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9222 B.   Standard Total Coliform Membrane Filter Procedure

1. Laboratory Apparatus

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

a. Sample bottles: See Section 9030B.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

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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	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:†#(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	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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3. Samples

Collect samples as directed in Section 9060A and Section 9060B.

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 noncoliform growth on the medium (Table 9222:I). For regulation purposes, 100 mL is the official sample size.

An ideal sample volume will yield 20 to 80 coliform colonies and not more than 200 colonies of all types on a membrane-filter surface. Analyze drinking waters by filtering 100 to 1000 mL, or by filtering replicate smaller sample volumes such as duplicate 50-mL or four replicates of 25-mL portions. Analyze other waters by filtering three different volumes (diluted or undiluted), depending on the expected bacterial density. See Section 9215B.2 for preparation of dilutions. When less than 10 mL of sample (diluted or undiluted) is to be filtered, add approximately 10 mL sterile dilution water to the funnel before filtration or pipet the sample volume into a sterile dilution 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.3*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 $35 \pm 0.5°C$.

 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 $35 \pm 0.5°C$.

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.

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 Section 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 $35 \pm 0.5°C$ 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 $35 \pm 0.5°C$. 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 pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red, mucoid, or nucleated without sheen. Generally pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms. The total count of colonies (coliform and noncoliform) on Endo-type medium has no consistent relationship to the total number of bacteria present in the original sample. A high count of noncoliform colonies may interfere with the maximum development of coliforms. 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 least one colony from each typical or atypical colony type from a 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.''

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

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 (disregarding the lower limit of 20 cited above) and use the formula given above to obtain coliform density.

If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as ''confluent growth with (or without) coliforms.'' If the total number of bacterial colonies, coliforms plus noncoliforms, exceeds 200 per membrane, or if the colonies are not distinct enough for accurate counting, 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

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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.

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

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

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.

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

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Endnotes

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

2 (Popup - Footnote)
† 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.