IDEXX
Literature Cover Sheet

IDEXX #: 5K

Title: 9222 Membrane Filter Technique for Members of the Coliform Group

Author(s): APHA, AWWA, WEF

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Source: Standard Methods for the Examination of Water and Wastewater

Topic: Membrane Filtration (MF) total coliform and fecal coliform test procedure

Highlights:

- MF is a 1 to 5-day test.
- It involves numerous steps (see pages 9-57 - 9-62):
  1. Media Preparation
  2. Filtration
  3. Incubation of filter for 24 hours at 35°C on Endo-type media
  4. Observation of colony growth for typical and atypical morphology
  5. Confirmation of potential coliform positive colonies in BGLB for 48 hours at 35°C.
  6. A fecal coliform test using EC Medium for 24 hours at 44.5°C.
- It is recommended to read colony counts with 20-80 coliform colonies and not more than 200 total colonies. (page 9-60)
- Membrane colony counts are not absolute (Table 9222:II illustrates 95% confidence limits). (page 9-61)
- A verification step on typical colonies is required and verification on atypical colonies is recommended. (page 9-56 & 9-60)
- The fecal coliform test uses an enriched lactose medium and an incubation temperature of 44.5 ± 0.2°C for 24 ± 2 hours to differentiate between coliforms found in the feces of warm blooded animals and those from other environmental sources. (page 9-63 – 9-65)
- EC-MUG is a specific procedure used to test for *E. coli*. (page 9-67 9-68)

Standard Methods

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Examination of Water and Wastewater

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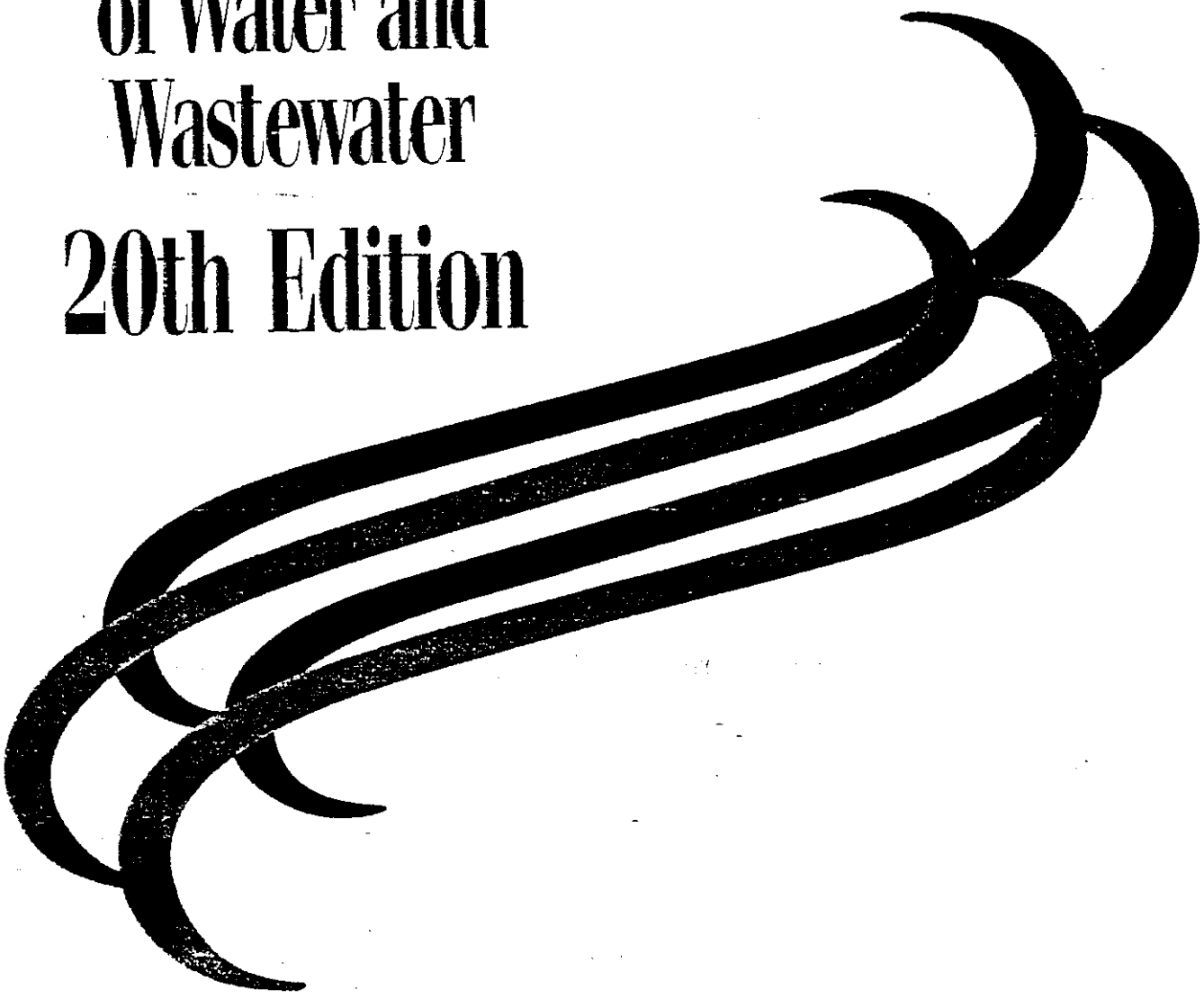

Potassium dihydrogen phosphate, KH_2PO_4	1.5 g
Sodium chloride, NaCl	5.0 g
4-methylumbelliferyl-β-D-glucuronide (MUG)	0.05 g
Reagent-grade water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. pH should be 6.9 ± 0.2 after sterilization. Before sterilization, dispense in tubes that do not fluoresce under long-wavelength (366 nm) ultraviolet (UV) light. An inverted tube is not necessary. Close tubes with metal or heat-resistant plastic caps.

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

1) Gently shake or rotate presumptive fermentation tubes or bottles showing growth, gas, or acidity. Using a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from presumptive fermentation tube or bottle to EC-MUG broth.

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

c. Interpretation: Examine all tubes exhibiting growth for fluorescence using a long-wavelength UV lamp (preferably 6 W). The presence of bright blue fluorescence is considered a positive response for *E. coli*. A positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret the results and to avoid confusion of weak auto-fluorescence of the medium as a positive response. If multiple tubes are used, calculate MPN from the number of positive EC-MUG broth tubes as described in Section 9221C. When using only one tube or subculturing from a single presumptive bottle, report as presence or absence of *E. coli*.

2. Bibliography

FENG, P.C.S. & P.A. HARTMAN. 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli*. *Appl. Environ. Microbiol.* 43:1320.

HARTMAN, P.A. 1989. The MUG (glucuronidase) test for *E. coli* in food and water. *In* A. Balows et al., eds., Rapid Methods and Automation in Microbiology and Immunology. Proc. 5th Intl. Symp. on Rapid Methods and Automation in Microbiology & Immunology, Florence, Italy, Nov. 4–6, 1987.

SHADIX, L.C. & E.W. RICE. 1991. Evaluation of β-glucuronidase assay for the detection of *Escherichia coli* from environmental waters. *Can. J. Microbiol.* 37:908.

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 usually yields numerical results more rapidly than the multiple-tube fermentation 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 large numbers of noncoliform (background) bacteria. 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.

1. Definition

As related to the MF technique, the coliform group is defined as those 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, 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 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.

*Approved by Standard Methods Committee, 1997.

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

The standard volume to be filtered for drinking water samples is 100 mL. This may be distributed among multiple membranes if necessary. However, for special monitoring purposes, such as troubleshooting water quality problems or identification of 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 portions of 250 mL 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 the MF is more precise (compare Tables 9221: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).

3. Bibliography

CLARK, H.F., E.E. GELDREICH, H.L. JETER & P.W. KABLER. 1951. The membrane filter in sanitary bacteriology. *Pub. Health Rep.* 66:951.

KABLER, P.W. 1954. Water examinations by membrane filter and MPN procedures. *Amer. J. Pub. Health* 44:379.

THOMAS, H.A. & R.L. WOODWARD. 1956. Use of molecular filter membranes for water potability control. *J. Amer. Water Works Assoc.* 48: 1391.

MCCARTHY, J.A., J.E. DELANEY & R.J. GRASSO. 1961. Measuring coliforms in water. *Water Sewage Works* 108:238.

LIN, S. 1973. Evaluation of coliform test for chlorinated secondary effluents. *J. Water Pollut. Control Fed.* 45:498.

MANDEL, J. & L.F. NANNI. 1978. Measurement evaluation. *In* S.L. Inhorn, ed. Quality Assurance Practices for Health Laboratories, p. 209. American Public Health Assoc., Washington, D.C.

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.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. 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. Incubate loose-lidded glass and disposable plastic culture dishes in tightly closed containers with wet paper or cloth 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 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 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.

Wrap the assembly (as a whole or separate parts) in heavy wrapping paper or aluminum foil, sterilize by autoclaving, and store until use. Alternatively expose all surfaces of the previously cleaned assembly to ultraviolet radiation (2 min exposure) for the initial sanitization before use in the test procedure, or before reusing units between successive filtration series. Field units may be sanitized by dipping or spraying with alcohol and then igniting 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 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 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 flat 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 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 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. Commercially prepared media in liquid form (sterile ampule or other) 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 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 resample.

*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 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 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 of 4 to 5 m. 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, or medium deterioration (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 product 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.

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 absorbent pads (see absorbent pad specifications, Section 9222B.1) 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

* Rehydrated Difco M-Endo Agar LES (No. 0736), dehydrated BBL M-Endo Agar (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.

agents at a concentration that could inhibit bacterial growth. Refrigerated broth 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. Coliform Definition

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.

5. Procedures

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 non[illegible]orm growth on the medium (Table 9222:I). For regulation purpo[illegible]s, 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 bottle, 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. See Section 9222B.1*f* for sterilization procedures and Section 9020B.2*m* and *n* 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 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. If the agar-based medium is used, place prepared filter directly on agar, 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 at least 2.0 mL M-Endo medium and carefully remove excess medium by decanting the plate. 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.

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 decontaminate filter unit after each sample (as described above) because of the high number of coliform bacteria present in these samples. Alternatively, use an additional buffer rinse of the filter unit after the filter is removed to prevent carryover between samples.

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	
Chlo[illegible]d sewage				X	X	X		
Raw [illegible]ge					X	X	X	X

d. Alternative enrichment technique: 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 9221B.1.*a*1), 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 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, 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 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 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 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 with a metallic surface sheen. Count both typical and atypical coliform colonies. The sheen area may vary in size from a small nhead to complete coverage of the colony surface. Atypical coorm 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. 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.

f. Coliform verification: Occasionally, typical sheen colonies may be produced by noncoliform organisms and atypical colonies (dark red or nucleated colonies without sheen) may be coliforms. Preferably verify all typical and atypical colony types. For drinking water, verify all suspect colonies by swabbing the entire membrane or pick at least five typical colonies and five atypical colonies from a given membrane filter culture. For waters other than drinking water, at a minimum, verify at least 10 sheen colonies (and representative atypical colonies of different morphological types) from a positive water sample monthly. See Section 9020B.8. Based on need and sample type, laboratories may incorporate more stringent quality control measures (e.g., verify at st one colony from each typical or atypical colony type from given membrane filter culture, verify 10% of the positive samples). Adjust counts on the basis of 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 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 ± 0.2°C incubation will provide information on the presence of fecal 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 Section 9222G for MF partition procedures.

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:

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

If no coliform colonies are observed, report the coliform colonies counted as "<1 coliform/100 mL."

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

With water of good quality, the occurrence of coliforms generally will be minimal. Therefore, count all coliform colonies (dis-

regarding 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, report results as "too numerous to count" (TNTC) or "confluent," respectively. For drinking water, the presence of coliforms in such cultures showing no sheen may be confirmed by either transferring a few colonies or 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 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 (which is verified) 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 each of four membranes, etc. Total the coliform counts observed on all membranes and report as number per 100 mL.

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.,

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

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.,

$$\frac{[(15 + 6 + 0) \times 100]}{(50 + 25 + 10)} = 25 \text{ coliforms/100 mL}$$

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, 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.,

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

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. 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

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 precision 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:

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

7. Bibliography

FIFIELD, C.W. & C.P. SCHAUFUS. 1958. Improved membrane filter medium for the detection of coliform organisms. *J. Amer. Water Works Assoc.* 50:193.

MCCARTHY, J.A. & J.E. DELANEY. 1958. Membrane filter media studies. *Water Sewage Works* 105:292.

RHINES, C.E. & W.P. CHEEVERS. 1965. Decontamination of membrane filter holders by ultraviolet light. *J. Amer. Water Works Assoc.* 57: 500.

GELDREICH, E.E., H.L. JETER & J.A. WINTER. 1967. Technical considerations in applying the membrane filter procedure. *Health Lab. Sci.* 4:113.

WATLING, H.R. & R.J. WATLING. 1975. Note on the trace metal content of membrane filters. *Water SA* 1:28.

LIN, S.D. 1976. Evaluation of Millipore HA and HC membrane filters for the enumeration of indicator bacteria. *Appl. Environ. Microbiol.* 32:300.

STANDRIDGE, J.H. 1976. Comparison of surface pore morphology of two brands of membrane filters. *Appl. Environ. Microbiol.* 31:316.

GELDREICH, E.E. 1976. Performance variability of membrane filter procedure. *Pub. Health Lab.* 34:100.

GRABOW, W.O.K. & M. DU PREEZ. 1979. Comparison of m-Endo LES, MacConkey and Teepol media for membrane filtration counting of total coliform bacteria in water. *Appl. Environ. Microbiol.* 38:351.

DUTKA, B.D., ed. 1981. Membrane Filtration Applications, Techniques and Problems. Marcel Dekker, Inc., New York, N.Y.

EVANS, T.M., R.J. SEIDLER & M.W. LECHEVALLIER. 1981. Impact of verification media and resuscitation on accuracy of the membrane filter total coliform enumeration technique. *Appl. Environ. Microbiol.* 41: 1144.

FRANZBLAU, S.G., B.J. HINNEBUSCH, T.M. KELLEY & N.A. SINCLAIR. 1984. Effect of noncoliforms on coliform detection in potable groundwater: improved recovery with an anaerobic membrane filter technique. *Appl. Environ. Microbiol.* 48:142.

MCFETERS, G.A., J.S. KIPPIN & M.W. LECHEVALLIER. 1986. Injured coliforms in drinking water. *Appl. Environ. Microbiol.* 51:1.

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.

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 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*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 (50 × 12 mm) with tight-fitting lids. 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 M-Endo medium 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. 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. 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

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 mL to tight-lidded plastic culture dishes containing an absorbent pad and carefully remove excess liquid from pad by decanting plate. Store in refrigerator for use within 96 h.

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

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 to 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 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.

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.

5. Bibliography

GELDREICH, E.E., P.W. KABLER, H.L. JETER & H.F. CLARK. 1955. A delayed incubation membrane filter test for coliform bacteria in water. *Amer. J. Pub. Health* 45:1462.

PANEZAI, A.K., T.J. MACKLIN & H.G. COLES. 1965. *Coli-aerogenes* and *Escherichia coli* counts on water samples by means of transported membranes. *Proc. Soc. Water Treat. Exam.* 14:179.

BREZENSKI, F.T. & J.A. WINTER. 1969. Use of the delayed incubation membrane filter test for determining coliform bacteria in sea water. *Water Res.* 3:583.

CHEN, M. & P.J. HICKEY. 1986. Elimination of overgrowth in delayed-incubation membrane filter test for total coliforms by M-ST holding medium. *Appl. Environ. Microbiol.* 52:778.

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. 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.

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 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 5- to 7-mL quantities to 50- × 12-mm petri plates 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, 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.

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

* 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.

water through filter. If controls indicate contamination, reject all data from affected samples and request resample.

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, 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 water baths that also are equipped with a gable top for the reduction of water and heat losses.

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 at least 2.0 mL M-FC medium, prepared as directed above, to saturate pad. Carefully remove any excess liquid from culture dish by decanting the plate. 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 dishes 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 coliform colonies will be observed on M-FC 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 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 M-FC medium. Calculate fecal coliform density as directed in Section 9222B.6 above. Record densities as fecal coliforms per 100 mL.

b. Sediment and biosolid samples: For total solid (dry weight basis) see Section 2540G.

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

$$\text{Fecal coliforms per gram dry weight} = \frac{\text{colonies counted}}{(\text{dilution chosen}) \times (\%\text{ dry solids})}$$

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 biosolid with 4% dry solids.

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

If no filter has a coliform count falling in the ideal range (20

Table 9222:III. Suggested Samples Volumes for Membrane Filter Fecal Coliform Test

	Volume (*X*) To Be Filtered, *mL*							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
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

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 biosolid 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 (usually seven) by averaging the $\log_{10}$ values of the coliform densities and taking the antilog of that value.

4. Bibliography

GELDREICH, E.E., H.F. CLARK, C.B. HUFF & L.C. BEST. 1965. Fecal-coliform-organism medium for the membrane filter technique. *J. Amer. Water Works Assoc.* 57:208.

ROSE, R.E., E.E. GELDREICH & W. LITSKY. 1975. Improved membrane filter method for fecal coliform analysis. *Appl. Microbiol.* 29:532.

LIN, S.D. 1976. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. *Appl. Environ. Microbiol.* 32:547.

PRESSWOOD, W.G. & D.K. STRONG. 1978. Modification of M-FC medium by eliminating rosolic acid. *Appl. Environ. Microbiol.* 36:90.

GREEN, B.L., W. LITSKY & K.J. SLADEK. 1980. Evaluation of membrane filter methods for enumeration of faecal coliforms from marine waters. *Mar. Environ. Res.* 67:267.

SARTORY, D.P. 1980. Membrane filtration faecal coliform determinations with unmodified and modified M-FC medium. *Water SA* 6:113.

GRABOW, W.O.K., C.A. HILNER & P. COUBROUGH. 1981. Evaluation of standard and modified M-FC, MacConkey, and Teepol media for membrane filter counting of fecal coliform in water. *Appl. Environ. Microbiol.* 42:192.

RYCHERT, R.C. & G.R. STEPHENSON. 1981. Atypical *Escherichia coli* in streams. *Appl. Environ. Microbiol.* 41:1276.

PAGEL, J.E., A.A. QURESHI, D.M. YOUNG & L.T. VLASSOFF. 1982. Comparison of four membrane filter methods for fecal coliform enumeration. *Appl. Environ. Microbiol.* 43:787.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1992. Environmental Regulations and Technology. Control of Pathogens and Vector Attraction in Sewage Sludge. EPA-626/R-92-013, Washington, D.C.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1993. Standards for the Use or Disposal of Sewage Sludge: Final Rule. 40 CFR Part 257; *Federal Register* 58:9248, Feb. 19, 1993.

9222 E. Delayed-Incubation Fecal Coliform Procedure

This delayed-incubation procedure is similar to the delayed-incubation total coliform procedure (Section 9222C). Use the delayed-incubation test only when the standard immediate fecal coliform test cannot be performed (i.e., 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.

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 (grey to green) 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

CHEN, M. & P.J. HICKEY. 1983. Modification of delayed-incubation procedure for detection of fecal coliforms in water. *Appl. Environ. Microbiol.* 46:889.

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. Refrigerate 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. Refrigerated medium can be held for 20 d at 4 to 8°C.

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), 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.

4. Bibliography

DUNCAN, D.W. & W.E. RAZELL. 1972. *Klebsiella* biotypes among coliforms isolated from forest environments and farm produce. *Appl. Microbiol.* 24:933.

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

STRAMER, S.L. 1976. Presumptive identification of *Klebsiella pneumoniae* on M-FC medium. *Can. J. Microbiol.* 22:1774.

BAGLEY, S.T. & R.J. SEIDLER. 1977. Significance of fecal coliform-positive *Klebsiella*. *Appl. Environ. Microbiol.* 33:1141.

KNITTEL, M.D., R.J. SEIDLER, C. EBY & L.M. CABE. 1977. Colonization of the botanical environment by *Klebsiella* isolates of pathogenic origin. *Appl. Environ. Microbiol.* 34:557.

EDMONSON, A.S., E.M. COOK, A.P.D. WILCOCK & R. SHINEBAUM. 1980. A comparison of the properties of *Klebsiella* isolated from different sources. *J. Med. Microbiol.* (U.K.) 13:541.

SMITH, R.B. 1981. A Critical Evaluation of Media for the Selective Identification and Enumeration of *Klebsiella*. M.S. thesis, Dep. Civil & Environmental Engineering, Univ. Cincinnati, Ohio.

NIEMELA, S.I. & P. VAATANEN. 1982. Survival in lake water of *Klebsiella pneumoniae* discharged by a paper mill. *Appl. Environ. Microbiol.* 44:264.

GELDREICH, E.E. & E.W. RICE. 1987. Occurrence, significance, and detection of *Klebsiella* in water systems. *J. Amer. Water Works Assoc.* 79:74.

DUNCAN, I.B.R. 1988. Waterborne *Klebsiella* and human disease. *Toxicity Assess.* 3:581.

9222 G. MF Partition Procedures

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.

In the examination of drinking water samples, use this method to verify the presence of *E. coli* from a total-coliform-positive MF on Endo-type media. 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. 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	1.4 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. See Section 9222B.5 for total coliform verification procedures.

Alternatively, after transfer and incubation on NA-MUG, swab the surface growth on the filter and transfer to the appropriate total coliform verification medium. Aseptically transfer the membrane from the Endo-type medium to NA-MUG or EC-MUG medium. If differentiation 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 ±2 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.

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 nec-

essary to interpret the results and to avoid confusion of weak autofluorescence of the medium as a positive response. See Section 9221F.

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.

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 Endotype 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*f*). 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

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1989. Drinking Water; National Primary Drinking Water Regulations; Total Coliforms (Including Fecal Coliforms and *E. coli*); Final Rule. 40 CFR Parts 141 and 142. *Federal Register* 54:27544, June 29, 1989.

MATES, A. & M. SHAFFER. 1989. Membrane filtration differentiation of *E. coli* from coliforms in the examination of water. *J. Appl. Bacteriol.* 67:343.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1991. National Primary Drinking Water Regulations; Analytical Techniques; Coliform Bacteria. 40 CFR Part 141. *Federal Register* 56:636, Jan. 8, 1991.

MATES, A. & M. SHAFFER. 1992. Quantitative determination of *Escherichia coli* from coliforms and fecal coliforms in sea water. *Microbios* 71:27.

SARTORY, D. & L. HOWARD. 1992. A medium detecting beta-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Lett. Appl. Microbiol.* 15:273.

SHADIX, L.C., M.E. DUNNIGAN & E.W. RICE. 1993. Detection of *Escherichia coli* by the nutrient agar plus 4-methylumbelliferyl-β-D-glucuronide (MUG) membrane filter method. *Can. J. Microbiol.* 39: 1066.

9223 ENZYME SUBSTRATE COLIFORM TEST*

9223 A. Introduction

The enzyme substrate test utilizes hydrolyzable substrates for the simultaneous detection of total coliform bacteria and *Escherichia coli* enzymes. When the enzyme 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, multi-well, 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) or chlorophenol red-β-D-galactopyranoside (CPRG), 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 a positive test for total coliforms at 24 h (ONPG) or 28 h (CPRG) without additional procedures. Noncoliform bacteria, such as *Aeromonas* and *Pseudomonas* species, may produce small amounts of the enzyme β-D-galactosidase, but are suppressed and generally will not produce a positive response within the incubation time unless more than 10^4 colony-forming units (CFU)/mL (10^6 CFU/100 mL) are present.

b. Escherichia coli: A fluorogenic substrate, such as 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 enzyme substrate coliform test is recommended for the analysis of drinking and source water samples. Formulations also are available for the analysis of marine waters. Initially, laboratories planning to use this procedure should conduct parallel quantitative testing (including seasonal variations) with one of the standard coliform tests to assess the effectiveness of the test for

* Approved by Standard Methods Committee, 1997.