recommended for the analysis of drinking and fresh source water samples. Formulations also are available for the analysis of marine waters. Initially, laboratories planning to use this procedure should conduct parallel tests with one of the standard coliform tests over a period of several months to assess the effectiveness of the test for the specific water type being analyzed and to determine the comparability of the two techniques.

Water samples containing humic or other material may be colored. If there is background color, compare inoculated tubes to a control tube containing only water sample. In certain waters, high calcium salt content can cause precipitation but this should not affect the reaction.

Do not use the chromogenic substrate test to verify presumptive coliform cultures or membrane filter colonies, because the substrate may be overloaded by the heavy inoculum of weak β-D-galactosidase-producing noncoliforms, causing false-positive results.

9223 B. Chromogenic Substrate Test

1. Substrate

Formulations are available commercially* in disposable tubes for the multiple-tube procedure or in containers that will hold 100-mL samples for the presence-absence approach. Appropriate preweighed portions of the reagent for mixing and dispensing into multiple tubes for 10-mL test portions or other containers for 100-mL samples also are available. The need for good quality assurance and uniformity requires the use of a commercial substrate reagent. Avoid prolonged exposure of the substrate to direct sunlight.

2. Procedure

a. Multiple-tube procedure: Select the appropriate number of tubes per sample with predispensed media for the multiple-tube test and label. Follow manufacturer's instructions for preparing serial dilutions for various formulations. Aseptically add 10 mL sample to each tube, cap tightly, and mix vigorously to dissolve. The mixture remains colorless. Some particles may remain undissolved throughout the test; this will not affect test performance.

The procedure also can be performed by adding appropriate amounts of the substrate reagent to the sample, mixing thoroughly, and dispensing into five or ten sterile tubes. Incubate at 35 ± 0.5°C for 24 h.

b. Presence-absence procedure: Aseptically add preweighed enzymatic substrate to 100 mL sample in a sterile, transparent, nonfluorescent borosilicate glass or equivalent bottle or container. Optionally, add 100 mL sample to the enzymatic substrate in a sterile container provided by the manufacturer. Aseptically cap and mix thoroughly to dissolve. Incubate at 35 ± 0.5°C for 24 h.

3. Interpretation

a. Total coliform bacteria: After 24 h incubation, examine tubes or containers for a color change. When the substrate is ortho-nitrophenyl-β-D-galactopyranoside (ONPG) it is hydrolyzed by the bacterial enzyme to yield yellow orthonitrophenol; substrates used in other formulations may yield a different color response. A distinct chromogenic response is a positive reaction for total coliforms. If the color response is not uniform throughout the tube, mix by inversion before reading. Compare each tube against the color comparator available from a commercial source of the substrate. If the color intensity is greater than or equal to that of the comparator, total coliforms are present. Samples are negative for total coliforms if no color is observed. If a chromogenic response is questionable after 24 h, incubate up to an additional 4 h. If the chromogen intensifies, the sample is total-coliform positive; if it does not, the sample is negative.

b. Escherichia coli: Examine positive total coliform tubes or containers for fluorescence using a long-wavelength (366-nm) ultraviolet lamp (preferably 6-W bulb). Compare each tube against the reference comparator available from a commercial source of the substrate. The presence of fluorescence is a positive test for *E. coli*. If fluorescence is questionable, incubate for an additional 4 h; intensified fluorescence is positive test result.

4. Reporting

If performing an MPN procedure, calculate the MPN value for total coliforms and *E. coli* from the number of positive tubes as described in Section 9221C. If using the presence-absence procedure, report results as total coliform and *E. coli* present or absent in 100 mL sample.

5. Quality Control

Test each lot of substrate purchased for performance by inoculation with three control bacteria: *Escherichia coli*, a total coliform other than *E. coli* (e.g., *Enterobacter cloacae*), and a noncoliform. Avoid using a heavy inoculum. If *Pseudomonas* is

* Colilert and Colilert-MW, Idexx Laboratories, Inc., Westbrook, Maine, or demonstrably equivalent product.

used as the representative noncoliform, select a nonfluorescent species. Incubate these controls at 35 ± 0.5°C for 24 h. Read and record results. Other quality-control guidelines are included in Section 9020.

6. Bibliography

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9225 DIFFERENTIATION OF THE COLIFORM BACTERIA*

9225 A. Introduction

Identification of bacteria that constitute the coliform group sometimes is necessary to determine the nature of pollution. It is of particular importance in reference to distinguishing the presence of *Escherichia coli*. Special procedures for detection of *E. coli* are given in Sections 9221F, 9222G, and 9223. Differential tests for identification must be used with the knowledge that all strains taxonomically assigned to the coliform group do not conform necessarily to the coliform definition stated in this manual because they may not ferment lactose, or if they do, they may not produce gas. Furthermore, gram-negative bacteria other than coliforms ferment lactose and produce sheen (e.g., *Aeromonas* spp.) and not all strains of a species will react uniformly in media. Unusual strains (such as *E. coli*, inactive, Table 9225:I), mutants, and injured organisms may not give classical responses. The traditional "IMViC" tests (i.e., indole, methyl red, Voges-Proskauer, and citrate utilization) are useful for coliform differentiation, but do not provide complete identification. Additional biochemical tests often are necessary. Commercial kits for identification are available and may serve as economical alternatives to traditional differential media. Automated systems of identifying large numbers of isolates also are available.

The significance of various coliform organisms in water has been and is a subject of considerable study. Collectively, the coliforms are referred to as indicator organisms. The genera *Enterobacter, Klebsiella, Citrobacter,* and *Escherichia* usually are represented in the majority of isolations made from raw and treated municipal water supplies.

* Approved by Standard Methods Committee, 1994.

9225 B. Culture Purification

1. Procedure

A pure culture is essential for accurate identification. Obtain a pure culture by carefully picking a well-isolated colony that gives typical responses on an appropriate solid medium or membrane filter, and streaking on a tryptic soy or nutrient agar plate. Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v) and then streaked. When picking a colony from a primary culture on a selective medium, be aware that viable cells, which have not formed colonies themselves, may surround the picked colony. Incubate the subculture at 35 ± 0.5°C for 24 h and test a single well-isolated colony by the Gram stain to confirm the sole presence of gram-negative, non-spore-forming rods (Section 9221B). Also determine that the culture is oxidase-negative (Section 9225E). Oxidase-positive, gram-negative, non-spore-forming rods are not coliform bacteria, but may be organisms such as *Aeromonas*, which is not regarded as an indicator of fecal pollution.

Variation in organisms of the coliform group occurs occasionally and mixed reactions in differential media may indicate a pure culture undergoing variation. Persistent variations of reactions in differential media indicate a mixed culture caused by inadequate purification.

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9225 C. Identification

1. Definition

Coliforms are defined here as facultative anaerobic, gram-negative non-spore-forming rods that ferment lactose with gas formation within 48 h at 35°C or, as applied to the membrane filter method, produce a dark red colony with a metallic sheen within 24 h on an Endo-type medium containing lactose. However, anaerogenic (non-gas-producing) lactose-fermenting strains of *Escherichia coli* and coliforms that do not produce metallic sheen on Endo medium may be encountered. These organisms, as well as typical coliforms, can be considered indicator organisms, but they are excluded from the current definition of coliforms. More extensive testing may be required for proper identification.

2. Characteristics and Tests

Coliforms belong to the bacterial taxonomic family Enterobacteriaceae. Table 9225:I provides data on some of the biochemical reactions used for differentiating these organisms.

Preparing differential media and reagents may not be as economical for many laboratories as using commercially prepared and prepackaged multiple-test kits, which reduce quality-control work. These commercial kits are simple to store and use, and give reproducible and generally accurate results. Periodically test reactions with known stock cultures of bacteria to assure accuracy and reproducibility of results. Make further tests if the kit provides equivocal results.

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TABLE 9225:I. BIOCHEMICAL REACTIONS OF KEY SPECIES OF THE FAMILY ENTEROBACTERIACEAE*

Species	Percentage Isolates Positive in 1 to 2 Days†											
	Lactose Fermentation	ONPG Hydrolysis	Indole Production	Methyl Red	Voges-Proskauer	Simmons' Citrate	Ornithine Decarboxylase	Lysine Decarboxylase	Sorbitol Fermentation	Cellobiose Fermentation	Motility, 35–37°C	Yellow Pigment
Citrobacter diversus	35	96	99	100	0	99	99	0	99	99	95	0
Citrobacter freundii	50	95	5	100	0	95	20	0	98	55	95	0
Enterobacter aerogenes	95	100	0	5	98	95	98	98	100	100	97	0
Enterobacter agglomerans	40	90	20	50	70	50	0	0	30	55	85	75
Enterobacter cloacae	93	99	0	5	100	100	96	0	95	99	95	0
Escherichia coli	95	95	98	99	0	1	65	90	94	2	95	0
Escherichia coli, inactive	25	45	80	95	0	1	20	40	75	2	5	0
Escherichia fergusonii	0	83	98	100	0	17	100	95	0	96	93	0
Escherichia hermannii	45	98	99	100	0	1	100	6	0	97	99	98
Escherichia vulneris	15	100	0	100	0	0	0	85	1	100	100	50
Hafnia alvei	5	90	0	40	85	10	98	100	0	15	85	0
Klebsiella oxytoca	100	100	99	20	95	95	0	99	99	100	0	1
Klebsiella ozaenae	30	80	0	98	0	30	3	40	65	92	0	0
Klebsiella pneumoniae	98	99	0	10	98	98	0	98	99	98	0	0
Klebsiella rhinoscleromatis	0	0	0	100	0	0	0	0	100	100	0	0
Serratia fonticola	97	100	0	100	9	91	97	100	100	6	91	0
Serratia marcescens	2	95	1	20	98	98	99	99	99	5	97	0

* Modified after Farmer, J.J. III, 1985. Clinical identification of new species and biogroups of Enterobacteriaceae. *J. Clin. Microbiol.* 21:46.
† Reactions that become positive after 2 d are not considered.

9225 D. Media, Reagents, and Procedures

Commercially available media and reagents can reduce work and cost; however, include negative and positive controls with known stock cultures to assure accuracy and reliability. Detailed methods are available. Expected test results are shown in Table 9225:I.

1. Lactose, Sorbitol, and Cellobiose Fermentation Tests

Suspend 16 g phenol red broth base and 5 g of the desired carbohydrate in 1 L reagent-grade water and stir to dissolve completely. Dispense in tubes to a depth of one-third tube length. To determine gas production place a small inverted vial (Durham tube) in the tubes of media at the time of preparation. Close tubes and sterilize at 121°C for 15 min. Store tubes in the dark (refrigeration preferred) and discard if evaporation exceeds 10% of the volume.

To conduct a test, inoculate with a loopful of growth from a well-isolated colony or slant and incubate for 24 to 48 h at 35 ± 0.5°C. Carbohydrate fermentation (acid production) is indicated by a decrease in pH, resulting in a change in color of the pH indicator, phenol red, from red-orange to yellow (pH <6.6). Alternatively, for lactose fermentation, lauryl tryptose broth (Section 9221B) may be used.

2. ONPG Hydrolysis

Numerous commercial test kits and disks for determining ONPG hydrolysis are available, or an ONPG-containing medium (Section 9222) can be used. Alternatively, prepare peptone water by dissolving 1 g peptone and 0.5 g NaCl in 100 mL reagent-grade water. Sterilize at 121°C for 15 min. Also prepare ONPG solution by dissolving 0.6 g *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 100 mL 0.01*M* Na_2HPO_4, sterilize by filtration, and store in the dark at 4 to 10°C. To prepare ONPG broth, aseptically combine 25 mL ONPG solution and 75 mL peptone water, dispense aseptically in 2.5-mL amounts in sterile 13- × 100-mm tubes, and store in the dark for up to 1 month at 4 to 10°C. Do not use the ONPG solution if it becomes yellow.

To conduct the test, inoculate 0.5 mL ONPG broth with a heavy loopful of growth from a slant and incubate at 35 ± 0.5°C for up to 24 h. A yellow color, compared with an uninoculated tube or (preferably) a tube inoculated with an ONPG-negative culture, is a positive test. Interpret tests of yellow-pigmented organisms with caution. Do not use the chromogenic substrate method (Section 9223) to test ONPG hydrolysis.

3. Indole Test

Indole is a product of the metabolism of tryptophane.

a. Reagents:

1) *Medium:* Use tryptophane broth. Dissolve 10.0 g tryptone or trypticase/L reagent-grade water. Dispense in 5-mL portions in test tubes and sterilize.

2) *Test reagent:* Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 mL isoamyl (or normal amyl) alcohol, ACS grade, and add 25 mL conc HCl. The reagent should be yellow. Some brands

of *p*-dimethylaminobenzaldehyde are not satisfactory and some good brands become unsatisfactory on aging.

The amyl alcohol solution should have a pH value of less than 6.0. Purchase both amyl alcohol and benzaldehyde in as small amounts as will be consistent with the volume of work to be done.

b. Procedure: Inoculate 5-mL portions of medium from a pure culture and incubate at 35 ± 0.5°C for 24 ± 2 h. Add 0.2 to 0.3 mL test reagent and gently shake. Let stand for about 10 min and observe results.

A dark red color in the amyl alcohol surface layer constitutes a positive indole test; the original color of the reagent, a negative test. An orange color probably indicates the presence of skatole, a breakdown product of indole.

4. Methyl Red Test

The methyl red test measures the ability of organisms to produce stable acid end products from glucose fermentation.

a. Reagents:

1) *Medium:* Use buffered glucose broth. Dissolve 7.0 g proteose peptone or equivalent peptone, 5.0 g glucose, and 5.0 g dipotassium hydrogen phosphate (K_2HPO_4) in 1 L reagent-grade water. Dispense in 5-mL portions in test tubes and sterilize by autoclaving at 121°C for 12 to 15 min, making sure that total time of exposure to heat is not longer than 30 min.

2) *Indicator solution:* Dissolve 0.1 g methyl red in 300 mL 95% ethyl alcohol and dilute to 500 mL with reagent-grade water.

b. Procedure: Inoculate 10-mL portions of medium from a pure culture. Incubate at 35 ± 0.5°C for 5 d. To 5 mL of the culture add 5 drops methyl red indicator solution.

Incubation for 48 h is adequate for most cultures, but do not incubate for less than 48 h. If test results are equivocal at 48 h repeat with cultures incubated for 4 or 5 d. In such cases incubate duplicate cultures at 22 to 25°C. Testing of culture portions at 2, 3, 4, and 5 d may provide positive results sooner.

Record a distinct red color as methyl-red-positive and a distinct yellow color as methyl-red-negative. Record a mixed shade as questionable and possibly indicative of incomplete culture purification.

5. Voges-Proskauer Test

The Voges-Proskauer test measures the ability of organisms to produce a neutral end product (acetoin) from glucose fermentation.

a. Reagents:

1) *Medium:* See ¶ 4*a*1) above.

2) *Naphthol solution:* Dissolve 5 g purified α-naphthol (melting point 92.5°C or higher) in 100 mL absolute ethyl alcohol. When stored at 5 to 10°C, this solution is stable for 2 weeks.

3) *Potassium hydroxide, 7N:* Dissolve 40 g KOH in 100 mL reagent-grade water.

b. Procedure: Inoculate 5 mL medium and incubate for 48 h at 35 ± 0.5°C. To 1 mL of culture add 0.6 mL naphthol solution and 0.2 mL KOH solution. Shake well after the addition of each reagent. Development of a pink to crimson color at the surface within 5 min constitutes a positive test. Do not read after 10 min. Disregard tubes developing a copper color.

6. Simmons' Citrate Test

The citrate test measures the ability of bacteria to utilize citrate as the sole source of carbon.

a. Medium: Use Simmons' citrate agar. To make Simmons' citrate agar, add 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g ammonium dihydrogen phosphate ($NH_4H_2PO_4$), 1.0 g K_2HPO_4, 2.0 g sodium citrate dihydrate, 5.0 g NaCl, 15.0 g agar, and 0.08 g bromthymol blue to 1 L reagent-grade water. Tube for long slants.

b. Procedure:

Inoculate agar medium by the streak technique using a light inoculum.

Incubate 48 h at 35 ± 0.5°C. Record growth on the medium with a blue color as a positive reaction; record absence of growth or color change as negative.

7. Motility Test

The motility test measures whether an organism is motile in a semi-solid medium.

a. Medium: Use motility test medium made by adding 3.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, and 4.0 g agar to 1 L reagent-grade water. Adjust pH to 7.4, dispense in 3-mL portions in 13- × 100-mm tubes or 8-mL portions in 16- × 125-mm tubes, and sterilize.

b. Procedure: Inoculate by stabbing into the center of the medium, using an inoculating needle, to a depth of 5 mm. Incubate for 1 to 2 d at 35°C. If negative, incubate an additional 5 d at 22 to 25°C.

Diffuse growth through the medium from the point of inoculation is positive. In a negative test, growth is visible only along the stab line and the surrounding medium stays clear. Alternatively, prepare the medium without agar and examine a young culture using the hanging drop slide technique for motile organisms.

8. Lysine and Ornithine Decarboxylase Tests

This procedure tests the ability of bacteria to metabolize the amino acids lysine and ornithine.

a. Reagents:

1) *Media:* Use a basal medium made according to the Moeller or Falkow methods. For the Moeller method, dissolve 5.0 g peptone (Orthana special, thiotone, or equivalent), 5.0 g beef extract, 0.625 mL bromcresol purple (1.6%), 2.5 mL cresol red (0.2%), 0.5 g glucose, and 5.0 mg pyridoxal in 1 L reagent-grade water and adjust to pH 6.0 to 6.5. For the Falkow method, dissolve 5.0 g peptone, 3.0 g yeast extract, 1.0 g glucose, and 1.0 mL bromcresol purple (1.6%) in 1 L reagent-grade water and adjust to pH 6.7 to 6.8. For either decarboxylase test divide into three portions: make no addition to the first portion, add enough L-lysine dihydrochloride to the second portion to make a 1% solution, and add L-ornithine dihydrochloride to the third to make 1% (for the Falkow method, add only 0.5% of the L-amino acid). After adding ornithine readjust pH of the medium to 6.0 ± 0.2. Dispense in 3- to 4-mL portions in screw-capped test tubes and sterilize by autoclaving at 121°C for 10 min. A floccular precipitate in the ornithine medium does not interfere with its use.

2) *Mineral oil:* Use mineral oil sterilized by autoclaving at 121°C for 30 to 60 min depending on the size of the container.

b. Procedure: Lightly inoculate each of the three media, add a layer of about 10 mm thickness of mineral oil, and incubate at 37°C for up to 4 d. Examine tubes daily. A color change from yellow to violet or reddish-violet constitutes a positive decarboxylase test; a change to bluish gray indicates a weak positive; no color change or a yellow color represents a negative test. See Table 9225:I.

9. Oxidase Test

The oxidase test determines the presence of oxidase enzymes. Coliform bacteria are oxidase-negative.

a. Reagents:

1) *Media:* Use either nutrient agar or tryptic soy agar plates to streak cultures and produce isolated colonies. From these obtain the inoculum for oxidase testing on impregnated filter paper. Do not use any medium that includes a carbohydrate in its formulation. Use only tryptic soy agar if reagent is dropped on colonies.

Tryptic soy agar:

Tryptone	15.0 g
Soytone	5.0 g
Sodium chloride, NaCl	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

pH should be 7.3 ± 0.2 after sterilization.

2) *Tetramethyl* p-*phenylenediamine dihydrochloride*, 1% aqueous solution, freshly prepared or refrigerated for no longer than 1 week. Impregnate a filter paper strip* with this solution. Alternatively, prepare a 1% solution of dimethyl *p*-phenylenediamine hydrochloride. Single-use reagent ampules, commercially available, are convenient and economical, but use them with caution. When the reagent is to be dropped directly on colonies, use tryptic soy agar plates because nutrient agar plates give inconsistent results; when smearing a portion of a picked colony on reagent-impregnated filter paper, do not transfer any medium with the culture material.

b. Procedure: Remove some of a colony from agar plate with a platinum wire, a wooden or plastic applicator stick, or a glass rod and smear on the test strip. Do not use iron or other reactive wire because it will cause false positive reactions. A dark purple color that develops within 10 s is a positive oxidase test. Test positive and negative cultures concurrently. If the liquid reagent is used, drop it on colonies on the culture plate. Oxidase-positive colonies develop a pink color that successively becomes maroon, dark red, and finally, black.

* Whatman No. 1 or equivalent.

10. Yellow Pigment

Observe isolated colonies on nutrient agar slants and plates or plates of tryptic soy agar incubated at 35 ± 0.5°C for up to 48 h. Pigmentation often intensifies as time of incubation proceeds.

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9230 FECAL STREPTOCOCCUS AND ENTEROCOCCUS GROUPS*

9230 A. Introduction

1. Fecal Streptococcus Group

The fecal streptococcus group consists of a number of species of the genus *Streptococcus*, such as *S. faecalis*, *S. faecium*, *S. avium*, *S. bovis*, *S. equinus*, and *S. gallinarum*. They all give a positive reaction with Lancefield's Group D antisera[1] and have been isolated from the feces of warm-blooded animals. In addition, *S. avium* sometimes reacts with Lancefield's Group Q antisera. *S. faecalis* subsp. *liquefaciens* and *S. faecalis* subsp. *zymogenes* are differentiated based on the ability of these strains to liquefy gelatin and hemolyze red cells. However, the validity of these subspecies is questionable.[2,3]

The normal habitat of fecal streptococci is the gastrointestinal tract of warm-blooded animals. *S. faecalis* and *S. faecium* once were thought to be more human-specific than other *Streptococcus* species. Other species have been observed in human feces but less frequently.[4] Similarly, *S. bovis*, *S. equinus*, and *S. avium* are not exclusive to animals, although they usually occur at higher densities in animal feces.[5] Certain streptococcal species predominate in some animal species and not in others, but it is not possible to differentiate the source of fecal contamination based on speciation of fecal streptococci.

The fecal streptococci have been used with fecal coliforms to differentiate human fecal contamination from that of other warm-blooded animals. Editions of *Standard Methods* previous to the 17th suggested that the ratio of fecal coliforms (FC) to fecal streptococci (FS) could provide information about the source of contamination. A ratio greater than four was considered indicative of human fecal contamination, whereas a ratio of less than 0.7 was suggestive of contamination by nonhuman sources. The

* Approved by Standard Methods Committee, 1993.

value of this ratio has been questioned because of variable survival rates of fecal streptococcus group species. *S. bovis* and *S. equinus* die off rapidly, once exposed to aquatic environments, whereas *S. faecalis* and *S. faecium* tend to survive longer.[6] Furthermore, disinfection of wastewaters appears to have a significant effect on the ratio of these indicators, which may result in misleading conclusions regarding the source of contaminants.[7] The ratio is affected also by the methods for enumerating fecal streptococci. The KF membrane filter procedure has a false-positive rate ranging from 10 to 90% in marine and fresh waters.[8-10] For these reasons, the FC/FS ratio cannot be recommended, and should not be used as a means of differentiating human and animal sources of pollution.

2. Enterococcus Group

The enterococcus group is a subgroup of the fecal streptococci that includes *S. faecalis, S. faecium, S. gallinarum*, and *S. avium*. The enterococci are differentiated from other streptococci by their ability to grow in 6.5% sodium chloride, at pH 9.6, and at 10°C and 45°C.

The enterococci portion of the fecal streptococcus group is a valuable bacterial indicator for determining the extent of fecal contamination of recreational surface waters. Studies at marine and fresh water bathing beaches indicated that swimming-associated gastroenteritis is related directly to the quality of the bathing water and that enterococci are the most efficient bacterial indicator of water quality.[11,12] Water quality guidelines based on enterococcal density have been proposed for recreational waters.[13] For recreational fresh waters the guideline is 33 enterococci/100 mL while for marine waters it is 35/100 mL. Each guideline is based on the geometric mean of at least five samples per 30-d period during the swimming season.

3. Selection of Method

The multiple-tube technique is applicable primarily to raw and chlorinated wastewater and sediments, and can be used for fresh and marine waters. The membrane filter technique also may be used for fresh and saline water samples, but is unsuitable for highly turbid waters.

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9230 B. Multiple-Tube Technique

1. Materials and Culture Media

a. Azide dextrose broth:[1]

Beef extract	4.5	g
Tryptone or polypeptone	15.0	g
Glucose	7.5	g
Sodium chloride, NaCl	7.5	g
Sodium azide, NaN_3	0.2	g
Reagent-grade water	1	L

pH should be 7.2 ± 0.2 at 25°C after sterilization.

b. Pfizer selective enterococcus (PSE) agar:[2]

Peptone C	17.0	g
Peptone B	3.0	g
Yeast extract	5.0	g
Bacteriological bile	10.0	g
Sodium chloride, NaCl	5.0	g
Sodium citrate	1.0	g
Esculin	1.0	g
Ferric ammonium citrate	0.5	g
Sodium azide, NaN_3	0.25	g
Agar	15.0	g
Reagent-grade water	1	L

pH should be 7.1 ± 0.2 after sterilization. Hold medium for not more than 4 h at 45 to 50°C before plates are poured.

2. Presumptive Test Procedure

Inoculate a series of tubes of azide dextrose broth with appropriate graduated quantities of sample. Use sample of 10 mL portions or less. Use double-strength broth for 10-mL inocula. The portions used will vary in size and number with the sample character. Use only decimal multiples of 1 mL (see Section 9221 for suggested sample sizes).

Incubate inoculated tubes at 35 ± 0.5°C. Examine each tube for turbidity at the end of 24 ± 2 h. If no definite turbidity is present, reincubate, and read again at the end of 48 ± 3 h.

3. Confirmed Test Procedure

Subject all azide dextrose broth tubes showing turbidity after 24- or 48-h incubation to the confirmed test.

Streak a portion of growth from each positive azide dextrose broth tube on PSE agar. Incubate the inverted dish at 35 ± 0.5°C for 24 ± 2 h. Brownish-black colonies with brown halos confirm the presence of fecal streptococci.

Brownish-black colonies with brown halos may be transferred to a tube of brain-heart infusion broth containing 6.5% NaCl. Growth in 6.5% NaCl broth and at 45°C indicates that the colony belongs to the enterococcus group.

4. Computing and Recording of MPN

Estimate fecal streptococci densities from the number of tubes in each dilution series that are positive on PSE agar. Similarly, estimate enterococci densities from the number of tubes in each dilution series containing streptococci that can grow in 6.5% NaCl broth. Compute the combination of positives and record as the most probable number (MPN). Refer to Section 9221D.

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9230 C. Membrane Filter Techniques

1. Laboratory Apparatus

See Section 9222B.1.

2. Materials and Culture Media

a. mE agar for enterococci:[1]

Peptone	10.0	g
Sodium chloride, NaCl	15.0	g
Yeast extract	30.0	g
Esculin	1.0	g
Actidione (cycloheximide)	0.05	g
Sodium azide, NaN_3	0.15	g
Agar	15.0	g
Reagent-grade water	1	L

Heat to dissolve ingredients, sterilize, and cool in a water bath at 44 to 46°C. Mix 0.25 g nalidixic acid in 5 mL reagent-grade water, add a few drops of 0.1*N* NaOH to dissolve the antibiotic, and add to the basal medium. Add 0.15 g 2,3,5-triphenyl tetrazolium chloride and mix well to dissolve. Pour the agar into 9- × 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL), and let solidify. The final pH should be 7.1 ± 0.2. Store poured plates in the dark at 2 to 10°C. Discard after 30 d. (NOTE: This medium is recommended for culturing enterococci in fresh and marine recreational waters.)

b. EIA substrate:[1]

Esculin	1.0	g
Ferric citrate	0.5	g
Agar	15.0	g
Reagent-grade water	1	L

The pH should be 7.1 ± 0.2 before autoclaving. Heat to dissolve ingredients, sterilize, and cool in a water bath at 44 to 46°. Pour medium into 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Store poured plates in the dark at 2 to 10°C. Discard after 30 d.

c. m Enterococcus agar for fecal streptococci:[2]

Tryptose	20.0	g
Yeast extract	5.0	g
Glucose	2.0	g
Dipotassium phosphate, K_2HPO_4	4.0	g
Sodium azide, NaN_3	0.4	g
2,3,5-Triphenyl tetrazolium chloride	0.1	g
Agar	10.0	g
Reagent-grade water	1	L

Heat to dissolve ingredients. Do not autoclave. Dispense into 9- × 50-mm petri plates to a depth of 4 to 5 mm (approximately 4 to 6 mL), and let solidify. Prepare fresh medium for each set of samples. (NOTE: This medium is recommended for Group D streptococci in fresh and marine waters.)

d. Brain-heart infusion broth:

Infusion of calf brain	200	g
Infusion of beef heart	250	g
Proteose peptone	10.0	g
Glucose	2.0	g
Sodium chloride, NaCl	5.0	g
Disodium hydrogen phosphate, Na_2HPO_4	2.5	g
Reagent-grade water	1	L

The pH should be 7.4 after sterilization.

e. Brain-heart infusion agar: Add 15.0 g agar to the ingredients for brain-heart infusion broth. The pH should be 7.4 after sterilization. Tube for slants.

f. Bile esculin agar:[3]

Beef extract	3.0	g
Peptone	5.0	g
Oxgall	40.0	g
Esculin	1.0	g
Ferric citrate	0.5	g
Agar	15.0	g
Reagent-grade water	1	L

Heat to dissolve ingredients. Dispense 8 to 10 mL into tubes for slants or an appropriate volume into a flask for subsequent pouring into plates. Autoclave at 121°C for 15 min. Do not overheat because this may cause darkening of the medium. Cool to 44 to 46°C and slant the tubes or dispense 15 mL into 15- × 100-mm petri dishes. The final pH should be 6.6 ± 0.2 after sterilization. Store at 4 to 10°C.

3. Procedures

a. mE Method:[1]

1) Selection of sample size and filtration—Filter appropriate sample volumes through a 0.45-µm, gridded, sterile membrane to give 20 to 60 colonies on the membrane surface. Transfer filter to agar medium in petri dish, avoiding air bubbles beneath the membrane.

2) Incubation—Invert culture plates and incubate at 41°C ± 0.5°C for 48 h.

3) Substrate test—After 48 h incubation, carefully transfer filter to EIA medium. Incubate at 41°C ± 0.5°C for 20 min.

4) Counting—Pink to red enterococci colonies develop a black or reddish-brown precipitate on the underside of the filter. Count colonies using a fluorescent lamp and a magnifying lens.

b. m Enterococcus method:[2]

1) Selection of sample size and filtration—See ¶ 3*a*.

2) Incubation—Let plates stand for 30 min, then invert and incubate at 35 ± 0.5°C for 48 h.

3) Counting—Count all light and dark red colonies as enterococci. Count colonies using a fluorescent lamp and a magnifying lens.

4. Calculation of Fecal Streptococci or Enterococci Density

Compute density from sample quantities producing membrane filter counts within the desired 20- to 60-fecal streptococcus or enterococcus colony range. Calculate as in Section 9222B.6. Record densities as fecal streptococci or enterococci per 100 mL.

5. Verification Tests

Pick selected typical colonies from a membrane and streak for isolation onto the surface of a brain-heart infusion agar plate. Incubate at 35°C ± 0.5°C for 24 to 48 h.

Transfer a loopful of growth from a well-isolated colony on brain-heart infusion agar into a brain-heart infusion broth tube and to each of two clean glass slides. Incubate the brain-heart infusion broth at 35 ± 0.5°C for 24 h. Add a few drops of freshly prepared 3% hydrogen peroxide to the smear on a slide. The appearance of bubbles constitutes a positive catalase test and indicates that the colony is not a member of the fecal streptococcus group. If the catalase test is negative, i.e., no bubbles, make a Gram stain of the second slide. Fecal streptococci and enterococci are gram-positive, ovoid cells, 0.5 to 1.0 µm in diameter, mostly in pairs or short chains.

Transfer a loopful of growth from the brain-heart infusion broth to each of the following media: bile esculin agar (incubate at 35 ± 0.5°C for 48 h); brain-heart infusion broth (incubate at 45 ± 0.5°C for 48 h); brain-heart infusion broth with 6.5% NaCl (incubate at 35 ± 0.5°C for 48 h).

Growth of catalase-negative, gram-positive cocci on bile esculin agar and at 45°C in brain-heart infusion broth verifies that the colony is of the fecal streptococcus group. Growth at 45°C and in 6.5% NaCl broth indicates that the colony belongs to the enterococcus group.

6. Serological Verification of Group D Fecal Streptococci

An alternate verification test for Group D streptococci can be performed using the precipitin method of Lancefield.[4] This test is highly specific for *S. faecalis, S. faecium, S. avium, S. gallinarum, S. bovis,* and *S. equinus.*

a. Antigen preparation: Pick typical single colonies from the membrane filter and streak for isolation on brain-heart infusion agar or blood agar plates. Pick a well-isolated colony and inoculate into 30 to 50 mL of Todd-Hewitt broth.[5] Incubate at 35°C for 24 h under aerobic conditions. Concentrate bacterial suspension by centrifuging (3000 × *g* for 5 min). Draw supernatant off and resuspend cells in 0.5 mL saline solution. Autoclave resuspended cells for 15 min at 121°C. Centrifuge the bacteria and decant clear supernatant fluid containing the group antigen.

b. Capillary precipitin test: Antisera for this test may be obtained from commercial sources.

Dip a 1.2- to 1.5-mm-OD capillary tube into antiserum and draw up about 1 cm of serum. Place a finger over upper end of tube so that no air will be drawn up and carefully wipe off excess antiserum. Dip tube into streptococcal antigen extract solution and draw up an equal volume of antigen. Carefully wipe off excess extract. Place a finger over upper end of tube and force lower end into plasticine to plug lower opening. Invert tube and place it in plasticine groove of a capillary holding rack.

A positive test for Group D antigen is characterized by a white precipitate that appears at the antigen-antiserum interface within 15 min and usually by 5 min. If no reaction has occurred by 30 min, the test is negative. Examination of the tubes is more effective if they are read in a bright light against a dark background.

Serological verification of Group D streptococci also can be done using commercially available agglutination tests.* The slide agglutination tests are simple and appear to be reliable. Group D streptococci are verified directly from isolated colonies on membrane filter plates or from broth culture tubes. To verify presumptive enterococci cultures, test also for salt tolerance (growth in 6.5% NaCl broth).

* Phadebact Strep D test, Pharmacia Diagnostics, Piscataway, N.J. and Streptex Test, Burroughs-Wellcome Co., Research Triangle Park, N.C.

TABLE 9230:I. SELECTED KEY BIOCHEMICAL CHARACTERISTICS OF THE STREPTOCOCCUS SPECIES WITHIN THE FECAL STREPTOCOCCUS AND ENTEROCOCCUS GROUPS*

	Fecal Streptococcus Group					
	Enterococcus Group					
Test	*S. faecalis*	*S. faecium*	*S. avium*	*S. gallinarum*	*S. bovis*	*S. equinus*
Catalase	–	–	–	–	–	–
40% Bile	+	+	+	+	+	+
Esculin[6]	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	–	–
Growth at 10°C	+	+	+	+	–	–
Pyruvate utilization[7]	+	–	–	–	–	–
Phosphatase activity[8]	+	–	+	+	–	–
Arginine hydrolysis[8]	+	+	–[d]	–	–	–
L-Sorbose fermentation[9]	–	–	+	–	–	–
Lactose fermentation[8]	+	+	+	+	+	+
n-Acetyl-β-glucoseaminidase activity[9]	–[d]	–	–	+	–	–
Starch[6]	–	–	–	–	+	–
Arabinose[6]	–	+	+	–	–	–

* + = 90% or more of strains are positive
– = 90% or more of strains are negative
[d] = reactions variable

7. Identification of Individual Species within Fecal Streptococcus and Enterococcus Groups

Table 9230:I shows some of the key biochemical reactions for identifying fecal streptococci, enterococci, and species within these two groups.

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9240 IRON AND SULFUR BACTERIA*

9240 A. Introduction

The group of nuisance organisms collectively designated "iron and sulfur bacteria" is morphologically and physiologically heterogeneous, having in common the ability to transform or deposit significant amounts of iron or sulfur, usually in the form of objectionable slimes. However, iron and sulfur bacteria are not the sole producers of bacterial slimes and in some cases may be associated with slimes of other bacteria.

The iron and sulfur bacteria may be filamentous or single-celled, autotrophic or heterotrophic, aerobic or anaerobic. The taxonomic position of these bacteria is very diverse. They are studied as iron and sulfur bacteria, because these elements and their transformations may be important in water treatment and distribution systems and may be especially bothersome in waters for industrial use, such as cooling and boiler waters. Iron bacteria may cause, or be associated with, fouling and plugging of wells. Also, the growth of these bacteria may result in consumer complaints of red water in distribution systems for potable water and sulfate-reducing bacteria may cause rusty water and tuberculation of pipes. These organisms also may cause odor, taste, frothing, color, and increases in turbidity in waters.

The nutrient supply for iron and sulfur bacteria may be wholly or partly inorganic. Many of these bacteria can grow under oligotrophic conditions when attached to a substrate in flowing water. This seems quite important in the case of certain sulfur bacteria utilizing small amounts of hydrogen sulfide or in the case of organisms such as *Gallionella*, which obtain their energy from the oxidation of ferrous iron. *Thiobacillus ferrooxidans* contributes to the problem of acid mine drainage and can be identified by tests for transformation of ferrous to ferric iron or oxidation of reduced sulfur compounds under conditions of low pH. Temperature, light, pH, and oxygen supply are critical to the growth of iron and sulfur bacteria. Under different environmental conditions some bacteria may appear either as iron or as sulfur bacteria.

* Approved by Standard Methods Committee, 1991.