



# 7.1 FECAL INDICATOR BACTERIA

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# FECAL INDICATOR BACTERIA 7.1

Fecal indicator bacteria are used to assess the quality of water because they are not typically disease causing, but are correlated to the presence of several waterborne disease-causing organisms (pathogens). The concentration of indicator bacteria (the term “indicator bacteria” is used synonymously with fecal indicator bacteria in this section) is a measure of water safety for body-contact recreation or for consumption.

- ▶ Wastes from warm-blooded animals contribute a variety of intestinal bacteria that are pathogenic to humans.
- ▶ Body contact with water that contains pathogens of the genera *Salmonella*, *Shigella*, and *Vibrio* can result in several types of disease in humans, including gastroenteritis and bacillary dysentery, typhoid fever, and cholera.
- ▶ The presence of *Escherichia coli* (*E. coli*) in water is direct evidence of fecal contamination from warm-blooded animals and indicates the possible presence of pathogens (Dufour, 1977).

The most widely used indicator bacteria are of the total coliform, fecal coliform, enterococci, and fecal streptococci groups, and *E. coli*. Bacteriological tests are used to assess the sanitary quality of water and the potential public health risk from waterborne diseases. This section describes five membrane filtration methods for identifying and enumerating fecal indicator bacteria (Britton and Greeson, 1989; U.S. Environmental Protection Agency, 1985 and 1991a).

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Fecal indicator bacteria:  
measures of the  
sanitary quality of  
water.

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Water-quality criteria have been developed by the U.S. Environmental Protection Agency (USEPA) for concentrations of indicator bacteria in recreational, shellfish growing, ambient, and potable waters.

- ▶ Typical concentrations of indicator bacteria in contaminated and uncontaminated surface waters (table 7.1–1) are often much higher than water-quality criteria established as safe levels by USEPA. For bathing water, the geometric mean concentration established by the U.S. Environmental Protection Agency (1976) for fecal coliform bacteria is 200 col/100 mL (colonies per 100 milliliters). In 1986, the USEPA modified the Federal criteria so that *E. coli* and enterococci bacteria are now the recommended indicator bacteria (U.S. Environmental Protection Agency, 1986).
- ▶ Ground water typically contains lower concentrations of indicator bacteria than surface water.

*E. coli* and enterococci are currently the preferred indicators for recreational waters because both are superior to fecal coliform and fecal streptococci bacteria as predictors of swimming-associated gastroenteritis in marine and fresh water (Cabelli, 1977; Dufour and Cabelli, 1984). The freshwater criterion for *E. coli* in bathing water is a geometric-mean concentration of 126 col/100 mL. The freshwater criterion for enterococci is a geometric-mean concentration of 33 col/100 mL (U.S. Environmental Protection Agency, 1986, p. 15). For potable waters, the detection of as few total coliforms as 4 col/100 mL, and the detection of 1 col/100 mL of either fecal coliform or *E. coli* warrant concern for public health.

Table 7.1–1. Ranges of fecal indicator bacteria typically found in surface water and contaminated surface water (modified from Bordner and Winter, 1978, p. 127; American Public Health Association and others, 1992, p. 9–56, 9–60; American Public Health Association and others, 1985, p. 905; U.S. Environmental Protection Agency, 1986)

Bacterial group	Surface water, colonies per 100 milliliters	Fecal-contaminated surface water, colonies per 100 milliliters
Total coliforms	<1 to 80,000	1,200 to > 4,000,000
Fecal coliforms	<1 to 5,000	200 to > 2,000,000
<i>Escherichia coli</i>	<1 to 576	126 to > 2,000,000
Fecal streptococcus	<1 to 1,000	400 to > 1,000,000

# EQUIPMENT AND EQUIPMENT STERILIZATION PROCEDURES 7.1.1

Specific equipment and supplies are needed for collection and analysis of indicator bacteria by membrane filtration procedures. The equipment listed in table 7.1–2 should be sufficient to begin analysis of fecal indicator bacteria using these procedures.

Table 7.1–2. Equipment and supplies used for membrane filtration analysis

[mL, milliliters; °C, degrees Celsius;  $\mu\text{m}$ , micrometers; TTC, triphenyltetrazolium chloride; mm, millimeters; TD, to deliver; NIST, National Institute of Standards and Technology; NA-MUG, nutrient agar-4-methylumbelliferyl- $\beta$ -D-glucuronide; nm, nanometers; lb/in<sup>2</sup>, pounds per square inch; cm, centimeters ]

Item	Description
✓ Absorbent pads	For use with total coliform and <i>Escherichia coli</i> methods
✓ Alcohol burner	Glass or metal, containing ethanol for flame sterilizing forceps
✓ Alcohol bottle	Wide mouth, 100 mL, containing ethanol for forceps
✓ Autoclave	For sterilization, capable of maintaining 121°C
✓ Bottles	Milk dilution, 99 mL with autoclavable screwcaps
✓ Counter	Handheld for counting bacterial colonies
✓ Filter disk	Sterile, disposable, 0.2 $\mu\text{m}$ , for sterilizing TTC, to fit on 5-mL barrel syringe
✓ Filtration assembly	Filter funnel, filter base, and stainless steel, glass, or plastic filter holder; wrapped in aluminum foil, autoclavable bag, or kraft paper, sterile; autoclavable
✓ Flasks	Narrow mouth, Erlenmeyer type with stir bars for media preparation, 250 or 500 mL
✓ Forceps	Stainless steel, smooth tips
✓ Hot plate	With magnetic stirrer or boiling water bath for media preparation
✓ Graduated cylinders	Borosilicate glass or plastic, 100 and 25 mL, wrapped in sterile aluminum foil, autoclavable bag, or kraft paper
✓ Incubator	Aluminum heat sink (heater block) or water bath, capable of maintaining specified temperature ranges during incubation
✓ Membrane filters	Sterile, white, gridded, 47-mm diameter, either 0.45- or 0.7-mm pore size, depending on test method
✓ Microscope	Wide field type with 10–20 x magnifications, dissecting type with fluorescent lamp
✓ Pipets	Sterile, TD, bacteriological or Mohr, glass or plastic, 1 and 10 mL
✓ Pipettor or pipet bulb	For use with pipets ( <b>no pipetting by mouth</b> )
✓ Petri dishes	Sterile, plastic, disposable, 50 x 12 mm
✓ Syringe	5 mL, disposable for delivering TTC
✓ Thermometer	Range of 40–110°C, glass mercury or dial, calibrated in 0.2°C increments checked against a NIST-certified thermometer
✓ Ultraviolet lamp, long wave	For use with NA-MUG test, 366 nm, 6-watt bulb
✓ Vacuum source	Either a hand pump with gage or electric vacuum or peristaltic pump, vacuum pressure not to exceed 5 lb/in <sup>2</sup> or 25 cm of mercury

**Equipment for collection and analysis of bacterial samples must be clean and sterile (table 7.1-3).** Wrap equipment in kraft paper, autoclavable bags, or aluminum foil. Sterilize and store the equipment in a clean area. Resterilize equipment if foil, bag, or kraft paper is torn.

**Add sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ )** to sample bottles before sterilization if the water to be collected contains residual chlorine or other halogens added for disinfection. Residual chlorine can be found in samples collected from sources such as treated potable-water taps, in effluents, and surface-water samples collected from the mixing zones of wastewater-treatment plants. A 10-percent solution of  $\text{Na}_2\text{S}_2\text{O}_3$  is prepared in the following manner. In a volumetric flask, dissolve 100 g  $\text{Na}_2\text{S}_2\text{O}_3$  into 500 mL of deionized or distilled water; stir until dissolved, and fill flask to 1,000 mL (Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1992, p. 9-18). Add 0.1 mL of 10-percent  $\text{Na}_2\text{S}_2\text{O}_3$  solution for every 100 mL of sample. Keep  $\text{Na}_2\text{S}_2\text{O}_3$  refrigerated and in a dark bottle; after 6 months prepare a fresh solution.

**Add ethylenediaminetetraacetic acid (EDTA)** to sample bottles when water to be collected contains trace elements such as copper, nickel, and zinc at concentrations greater than 10  $\mu\text{g}/\text{L}$  (Britton and Greeson, 1989, p. 5-6; Bordner and Winter, 1978, p. 6; American Public Health Association and others, 1992, p. 9-18). A 15-percent solution of EDTA is prepared by dissolving 372 mg in 1,000 mL of distilled or deionized water. Before sterilization, add 0.3 mL of the EDTA solution per 100 mL of sample to sample bottles. EDTA can be combined with the  $\text{Na}_2\text{S}_2\text{O}_3$  solution in the sample bottle before sterilization.

Autoclaving is the preferred method for sterilizing equipment.

Sterilize the filtration apparatus between sites or for each sample collected at the same site at different times. Autoclaving is the preferred method of sterilization. Use only autoclaves that have temperature, pressure, and liquid- and dry-utensil-cycle controls. Steam sterilizers and vertical autoclaves are not recommended because the temperature cannot be held constant.

Table 7.1–3. Equipment cleaning and sterilizing procedures

[ $\text{Na}_2\text{S}_2\text{O}_3$ , sodium thiosulfate; >, greater than value shown;  $\mu\text{g}/\text{L}$ , micrograms per liter; EDTA, ethylenediaminetetraacetic acid;  $^\circ\text{C}$ , degrees Celsius;  $\text{mg}/\text{L}$ , milligrams per liter]

Equipment	Cleaning and sterilizing procedures
All equipment	<p>Wash equipment thoroughly with a dilute nonphosphate, laboratory-grade detergent.</p> <p>Rinse three times with hot tap water.</p> <p>Rinse again three to five times with deionized or glass-distilled water.</p>
Glass, polypropylene, or Teflon™ bottles	<p>If sample will contain residual chlorine or other halogens, add <math>\text{Na}_2\text{S}_2\text{O}_3</math>.</p> <p>If sample will contain <math>&gt;10 \mu\text{g}/\text{L}</math> trace elements, add EDTA.</p> <p>Autoclave at <math>121^\circ\text{C}</math> for 15 minutes, or bake glass jars at <math>170^\circ\text{C}</math> for 2 hours.</p>
Stainless-steel field units	<p>Flame sterilize with methanol (Millipore Hydrosol™ units only), or autoclave, or bake at <math>170^\circ\text{C}</math> for 2 hours.</p>
Portable submersible pumps and pump tubing	<p>Autoclavable equipment (preferred): Autoclave at <math>121^\circ\text{C}</math> for 15 minutes.</p> <p>Non-autoclavable equipment: Submerge sampling system in a 200-mg/L laundry bleach solution and circulate solution through pump and tubing for 30 minutes; follow with thorough rinsing, inside and out, with sample water pumped from the well.</p> <p><b>DO NOT USE THIS METHOD TO DISINFECT EQUIPMENT USED TO COLLECT SAMPLES FOR SUBSEQUENT DETERMINATIONS OF TRACE ELEMENTS AND ORGANIC SUBSTANCES.</b></p>

Take care to ensure that materials to be autoclaved, such as tubing and containers, are thermally stable. Polymers (such as polycarbonate, polypropylene, polyallomer, and polymethylpentene) and Teflons™ and Tefzel™ (such as perfluoroalkoxy-polymers or PFA™, ethylenetetrafluoro-ethylene or ETFE™, fluorinated ethylene propylene or FEP™, and polytetrafluoroethylene polymers or PTFE™) can be autoclaved. Each has different thermal characteristics and tolerances to repeated autoclaving.

**CAUTION:** when flame sterilizing, have proper safety equipment such as a fire extinguisher on hand, and implement procedures carefully.

Only Millipore Hydrosol™ field filtration units are designed to be flame sterilized with methanol. Formaldehyde gas, a by-product of methanol combustion, kills all microorganisms in the unit. The following sterilization procedure is acceptable for the Hydrosol unit in field situations where other sterilization techniques are not practicable (Millipore, 1973, p. 48–49).

**Carefully:**

1. Remove the stainless steel flask from the base of the filter-holder assembly.
2. Saturate the asbestos ring (wick) around the base assembly with methanol.
3. Ignite the methanol on the asbestos wick and allow to burn for 30 seconds.
4. Invert the stainless steel flask over the funnel and the burning asbestos ring, and seat the flask on the base of the filter-holder assembly. Leave the flask in place for 15 minutes. Before filtering the next sample, rinse flask and funnel thoroughly with sterile buffered water to remove all residues of formaldehyde.
5. Repeat sterilization procedure before processing the next sample.

**Quality control.** Use a sterilization indicator, such as autoclave tape or StreamClox™, to help determine whether adequate temperature and pressure have been attained during autoclaving. Keep a log book of equipment and include quality-control procedures with the date, the results, and the name of the analyst. The 18th edition of *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association and others, 1992, p. 9–9, Table 9020:III) contains specifications for the length of time and temperature for autoclave sterilization of various media, apparatus, and cultures to be discarded. Overloading the autoclave with equipment or materials will result in incomplete sterilization. Make sure that steam can circulate around all equipment, utensils, and bottles. Cultures of bacteria to be discarded must be autoclaved for at least one-half hour.

## SAMPLE COLLECTION, PRESERVATION, AND STORAGE 7.1.2

Because sterile conditions must be maintained during collection, preservation, storage, and analysis of indicator bacteria samples, specific procedures have been developed that must be strictly followed. These procedures vary with types of sampling equipment and source of sample (surface water, ground water, treated water, or waste water).

A summary of requirements for sample-collection containers and procedures for sample preservation are given in table 7.1-4.

Adhere strictly to the 6-hour maximum holding-time requirement.

Table 7.1-4. Summary of equipment for sample collection and procedures for sample preservation of indicator bacteria

[EWI, equal-width increment; EDI, equal-discharge increment; L, liter; mL, milliliter; °C, degrees Celsius; EDTA, ethylenediaminetetraacetic acid]

<b>Equipment for sample collection</b>
<p>For EWI or EDI surface-water samples: sterile DH-77 3-L or DH-81 1-L wide-mouth bottle with sterile caps and nozzles.</p> <p>For surface-water and ground-water samples using point samplers: a sterile, narrow-mouth container, 250- to 500-mL capacity.</p> <p>All containers must be composed of sterilizable materials such as borosilicate glass, polypropylene, stainless steel, or Teflon™.</p>
<b>Procedures for sample preservation</b>
<p>Chill all samples at 1–4°C before analysis. If necessary, add 0.1 mL of a 10-percent sodium thiosulfate solution per 100 mL sample for halogen neutralization.</p> <p>Add 0.3 mL of a 15-percent EDTA solution per 100 mL sample for chelation of trace elements, if necessary.</p> <p>Do not exceed the 6-hour maximum holding time after sample collection.</p>

## 7.1.2.A SURFACE-WATER SAMPLE COLLECTION

The areal and temporal distribution of indicator bacteria in surface water can be as variable as the distribution of suspended sediment because bacteria commonly are associated with solid particles. To obtain representative data, use the same methods for collecting surface-water samples for bacteria analysis as for suspended sediment (Edwards and Glysson, 1988; NFM 4).

- ▶ For flowing water, use depth-and-width-integrating sampling methods.
- ▶ For still water or other surface-water conditions for which depth-and-width-integrating methods are not applicable, use the hand-dip method.

**Quality control.** Depending on the data-quality requirements, quality-control (QC) samples (blanks and replicates) can comprise from 5 to 30 percent or more of the total number of samples collected over a given period of time.

- ▶ Collect and analyze field blanks to document that sampling equipment has not been contaminated.
- ▶ Collect and analyze filter and procedure blanks to document that filtration equipment was sterile and not subsequently contaminated by the analyst. The filter blank is processed through the filtration equipment before the sample is filtered and the procedure blank is processed through the filtration equipment afterwards.

### ***Process field blanks before collecting the water sample:***

1. Rinse sterile sampling equipment and containers with sterile buffered water.
2. Process sterile buffered water through sampling equipment and into sterile sample bottle and analyze for colony growth. If no growth is observed, the sample was collected using sterile procedures.

### ***Depth-and-width-integrating methods***

Depth-and-width-integrating sampling methods include the equal-discharge increment (EDI) method and the equal-width increment (EWI) method. EDI or EWI methods are recommended unless study objectives or site characteristics dictate otherwise.

1. Select the EDI or EWI method. The EDI method is preferred to the EWI method for sites at which the velocity distribution across a stream section is well established; for example, at a gaging station (Edwards and Glysson, 1988).

2. Select the appropriate sampler and equipment. **Sampling equipment must be sterile**—polypropylene collection bottle, nozzle, and cap, or bags for the bag sampler.
  - For streams with depths of 5 m or less, use a US D-77 or a US DH-81 sampler.
  - For stream sections where depths exceed 5 m, use the bag version of the US D-77, either with autoclavable Teflon™ bags or autoclavable cooking bags. Thermotolerant polymers are described in more detail in 7.1.1 under "Equipment and Equipment Sterilization Procedures".
  - For compositing subsamples, use a sterile 3-L bottle with a US D-77 sampler and a sterile 1-L bottle with a US DH-81 sampler.
  - Use the proper nozzle size and transit rate for the velocity conditions in the section to ensure isokinetic collection of the sample.

The number of subsamples that can be collected by use of EDI and EWI collection methods are somewhat limited because churn and cone splitters cannot be autoclaved or easily kept sterile under field conditions.

- ▶ For wide channels, several samples, each composed of subsamples composited into 3-L bottles, may be needed.
- ▶ For narrow channels, collect subsamples at 5 to 10 or more vertical locations in the cross section without overfilling the bottle.

### ***Hand-dip method***

If the stream depth and (or) velocity is not sufficient to use a depth-and-width integrating method, collect a sample by a hand-dip method. Sampling still water or sampling at depth in lakes, reservoirs, estuaries, and oceans requires a sterile point sampler. Niskin, ZoBell, and Wheaton samplers hold a sterilizable bottle or bag. To collect a hand-dipped sample:

1. Open a sterile, narrow-mouth borosilicate glass or plastic bottle; grasp the bottle near the base, with hand and arm on downstream side of bottle.
2. Without rinsing, plunge the bottle opening downward, below the water surface. Allow the bottle to fill with the opening pointed slightly upward into the current.
3. Remove the bottle with the opening pointed upward from the water and tightly cap it, allowing about 2.5 to 5 cm of headspace (American Public Health Association and others, 1992, p. 9–19; Bordner and Winter, 1978, p. 8). This procedure minimizes collection of surface film and avoids contact with the streambed.

## 7.1.2.B GROUND-WATER SAMPLE COLLECTION

As with surface water, most bacteria in ground and well water are associated with solid particles. Stable values of field measurements (turbidity, temperature, dissolved-oxygen concentration, pH, and specific electrical conductance) are important criteria for judging if a well has been sufficiently purged for collection of a representative ground-water sample for indicator bacteria analysis (NFM 6.0.2).

**Quality control.** Depending on the data-quality requirements, quality-control (QC) samples (blanks and replicates) can comprise from 5 to 30 percent or more of the total number of samples collected over a given period of time.

- ▶ Collect and analyze field blanks to document that sampling equipment has not been contaminated.
- ▶ Collect and analyze filter and procedure blanks to document that filtration equipment was sterile and not subsequently contaminated by the analyst. The filter blank is processed through the filtration equipment before the sample is filtered and the procedure blank is processed through the filtration equipment afterwards.

### ***Process field blanks before collecting the water sample:***

1. Rinse sterile sampling equipment and containers with sterile buffered water.
2. Process sterile buffered water through sampling equipment and into sterile sample bottle and analyze for colony growth. If no growth is observed, the sample was collected using sterile procedures.

### ***Production and domestic water-supply wells***

- ▶ If samples are to be collected from a production well, select a tap that supplies water from a service pipe connected directly to the main and is not served from a cistern or storage tank (American Public Health Association and others, 1992, p. 9–18, 19; Britton and Greeson, 1989, p. 5; Bordner and Winter, 1978, p. 5–16).
- ▶ Before collecting the sample, remove screens, filters, or other devices from the tap.

- ▶ Do not sample from leaking taps.
- ▶ Avoid sampling after downhole chlorination, if possible. Dechlorination is required if you cannot avoid collecting the sample before it has passed through the treatment unit.

Collect a sample directly from the tap into a sterile bottle without splashing or allowing sample bottle to touch the tap.

- ▶ Supply wells ordinarily are equipped with permanently installed pumps. If the well is pumped daily, collect the sample directly from the tap into the sterile container (table 7.1-4) after running tap water to waste for a minimum of 5 minutes and after recording stable field measurements.
- ▶ If the well is infrequently used, run the tap to waste until a minimum of five sets of field measurements are obtained.

### ***Observation and monitoring wells***

If a well does not have an in-place pump, obtain samples by use of a portable sampler, such as a submersible pump or a bailer (U.S. Environmental Protection Agency, 1982). Samplers and sample line must be autoclaved, sterilized, or disinfected, followed by flushing with ground-water sample, before sample is collected into sterile bottles.

- ▶ Use autoclavable samplers, if possible. After flushing the sterilized pump lines with sample, collect the sample directly into the sterile sample bottles.
- ▶ Use of disinfectants can interfere with chemical analysis—check data-collection objectives before using a disinfectant.
- ▶ **Disinfectants are corrosive, can damage the metal parts of a pump, and render the pump inadequate for sampling trace elements and other constituents.**

### ***To disinfect a pump:***

1. Submerge the pump and pump tubing in a 200-mg/L solution of household laundry bleach. Most bleach is about 5 percent chlorine, but bleach in a container left open for more than 60 days may not be full strength.
2. Circulate the disinfectant through the pump and tubing for 30 minutes.

3. Rinse thoroughly by lowering the pump carefully in the well and pumping well water to waste until chlorine is removed (U.S. Environmental Protection Agency, 1982). **The pump must have a backflow check valve (an antibacksiphon device) to prevent residual disinfectant from flowing back into the well.**

The type of well, its use, construction, and condition, could alter samples for fecal-indicator bacteria analysis.

***If the pump cannot be disinfected:***

1. Handle the pump and tubing carefully to avoid contamination.
2. Purge the well with the pump used for sampling to allow the pump and tubing to be thoroughly flushed with aquifer water before sampling.
3. Collect blanks through the sampling equipment for quality control at each site.
4. An alternative to sampling with the pump is to withdraw the pump after purging and collect the sample with a sterile bailer (U.S. Environmental Protection Agency, 1982, p. 252–253). If using this method, evaluate the potential for bias from stirring up particulates during pump removal and bailing that otherwise would not be included in the sample.

Presampling activities, such as purging, must be carried out in such a way as to avoid contaminating the well, the equipment, or the samples. Avoid collecting surface film from the well water in the sample and ensure that the sampler intake is at the depth interval targeted for study. If using a nonpumping sampler, select a point-source sampler, such as a bailer with a double-check valve. **Do not use a bailer unless the bailer can be sterilized.**

**Sampling equipment that does not require chlorine disinfection:**

If water level is less than 7 to 10 m (roughly 20 to 30 ft) below land surface, a sample can be collected without contamination and without chlorine disinfection by use of a surface vacuum pump, a sterile vacuum flask, and two lengths of sterile silicon tubing (U.S. Environmental Protection Agency, 1982), as follows—

1. Obtain and assemble apparatus as shown on figure 7.1-1. To prevent standing water from entering the sampling tube upon insertion into the well, make the sampling tube at least 1 ft shorter than the flushing tube.
2. Autoclave flask and tubing inside a larger autoclavable container or bag before starting field work.
3. Attach tubing to the inlet side of the pump and lower into the well. **Handle the tubing with sterile gloves.**
4. Turn on the pump to the flushing system as the tubing is put into the well and purge the well (NFM 6.0).
5. After purging, turn on the pump to the sample line and collect the sample into the sterile vacuum flask.
6. Transfer the sample from the sterile flask to a sterile sample bottle and keep the sample chilled before processing.

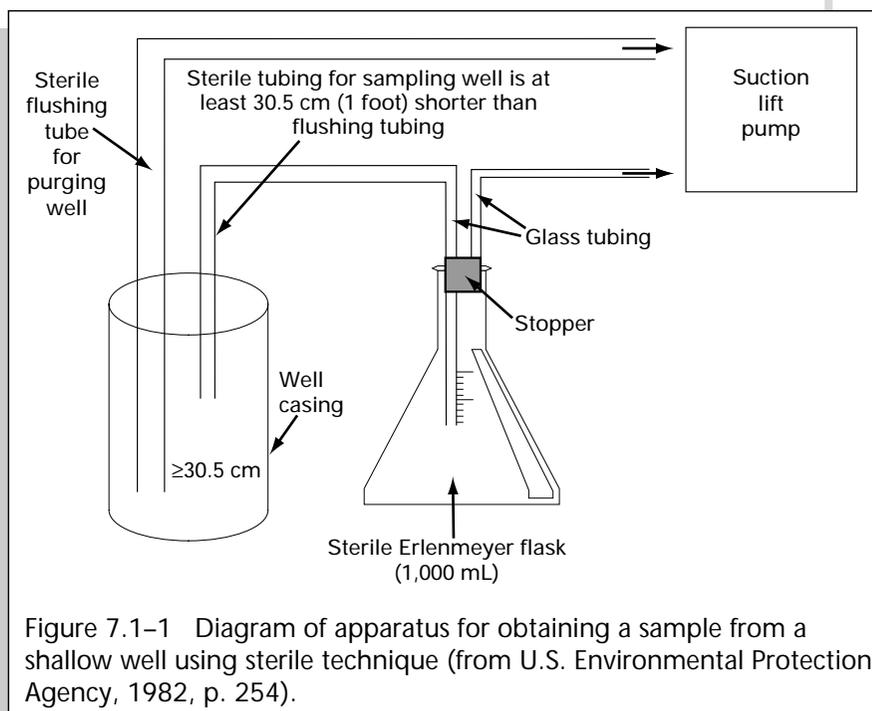


Figure 7.1-1 Diagram of apparatus for obtaining a sample from a shallow well using sterile technique (from U.S. Environmental Protection Agency, 1982, p. 254).

***Precautions for collecting samples for microbiological analysis from monitoring wells:***

- ▶ Avoid collecting samples from wells with casings made of galvanized materials; such casings can contain bacteriocidal metals. If samples must be collected from these types of wells, add 0.3 mL of a 15-percent EDTA solution per 100 mL of sample.
- ▶ Purge the well (see NFM 6.0.2) while monitoring field measurements. Measurements of turbidity and dissolved oxygen are especially relevant. For wells in which field measurements do not stabilize, proceed with sampling, and use replicate samples for quality control.

Avoid contamination—the maximum contaminant limit for most domestic and public supply wells is exceeded with four total or one fecal coliform or *E. coli* colony per 100 mL sample (USEPA, 1991b).

### **7.1.2.C SAMPLE PRESERVATION AND STORAGE**

After collection, immediately chill samples in an ice chest or refrigerator at 1 to 4°C. Do not freeze samples. Begin analysis as quickly as possible, preferably within 1 hour but not more than 6 hours after sample collection, to minimize changes in the concentration of indicator bacteria.

Chill and store samples in the dark until analysis.

## 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  $24 \pm 2$  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  $24 \pm 2$  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  $48 \pm 2$  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  $41.0^\circ\text{C}$  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.

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### 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- $\beta$ -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) <sup>1</sup>	Added as (mL) <sup>2</sup>	Sample volume (mL) <sup>1</sup>	Added as (mL) <sup>2</sup>	Sample volume (mL) <sup>1</sup>	Added as (mL) <sup>2</sup>
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				

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

<sup>2</sup>Sample 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.)

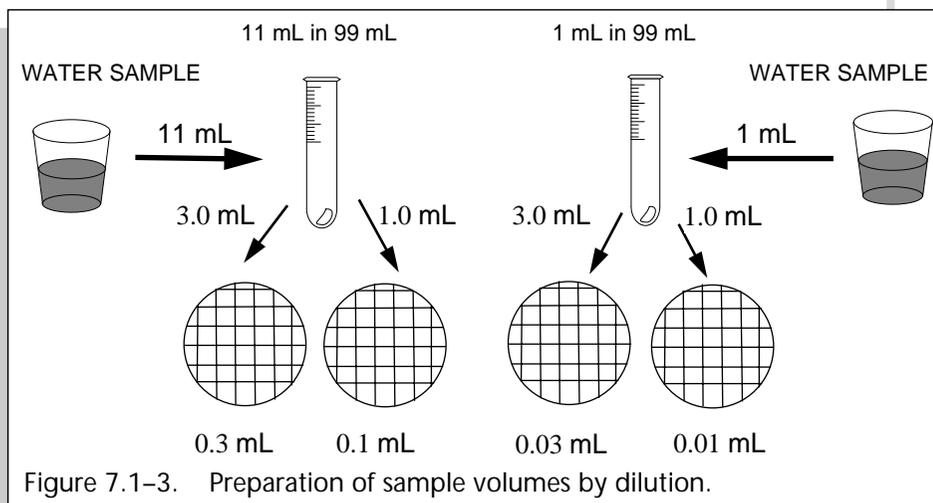
Using sterile forceps, place a sterile, gridded membrane filter (47-mm diameter) on top of the filter base, grid-side up. Be sure to use the correct pore-size membrane filter for the test procedure (table 7.1–7).

- 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/in<sup>2</sup> (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.

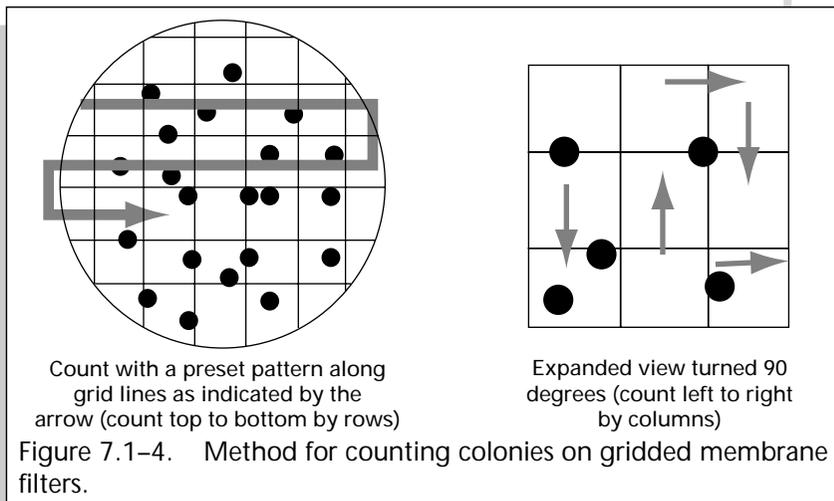
- The presence of colonies on blanks indicates that results of the bacterial analyses bracketed by positive blanks are suspect and should not be reported.
- It is not valid to subtract colony counts on blanks from results calculated for samples.

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- $\beta$ -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).



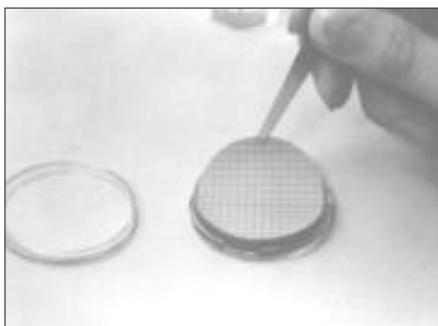
#### PROCEDURE

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).

## 7.1.4 CALCULATION AND REPORTING OF FECAL INDICATOR BACTERIA

The range of ideal colony counts differs depending on the fecal indicator group to be enumerated (table 7.1–9). Crowding and insufficient media to support full development of colonies can result if the bacterial concentration on the filter exceeds the upper limit of the ideal range. The lower limit of the ideal range is set as a number below which statistical validity becomes questionable (Britton and Greeson, 1989, p. 14). For potable waters, results are routinely reported from counts of less than 20 colonies per filter. The computation is the sum of the colony counts for each sample volume, multiplied by 100, and divided by the sum of the sample volumes.

- ▶ Enumeration results for the membrane-filter method are expressed as a concentration in units of col/100 mL.
- ▶ Whole numbers are reported for results less than 10, and two significant figures are reported for results greater than or equal to 10.

The examples that follow include most of the colony-count situations commonly experienced:

**Case 1: Ideal colony counts****Example 1:** Ideal colony count on one filter

<u>Sample volume</u>	<u>Colony count</u>
6.0	7 (do not use)
25	21
100	101 (do not use)
Sum 25	21

$$\text{col}/100 \text{ mL} = (21 \times 100) / 25 = 84$$

**Example 2:** Ideal colony counts on two or more filters

<u>Sample volume</u>	<u>Colony count</u>
6.0	7 (do not use)
25	21
100	58
Sum 125	79

$$\text{col}/100 \text{ mL} = (21 + 58) \times 100 / (100 + 25) = 63$$

**Example 3:** If dilutions of the original sample were made before filtration (sample volumes less than 1 mL), calculate the volume (in mL) of original sample as follows:

(TNTC, "Too numerous to count")

<u>Sample volume</u>	<u>Colony count</u>
0.1 (1:10)	50
0.3 (3:10)	TNTC (do not use)
1.0	TNTC (do not use)
Sum 0.1	50

$$\text{Colony count} = 50$$

$$\text{Volume of original sample} = 0.1 \text{ mL}$$

$$\text{Volume of diluted sample} = 1.0 \text{ mL}$$

$$\text{col}/100 \text{ mL} = 50 \times 100 / 1 \times 0.1 = 50,000$$

**Case 2: Colony counts less than or greater than the ideal range but not zero or TNTC (Too Numerous To Count).**

Results are reported with the statement,  
 "Estimated count based on non-ideal colony count."

Example 1: Less than the ideal range on all filters

<u>Sample volume</u>	<u>Colony count</u>
3.0	2
10	6
30	18
<hr/> Sum 43	<hr/> 26

$$\text{col}/100 \text{ mL} = (2 + 6 + 18) \times 100 / (3 + 10 + 30) = 60$$

Example 2: Either greater than or less than the ideal range

<u>Sample volume</u>	<u>Colony count</u>
4	18
20	101
100	TNTC (do not use)
<hr/> Sum 24	<hr/> 119

$$\text{col}/100 \text{ mL} = (18 + 101) \times 100 / (4 + 20) = 500$$

**Case 3: No typical colonies on any of the filters. Assume a colony count of one on the filter with the largest sample volume.**

Result is reported as less than the calculated number per 100 mL.  
 It is a maximum estimated number.

<u>Sample volume</u>	<u>Colony count</u>
3.0	0 (do not use)
10	0 (do not use)
30	assume 1
<hr/> Sum 30	<hr/> 1

$$\text{col}/100 \text{ mL} = (1 \times 100) / 30 = <3$$

**Case 4: Less than the ideal range—including some zero counts—and no filters that are TNTC (Too numerous to count).**

The result is reported as "Estimated count based on non-ideal colony count."

<u>Sample volume</u>	<u>Colony count</u>
3.0	0 (do not use)
10	0 (do not use)
30	5
<hr/> Sum 30	<hr/> 5

$$\text{col}/100 \text{ mL} = 5 \times 100 / 30 = 17$$

**Case 5: Colony counts on all filters are TNTC. Assume maximum ideal count on filter with the smallest volume filtered.**

(Result is reported as greater than the calculated number per 100 mL. It is a minimum estimated number.)

<u>Sample volume</u>	<u>Colony count</u>
3.0	TNTC assume 60
10	TNTC (do not use)
30	TNTC (do not use)
<hr/> Sum 3	<hr/> 60

$$\text{col}/100 \text{ mL} = 60 \times 100 / 3 = >2,000$$

## 7.1.5 INSTRUCTIONS FOR MEDIA PREPARATION

1. **m-Endo media for total coliform analysis.**
  - a. Empty the vial containing 4.8 g dehydrated m-Endo media into a 250-mL beaker or flask and add 100 mL of a 2-percent ethanol solution.
  - b. Stir the mixture well for several minutes to break up clumps and prevent agar from adhering to the flask.
  - c. Place the flask in a heated water bath or on a hot plate and heat slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. **Do not autoclave.**
  - d. When medium reaches the boiling point, promptly remove from heat. **Do not boil.**
  - e. Cool the medium to a temperature of about 50°C and pour 6 to 7 mL in 50-mm petri dish bottoms. Quickly place petri dish tops loosely on petri dish bottoms.
  - f. When the medium has solidified, close petri dishes by pressing on tops firmly. The dishes are suitable for use after the medium has solidified. About 15 to 20 petri dishes can be filled from 100 mL of media.
  - g. Plates not used immediately after preparation should be placed upside down in small plastic bags to prevent drying and stored in darkness in a refrigerator for a maximum of 5 days.
2. **NA-MUG media for confirmation of *Escherichia coli* after primary culturing of total coliform bacteria with m-Endo medium.**
  - a. Add 2.3 g of NA-MUG media to 100 mL of deionized or distilled reagent grade water in a 250-mL flask or beaker.
  - b. Stir the mixture well for several minutes to break up clumps and prevent media from adhering to the flask.
  - c. Place the flask in a heated water bath or on a hot plate and heat slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching.
  - d. Autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow to cool to 44 to 46°C, or when the flask is cool enough to pick up bare handed.
  - e. Pour 6 to 7 mL of medium into 50-mm petri dish bottoms.

About 15 to 20 petri dishes can be filled from 100 mL NAMUG media. Quickly place petri dish tops loosely on petri dish bottoms.

- f. When the medium has solidified, close petri dishes by pressing on tops firmly. The dishes are suitable for use after the medium has solidified. Prepared dishes, sealed in small plastic bags to prevent drying, can be stored in a refrigerator up to 2 weeks.

### 3. m-FC media for fecal coliform bacteria analysis.

- a. Prepare a rosolic acid solution by adding 10 mL of 0.2 N sodium hydroxide to 0.1 g rosolic acid crystals. Shake the mixture to dissolve crystals. **Do not heat.** The crystals will dissolve in 15 minutes. Prepare a new solution for each analysis.
- b. Empty the vial containing 5.2 g dehydrated media into 100 mL of deionized or distilled water in a 250-mL flask.
- c. Stir the mixture well for several minutes to break up clumps and prevent media from adhering to the flask.
- d. Place the flask in a heated water bath or on a hot plate and heat slowly to 90°C. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. **Do not autoclave.**
- e. With a clean pipet, add 1 mL rosolic acid solution per 100 mL of media when the medium reaches 90°C. Continue heating until boiling begins. Remove from heat.
- f. Cool the medium to a temperature of about 45 to 50°C and pour 6 to 7 mL in 50-mm petri dish bottoms. Place petri dish tops loosely on petri dish bottoms to allow condensation to escape.
- g. When the media has solidified (about 10 minutes), close petri dishes by pressing on tops firmly. The petri dishes are suitable for use after the medium has solidified.
- h. Petri dishes not used immediately after preparation should be placed upside down in small plastic bags to prevent drying and can be stored in a refrigerator for no more than 72 hours.

### 4. KF media for fecal streptococci analysis.

- a. Empty the vial containing 7.64 g dehydrated media into 100 mL of distilled or deionized water in a 250-mL beaker or flask.
- b. Stir the mixture well for several minutes to break up clumps and prevent media from adhering to the flask.
- c. Add 10 mL of deionized water to the 0.015 g TTC (triphenyltetrazolium chloride crystals).

- d. Place the flask in a heated water bath or on a hot plate and heat slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. After boiling begins, simmer at this temperature for 5 minutes.
- e. Remove media solution from heat and cool to 50 to 60°C. Sterilize the TTC solution by aseptically filtering through a disposable sterile membrane filter (0.22 µm). Add 1 mL of sterile TTC solution to 100 mL of media and stir. Prepare fresh TTC solution each time media is prepared.
- f. Cool the medium to a temperature of about 50°C and pour 6 to 7 mL in 50-mm petri dish bottoms. Place the petri dish tops loosely on the dish bottoms to allow condensation to escape.
- g. When the medium has solidified, close the petri dishes tightly by pressing firmly on the tops. Petri dishes are suitable for use after the medium has solidified.
- h. Petri dishes not used immediately after preparation should be placed in small plastic bags to prevent drying and can be stored in the refrigerator for up to 2 weeks if sterile TTC was used. If not, the medium should be used within 24 hours.

#### 5. m-TEC media for *E. coli* analysis.

- a. Empty the vial containing 4.53 g dehydrated m-TEC media into 100 mL of distilled or deionized water in a 250-mL beaker or flask.
- b. Stir this mixture for several minutes to break up clumps. Make sure that none of the media adheres to the bottom or side of the beaker.
- c. Place the beaker containing the media solution in a heated water bath or on a hot plate and begin heating slowly to boiling. If using a hot plate, stir the mixture constantly or use a stir bar and magnetic stirring hot plate to prevent scorching. After boiling begins, remove the beaker from the hot plate or boiling water bath and autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow to cool to 45 to 50°C, or when the beaker is cool enough to pick up bare handed.
- d. Pour 6 to 7 mL of the medium into 50-mm petri dish bottoms. Place the petri dish tops loosely on dish bottoms to allow condensation to escape.
- e. When the medium has solidified (in about 10 minutes) close the petri dishes tightly by pressing firmly on the tops. The petri dishes are suitable for use after the medium has solidified.

- f. Petri dishes not used immediately after preparation should be placed in small plastic bags to prevent drying and stored in the refrigerator (at about 5°C). The dishes can be stored up to 2 weeks.

#### 6. Urea-phenol broth for confirmation of *E. coli*.

- a. **Confirmation of *E. coli* by urea-phenol broth media is required.** After sample incubation, prepare the urea-phenol red broth by adding 100 mL of sterile distilled or deionized water to 2.0 g urea and 0.01 g phenol red crystals in a 250-mL beaker and mix thoroughly.
- b. When preparing the filter pads for counting colonies, use a clean pipet or dropper and add 2.0 mL of the solution to each absorbent pad before placing the filter on the pad. Before transferring the filters from m-TEC to urea-phenol broth, carefully drain excess solution from each pad by tilting the plate against a clean lab wipe.

#### 7. m-E media for enterococci bacteria analysis.

- a. Pour 5 to 10 mL distilled or deionized water into a bottle containing 0.015 g TTC crystals. Cap and shake the TTC to dissolve the crystals. If using a QWSU kit, retain the remaining 90 mL of distilled water. **Do not heat.**
- b. Empty the vial containing 7.12 g m-E medium into the remainder of the distilled water (approximately 90 mL) in a 250-mL flask or beaker.
- c. Stir this mixture for several minutes to break up clumps. It is important that none of the media adheres to the bottom or the side of the beaker.
- d. Prepare the nalidixic acid solution by adding 10 mL of 0.2 N NaOH solution to 0.25 g nalidixic acid crystals. Shake the mixture to dissolve crystals. **Do not heat.** Make sure all the crystals dissolve.
- e. Place the beaker containing the media solution in a heated water bath or on a hot plate and stir the solution constantly, or use a stir bar and magnetic stirring hot plate to prevent scorching. After ingredients dissolve, autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow to cool enough to pick up bare handed.
- f. The dissolved reagents can now be added. Using a sterile pipet, add 1.3 mL of the nalidixic acid solution. If the medium is intended to be used within 24 hours, add all the TTC solution directly from the bottle. If the prepared medium will be stored for a longer period before use, the TTC solution should

be sterilized by passage through a 0.22-mm membrane filter in an aseptic manner.

- g. Pour 6 to 7 mL of the medium into 50-mm petri dish bottoms. Place the petri dish tops loosely on the dish bottoms to allow condensation to escape.
- h. When the medium has solidified (about 10 minutes), close the petri dishes by pressing firmly on the tops. The plates are suitable for use after the medium has solidified. Prepared petri dishes, sealed in small plastic bags to prevent drying, can be stored in a refrigerator for up to 2 weeks if sterile TTC was used. If not, the medium should be used within 24 hours.

#### 8. EIA media for confirmation of enterococci bacteria.

- a. **Confirmation of enterococci bacteria by EIA is required.** If using the QWSU kit, the second bottle of 100 mL distilled water is used to prepare the EIA medium. This medium is used for confirmation of enterococci colonies after primary incubation of the filters on m-E medium.
- b. Empty the vial containing 1.65 g EIA media into 133 mL of distilled or deionized water in a 250-mL flask or beaker.
- c. Stir this mixture for several minutes to break up clumps. Make sure that none of the media adheres to the bottom or the side of the beaker.
- d. Place the beaker containing the media solution in a heated water bath or on a hot plate and stir the solution constantly, or use a stir bar and magnetic stirring hot plate to prevent scorching.
- e. After ingredients dissolve, autoclave at 121°C and 15 lb/in<sup>2</sup> for 15 minutes. Allow beaker to cool enough to pick up bare handed.
- f. Pour the 6 to 7 mL of EIA medium into 50-mm petri dish bottoms. Place the petri dish tops loosely on dish bottoms to allow condensation to escape.
- g. When the medium has solidified (in about 10 minutes) close the petri dishes tightly by pressing on the dish tops. The petri dishes are suitable for use after the medium has solidified.
- h. Prepared petri dishes, sealed in small plastic bags to prevent drying, can be stored in a refrigerator for up to 2 weeks.