

IDENTIFICATION AND ENUMERATION METHODS 7.1.3

The membrane filtration (MF) and most probable number (MPN) methods are used for the presumptive identification, confirmation, and enumeration of indicator bacteria. For general use, the MF method is preferable to the MPN method. The MPN method is preferred if toxic substances are present in the sample or if, after filtration, a residue heavy enough to block the micropores of the membrane filter is visible. The MPN method is described in *Standard Methods for the Examination of Water and Wastewater*, 18th edition (American Public Health Association and others, 1992, p. 9–45 to 9–53) and in Britton and Greeson (1989). Procedures for analyzing water samples by use of MF methods are described below.

Indicator bacteria for presumptive identification and enumeration are cultured on selective media after filtration of several different sample volumes onto gridded membrane filters. Detailed confirmation, identification, and enumeration of these bacteria require additional culturing and biochemical testing, the details of which are beyond the scope of this manual. However, additional confirmation procedures are needed under certain circumstances, such as use of the data in support of environmental regulation and enforcement.

The fecal indicator bacteria are operationally defined by the method employed for identification and enumeration, as follows:

- ▶ The total coliform bacteria are defined as the organisms that produce red colonies with a golden-green metallic sheen within 24 ± 2 hours when incubated at $35.0 \pm 0.5^\circ\text{C}$ on m-Endo medium.
- ▶ The fecal coliform bacteria are defined as the organisms that produce blue colonies in whole or part within 24 ± 2 hours when incubated at $44.5 \pm 0.2^\circ\text{C}$ on m-FC medium.
- ▶ *E. coli* are defined as the organisms that produce yellow or yellow-brown colonies that remain so when placed on a filter pad saturated with urea substrate broth for 15 minutes after resuscitation at $35.0 \pm 0.5^\circ\text{C}$ for 2 hours and incubation for 22 to 24 hours at $44.5 \pm 0.2^\circ\text{C}$ on m-TEC medium.

- ▶ *E. coli* are defined as the organisms that produce a blue fluorescent margin around a darker colony center within 4 hours when incubated at $35 \pm 0.5^\circ\text{C}$ on NA-MUG medium after primary culturing as total coliform bacteria on m-Endo medium.
- ▶ The fecal streptococci are defined as the organisms that produce red or pink colonies within 48 ± 2 hours when incubated at $35.0 \pm 0.5^\circ\text{C}$ on KF medium.
- ▶ Enterococci are defined as the organisms that produce pink to red colonies with a black or reddish-brown precipitate after primary culture for 48 to 50 hours at $41.0 \pm 0.5^\circ\text{C}$ on m-E medium followed by incubation for 20 minutes at 41.0°C on EIA medium.

7.1.3.A PREPARATION OF MEDIA AND REAGENTS

MF analysis requires the use of several types of media and reagents, the types being dependent on the indicator. The necessary media and reagents include sterile buffered water, agar- or broth-based selective and differential growth media, and media and reagents for additional biochemical identification.

Sterile buffered water (buffer) is used to dilute samples and to rinse the membrane-filtration apparatus and utensils. **Purchase sterile buffered water from the Quality of Water Service Unit (QWSU).** It is provided in 250-mL bottles and in 99-mL dilution bottles. There are two types: phosphate buffer to be used for total and fecal coliform, and fecal streptococci tests; and saline buffer to be used for *E. coli* and enterococci tests. Buffer exceeding the expiration date should not be used. When sterile buffered water is not obtained from the QWSU, it can be prepared ahead of time and sterilized by autoclaving. Preparation instructions for sterile buffered water are described in Britton and Greeson (1989, p. 18) and *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association and others, 1992, p. 9–17).

Culture media for enumeration of fecal indicator bacteria should be purchased in kits from the QWSU. The QWSU provides instructions for media preparation with each kit. Otherwise, dehydrated media can be purchased from scientific suppliers. Guidelines for storage of media and reagents are as follows:

- ▶ Store media kit (supplied by QWSU) and dehydrated, commercially prepared media in a desiccator. Store other reagents in a dust-free laboratory cabinet (not in a field vehicle).

- ▶ Label all media with the date received, date opened, and preparer's initials. Discard media and reagents with an expired shelf life.
- ▶ Refrigerate reagents when necessary. Use buffered dilution water immediately after opening; discard any remainder. Storing an opened bottle is not recommended.
- ▶ Mark all plates to identify the media type, the preparation date, and the preparer.
- ▶ Store prepared petri dishes upside down in a plastic bag before use and refrigerate.

PREPARATION, HOLDING TIMES, AND SPECIFICATIONS FOR CULTURE MEDIA 7.1.3.B

The preparation of selective and differential culture media for indicator bacteria is an important part of analysis. Adhering to proper preparation, storage, and holding-time requirements will help ensure the quality of the analysis. Instructions for the preparation of 100 mL of primary culture media for five MF tests and additional confirmation media or broth for three MF confirmation tests are described in section 7.1.5, entitled "Instructions for Media Preparation."

Quality control. Supplies of dehydrated media purchased from the QWSU or through catalogs have been quality-control tested. Media prepared fresh by the analyst must also be quality-control tested. If sterile buffered water is prepared in the laboratory, quality-control procedures must be used to ensure it will provide a suitable medium for transfer of bacteria from samples to filters. **Sterile buffered water should be tested for sterility by use of blanks of 100 mL, processed along with each set of samples.** Quality-control procedures applicable to microbiological testing can be found in the 18th edition of "*Standard Methods for the Examination of Water and Wastewater*" (American Public Health Association and others, 1992, p. 9-7 to 9-13).

7.1.3.C MEMBRANE FILTRATION PROCEDURE

After collecting the sample and selecting the appropriate sample volumes, label the petri dishes with the station number (or other identifiers), the volume of sample filtered, date, and time. Select those sample volumes that are anticipated to yield one or two plates in the ideal colony count range. General information on the concentrations of fecal indicator bacteria in surface water and contaminated surface water is given in table 7.1-1.

A suitable work area inside the field vehicle and out of direct sunlight and wind is best.

- ▶ Before and after processing the samples, clean countertops in field vehicles with an antibacterial cleaning solution; for example, a 7-percent phenolic solution, 50 to 70 percent isopropyl or ethyl alcohol; 5 percent bleach; or a 7-percent ammonia solution.
- ▶ Preheat incubators for at least 2 hours before beginning analysis, according to specifications for each test (table 7.1-5). Portable heater-block incubators must not be left on in closed, unventilated vehicles when the outside temperature is less than 15°C or greater than 37°C.

TECHNICAL NOTE: Review past analyses for the site to help determine the number of sample volumes to be filtered. Where past analyses of samples from a site have shown a small variation in the number of fecal indicator bacteria, the filtration of as few as three or four different sample volumes may suffice. However, where past analyses have shown the variation to be large or where the variation is not known, the filtration of five or more different sample volumes is recommended.

The steps required for membrane filtration are depicted in figure 7.1-2 (pages 28 and 29) and listed below. Quality-control samples must be collected as part of the filtration procedure (see Technical Note, step 16).

Table 7.1–5. Incubation times and temperatures for fecal indicator tests [m-Endo, total coliform media; \pm , plus or minus; $^{\circ}\text{C}$, degrees Celsius; NA-MUG, *E. coli* confirmation media (nutrient agar-4-methylumbelliferyl- β -D-glucuronide; m-FC, fecal coliform media; m-TEC, *E. coli* media; KF, fecal streptococcus media; m-E, enterococcus media; EIA, enterococcus confirmation media]

Test (media)	Incubation time and temperature
Total coliform bacteria (m-Endo)	24 \pm 2 hours at 35.0 \pm 0.5 $^{\circ}\text{C}$
<i>Escherichia coli</i> (NA-MUG)	4 hours at 35 \pm 0.5 $^{\circ}\text{C}$ after primary culture on m-Endo media
Fecal coliform bacteria (m-FC)	24 \pm 2 hours at 44.5 \pm 0.2 $^{\circ}\text{C}$
<i>Escherichia coli</i> (on urea substrate broth after primary culture on m-TEC media)	First resuscitate for 2 hours at 35.0 \pm 0.5 $^{\circ}\text{C}$, and then incubate for 22 to 24 hours at 44.5 \pm 0.2 $^{\circ}\text{C}$ After 22 to 24 hours, transfer filter to urea substrate broth for 15 to 20 minutes before counting
Fecal streptococci (KF media)	48 \pm 2 hours when incubated at 35.0 \pm 0.5 $^{\circ}\text{C}$
Enterococci (m-E and EIA)	48 to 50 hours at 41.0 \pm 0.5 $^{\circ}\text{C}$ on m-E medium. Transfer filter to EIA medium for 20 minutes at 41.0 $^{\circ}\text{C}$ before counting

Steps to follow when filtering samples and making colony counts are listed below (and summarized in fig. 7.1–2):

1. Select sample volumes (table 7.1–6) to result in at least one filter having colonies in the ideal counting range. The ideal range and number of sample volumes to filter depend on the test and the expected bacterial concentrations. Record on the petri dish and on the record sheet the site name, date, time of sample collection, and sample volume. Record the time of sample processing on the record sheet. Also label equipment and procedure blanks and other quality-control samples.
2. Assemble filtration equipment by inserting the base of the filter-holder assembly into a flask. Vacuum is supplied by use of a hand-held pump, vacuum, or battery-operated peristaltic pump. If flame sterilization was used, rinse the inside of the filtration apparatus with sterile buffered water to remove any residue of formaldehyde.
3. Sterilize stainless steel forceps by immersing tips in a small bottle or flask containing 70 or 90 percent ethanol; then pass forceps through the open flame of an alcohol burner. Allow alcohol to burn out and allow the forceps to cool for several seconds to prevent heat damage to the membrane filter. Resterilize forceps before each use. Return cooled forceps to alcohol container between transfers. **Do not set forceps on the countertop.**

Table 7.1–6. Recommended sample volumes for membrane filtration analyses based on ideal colony count and concentration range

[<, less than; col/100 mL; colonies per 100 milliliters; mL, milliliters]

Ranges of observed fecal indicator concentrations					
<1 to 60,000 col/100 mL		<1 to 80,000 col/100 mL		<1 to 200,000 col/100 mL	
Ideal counting ranges for number of colonies per membrane filter					
20–60 colonies		20–80 colonies		20–100 colonies	
Sample volume (mL) ¹	Added as (mL) ²	Sample volume (mL) ¹	Added as (mL) ²	Sample volume (mL) ¹	Added as (mL) ²
100	100	100	100	100	100
30	30	25	25	20	20
10	10	6.0	6.0	5.0	5.0
3.0	3.0	1.5	1.5	1.0	1.0
1.0	1.0	0.4	4.0 of 1:10 dilution	0.25	2.5 of 1:10 dilution
0.3	3.0 of 1:10 dilution	0.1	10 of 1:100 dilution	0.05	5.0 of 1:100 dilution
0.1	10 of 1:100 dilution				

¹All sample volumes less than 1.0 mL require dilution in a 99-mL bottle.

²Sample volumes smaller than those indicated may be needed when bacterial concentrations are greater than those listed.

4. Remove the sterilized funnel from the filtration apparatus. Always hold the funnel in one hand while placing or removing the membrane filter. (Placing the funnel on anything but the filtration apparatus might result in contamination of the funnel.)

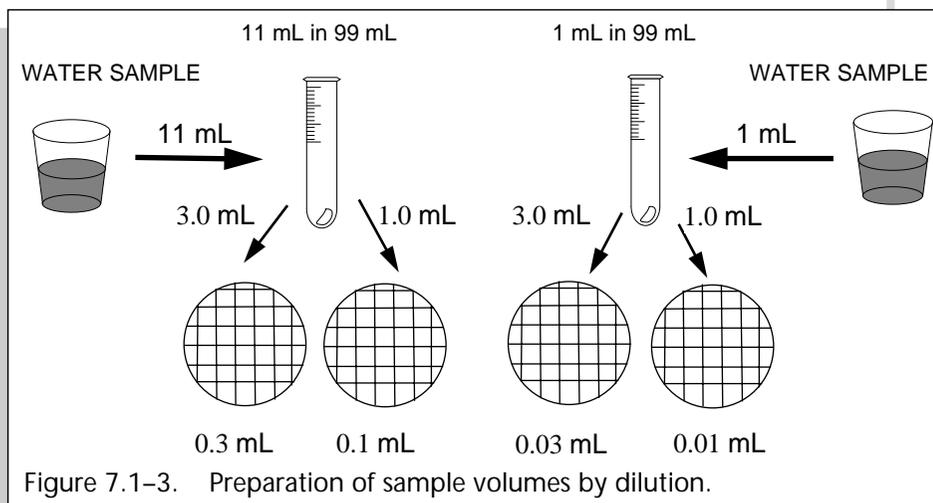
Using sterile forceps, place a sterile, gridded membrane filter (47-mm diameter) on top of the filter base, grid-side up. Be sure to use the correct pore-size membrane filter for the test procedure (table 7.1–7).

- Carefully replace and secure the filter funnel on filter base. Avoid tearing or creasing the membrane filter.

Table 7.1–7. Membrane-filter pore sizes for fecal indicator bacteria tests

Test	Pore size of membrane filter (in micrometers)
Total coliform	0.45, 0.65 or 0.7
Fecal coliform	0.65 or 0.7
<i>Escherichia coli</i>	0.45
Fecal streptococci	0.45
Enterococci	0.45

- Rinse funnel with 100 mL of sterile buffered water before filtering sample volumes to obtain a filtration assembly equipment blank (filter blank).
 - Filter sample in order of smallest to largest sample volume.
5. If the sample volume is less than 1.0 mL, prepare dilutions with sterile buffered water in a 99-mL dilution bottle and transfer appropriate volume of dilution to the membrane filter (fig. 7.1–3 and table 7.1–3 and table 7.1–8).
- When preparing dilutions, use a sterile pipet to measure each sample volume.
 - After each sample-volume transfer, close and shake the dilution bottle vigorously at least 25 times.
 - Filter diluted samples within 20 minutes after preparation. Keep dilution bottles out of sunlight and do not transfer dilute sample volumes with pipets used to transfer concentrated volumes.



6. Shake the sample vigorously at least 25 times before each sample volume is withdrawn in order to break up particles and ensure an even distribution of indicator bacteria in the sample container. Proceeding from smallest to largest sample volume, deliver the sample volume to the membrane filter by use of a pipettor or pipet bulb with a valve for volume control.
- Allow the pipet to drain, and touch the tip to the inside of the funnel to remove remaining sample. Pipets of the TD (to deliver) type will have a small amount of liquid left in the tip after dispensing the liquid.

- If the volume of sample to be filtered is 10 mL or more—transfer the sample with a sterile pipet or graduated cylinder directly into the funnel.
- If the volume of sample to be filtered is between 1.0 and 10.0 mL—pour about 20 mL of sterile buffered water into the funnel before pipetting the sample to facilitate distribution of bacteria on the membrane filter. Refer to table 7.1–6 for appropriate sample volumes for each test.

Table 7.1–8. Preparation guidelines for dilution of samples to volumes less than 1.0 milliliter for fecal indicator bacteria analysis
[mL, milliliter]

Dilution factor	Volume (mL) of sample added to 99 mL sterile dilution water	To obtain this dilution, filter this volume
1:10	11.0 mL of original sample	1.0 mL of 1:10 = 0.1 mL 3.0 mL of 1:10 = 0.3 mL
1:100	1.0 mL of original sample	1.0 mL of 1:100 = 0.01 mL 3.0 mL of 1:100 = 0.03 mL
1:1,000	1.0 mL of 1:10 dilution	1.0 mL of 1:1,000 = 0.001 mL 3.0 mL of 1:1,000 = 0.003 mL

7. Apply vacuum with a hand, peristaltic, or vacuum pump. To avoid damage to bacteria, do not exceed a pressure of about 5 lb/in² (25 cm of mercury).
8. Rinse inside of funnel twice with 20 to 30 mL of sterile buffered water while applying vacuum. If a graduated cylinder was used, rinse the cylinder with sterile buffered water and deliver rinse water to the filtration apparatus.
9. Remove the funnel and hold it in one hand. Do not set the funnel on the countertop. Remove the membrane filter with sterile forceps. Release the vacuum. Releasing the vacuum after removing the filter prevents backflow of sample water onto the filter. Unnecessarily wet filters promote confluent growth of colonies and poor results. Replace funnel on filter base.
10. Open petri dish and place membrane filter grid side up on medium by use of a rolling action, starting at one edge. Avoid trapping air bubbles under the membrane filter. If air is trapped, use sterile forceps to remove the membrane filter and roll it onto the medium again. **Do not expose prepared plates to direct sunlight.**

Do not pipet by mouth.

11. Close petri dish by pressing top firmly onto bottom. Invert the petri dish. To avoid growth of interfering microorganisms, incubate within 20 minutes.
12. Continue to filter the other sample volumes in order, from smallest to largest volume. Record on the field forms the volumes filtered and time of processing.
13. After filtrations are complete, place a sterile, gridded-membrane filter on the funnel base and rinse the funnel with 100 mL of sterile buffered water to obtain a procedure blank.
14. After the sample volumes and blanks have been filtered, place the inverted petri dishes in a preheated aluminum heater-block or water-bath incubator. Incubate at the prescribed times and temperatures (table 7.1–5). Wash, then flame sterilize or autoclave filtration apparatus. **Wash countertop between each sample and wash hands with bacteriocidal soap.**
15. After incubation, remove the petri dishes from the incubator. Count and record on the field forms, for each sample volume filtered, the number of typical colonies (table 7.1–9). Recount until results agree within 5 percent. Recounting is done by turning the plate 90 degrees to obtain a slightly different angle. Count by use of a preset plan (a side-to-side pattern along grid lines is suggested) (fig. 7.1–4). **Make the counts with the aid of 5 to 15 magnifications and a fluorescent illuminator placed as directly above the filter as possible.**
 - For total coliform colonies, enhance sheen production by removing filters from media and placing them on absorbent pads to dry for at least 1 minute before counting.
 - If the optional NA-MUG test is done for *E. coli*, transfer the total coliform filter onto NA-MUG plates and incubate for 4 hours at 35°C. Afterward, count under a long-wave ultraviolet light in a completely darkened room (U.S. Environmental Protection Agency, 1991b).
 - For *E. coli* and enterococci, additional biochemical tests are required by use of confirmation media. For *E. coli*, transfer the filter to a filter pad saturated with urea-phenol reagent; count only yellow colonies after 15 to 20 minutes at room temperature (U.S. Environmental Protection Agency, 1985).
 - For enterococci, transfer the filter to EIA media after incubation for 20 minutes at 41°C; count colonies from the underside of the plate placed over a fluorescent illuminator.
16. Check quality-control blanks for colony growth, and report results on the field forms.

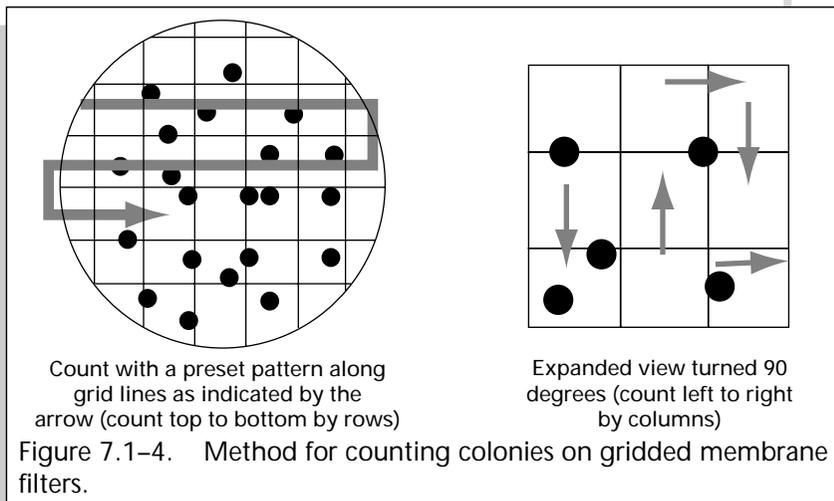
- The presence of colonies on blanks indicates that results of the bacterial analyses bracketed by positive blanks are suspect and should not be reported.
- It is not valid to subtract colony counts on blanks from results calculated for samples.

TECHNICAL NOTE: It is necessary to collect equipment, filter, and procedure blanks. The equipment and filter blanks measure the effectiveness of sterilization. One or more colonies on this type of blank indicates inadequate sterilization of either the equipment or the buffered water. The procedure blank measures the effectiveness of the analyst's rinsing technique. One or more colonies on the procedure blank indicates either inadequate rinsing or contamination of equipment or buffered water during sample processing.

Table 7.1–9. Test (media type), ideal colony count, and typical colony color, size, and morphology for indicator bacteria colonies

[m-Endo, total coliform media; mm, millimeters; NA-MUG, nutrient agar-4-methylumbelliferyl- β -D-glucuronide; m-FC, fecal coliform media; m-TEC, *E. coli* media; KF, fecal streptococcus media; m-E, enterococcus media; EIA; enterococcus confirmation media]

Test (media type)	Ideal count range (colonies per filter)	Typical colony color, size, and morphology
Total coliform bacteria (m-Endo)	20–80	Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and red with a golden-green metallic sheen.
<i>Escherichia coli</i> After primary culture as total coliform colonies on m-Endo (NA-MUG)	None given but much fewer in number than total coliforms on same filter	Colonies are cultured on m-Endo media as total coliform colonies. After incubation on NA-MUG, colonies have blue fluorescent margins with a dark center. Count under a long-wave ultraviolet lamp in a completely darkened room.
Fecal coliform bacteria (m-FC)	20–60	Colonies are round, raised, and smooth with even to lobate margins; 1 to 6 mm in diameter, and light to dark blue in whole or part. Some may have brown or cream colored centers.
<i>Escherichia coli</i> (m-TEC)	20–80	Colonies are round, raised, and smooth; 1 to 4 mm in diameter, yellow to yellow brown; may have darker raised centers.
Fecal streptococci (KF media)	20–100	Colonies are small, raised, and spherical; about 0.5 to 3 mm in diameter; glossy pink or red in color.
Enterococci (m-E and EIA)	20–60	Colonies are round, smooth, and raised; 1 to 6 mm in diameter; pink to red with a black or reddish-brown precipitate on underside.



17. Calculate the number of colonies per 100 mL of sample as shown in section 7.1.4, "Calculation and Reporting of Fecal Indicator Bacteria."
18. Put all plates to be discarded in an autoclavable bag. Freeze or chill the plates to be discarded until they can be autoclaved in the laboratory. Autoclave all cultures at 121°C for a minimum of 30 minutes before discarding.

Quality control. In addition to blanks, collect and analyze samples in duplicate at a minimum frequency of 5 percent (1 in every 20 samples). Periodically purchase and analyze a pure culture containing *Escherichia coli* or *Enterococcus faecalis* (formerly *Streptococcus faecalis*) to ensure that the test procedure is acceptable.



PROCEDURE

1. Preheat incubator, prepare work areas.
2. Select sample volumes. If needed, prepare dilutions for filtration of sample volumes less than 1.0 mL. (Tables 7.1–6 and 7.1–8; and figure 7.1–3.)
3. Label petri dishes.
4. Assemble, and if not sterile, sterilize filtration apparatus.



PROCEDURE

5. Place sterile filter on filtration apparatus using sterile forceps.

TECHNICAL NOTE: a small hand pump is preferred over a syringe as a vacuum source.



PROCEDURE

6. Shake sample 25 times and deliver to filtration apparatus by use of graduated cylinder or pipet. Add 20 mL sterile buffered water to filtration apparatus before filtering sample volumes less than 10 mL.

Figure 7.1–2. Steps in membrane-filtration procedure (taken from Millipore, 1973, and published with permission).



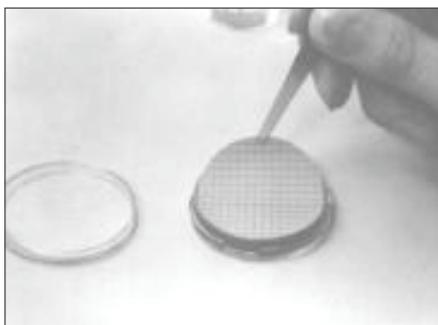
PROCEDURE

7. Apply vacuum, and afterwards, rinse filtration apparatus and cylinder twice with sterile buffered water.



PROCEDURE

8. Sterilize forceps and remove filter. Replace funnel on filtration apparatus.



PROCEDURE

9. Roll filter onto media in petri dish. Place inverted petri dish in incubator.

PROCEDURE

10. Repeat steps 4–9 for each sample volume in order of the smallest to the largest volume. A filter blank is processed before each sample. Filter a procedure blank after every 20 samples or once per day or at each site, according to study objective. Filter a duplicate sample after every 20 samples or at each site, according to study objective. Use a hand pump instead of a syringe.

Figure 7.1–2—Continued. Steps in membrane-filtration procedure (taken from Millipore, 1973, and published with permission).