9222 MEMBRANE FILTER TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9222 A. Introduction

The membrane filter (MF) technique is reproducible, can be used to test relatively large sample volumes, and usually yields numerical results more rapidly than the multiple-tube fermentation procedure. The MF technique is 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 large numbers of noncoliform (background) bacteria. If heterotrophic bacteria interference is exhibited, for example, sample results may need to be invalidated and new samples collected. When the MF technique has not been used previously, it is desirable to conduct parallel tests with the method the laboratory is using currently to demonstrate applicability and comparability. There have been many coliform performance studies reported in the literature, and the rates of false positive and negative results can differ among various media. Users should carefully select the medium and procedure that best fits their needs.

1. Definition

As related to the MF technique, the *coliform group* is defined as facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that develop colonies with distinctive characteristics on specific media. Occasionally on m-Endo media, typical sheen colonies may be produced by noncoliform organisms, and atypical colonies (pink, dark red, or nucleated colonies without sheen) may be coliform bacteria; thus, verification of all typical and atypical colonies is recommended. Details of these characteristics are given below for the standard total coliform MF procedure (9222B) and for two procedures for simultaneous detection of total coliforms and *E. coli* (9222H and I).

a. Endo-type agar medium: Coliform bacteria are defined as bacteria that develop red colonies with a metallic (golden-green) sheen within 24 h at 35°C on an Endo-type medium containing lactose. Some members of the total coliform group also produce dark red, mucoid, 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, pink (non-mucoid), blue, white, or colorless colonies lacking sheen on Endo media are considered noncoliforms by this technique.

b. Dual-chromogen agar medium (mColiBlue24): Coliform bacteria (other than *E. coli*) are defined as those that produce red colonies within 24 h at 35°C on a medium containing lactose and a nonselective dye [2,3,5-triphenoltetrazolium chloride (TTC)]. *E. coli* are distinguished from other coliform bacteria by blue to purple colonies from the action of β-glucuronidase enzyme on 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG), also present in the medium.

c. Fluorogen/chromogen MI medium: Coliform bacteria are defined as bacteria that produce fluorescent colonies upon exposure to longwave ultraviolet (UV) light within 24 h at 35°C on MI medium containing the fluorogen 4-methylumbelliferyl-β-D-galactopyranoside (MUGal). This differential membrane filter medium simultaneously detects and enumerates both total coliforms (TC) and *Escherichia coli* (*E. coli*) in water samples in 24 h based on their specific enzyme activities. Two enzyme substrates—the fluorogen MUGal and a chromogen indoxyl-β-D-glucuronide (IBDG)—are included in the medium to detect the enzymes β-galactosidase and β-glucuronidase produced by TC and *E. coli*, respectively.

2. Applications

The MF technique may be used for testing drinking, surface, ground, swimming pool, and marine waters. Do not use the MF technique to test wastewater receiving only primary treatment unless the sample is diluted, because the high turbidity level may quickly clog the membrane filter before sufficient sample is collected. Chlorinated effluents should have low counts and turbidity. Also, do not use the MF technique to test wastewater containing high levels of toxic metals or toxic organic compounds (e.g., phenols) because such substances may be concentrated by the filter and inhibit coliform growth. For waters other than wastewater, high turbidity levels may clog the filter and high levels of heterotrophic bacteria may interfere with the growth of coliforms on the filter, possibly requiring the use of more than one filter for a sample. This is not a problem with MI medium. To detect 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 thermotolerant coliforms, some of which are of fecal origin, in chlorinated wastewater (Section 9212) 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 ± 2.5 mL. This may be distributed among multiple membranes, if necessary. However, for special monitoring purposes (e.g., troubleshooting water quality problems or identifying coliform breakthrough in low concentrations from treatment barriers), it may be desirable to test 1-L samples. If particulates prevent filtering a 1-L sample through a single filter, divide sample into four 250-mL portions for analysis. Total the coliform counts on each membrane to report the number of coliforms per liter. Smaller sample volumes will be necessary for source or recreational waters and wastewater effluents that have much higher coliform densities.

Statistical comparisons of results obtained by the multiple-tube method and the MF technique show that MF is more

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

precise (compare Tables 9221:III and IV with Table 9222:III). Data from each test yield approximately the same water quality information, although numerical results are not identical.

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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.

a. Sample bottles: See Section 9030B.19.

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. 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, 15- × 60-mm, 9- × 50-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. Incubate loose-lidded glass and disposable plastic culture dishes in tightly closed containers to prevent moisture evaporation with resultant drying of medium and to maintain a humid environment for optimum colony development.

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

f. Filtration units: The filter-holding assembly (constructed of glass, autoclavable plastic, porcelain, stainless steel, or disposable plastic) consists of a seamless funnel fastened to a base by a locking device or by magnetic force. The design should permit the membrane filter to be held securely on the receptacle's porous plate 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. Replace damaged screens on stainless steel units.

Wrap the assembly (as a whole or separate parts) in heavy wrapping paper or aluminum foil, or place in commercially available autoclave bags, sterilize via autoclaving, and store until use. Alternatively, expose all surfaces of the previously cleaned and sterilized assembly to UV radiation (2 min exposure) for the initial sanitization before use in the test procedure, or before reusing units in successive filtration series. Field units may be sanitized by dipping in or spraying with alcohol and then igniting or immersing in boiling water for 2 min. Reagent water should be used to avoid hard-water deposits. After submerging unit in boiling water, cool to room temperature before reuse. Do not ignite plastic parts. Sterile, disposable field units also may be used.

For filtration, mount receptacle of filter-holding assembly on 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 a vacuum line, 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 filters:* Use membrane filters with a rated pore diameter to provide complete retention of coliform bacteria (usually 0.45 μm) (for additional specifications, see Section

9020). Only use filter membranes that have been found, through adequate quality control (QC) testing and certification by the manufacturer, to exhibit the following: full retention of the organisms to be cultivated, stability in use, freedom from chemical extractables that may inhibit bacterial growth and development, a satisfactory filtration speed (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 so bacterial growth is neither inhibited nor stimulated along the grid lines when 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 temperature and humidity extremes. 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 membrane's chemical or physical properties. 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: Disks of filter paper or other material that the manufacturer has certified, by lot, to be of high quality and free of sulfites or other substances 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. Some pads may require 3.0 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 endpoint, pH 8.3, using 0.02 *N* NaOH and produce pH levels of 7.0 ± 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.

i. Forceps: Use smooth blunt forceps, 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× 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 commercial 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 (if necessary). Commercially prepared media in liquid form (sterile ampule or other) may be used if known to give equivalent results. See Section 9020 for media QC specifications.

Test each new medium lot against a previously acceptable lot for satisfactory performance (as described in Section 9020B.5*j*). With each new lot of Endo-type medium, verify a minimum 10% of coliform colonies (obtained from natural samples or samples with known additions) to establish the comparative recovery of the medium lot.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls. Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request new samples.

*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

CAUTION: Basic fuchsin is a suspected carcinogen and mutagen. Avoid skin contact, ingestion, and exposure to mucous membrane. Follow manufacturer's and Material Safety Data Sheet (MSDS) instructions.

Rehydrate product 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 between 45 and 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 petri dishes or 4- to 6-mL quantities into lower section of 50-mm plastic petri dishes and allow to solidify. If dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm. Do not expose poured plates to direct sunlight; refrigerate in the dark, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused medium after 2 weeks [or sooner if there is evidence of moisture loss, medium contamination, medium deterioration (darkening of the medium), or surface sheen formation].

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

* Dehydrated Difco m-Endo Agar LES (No. 273610), or equivalent
† Dehydrated Difco m-Endo Broth MF (No. 274920), dehydrated BBL m-Endo Broth (No. 21119), or equivalent may be used if absorbent pads are used.

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

CAUTION: Basic fuchsin is a suspected carcinogen and mutagen. Avoid skin contact, ingestion, or exposure to mucous membranes. Follow manufacturer's and MSDS instructions.

1) Agar preparation—Rehydrate product in 1 L water containing 20 mL 95% ethanol. Heat to near boiling to dissolve agar, promptly remove from heat, and cool to between 45 and 50°C. Dispense 5- to 7-mL quantities into 60-mm sterile glass or 4- to 6-mL quantities into 50-mm 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.

Refrigerate finished medium in the dark, and discard unused agar after 2 weeks.

2) Broth preparation—Prepare as above, omitting agar. Dispense liquid medium (at least 2.0 mL per plate) onto sterile absorbent pads (see absorbent pad specifications, 9222B.1*h*) and carefully remove excess medium by decanting the plate. 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. Refrigerated broth in screw-capped bottles or flasks may be stored 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. Procedures

a. Selection of sample size: Size of sample will be governed by expected bacterial density and, if applicable, by regulatory requirements.‡ 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).

An ideal sample volume will yield 20 to 80 total coliform colonies and not more than 200 colonies of all types (typical, atypical, and noncoliform background colonies) on a membrane-filter surface (Table 9222:II). Analyze drinking waters by filtering 100 to 1000 mL, or by filtering replicate smaller sample volumes (e.g., duplicate 50-mL portions 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 buffered dilution water to the funnel and then add sample followed by another 25 to 50 mL dilution water before filtration or pipet the sample volume into sterile dilution water and then filter the entire contents of the dilution bottle. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

‡ U.S. EPA Total Coliform Rule (June 29, 1989, *Fed. Reg.* 54:27544) prescribes 100 ± 2.5 mL for sample size.

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

	Volume (X) To Be Filtered *m*L							
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

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 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. (See 9222B.1*f* for sterilization procedures and Section 9020B.4*l* and *m* for UV cleaning and safety guidelines.)

c. Filtration of sample: Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of the base. Carefully place matched funnel unit over base 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 buffered dilution water. Alternatively, rinse funnel by a flow of sterile buffered dilution water from a squeeze bottle (or other appropriate device). This is satisfactory only if the squeeze bottle and its contents do not become contaminated during use. Do not reuse partially filled dilution water bottles. Rinsing between samples prevents carryover contamination. Upon completion of final rinse and filtration process, disengage vacuum, unlock and remove funnel, immediately remove membrane filter with a sterile forceps, and place filter on selected medium with a rolling motion to avoid entrapment of air. 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.

TABLE 9222:II. NUMBERS OF COLONIES IN THE IDEAL RANGE FOR QUANTITATIVE DETERMINATIONS

	Colony Counting Range	
Test	Minimum	Maximum
Total coliform	20	80
Fecal coliform	20	60
Fecal streptococci	20	100
Enterococci	20	60
E. coli	20	60

Place only one membrane filter per plate. Invert dish, and incubate for 22 to 24 h at 35 ± 0.5°C.

If liquid Endo medium is used, aseptically place a sterile pad in the culture dish and saturate it with at least 2.0 to 3.0 mL of medium (depending on the pad manufacturer), and carefully remove excess medium by decanting the plate. Place prepared filter directly on the pad, invert dish, and incubate Endo plate for 22 to 24 h at 35 ± 0.5°C. If loose-lidded plates are used, place plates in a humid chamber (or humidified incubator).

Differentiation of some colonies may be lost if cultures are incubated beyond 24 h.

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.

For nonpotable water samples, preferably rinse and then sanitize the filter unit after each sample (as described above) because of the high number of coliform bacteria present in these samples

d. Alternative enrichment technique: Use this technique with m-Endo media only. Place a sterile absorbent pad in the lid of a sterile culture dish, and pipet at least 2.0 mL lauryl tryptose broth (prepared as directed in Section 9221B.2*a*) to saturate pad. Carefully remove any excess liquid from absorbent pad by decanting plate. Aseptically place filter—through which the sample has been passed—on the 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.

Remove enrichment culture from incubator, lift filter from enrichment pad, and roll it onto the m-Endo agar surface, which has been allowed to equilibrate to room temperature. 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 liquid medium is used, prepare final culture by removing enrichment culture from incubator and separating the dish halves. Place a fresh sterile pad in dish bottom, saturate with at least 2.0 mL of m-Endo medium, and carefully remove excess liquid from absorbent pad by decanting plate. 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 ¶ *e* below.

e. Counting: To determine colony counts on m-Endo membrane filters, use a low-power (10 to 15× magnification) binocular wide-field dissecting microscope or other optical device, with a cool white fluorescent light source directed to provide optimal viewing of sheen. The angle of light on the colony affects the detection of sheen for coliform colonies growing on m-Endo plates. Rocking and turning the petri plate reflects light at different angles and aids in detecting sheen on the colony. The typical coliform colony on m-Endo media has a pink to dark-red color with a metallic surface sheen. Count both typical and atypical coliform colonies. 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, mucoid, or nucleated without sheen. Generally pink, blue, white, or colorless colonies lacking sheen are considered 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. After 22 h incubation, refrigerating cultures with high densities of noncoliform colonies for 0.5 to 1 h before counting may deter spread of noncoliform growth 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.

f. Coliform verification: Occasionally on m-Endo media, typical sheen colonies may be produced by noncoliform organisms, and atypical colonies (pink, dark red, or nucleated colonies without sheen) may be coliforms. Preferably, verify all typical and atypical colony types, but at a minimum, verify at least five typical and five atypical colonies per membrane. For drinking water, verify all colonies on Endo media by swabbing the entire membrane or picking at least five typical colonies and five atypical colonies from a given membrane filter culture. (See Section 9020B.9.) Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, or verify 10% of positive samples). Adjust counts based on verification results. Verification tests are listed below.

1) Lactose fermentation—Transfer growth from each colony, or swab the entire membrane with a sterile cotton swab (for presence–absence results in drinking water samples) and place in lauryl tryptose broth; incubate the lauryl tryptose broth at 35 ± 0.5°C for up to 48 h. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose broth (see Section 9221B.3*a* 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 ± 0.2°C incubation will provide information on the presence of thermotolerant coliforms. Use of EC-MUG with incubation at 44.5 ±0.2°C for 24 h will provide information on presence of *E. coli*. (See 9222G for MF partition procedures.)

2) Alternative coliform verifications—Apply this alternative coliform verification procedure to isolated colonies on the Endo membrane filter media. 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 ensure culture purity or submit the mixed growth to the fermentation tube method.

a) Rapid test—A rapid verification of colonies uses 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 coliform colony by selecting a well-isolated colony, streaking for isolation, and inoculating a pure colony into a multi-test identification system for *Enterobacteriaceae* that includes lactose fermentation and/or β-galactosidase and CO test reactions.

5. Calculation of Coliform Density

Quantitative information only provides a gross estimation of the actual coliform population at collection time due to nonuniform distribution within the matrix. Select the membrane(s) with acceptable number of colonies (Table 9222:II) and not more

than 200 colony-forming units (CFU) of all types per membrane, by the following equation:

$$\text{(Total) coliforms, No./100 mL} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered}} = \text{No. CFU/100 mL}$$

For drinking water samples, if no total coliform colonies are observed, then report the total coliform colonies counted as "<1 CFU/100 mL."

For nonpotable water samples, if 10.0-, 0.1-, and 0.01-mL portions are examined and all counts are 0, then calculate the number of coliforms per 100 mL that would have been reported

$$1/10 \times 100 = <10 \text{ CFU/100 mL}$$

For verified coliform counts, adjust the initial count based on the positive verification percentage and report as follows:

$$\text{Verified (total) coliforms, No./100 mL} = \frac{\text{number of verified colonies}}{\text{total number of coliform colonies subjected to verification}} \times 100$$

If no colonies are found in a drinking water sample, report "total coliforms absent per 100 mL sample."

a. Water of drinking water quality: While the EPA Total Coliform Rule for drinking water samples requires only a record of total coliform presence or absence in 100-mL samples, quantitative information may sometimes be useful in providing an indication of the magnitude of a contaminating event.

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, 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." 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, then report results as "too numerous to count" (TNTC) or "confluent," respectively. For drinking water samples using Endo-type media, the presence of coliforms in such cultures may be confirmed (see Section 9224). As an alternative, brush entire filter surface with a sterile loop, applicator stick, or cotton swab and inoculate this growth into a tube of brilliant green lactose bile broth. If gas is produced from the brilliant green bile broth tube within 48 h at 35 ± 0.5°C, coliforms are present. For compliance with the EPA Total Coliform Rule, report confluent growth or TNTC with at least one detectable coliform colony (verification only required with Endo media) as a total coliform positive sample. Report confluent growth or TNTC without detectable coliforms as invalid. For invalid samples, 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 two separate membranes, 25-mL portions through four separate membranes, etc. For the Total Coliform Rule, report the sample as total coliform positive if any membrane contains a verified total coliform colony. If a density determination is desired, 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, then report the total coliform count as 8 CFU per 100 mL:

$$\frac{[(5 + 3) \times 100]}{(50 + 50)} = 8 \text{ CFU/100 mL}$$

Similarly, if 50-, 25-, and 10-mL portions were examined and the counts were 15, 6, and <1 coliform colonies, respectively, then calculate on the basis of the most nearly acceptable value and report the total coliform count with a qualifying remark as "estimated 30 CFU/100 mL":

$$\frac{[(15) \times 100]}{(50)} = \text{estimated 30 CFU/100 mL}$$

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, then select the 10-mL portion only for calculating the coliform density because this filter had a coliform count falling in the acceptable range (see Table 9222:II), and report result as 400 CFU/100 mL:

$$\frac{(40 \times 100)}{10} = 400 \text{ CFU/100 mL}$$

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

If 10.0-, 1.0-, and 0.1-mL portions were examined with counts of TNTC, 150, and 92 coliform colonies, respectively, then calculate on the basis of the most nearly acceptable value and report, with a qualifying remark, as estimated 92 000 CFU/100 mL:

$$\frac{(92 \times 100)}{0.1} = \text{estimated 92 000 CFU/100 mL}$$

If 1.0-, 0.3-, 0.1-, and 0.03-mL portions were examined with counts of TNTC, TNTC, 78, and 21 coliform colonies, respectively, then sum the total coliform counts on the two filters and divide by the sum of their volume to obtain the final reported value of 76 000 CFU/100 mL:

$$\frac{[(78 + 21) \times 100]}{(0.1 + 0.03)} = 76\,000 \text{ CFU/100 mL}$$

TABLE 9222:III. 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	9.2	26.0
17	9.9	27.2
18	10.7	28.4
19	11.5	29.6
20	12.2	30.8

If 1.0-, 0.3-, and 0.01-mL portions were examined with counts on all portions of TNTC coliform colonies, then calculate using the maximum number of colonies acceptable for quantitative determination for that indicator with the smallest filtration volume and report result as >800 000 CFU/100 mL (for total coliform):

$$\frac{(80 \times 100)}{0.01} = \ > 800\ 000\ \text{CFU/100 mL}$$

c. Statistical reliability of membrane filter results: Although MF results are considered more precise than most probable number (MPN) results, membrane counts may underestimate the number of viable coliform bacteria. Table 9222:III 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:

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

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

6. Bibliography

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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; or (*c*) where the sampling location is remote from laboratory services. (See Section 9060B, Preservation and Storage.)

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 conventional MF methods.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on transport medium, and ship to laboratory. Complete 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 the 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 9222B.4*f*.

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, plastic petri dishes (9 × 50 mm) with tight-fitting lids. Such containers are lightweight and 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 9222B.1*e* for specifications.)

b. Field filtration units: See 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 reagent water to avoid hard-water deposits. Use a hand aspirator to obtain necessary vacuum.

2. Materials and Transport Media

a. m-Endo methods:

1) *m-Endo preservative medium:* Prepare m-Endo medium as described in 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 9- × 50-mm petri plates. Refrigerate poured plates. Discard unused medium after 96 h.

2) *Sodium benzoate solution*: Dissolve 12 g $NaC_7H_5O_2$ in sufficient reagent 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 by aseptically adding 50 mg cycloheximide/100 mL to m-Endo preservative medium. Store cycloheximide solution in refrigerator, and discard after 6 months. **CAUTION: Cycloheximide is a powerful skin irritant. Follow manufacturer's Material Safety Data Sheet (MSDS) instructions for proper handling and storage of this chemical.**

b. m-ST method:

m-ST holding medium:

Sodium phosphate, monobasic, $NaH_2PO_4 \cdot H_2O$	0.1	g
Dipotassium hydrogen phosphate, K_2HPO_4	3.0	g
Sulfanilamide	1.5	g
Ethanol (95%)	10	mL
Tris (hydroxymethyl) aminomethane	3.0	g
Reagent-grade water	1	L

Dissolve ingredients by rehydrating in water. Sterilize by autoclaving at 121°C for 15 min. Final pH should be 8.6 ± 0.2. Dispense at least 2.0 to 3.0 mL (depending on pad manufacturer) to tight-lidded plastic culture dishes containing an absorbent pad, and carefully remove excess liquid from pad by decanting the plate. Store plates in the 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 9222C.2). 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 to prevent membrane dehydration during transit. Place culture dish containing membrane in an appropriate shipping container and send to laboratory for test completion. The sample can be held without visible growth for a maximum of 72 h at ambient temperature on the holding/preservative medium. Visible growth occasionally begins on transport medium when high temperatures are encountered during transit.

b. Transfer and incubation: At the laboratory, transfer filter from holding medium on 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.

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

4. Estimation of Coliform Density

Proceed as in 9222B.5 above. Record times of collection, filtration, and laboratory examination, and calculate the elapsed time. Report elapsed time with coliform results.

5. Bibliography

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9222 D. Thermotolerant (Fecal) Coliform Membrane Filter Procedure

Thermotolerant coliform (also known as *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 thermotolerant coliform.) The thermotolerant coliform MF procedure uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2°C for selectivity. 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 solid heat sink incubator or other incubator that is documented to 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 9221E).

There are limitations to the interpretation of a thermotolerant coliform result from thermal waters (e.g., the tropics), drinking water biofilms, and pulp and paper mill effluent samples where thermotolerant *Klebsiella* have predominated and not been indicative of a sewerage source. As with all coliform results, a sanitary survey should be conducted to identify the most plausible source and public health risk interpretation (see 9222F, *Klebsiella* Membrane Filter Procedure).

According to the World Health Organization, *Klebsiella* spp. do not represent a source of gastrointestinal illness via ingestion of drinking water in the general population. *Klebsiella* spp. detected in drinking water are generally biofilm organisms and are unlikely to represent a health risk.

1. Materials and Culture Medium

a. mFC 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 QC specifications.

mFC 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 product 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 4- to 6-mL quantities to 9- × 50-mm petri plates (approximately 4 to 5 mm deep) and let solidify. Final pH should be 7.4 ± 0.2. Refrigerate finished medium (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.5*j*) 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, mFC 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.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls. Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through filter. If controls indicate contamination, reject all data from affected samples and request new samples.

b. Culture dishes: Tight-fitting plastic dishes are preferred because the membrane filter culture plates are submerged in a water bath during incubation. Place fecal coliform culture plates in plastic bags or seal individual dishes with waterproof (freezer) tape to prevent leakage during submersion. A dry recirculating incubator that can maintain this ±0.2 tolerance can also be used. (Specifications for plastic culture dishes are given in 9222B.1*e*.)

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

TABLE 9222:IV. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER THERMOTOLERANT COLIFORM OR *E. COLI* TEST

	Volume (X) To Be Filtered *m*L							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	X							
Lakes, reservoirs	X	X						
Wells, springs	X	X						
Water supply intake		X	X	X				
Natural bathing waters		X	X	X				
Sewage treatment plant			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	
Sewage sludge						X	X	X

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, slower heat transfer from air to the medium, 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 shown to give equivalent results. A temperature tolerance of 44.5 ± 0.2°C can be obtained with most types of circulating water baths that are also equipped with a gable top for the reduction of water and heat loss.

2. Procedure

a. Selection of sample size: Select volume of water sample to be examined in accordance with the information in Table 9222:IV. 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 the volume and/or dilution expected to yield a countable membrane, and select two additional quantities representing one-tenth and ten times (or one-third and three times) this volume, respectively.

b. Filtration of sample: Follow the same procedure and precautions as prescribed under 9222B.4*c*.

c. Preparation of culture dish: Using aseptic technique, place a sterile absorbent pad in each culture dish and pipet at least 2.0 mL mFC medium (prepared as directed above) to saturate pad. Carefully remove any excess liquid from culture dish by decanting the plate. After filtration, aseptically place sample filter on medium-impregnated pad (as described in 9222B).

As a substrate substitution for the nutrient-saturated absorbent pad, add 1.5% agar to mFC broth (as described in 9222B).

d. Incubation: Place prepared dishes in waterproof plastic bags and seal, invert, and submerge petri dishes in water bath; 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 mFC medium are various shades of blue. Nonfecal coliform colonies are gray to cream-colored. Normally, few nonfecal coliform colonies will be observed on mFC medium because of selective action of the elevated temperature and addition of rosolic acid salt reagent. Count colonies with a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device.

f. Verification: Verify at a frequency established by the laboratory. Verify typical blue colonies and any atypical grey to green colonies as described in Section 9020 for fecal coliform analysis. Simultaneous inoculation at both temperatures is acceptable.

3. Calculation of Fecal Coliform Density

a. General: 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 mFC medium. Calculate fecal coliform density as directed in 9222B.5. Record fecal coliform densities as CFU per l00 mL.

b. Sediment and biosolids samples: For total solids (dry weight basis), see Section 2540G.

Calculate fecal coliforms per gram dry weight for biosolids analysis as follows:

$$\text{Fecal coliform, CFU /g dry weight} = \frac{\text{colonies counted}}{(\text{dilution chosen}) \times (\%\ \text{dry solids})}$$

where dilution and % dry solids are expressed in decimal form.

Example 1: There were 22 colonies observed on the 1:10 000 dilution plate of a biosolids with 4% dry solids.

$$\frac{22}{(0.0001)\ (0.04)} = 5.5 \times 10^6\ \text{CFU / g dry weight}$$

If no filter has a coliform count falling in the ideal range (20 to 60), total the coliform counts on all countable filters, and report as fecal coliforms per gram dry weight:

Example 2: There were 18 colonies observed on the 1:10 000 dilution plate and 2 colonies observed on the 1:100 000 dilution plate of a biosolids sample with 4% dry solids.

$$\frac{(18 + 2)}{(0.0001 + 0.00001)\ (0.04)} = 4.5 \times 10^6$$

To compute a geometric mean of samples, convert coliform densities of each sample to $\log_{10}$ values. Determine the geometric mean for the given number of samples† by averaging the

† Usually seven if collecting for the EPA Pathogen Reduction Rule, 40 CFR Part 503.

$\log_{10}$ values of the coliform densities and taking the antilog of that value.

4. Bibliography

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9222 E. Delayed-Incubation Thermotolerant (Fecal) Coliform Procedure

This delayed-incubation procedure is similar to the delayed-incubation total coliform procedure (9222C). Use the delayed-incubation test only when the standard immediate thermotolerant coliform test cannot be performed (i.e., where the appropriate field incubator is unavailable, 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.

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

The m-ST medium keeps thermotolerant 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 thermotolerant coliform counts.

1. Apparatus

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

2. Materials and Transport Medium

a. m-ST medium: Prepare as described in 9222C.2*b*.
b. mFC medium: Prepare as described in 9222D.1*a*.

3. Procedure

a. Membrane filter transport: Using aseptic technique, place an absorbent pad in a tight-lid plastic petri dish and saturate with m-ST holding medium. After filtering sample, remove the 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 the filter 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 mFC medium.

c. Incubation: After transfering filter to mFC medium, place tight-lid dishes in waterproof plastic bags, invert, and submerge in a water bath at 44.5 ± 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× magnification.

e. Verification: Verify colonies at a frequency established by the laboratory. Verify typical blue colonies and any atypical (grey to green) colonies as described in Section 9020 for fecal coliform analysis.

4. Estimation of Fecal Coliform Density

Count as directed in 9222D.2*e* above and compute fecal coliform density as described in 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.

Klebsiella bacteria may be opportunistic pathogens that can give rise to bacteremia, pneumonia, urinary tract, and several other types of human infection where spread is associated with frequent handling of hospital patients—especially those with impaired immune systems, patients with burns or excessive wounds, etc. *Klebsiella* spp. are also excreted in the feces of many healthy humans and animals, and they are readily detected in sewage-polluted waters. 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 *Klebsiella* sp. 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 mFC agar base through substitution of inositol for lactose and adding carbenicillin or by using mKleb 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 9222B.1*e* for specifications.
b. Filtration units: See 9222B.1*f*.

2. Materials and Culture Medium

a. Modified mFC agar (mFCIC 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 4- to 6-mL quantities into 9- × 50-mm plastic petri dishes (approximate depth of 4 to 5 mm). Refrigerate until needed. Discard unused agar medium after 2 weeks. Do not sterilize by autoclaving. Final pH should be 7.4 ± 0.2.

b. mKleb 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 9- × 50-mm plastic culture plates. The final pH should be 7.4 ± 0.2. Refrigerated medium can be held for 20 d at 4 to 8°C.

3. Procedure

a. See 9222B.4 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 mFCIC 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 mKleb 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× magnification) 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), 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

1. *Escherichia coli* Partition Methods

a. Applications: Escherichia coli is a member of the thermotolerant coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification for *E. coli* may be achieved for a total-coliform- or fecal-coliform-positive MF sample by using media 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.

When examining drinking water samples, use one of the partition methods to determine the presence of *E. coli* from a total-coliform-positive MF sample on Endo-type media. When examining wastewater and other nonpotable water samples, use one of the partition methods to determine the presence of *E. coli* from thermotolerant (fecal)-coliform-positive MF samples on mFC media.

b. Apparatus:

1) *Culture dishes:* See 9222B.1*e*.
2) *Filtration units:* See 9222B.1*f*.
3) *Forceps:* See 9222B.1*i*.
4) *Incubator:* See 9222B.1*j*.
5) *Ultraviolet lamp,* long wave (366 nm), 6 W.
6) *Microscope* and *light source:* See 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 4- to 6-mL quantities aseptically into 50-mm plastic culture plates (approximate depth of 4 to 5 mm) and allow to solidify. Final pH should be 6.8 ± 0.2. Refrigerated prepared medium may be held for 2 weeks.

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	1.5	g
Sodium chloride, NaCl	5.0	g
4-methylumbelliferyl-β-D-glucuronide	0.05	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:

1) Selection of sample size and filtration procedure—See 9222B.4.

2) Total coliform verification—For drinking water samples using Endo-type medium, total coliform verification procedures can be performed before or after the partition method. Swab surface growth on the m-Endo filter or, if quantification is desired, transfer small portions of each target colony on m-Endo filter to the appropriate total coliform verification medium using a sterile needle. Alternatively, after transferring filter to NA-MUG media, incubating, and reading the results on this media, either transfer individual colonies, swab surface growth on filter, or place whole filter into appropriate total coliform verification medium. (See 9222B.4 for total coliform verification procedures.)

3) Partition method for *E. coli* determination—If NA-MUG medium is used, aseptically transfer membrane filter with at least one coliform-positive colony to NA-MUG plate. If quantification is desired, mark each sheen colony with a fine-tipped marker on reverse side of plate or puncture a hole in membrane filter adjacent to the colony with a sterile needle after transfer of membrane to NA-MUG medium. Incubate NA-MUG at 35 ± 0.5°C for 4 h. If EC-MUG medium is used, use aseptic technique to transfer total coliform-positive colonies on the membrane filter to a tube containing EC-MUG medium by one of the following methods: (*a*) remove membrane containing total coliform colonies from the substrate with sterile forceps and carefully curl and insert membrane into tube of EC-MUG medium, (*b*) swab entire membrane filter surface with a sterile cotton swab and transfer the inoculum to EC-MUG medium (do not leave cotton swab in EC-MUG medium), or (*c*) if quantification is desired, inoculate individual total coliform-positive colonies into separate EC-MUG tubes. Incubate EC-MUG media at 44.5 ± 0.2°C for 24 ± 2 h.

Observe individual colonies or tubes using a long-wavelength (366-nm) UV light source, preferably containing a 6-W bulb. The presence of a bright blue fluorescence in the tube, on the

periphery (outer edge) of a colony, or observed from the back of the plate is a positive response for *E. coli*. Record presence or absence of fluorescence, or if quantification is desired, count and record the number of target colonies. For nonpotable water samples, this partition method can be used to determine *E. coli* from the fecal coliform MF procedure using 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.

For the EC-MUG method, a positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret sample results and to avoid confusion of weak autofluorescence of the medium as a positive response. (See Section 9221F.)

2. Thermotolerant (Fecal) Coliform Partition Method

a. Applications: In a drinking water sample, thermotolerant coliform determination can be performed from a total-coliform-positive MF filter within 24 h. This technique may be applicable to other waters if warranted.

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

c. Procedure:

1) Selection of sample size and filtration procedure—See 9222B.4.

2) Total coliform verification—Verify total coliforms before using the fecal coliform partition method. Swab surface growth on the total-coliform-positive filter or, if quantification is desired, transfer small portions of each target colony on the filter to the appropriate total coliform verification medium using a sterile needle. See 9222B.4*f* for total coliform verification procedures.

3) Partition method for fecal coliform determination—Using aseptic technique, transfer total-coliform-positive colonies from the membrane filter to a tube containing EC medium by one of the following methods: (*a*) remove membrane containing the total coliform colonies from the substrate with sterile forceps and carefully curl and insert membrane into tube of EC medium, (*b*) swab the entire membrane filter surface with a sterile cotton swab and transfer the inoculum to EC medium (do not leave the cotton swab in the medium), or (*c*) if quantification is desired, inoculate individual total coliform-positive colonies into separate EC tubes. 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.

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9222 H. Simultaneous Detection of Total Coliform and *E. coli* by Dual–Chromogen Membrane Filter Procedure (PROPOSED)

1. Laboratory Apparatus

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

a. Sample bottles: See Section 9030B.19.

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

c. Pipets, sample containers, and graduated cylinders: See 9222B.1*c*.

d. Culture dishes: See 9222B.1*e*.

e. Filtration units: See 9222B.1*f*.

f. Membrane filters: See 9222B.1*g*.

g. Absorbent pads: See 9222B.1*h*.

h. Forceps: See 9222B.1*i*.

i. Incubators: See 9222B.1*j*.

2. Culture Medium

Purchase this medium* from a commercial vendor; it cannot be prepared from basic ingredients. See Section 9020 for media QC specifications.

Before use, test each lot with positive and negative culture controls. Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request new samples.

* m-ColiBlue24® broth, Hach Company, Loveland, CO; Millipore Corporation, Billerica, MA.

L-Methionine	0.1	g
Methylene blue	0.016	g
Casitone	8.0	g
Yeast extract	0.5	g
Lactose	0.6	g
Sodium chloride, NaCl	3.0	g
Dipotassium hydrogen phosphate, K_2HPO_4	1.75	g
Potassium dihydrogen phosphate, KH_2PO_4	1.25	g
Triphenyl tetrazolium chloride	0.07	g
Sodium pyruvate	1.0	g
Erythromycin	3.0	g
Octyphenol ethoxylate†	0.5	g
Magnesium sulfate, $MgSO_4$	0.3	g
5-bromo-4-chloro-3-indolyl-β-D–glucuronic acid (proprietary)		
Sodium azide	0.02	g
Cyclohexylammonium salt	0.2	g
Reagent-grade water	1	L

CAUTION: Sodium azide is highly toxic and mutagenic. Follow manufacturer's MSDS instructions for proper storage and handling of this medium.

Mix broth gently by inverting ampules two or three times before dispensing. Pour liquid medium (approximately 2.0 mL per plate) evenly onto sterile absorbent pads and place lid on petri dish. Final pH should be 7.0 ± 0.2.

3. Procedure

a. Selection of sample size: See 9222B.4*a.*

b. Sterile filtration units: See 9222B.4*b.*

c. Filtration of sample: See 9222B.4*c*.

d. Counting: To count colonies on membrane filters, use a low-powered (10 to 15×) binocular wide-field dissecting microscope or other optical device with a cool white fluorescent light source directed to provide optimal viewing. Count all red and blue to purple colonies under normal/ambient light and record as the total coliform result. Count only blue to purple colonies and record as *E. coli* result. Clear or white colonies are considered noncoliform colonies. A high noncoliform count may interfere with the development of coliform colonies.

† Triton X-114.

e. Coliform verification: For drinking water, total coliform and colony verification is not required for this medium. For waters other than drinking water, verify at a frequency established by the laboratory (see Section 9020B.9). Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of the positive samples) (see Section 9020B.9). Adjust counts based on verification results. Verification tests are listed in 9222B.4*f.*

4. Calculation of Coliform Density

See 9222B.5. Calculate the final values using the formula:

$$E.coli/100\text{ mL} = \frac{\text{number of blue-purple colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

$$\text{Total coliforms/100 mL} = \frac{\text{number of red } \textit{and} \text{ blue to purple colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

5. Bibliography

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9222 I. Simultaneous Detection of Total Coliform and *E. coli* by Fluorogen/Chromogen Membrane Filter Procedure (PROPOSED)

1. Laboratory Apparatus

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

a. Sample bottles: See Section 9030B.19.

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

c. Pipets, sample containers, and graduated cylinders: See 9222B.1*c*.

d. Culture dishes: See 9222B.1*e*.

e. Filtration units: See 9222B.1*f.*

f. Membrane filters: See 9222B.1*g*.

g. Absorbent pads: See 9222B.1*h*.

h. Forceps: See 9222B.1*i*.
i. Incubators: See 9222B.1*j*.

2. Culture Media

The need for uniformity dictates the use of commercial 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 (if necessary). Commercially prepared media in liquid form (sterile ampule or other) may be used if known to give equivalent results. See Section 9020 for media QC specifications.

Test each new medium lot against a previously acceptable lot for satisfactory performance (as described in Section 9020B).

a. Cefsulodin solution, 1 mg/1 mL: Add 0.02 g of cefsulodin to 20 mL reagent-grade distilled water, sterilize using a 0.22-μm syringe filter, and store in a sterile tube at 4°C until needed. Prepare fresh solution each time MI medium is made. Do not save the unused portion.

*b. MI agar:**

Proteose peptone No. 3	5.0	g
Yeast extract	3.0	g
β-D-Lactose	1.0	g
4-Methylumbelliferyl-β-D-galactopyranoside (MUGal) (final concentration 100 μg/mL)	0.1	g
Indoxyl-β-D-glucuronide (IBDG) (final concentration 320 μg/mL)	0.32	g
NaCl	7.5	g
K_2HPO_4	3.3	g
KH_2PO_4	1.0	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Agar	15.0	g
Reagent-grade distilled water	1	L

Autoclave medium for 15 min at 121°C, and add 5 mL of freshly-prepared cefsulodin (¶ *a* above) solution (5 μg/mL final concentration) per liter of tempered agar medium. Final pH should be 6.95 ± 0.2. Pipet medium into 9- × 50-mm petri dishes (5 mL/plate). Store plates at 4°C for up to 2 weeks.

c. MI broth:† Use same ingredients as MI agar, but omit agar. Prepare and sterilize, and add cefsulodin by the methods described for MI agar. Alternatively, the broth can be filter-sterilized. Final pH should be 7.05 ± 0.2. Place absorbent pads in 9- × 50-mm petri dishes and saturate with 2.0 to 3.0 mL MI broth containing 5 μg/mL final concentration of cefsulodin. Store plates in the refrigerator and discard after 96 h. Pour off excess broth before using the plates.

3. Procedure

a. Selection of sample size: See 9222B.4*a*.
b. Sterile filtration units: See 9222B.4*b*.
c. Filtration of sample: See 9222B.4*c*, with the following exception: incubate MI broth plates *grid-side up* at 35 ± 0.5°C for 22 to 24 h.

d. Counting: To count colonies on membrane filters, use a low-powered (10 to 15×) binocular wide-field dissecting microscope or other optical device with a cool white fluorescent light source directed to provide optimal viewing. Count all blue colonies on each MI plate under normal/ambient light and record as *E. coli* results. Positive results that occur in less than 24 h are valid, but results cannot be recorded as negative until the 24-h incubation period is complete. Expose each MI plate to long-wave UV light (366 nm), and count all fluorescent colonies [blue/green fluorescent *E. coli*, blue/white fluorescent TC other than *E. coli*, and blue/green with fluorescent edges (also *E. coli*)] to obtain the TC count. Record the data. Add any blue, nonfluorescent colonies (if any) found on the same plate to the TC count.

Calculate the final values using the formula:

$$E.coli/100\text{ mL} = \frac{\text{number of blue colonies}}{\text{volume of sample filtered (mL)}} \times 100$$

$$\text{TC}/100\text{ mL} = \frac{\text{number of fluorescent colonies} + \text{number of blue, non-fluorescent colonies (if any)}}{\text{volume of sample filtered (mL)}} \times 100$$

e. Coliform verification: For drinking water, total coliform colony verification is not required for this medium on coliform colonies from MI media. For waters other than drinking water, verify at a frequency established by the laboratory (see Section 9020B.9). Based on need and sample type, laboratories may incorporate more stringent QC measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of positive samples) (see Section 9020B.9). Adjust counts based on verification results. Verification tests are listed in 9222B.4*f*.

4. Calculation of Coliform Density

See 9222B.5.

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* BBL™ MI prepared plates (No. 214986), or equivalent.
† Dehydrated Difco™ MI Broth (No. 214882), or equivalent.