STANDARD METHODS 9222 B. STANDARD TOTAL COLIFORM MEMBRANE FILTER PROCEDURE,

STANDARD METHODS 9222 D. FECAL COLIFORM MEMBRANE FILTER PROCEDURE, AND

STANDARD METHODS 9222G: MF PARTITION PROCEDURES *ESCHERICHIA COLI* PARTITION METHODS

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STANDARD METHODS 9222 B. STANDARD TOTAL COLIFORM MEMBRANE FILTER PROCEDURE

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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:[1]

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	1.0 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).

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

b. M-Endo medium:[2]

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..... .1.0 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 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.

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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..... ..1.0 L

Rehydrate product in 1 L water containing 10 mL 1% rosolic acid in 0.2*N* NaOH.[3] 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

[3] 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.

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 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.

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9222 G. MF PARTITION PROCEDURES
ESCHERICHIA COLI PARTITION METHODS

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-$-D-glucuronide (MUG). In this method *E. coli* is defined as any coliform that produces the enzyme $-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.

1. Laboratory Apparatus

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

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

c. Forceps: See Section 9222B.1*i*.

d. Incubator: See Section 9222B.1*j*.

e. Ultraviolet lamp, long wave (366 nm), preferably 6 W.

f. Microscope and *light source*: See Section 9222B.1 *k*.

2. Materials and Culture Media

a. Nutrient agar with MUG (NA-MUG):

Peptone.....5.0 g
Beef extract.....3.0 g
Agar.....15.0 g
4-methylumbelliferyl-$-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.

b. EC broth with MUG (EC-MUG):

Tryptose or trypticase..... 20.0 g
Lactose..... .5.0 g
Bile salts mixture or bile salts No.....31.5 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-$-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.

3. Procedures

a. Selection of sample size: 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.

b. Alternative coliform verifications: 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 necessary to interpret the results and to avoid confusion of weak autofluorescence of the medium as a positive response. See Section 9221F.

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