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Method 1103.2: *Escherichia coli (E. coli)* in Water by Membrane Filtration Using membrane-Thermotolerant *Escherichia coli* Agar (mTEC)

U.S. Environmental Protection Agency
Office of Water (4303T)
1200 Pennsylvania Avenue, NW
Washington, DC 20460

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- EPA Office of Research and Development, National Risk Management Research Lab: Mark C. Meckes
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Method 1103.2 revisions include incorporation of editorial changes, standardizing language between the EPA membrane filtration methods, current lab practices and clarifying edits.

Questions concerning this method, or its application should be addressed to:

Engineering and Analysis Division (4303T)
U.S. EPA Office of Water, Office of Science and
Technology 1200 Pennsylvania Avenue, NW
Washington, DC 20460
https://www.epa.gov/cwa-methods/forms/contact-us-about-cwa-analytical-methods

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Method 1103.2 *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using membrane-Thermotolerant *Escherichia coli* Agar (mTEC)

September 2023

1.0 Scope and Application

1.1 Method 1103.2 describes a membrane filter (MF) procedure for the detection and enumeration of *Escherichia coli* bacteria in ambient (fresh) water. This is a two-step method which requires transferring the membrane filter after incubation to a pad saturated with a substrate.

1.2 *E. coli* is a common inhabitant of the intestinal tract of warm-blooded animals, and its presence in ambient water samples is an indication of fecal pollution and the possible presence of enteric pathogens.

1.3 The use of *E. coli* as an indicator is recommended as a measure of ambient water quality. Epidemiological studies have led to the development of criteria which can be used for recreational water quality standards based on established relationships between health effects and water quality. The significance of finding *E. coli* in ambient water samples is the direct relationship between the density of *E. coli* and the risk of gastrointestinal illness associated with swimming in the water (Reference 18.1).

1.4 Method 1103.1 and 1603 were submitted to interlaboratory validation in wastewater matrices and study results compared. The estimated percent of total colonies that would have resulted in false positives for disinfected, secondary, and combined disinfected/secondary wastewater were significantly lower for Method 1603 (0.6%, 1.4%, and 1.0%, respectively) compared to Method 1103.1 (6.2%, 11.2%, and 10.3%, respectively). The estimated percent of total colonies that would have resulted in false negatives for disinfected and combined disinfected/secondary wastewater were also significantly lower for Method 1603 (2.5%, and 2.2%, respectively) compared to Method 1103.1 (12.3% and 6.0%, respectively). The estimated percent of total colonies that would have resulted in false negatives were not significantly different for secondary wastewater samples for Methods 1603 and 1103.1 (2.6% and 2.0%, respectively).

Based on the high 1103.1 false positive and false negative levels when compared to Method 1603, Method 1103.1 is not approved for the analysis of disinfected wastewater. Laboratories wishing to test for *E. coli* in wastewater using a membrane filtration method are referred to EPA Method 1603.1. A summary of Method 1103.1 false positive and negative results is provided in Section 15.3.2.

1.5 For method application please refer to Title 40 Code of Federal Regulations Part 136 (40 CFR Part 136).

2.0 Summary of Method

Method 1103.2 provides a direct count of bacteria in ambient water based on the development of colonies on the surface of the membrane filter (Reference 18.2). A water sample is filtered through the membrane filter which retains the bacteria. After filtration, the membrane filter is placed on a selective and differential medium, mTEC, incubated at 35°C ± 0.5°C for 2 ± 0.5 hours to resuscitate injured or stressed bacteria, and then incubated at 44.5°C ± 0.2°C for 22 ± 2 hours. Following

incubation, the membrane filter is transferred to a filter pad saturated with urea substrate. After 15 minutes, yellow, yellow-green, or yellow-brown colonies are recorded as *E. coli*. A fluorescent lamp with a magnifying lens is used for counting to give maximum visibility of colonies.

3.0 Definition

In Method 1103.2, *E. coli* are those bacteria which produce colonies that remain yellow, yellow-green, or yellow-brown on a filter pad saturated with urea substrate broth after primary culturing on mTEC medium.

4.0 Interferences and Contamination

4.1 Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with enumeration and identification of target colonies.

4.2 The presence of high numbers of competing or inhibitory organisms, background debris, or toxic substances (e.g., metals or organic compounds) could interfere with enumeration of target colonies.

5.0 Safety

5.1 The analyst must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials and while operating sterilization equipment.

5.2 Mouth-pipetting is prohibited.

5.3 This method does not address all of the safety issues associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. A reference file of safety data sheets (SDSs) should be available to all personnel involved in Method 1103.2 analyses.

6.0 Equipment and Supplies

6.1 Glass lens with magnification of 2-5X, or stereoscopic microscope

6.2 Lamp, with a cool, white fluorescent tube

6.3 Hand tally or electronic counting device

6.4 Pipet container, stainless steel or aluminum, for reusable pipets

6.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume

6.6 Sterile graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper
***Note**: Graduated cylinders should be accurate to within ≤2.5% (Class B or better).*

6.7 Sterile membrane filtration units (filter base and funnel), glass, plastic, disposable, or stainless steel; wrapped with aluminum foil, kraft paper, or in sterilization pouches

6.8 Ultraviolet unit for sanitization of the filter funnel between filtrations (optional)

6.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source (In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used)

6.10 Filter manifold to hold a number of filter bases or vacuum filter flask (1 L) with appropriate tubing

6.11 Flask for safety trap placed between the filtration apparatus and the vacuum source

6.12 Forceps, straight or curved, with smooth tips to handle filters without damage

6.13 Ethanol, methanol, or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps

6.14 Burner, Bunsen, or Fisher type, or electric incinerator unit for sterilizing loops and needles

6.15 Thermometer (digital or non-mercury), verified against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Handbook 105-6

6.16 Balance, analytical balance capable of weighing 0.1 mg

6.17 pH meter

6.18 Petri dishes, sterile, plastic, 9 × 50 mm with tight-fitting lids; and 15 × 100 mm with loose fitting lids

6.19 Dilution bottles, (e.g., milk dilution), borosilicate glass, screw cap with neoprene liners, 125 mL volume with markings at 99 mL or 90 mL to prepare 1:100 and 1:10 dilutions, respectively

6.20 Flasks, borosilicate glass, screw cap, 250-2000 mL volume

6.21 Cornwall syringe, sterile, to deliver at least 5 mL or media dispensing pump

6.22 Cellulose ester membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 µm pore size

6.23 Absorbent pads, sterile, 47 mm diameter

6.24 Platinum wire inoculation loops, at least 3 mm diameter in suitable holders; or sterile plastic loops

6.25 Sterile disposable applicator sticks

6.26 Incubator maintained at 35°C ± 0.5°C

6.27 Water bath maintained at 44.5°C ± 0.2°C

6.28 Water bath for tempering media

6.29 Test tubes, borosilicate glass, 16 × 150 mm or other appropriate size(s), and autoclavable caps

6.30 Durham tubes, borosilicate glass, 10 × 75 mm

6.31 Whirl-Pak® bags or equivalent

6.32 Autoclave or steam sterilizer capable of achieving 121°C (15 lb pressure per square inch [PSI]) for 15 minutes

7.0 Reagents and Standards

7.1 Purity of reagents: Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee of Analytical Reagents of the American Chemical Society (Reference 18.3). The agar used in preparation of the culture media must be of microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control.

7.3 Purity of reagent water: Reagent-grade water conforming to specifications in: *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 136), Section 9020 (Reference 18.4).

7.4 Phosphate buffered saline (PBS)

7.4.1 Composition:

Monosodium phosphate (NaH_2PO_4)	0.58 g
Disodium phosphate (Na_2HPO_4)	2.5 g
Sodium chloride	8.5 g
Reagent-grade water	1.0 L

7.4.2 Dissolve the ingredients in 1 L of reagent-grade water and dispense in appropriate amounts for dilutions in screw cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap containers.

***Note**: The initial and ongoing precision and recovery (IPR and OPR) performance criteria established for Method 1103.2 were determined using spiked PBS samples (Section 9.3, **Table 1**). Laboratories must use PBS when performing IPR and OPR sample analyses. However, phosphate-buffered dilution water (Section 7.5) may be substituted for PBS as a sample diluent and filtration rinse buffer.*

7.5 Phosphate buffered dilution water (Reference 18.5)

7.5.1 Composition:

Stock phosphate buffer solution (Section 7.5.2.1)	1.25 mL
Stock magnesium chloride ($MgCl_2$) solution (Section 7.5.2.2)	5.0 mL
Reagent-grade water	1.0 L

Mix 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade water. Dispense in appropriate amounts for dilutions. Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.0 ± 0.2. The amount of time in the autoclave must be adjusted for the volume of buffer in the containers and the size of the load. Store at <10°C, but above freezing, for up to three months in screw cap containers.

7.5.2 Stock Solutions

7.5.2.1 Preparation of stock phosphate buffer solution: Dissolve 34 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL reagent-grade water. Adjust the pH of the solution to 7.2 with 1 N sodium hydroxide (NaOH) and bring the volume to 1 L with reagent-grade water. Sterilize by filtration or autoclave at 121°C (15 PSI) for 15 minutes.

7.5.2.2 Preparation of stock magnesium chloride solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$ to 1 L reagent-grade water. Sterilize by filtration or autoclave at 121°C (15 PSI) for 15 minutes.

***Note**: After sterilization, store the stock solutions at <10°C, but above freezing, for up to three months in screw cap containers. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.*

7.6 mTEC agar

7.6.1 Composition:

Protease peptone #3	5.0	g
Yeast extract	3.0	g
Lactose	10.0	g
Sodium chloride	7.5	g
Dipotassium phosphate (K_2HPO_4)	3.3	g
Monopotassium phosphate (KH_2PO_4)	1.0	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Bromocresol purple	0.08	g
Bromophenol red	0.08	g
Agar	15.0	g
Reagent-grade water	1.0	L

7.6.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve. Autoclave at 121°C (15 PSI) for 15 minutes, and cool to 45°C - 50°C in a water bath according to manufacturer's instructions. Dispense ~4-6 mL of mTEC into 9 × 50 mm sterile Petri dishes to a 4-5 mm depth and allow to solidify. Final pH should be 7.3 ± 0.2. Invert and store prepared plates at <10°C, but above freezing, for up to two weeks.

7.7 Urea substrate medium

7.7.1 Composition:

Urea	2.0	g
Phenol red	0.01	g
Reagent-grade water	100.0	mL

7.7.2 Add reagents to 100 mL reagent-grade water and mix thoroughly to dissolve. Adjust to pH 5.0 ± 0.2 with 1 N HCl. The substrate solution should be a straw-yellow color at this pH (See **Photo 1**). Store at <10°C, but above freezing, for up to one week.

Photo 1. Urea substrate medium should be straw-yellow in color.

7.8 Tryptic soy agar (TSA)

7.8.1 Composition:

Pancreatic digest of casein	15.0 g
Enzymatic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.8.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 PSI) for 15 minutes and cool to 45°C - 50°C in a water bath according to manufacturer's instructions. After cooling, dispense ~12-15 mL of TSA into each 15 × 100 mm sterile Petri dish to a 4-5 mm depth and allow to solidify. Final pH should be 7.3 ± 0.2. Invert and store prepared plates at <10°C, but above freezing, for up to two weeks.

7.9 Lauryl tryptose broth (LTB)

7.9.1 Composition

Tryptose	20.0 g
Lactose	5.0 g
Dipotassium phosphate (K_2HPO_4)	2.75 g
Monopotassium phosphate (KH_2PO_4)	2.75 g
Sodium chloride	5.0 g
Sodium lauryl sulfate	0.1 g
Reagent-grade water	1.0 L

7.9.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and warm to dissolve completely. Dispense 10 mL of LTB into 16 × 150 mm tubes containing inverted Durham tubes (10 × 75 mm tubes). Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should 6.8 ±

0.2. Store at <10°C, but above freezing, for up to two weeks in loose cap tubes or up to three months in screw cap tubes.

***Note**: Do not use tubes if the inverted tubes (Durham tubes) are not completely filled with medium after sterilization.*

7.10 Nutrient agar (NA) slants

7.10.1 Composition:

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.10.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to boiling to dissolve completely. Dispense in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Remove the tubes and slant until solid. Final pH should be 6.8 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap tubes.

7.11 Tryptic/trypticase soy broth (TSB)

7.11.1 Composition:

Pancreatic digest of casein	17.0 g
Enzymatic/papaic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Dextrose	2.5 g
Dipotassium phosphate (K_2HPO_4)	2.5 g
Reagent-grade water	1.0 L

7.11.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense in screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.3 ± 0.2. Invert and store at <10°C, but above freezing, for up to three months in screw cap tubes.

7.12 Simmons citrate agar slants

7.12.1 Composition:

Magnesium sulfate ($MgSO_4$)	0.2 g
Monoammonium phosphate ($NH_4H_2PO_4$)	1.0 g
Dipotassium phosphate (K_2HPO_4)	1.0 g
Sodium citrate (Citric acid)	2.0 g
Sodium chloride	5.0 g
Bromothymol blue	0.08 g
Agar	15.0 g

Reagent-grade water	1.0 L

7.12.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense 10 mL volumes into 16 × 125 mm screw cap tubes for slants. Autoclave at 121°C (15 PSI) for 15 minutes. After sterilization, slant until solid. Final pH should be 6.9 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap tubes.

7.13 Tryptone water

7.13.1 Composition:

Tryptone	10.0 g
Sodium chloride	5.0 g
Reagent-grade water	1.0 L

7.13.2 Add reagents to 1 L of reagent-grade water and mix thoroughly to dissolve. Dispense in 5-mL volumes into 16 × 125 mm screw cap tubes, and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.3 ± 0.2. Store at <10°C, but above freezing, for up to three months in screw cap tubes.

7.14 EC Broth

7.14.1 Composition:

Tryptose or trypticase peptone	20.0 g
Lactose	5.0 g
Bile salts No. 3	1.5 g
Dipotassium phosphate (K_2HPO_4)	4.0 g
Monopotassium phosphate (KH_2PO_4)	1.5 g
Sodium chloride	5.0 g
Reagent-grade water	1.0 L

7.14.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense into fermentation tubes (16 × 150 mm tubes containing inverted 10 × 75 mm Durham tubes). Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 6.9 ± 0.2. Store at <10°C, but above freezing, for up to two weeks in loose cap tubes or up to three months in screw cap tubes.

***Note**: Do not use tubes if the inverted tubes (Durham tubes) are not completely filled with medium after sterilization.*

7.15 Oxidase reagent

Composition:

N, N, N',N'-tetramethyl-p-phenylenediamine dihydrochloride, 1% aqueous solution (1 g per 100 mL sterile reagent-grade water)

***Note**: Prepared oxidase test slides are commercially available and are recommended for colony verification (Section 12.0).*

7.16 Kovacs indole reagent

7.16.1 Composition:

ρ-dimethylaminobenzaldehyde	10.0 g
Amyl or Isoamyl alcohol	150.0 mL
Concentrated (12 M) hydrochloric acid	50.0 mL

7.16.2 Dissolve ρ-dimethylaminobenzaldehyde in alcohol, slowly add hydrochloric acid, and mix.

7.17 Control cultures

7.17.1 Positive control and/or spiking organism (either of the following are acceptable)

- Stock cultures of *Escherichia coli* (*E. coli*) ATCC® # 11775™
- *E. coli* ATCC® # 11775™ BioBalls (bioMérieux Inc., Durham, NC)

7.17.2 Negative control organism (either of the following are acceptable)

- Stock cultures of *Enterococcus faecalis* (*E. faecalis*) ATCC® # 19433™
- *E. faecalis* ATCC® # 19433™ BioBalls (bioMérieux Inc., Durham, NC)

OR

- Stock cultures of *Enterobacter aerogenes* (*E. aerogenes*) ATCC® # 13048™

8.0 Sample Collection, Handling, and Storage

Sampling procedures are briefly described below. Detailed sampling methods can be found in *Microbiological Methods for Monitoring the Environment. Water and Wastes*, Part II, Section A Reference 18.5, and Appendix A. Adherence to sample handling procedures and holding time limits is critical to the production of valid data. Samples should not be analyzed if these conditions below are not met.

8.1 Sampling techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge, or bank adjacent to a surface water. Composite samples should not be collected, since such samples do not display the range of values found in individual samples.

The sampling depth for surface water samples should be 6-12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to allow for proper mixing before analyses.

8.2 Storage temperature and handling conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

8.3 Holding time limitations

Sample analysis should begin immediately, preferably within 2 hours of collection. Sample incubation must be started no later than 8 hours from time of collection.

9.0 Quality Control

Each laboratory that uses Method 1103.2 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. General requirements and recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 18.5.

9.1 The minimum analytical QC requirements for the analysis of samples using Method 1103.2 include routine analysis of positive and negative controls (Section 9.5), filter sterility checks (Section 9.7), method blanks (Section 9.8), and media sterility checks (Section 9.10). Additional analytical QC for the analysis of samples using Method 1103.2 include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.3), ongoing demonstration of laboratory capability through performance of ongoing precision and recovery (OPR) analysis (Section 9.4). For the IPR and OPR analyses, it is necessary to spike PBS samples with either laboratory-prepared spiking suspensions or BioBalls as described in Section 14.

9.2 **Initial precision and recovery (IPR)** – IPR analyses are used to demonstrate acceptable method performance (recovery and precision) by each laboratory before the method is used for monitoring field samples. EPA recommends but does not require that IPR analyses be performed by each analyst. IPR samples should be accompanied by an acceptable method blank (Section 9.8) and appropriate media sterility checks (Section 9.9). The IPR analyses are performed as follows:

9.2.1 Prepare four, 100-mL samples of sterile PBS and spike each sample with *E. coli* ATCC® # 11775™ according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each IPR sample according to the procedures in Section 11 and calculate the number of *E. coli* per 100 mL according to Section 13.

9.2.2 Calculate the percent recovery (R) for each IPR sample using the appropriate equation in Section 14.2.4.3 or 14.3.2 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

9.2.3 Using the percent recoveries of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.

9.2.4 Compare the mean recovery and RSD with the corresponding IPR criteria in Table 1, below. If the mean and RSD for recovery of *E. coli* meet acceptance criteria, system performance is acceptable, and analysis of field samples may begin. If the mean or the RSD

fall outside of the required range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat IPR analyses.

9.3 Ongoing precision and recovery (OPR) – To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked PBS samples. The laboratory should analyze one OPR sample after every 20 field and matrix spike samples or one per week that samples are analyzed, whichever occurs more frequently. OPR samples should be accompanied by an acceptable method blank (Section 9.8) and appropriate media sterility checks (Section 9.10). The OPR analysis is performed as follows:

9.3.1 Spike a 100-mL sterile PBS sample with *E. coli* ATCC® # 11775™ according to the spiking procedure in Section 14. Spiking with laboratory-prepared suspensions is described in Section 14.2 and spiking with BioBalls is described in Section 14.3. Filter and process each OPR sample according to the procedures in Section 11 and calculate the number of *E. coli* per 100 mL according to Section 13.

9.3.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equation in Section 14.2.4.3 or 14.3.2 for samples spiked with BioBalls or laboratory-prepared spiking suspensions, respectively.

9.3.3 Compare the OPR results (percent recovery) with the corresponding OPR criteria in Table 1, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable, and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the OPR analysis.

9.3.4 As part of the laboratory QA program, method recovery results for OPR and IPR samples should be charted, and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1103.2 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the accuracy as a recovery interval from R - $2s_r$ to R + $2s_r$.

Table 1. Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria

Performance test	Lab-prepared spike acceptance criteria	BioBall® spike acceptance criteria
Initial precision and recovery (IPR)		
• Mean percent recovery	76% - 124%	68% - 96%
• Precision (as maximum relative standard deviation)	41%	25%
Ongoing precision and recovery (OPR) as percent recovery	54% - 146%	58% - 106%

9.4 Culture controls

9.4.1 Negative controls – The laboratory should analyze negative controls to ensure that the mTEC agar and urea substrate are performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a negative control every day that samples are analyzed.

9.4.1.1 Negative controls are conducted by filtering a dilute suspension of viable *E. faecalis* (e.g., ATCC® # 19433™) and analyzing as described in Section 11. Viability of the negative controls should be demonstrated using a non-selective media (e.g., nutrient agar or TSA).

9.4.1.2 If the negative control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the negative control and reanalyze the appropriate negative control.

9.4.2 Positive controls – The laboratory should analyze positive controls to ensure that the mTEC agar and urea substrate are performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a positive control every day that samples are analyzed. An OPR sample (Section 9.4) may take the place of a positive control.

9.4.2.1 Positive controls are conducted by filtering a dilute suspension of viable *E. coli* (e.g., ATCC® # 11775™) and analyzing as described in Section 11.

9.4.2.2 If the positive control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the positive control and reanalyze the appropriate positive control.

9.4.3 Controls for verification media – All verification media should be tested with appropriate positive and negative controls whenever a new batch of media and/or reagents are used. On an ongoing basis, the laboratory should perform positive and negative controls on each of the verification media with each batch of samples submitted to verification. Examples of appropriate controls for verification media are provided in **Table 2**.

Table 2. Verification Controls

Medium	Positive Control	Negative Control
Cytochrome oxidase reagent	*P. aeruginosa*	*E. coli*
Kovacs indole reagent	*E. coli*	*E. aerogenes*
Simmons citrate agar	*E. aerogenes*	*S. flexneri*
EC broth (44.5°C ± 0.2°C)	*E. coli*	*E. aerogenes*

9.5 Colony verification – The laboratory should verify 10 typical colonies (positive) and 10 atypical colonies (negative) per month or 1 typical colony and 1 atypical colony from 10% of all positive samples, whichever is greater, to identify potential errors in lab procedure or change in matrix chemistry or biology. Verification procedures are provided in Section 12.

9.6 Filter sterility check – Place at least one membrane filter on a TSA plate and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the filter. On an ongoing basis, the laboratory should perform a filter sterility check every day that samples are analyzed.

9.7 Method blank – Filter a 50-mL volume of sterile PBS or phosphate-buffered dilution water, place the filter on an mTEC agar plate and incubate according to Section 11. Absence of growth indicates freedom of contamination from the target organism. On an ongoing basis, the laboratory should perform a method blank every day that samples are analyzed.

9.8 **Filtration blank** – Filter a 50-mL volume of sterile PBS or phosphate-buffered dilution water before beginning sample filtrations. Place the filter on a TSA plate and incubate for 24 ± 2 hours at 35°C ± 0.5°C. Absence of growth indicates sterility of the buffer and filtration assembly.

***Note**: If using UV to sanitize funnels in between samples, consider adding additional filtration blanks.*

9.9 **Media sterility check** – The laboratory should test media sterility by incubating one unit (tube or plate) from each batch of medium (TSA, mTEC, urea substrate, and verification media) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check, for each medium used, every day that samples are analyzed.

9.10 **Analyst colony counting variability** – Laboratories with two or more analysts should compare each analyst's colony counts from one positive field sample per month. Colony counts should be within 10% between analysts. Laboratories with a single analyst should have that analyst perform duplicate colony counts of a single membrane filter each month. Duplicate colony counts should be within 5% for a single analyst. If no positive field samples are available, an OPR sample may be substituted for these determinations.

10.0 Calibration and Standardization

10.1 Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.

10.2 Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST Handout 105-6.

10.3 Refrigerators used to store media and reagents should be monitored daily to ensure proper temperature control.

10.4 Calibrate the pH meter prior to each use period with the two standards (e.g., pH 4.0, 7.0, and 10.0) closest to the range being tested.

10.5 Calibrate top-loading balances monthly with reference weights of ASTM Class 2 (Standard E617-18).

11.0 Procedure

11.1 Prepare mTEC agar and urea substrate as directed in Sections 7.6 and 7.7, respectively. If media was prepared in advance and refrigerated, bring to room temperature prior to use.

11.2 Label the Petri dish and report form with the sample identification (e.g., field and QC samples) and sample volume.

11.3 Place a sterile membrane filter on the filter base, grid side up, and attach the funnel to the base so that the membrane filter is held between the funnel and the base.

11.4 Shake the sample bottle vigorously at least 25 times in 7 seconds in a one foot arc to distribute the bacteria uniformly and measure the desired volume of sample or dilution into the funnel.

11.5 Select sample volumes based on previous knowledge of *E. coli* concentration, to produce 20-80*E. coli* colonies on the membranes. It is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate (20-80 *E. coli* colonies) is obtained.

11.6 Smaller sample volumes or sample dilutions can be used to minimize the interference of turbidity or for high bacterial densities. Multiple volumes of the same sample or sample dilutions should be filtered.

***Note**: When analyzing smaller sample volumes (e.g., < 20 mL), add 20-30 mL of sterile PBS or phosphate buffered dilution water to the funnel prior to adding the sample, or add sample volume to a PBS or phosphate buffered water dilution blank (e.g., 20-30 mL), prior to filtration. This will allow even distribution of the sample on the membrane.*

11.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffer. Turn off the vacuum and remove the funnel from the filter base.

11.8 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the mTEC agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter outside the area of filtration, close to the edge of the dish, to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate 35°C ± 0.5°C for 2 ± 0.5 hours.

11.9 After a 2 ± 0.5 hour incubation at 35°C ± 0.5°C, transfer the plates to a bag (e.g., Whirl-Pak®). Seal the bag, removing as much air as possible, and submerge in a 44.5°C ± 0.2°C water bath for 22 ± 2 hours.

***Note**: Do not overfill the Whirl-Pak® bag because this will prevent proper sealing allowing liquid to enter the bag and possibly contaminating the plates.*

11.10 After 22 ± 2 hours, remove the plate from the water bath. Place an absorbent pad in the lid of the same Petri dish and saturate the pad with urea substrate medium. Aseptically transfer the membrane from mTEC agar to the absorbent pad saturated with urea substrate medium and allow to sit at room temperature for 15-20 minutes. (See **Photo 2**)

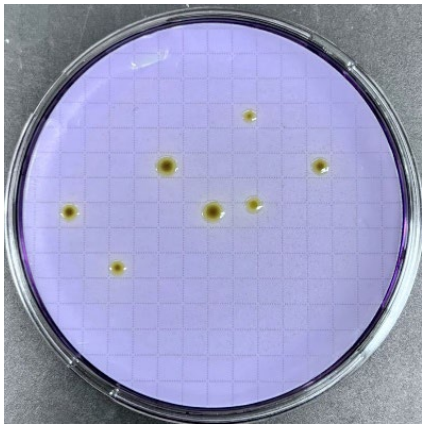

Photo 2. *E. coli* produces yellow, yellow-green, or yellow-brown colonies on mTEC agar.

11.11 After incubation on the urea substrate at room temperature, count and record the number of yellow, yellow-green, or yellow-brown colonies with the aid of an illuminated lens with a 2-5X magnification or a stereoscopic microscope. (See **Photo 3**)

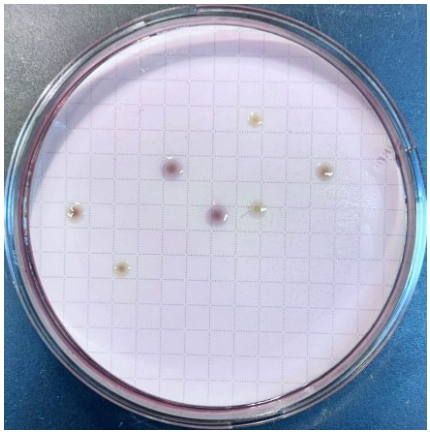

Photo 3. *E. coli* colonies remain yellow, yellow-green, or yellow-brown when the filter is placed on the urea substrate medium, while atypical colonies turn pink or purple.

12.0 Verification Procedure

12.1 Yellow, yellow-green, or yellow-brown colonies from the urease test are considered "typical" *E. coli.* Verification of typical and atypical colonies may be required in evidence gathering and is also recommended to identify potential errors in lab procedure or change in matrix chemistry or biology. The verification procedure is described below.

12.2 Using a sterile inoculating loop or needle, transfer growth from the centers of at least 10 well- isolated typical and 10 well-isolated atypical colonies to nutrient agar plates or slants and to tryptic/trypticase soy broth. Incubate the agar and broth cultures for 24 ± 2 hours at 35°C ± 0.5°C.

12.3 After incubation, transfer a loopful of growth from the nutrient agar plate or slant and perform the cytochrome oxidase test. If the area where the bacteria were applied turns deep purple within 15 seconds, the test is positive.

***Note**: Use only platinum or plastic loops, or wooden applicators, to perform the oxidase test. Do not use iron or other reactive wire because it may cause false positive reactions.*

12.4 Transfer a loopful of broth from each of the inoculated TSB tubes to tubes of Simmons citrate agar, tryptone water, and an EC broth fermentation tube.

12.4.1 Incubate the Simmons citrate agar for 4 days at 35°C ± 2.0°C with loosened caps. A positive reaction is indicated by growth with an intense blue color on the slant. *E. coli* is citrate negative, there should be either no growth or trace growth with no change in agar color (i.e., medium remains dark green).

12.4.2 Incubate the EC broth at 44.5°C ± 0.2°C in a water bath for 24 ± 2 hours. The water level must be above the level of the EC broth in the tube. A positive test is indicated by turbidity and production of gas in the inverted Durham tube.

12.4.3 Incubate the tryptone water for 18-24 hours at 35°C ± 2.0°C with loose caps. After the incubation period, add 0.5 mL of Kovacs indole reagent and shake the tube gently. Allow the tubes to stand for 5-10 minutes at room temperature. A positive test for indole is indicated by a deep red color which develops in the alcohol layer on top of the broth.

12.5 *E. coli* are oxidase-negative, citrate-negative, EC growth and gas-positive, and indole-positive.

12.6 Alternately, commercially available multi-test identification systems for *Enterobacteriaceae* that include lactose fermentation, σ-nitrophenyl-β-D-galactopyranoside (ONPG), and cytochrome oxidase test reactions may be used to verify colonies.

13.0 Data Analysis and Calculations

Use the following general rules to calculate the *E. coli* count (colony forming units [CFU]) per 100 ml of sample:

13.1 If possible, select a membrane filter with 20-80 yellow, yellow-green, or yellow-brown colonies on the urea substrate, and calculate the number of *E. coli* CFU per 100 mL according to the following formula:

$$E.coli\ CFU/100\ mL = \frac{Number\ of\ E.coli\ colonies\ (CFU)}{Volume\ of\ sample\ filtered\ (mL)}\ x\ 100$$

13.2 Refer to general counting rules in Reference 18.5 and Appendix B.

13.3 Report results as *E. coli* CFU per 100 mL of sample.

14.0 Sample Spiking Procedure

14.1 Method 1103.2 QC requirements (Section 9) include the preparation and analysis of spiked reference (PBS) samples in order to monitor initial and ongoing method performance. For the IPR (Section 9.3) and OPR (Section 9.4) tests it is necessary to spike samples with either laboratory-prepared spiking suspensions (Section 14.2) or BioBalls (Section 14.3) as described below.

14.2 Laboratory-Prepared Spiking Suspensions

14.2.1 Preparation

14.2.1.1 Stock Culture. Prepare a stock culture by inoculating a TSA slant (or other non-selective media) with *Escherichia coli* ATCC® # 11775™ and incubating at 35°C ± 3°C for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

14.2.1.2 Undiluted Spiking Suspension. Prepare a 1% solution of LTB by combining 99 mL of sterile PBS and 1 mL of sterile single- strength LTB in a sterile screw cap bottle or re-sealable dilution water container. Inoculate the 1% LTB using a small amount of growth from the stock culture. Disperse the inoculum by vigorously shaking the broth culture and incubate at 35°C ± 3°C for 20 ± 4 hours. This culture is referred to as the undiluted spiking suspension and should contain approximately 1.0×10^7 - 1.0×10^8 *E. coli* CFU per mL of culture.

14.2.1.3 Mix the undiluted spiking suspension thoroughly by shaking the bottle a minimum of 25 times and prepare a series of dilutions (4 total) in the following manner:

14.2.1.3.1 Dilution "A" – Aseptically transfer 1.0 mL of the undiluted spiking suspension to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "A" and 1 mL contains 10^{-2} mL of the original undiluted spiking suspension.

14.2.1.3.2 Dilution "B" – Aseptically transfer 1.0 mL of dilution "A" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "B" and 1 mL contains 10^{-4} mL of the original undiluted spiking suspension.

14.2.1.3.3 Dilution "C" – Aseptically transfer 11.0 mL of dilution "B" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "C" and 1 mL contains 10^{-5} mL of the original undiluted spiking suspension.

14.2.1.3.4 Dilution "D" – Aseptically transfer 11.0 mL of dilution "C" to 99 mL of sterile PBS and mix thoroughly by shaking the bottle a minimum of 25 times. This is spiking suspension dilution "D" and 1 mL contains 10^{-6} mL of the original undiluted spiking suspension.

14.2.2 Sample spiking

14.2.2.1 Add 0.3 mL of the spiking suspension dilution "D" to 100 mL of PBS and mix thoroughly by shaking the bottle a minimum of 25 times. The volume of undiluted spiking suspension added to each 100 mL sample is 3.0×10^{-7} mL [(0.3 mL × 10^{-6} mL) per 100 mL of sample] which is referred to as $V_{\text{spiked per 100 mL sample}}$ in Section 14.2.4.2 below. Filter the spiked sample and analyze the filter according to the procedures in Section 11.

14.2.3 Enumeration

14.2.3.1 Prepare TSA spread plates, in triplicate, for spiking suspension dilutions "B", "C", and "D".

***Note**: Agar plates must be dry prior to use. To ensure that the agar surface is dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.*

14.2.3.2 Mix dilution "B" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "B" onto the surface of each TSA plate in triplicate.

14.2.3.3 Mix dilution "C" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "C" onto the surface of each TSA plate in triplicate.

14.2.3.4 Mix dilution "D" by shaking the bottle a minimum of 25 times. Pipet 0.1 mL of dilution "D" onto the surface of each TSA plate in triplicate.

14.2.3.5 Use a sterile bent glass rod or spreader to distribute the inoculum over the surface of plates by rotating the dish by hand or on a turntable.

***Note**: Ensure that the inoculum is evenly distributed over the entire surface of the plate.*

14.2.3.6 Allow the inoculum to completely absorb into the medium of each plate. Invert plates and incubate at 35°C ± 0.5°C for 20 ± 4 hours.

14.2.3.7 Count and record number of colonies per plate. Refer to Section 14.2.4 for calculation of *E. coli* concentration in the undiluted spiking suspension. The number of *E. coli* (CFU/mL) in the undiluted spiking suspension will be calculated using all TSA plates yielding counts within the countable range of 30 to 300 CFU per plate.

14.2.4 Recovery calculations for samples spiked with laboratory-prepared spiking suspensions

14.2.4.1 Calculate the concentration of *E. coli* (CFU/mL) in the undiluted spiking suspension (Section 14.2.1.2) according to the following equation. Example calculations are provided in **Table 3,** below.

$$E.coli_{\,undiluted\ spike} = \frac{(CFU_1 + CFU_2 + \ldots + CFU_n)}{(V_1 + V_2 + \ldots + V_n)}$$

Where,

CFU = Number of colony forming units from TSA plates yielding counts within the countable range of 30 to 300 CFU per plate

V = Volume of undiluted sample on each TSA plate yielding counts within the countable range of 30 to 300 CFU per plate

n = Number of plates with counts within the countable range of 30 to 300 CFU per plate

***Note**: The example calculated numbers provided in the tables below have been rounded at the end of each step for simplification purposes. Generally, rounding should only occur after the final calculation.*

Table 3. Example Calculations of *E. coli* Spiking Suspension Concentration

Examples	Plate Count (CFU) (triplicate analyses) from TSA plates in Section 14.2.3.7			*E. coli* CFU/mL in undiluted spiking suspension ($EC_{undiluted\ spike}$)*
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	TNTC, TNTC, TNTC	94, 106, 89	10, 0, 4	(94+106+89) / (10^{-6}+10^{-6}+10^{-6}) = 289 / (3.0 × 10^{-6}) = 96,333,333 = **9.6×10^7 CFU/mL**
Example 2	269, 289, 304	24, 30, 28	0, 2, 0	(269+289+30) / (10^{-5}+10^{-5}+10^{-6}) = 588 / (2.1 × 10^{-5}) = 28,000,000 = **2.8×10^7 CFU/mL**

*$EC_{undiluted\ spike}$ is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

14.2.4.2 Calculate true concentration (CFU/100 mL) of spiked *E. coli* ($T_{spiked\ E.\ coli}$) according to the following equation. Example calculations are provided in **Table 4,** below.

$$T_{spiked\ E.\ coli} = E.coli_{\,undiluted\ spike}\ x\ V_{spiked\ per\ 100\ mL\ sample}$$

Where,

$\mathbf{T}_{\text{spiked } E.\ coli}$ = Number of spiked *E. coli* (CFU/100 mL)

$\boldsymbol{E.\ coli}_{\text{undiluted spike}}$ = *E. coli* (CFU/mL) in undiluted spiking suspension

$\mathbf{V}_{\text{spiked per 100 mL sample}}$ = mL of undiluted spiking suspension per 100 mL sample

Table 4. Example Calculations of Spiked *E. coli*

$\boldsymbol{E.\ coli}_{\text{undiluted spike}}$	$\mathbf{V}_{\text{spiked per 100 mL sample}}$	$\mathbf{T}_{\text{Spiked } E.\ coli}$
9.6×10^7 CFU/mL	3.0×10^{-7} mL per 100 mL sample	(9.6×10^7 CFU/mL) × (3.0×10^{-7} mL/100 mL) = **28.8 CFU/100 mL**
2.8×10^7 CFU/mL	3.0×10^{-7} mL per 100 mL sample	(2.8×10^7 CFU/mL) × (3.0×10^{-7} mL/100 mL) = **8.4 CFU/100 mL**

14.2.4.3 Calculate percent recovery (R) of spiked *E. coli* (CFU/100 mL) according to the following equation. Example calculations are provided in **Table 5,** below.

$$R = 100\ x\ \frac{N_s - N_u}{T}$$

Where,

R = Percent Recovery

N_s = *E. coli* (CFU/100 mL) in the spiked sample (Section 13)

N_u = *E. coli* (CFU/100 mL) in the unspiked sample (Section 13)

T = True spiked *E. coli* (CFU/100 mL) in spiked sample (Section 14.2.4.2)

Table 5. Example Percent Recovery Calculations

N_s (CFU/100 mL)	N_u (CFU/100 mL)	T (CFU/100 mL)	Percent recovery (R)
42	<1	28.8	100 × (42 - 1) / 28.8 = **142%**
34	10	28.8	100 × (34 - 10) / 28.8 = **83%**
16	<1	8.4	100 × (16 - 1) / 8.4 = **179%**
10	<1	8.4	100 × (10 - 1) / 8.4 = **107%**

14.3 BioBall® spiking procedure

14.3.1 Aseptically add 1 BioBall® to 100 mL (or appropriate volume) of sample and mix by vigorously shaking the sample bottle a minimum of 25 times. Analyze the spiked sample according to the procedures in Section 11.

14.3.2 Recovery calculations for samples spiked with BioBalls – Calculate percent recovery (R) of spiked *E. coli* (CFU/100 mL) according to the following equation. Example calculations are provided in **Table 6**, below.

$$R = 100\ x\ \frac{N_s - N_u}{T}$$

Where,

R = Percent recovery

N_s = *E. coli* (CFU/100 mL) in the spiked sample (Section 13)

N_u = *E. coli* (CFU/100 mL) in the unspiked sample (Section 13)

T = True spiked *E. coli* (CFU/100 mL) in spiked sample based on the lot mean value provided by manufacturer

Table 6. Example Percent Recovery Calculations

N_S (CFU/100mL)	N_U (CFU/100mL)	T (CFU/100 mL)	Percent recovery (R)
24	<1	32	100 × (24 - 1)/32 = **72%**
36	10	32	100 × (36 - 10)/32 = **81%**

15.0 Method Performance

15.1 Performance characteristics

15.1.1 Precision – The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. The mTEC method precision was found to be fairly representative of what would be expected from counts with a Poisson distribution (References 18.2).

15.1.2 Bias – The persistent positive or negative deviation of the average value of the method from the assumed or accepted true value. The bias of the mTEC method has been reported to be -2% of the true value (References 18.2).

15.1.3 Specificity – The ability of a method to select and or distinguish the target bacteria under test from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. The false positive rate reported for mTEC medium averaged 9% for fresh and marine water samples. Less than 1% of the *E. coli* colonies observed gave a false negative reaction (References 18.2).

15.1.4 Upper Counting Limit (UCL) – That colony count above which there is an unacceptable counting error. The error may be due to overcrowding or antibiosis. The UCL for *E. coli* on mTEC medium has been reported as 80 colonies per filter (References 18.2).

15.2 Collaborative Study Data for Ambient Waters

15.2.1 A collaborative study was conducted among eleven volunteer laboratories, each with two analysts who independently tested local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate. The data were reported to the Environmental Monitoring and Support Laboratory - Cincinnati, U.S. Environmental Protection Agency, for statistical calculations.

15.2.2 The results of the study are shown in Figure 1 where S_O equals the pooled standard deviation among replicate counts from a single analyst for three groupings (counts less than 30, counts from 30 to 50, and counts greater than 50) and S_B equals the pooled standard deviation between means of duplicates from analysts in the same laboratory for the same groupings. The precision estimates from this study did not show any difference among the water types analyzed.

15.2.3 By linear regression, the precision of the method can be generalized as:

S_O = 0.028 count/100 mL + 6.11 (dilution factor) and
S_B = 0.233 count/100 mL + 0.82 (dilution factor)

$$Dilution\ factor = \frac{100}{Volume\ of\ original\ sample\ filtered}$$

15.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series, and no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of *E. coli*. The mean count (0) and the overall standard deviation of the counts (S_T) (which includes the variability among laboratories for this standardized *E. coli* sample) were 31.6 colonies/membrane and 7.61 colonies/membrane, respectively.

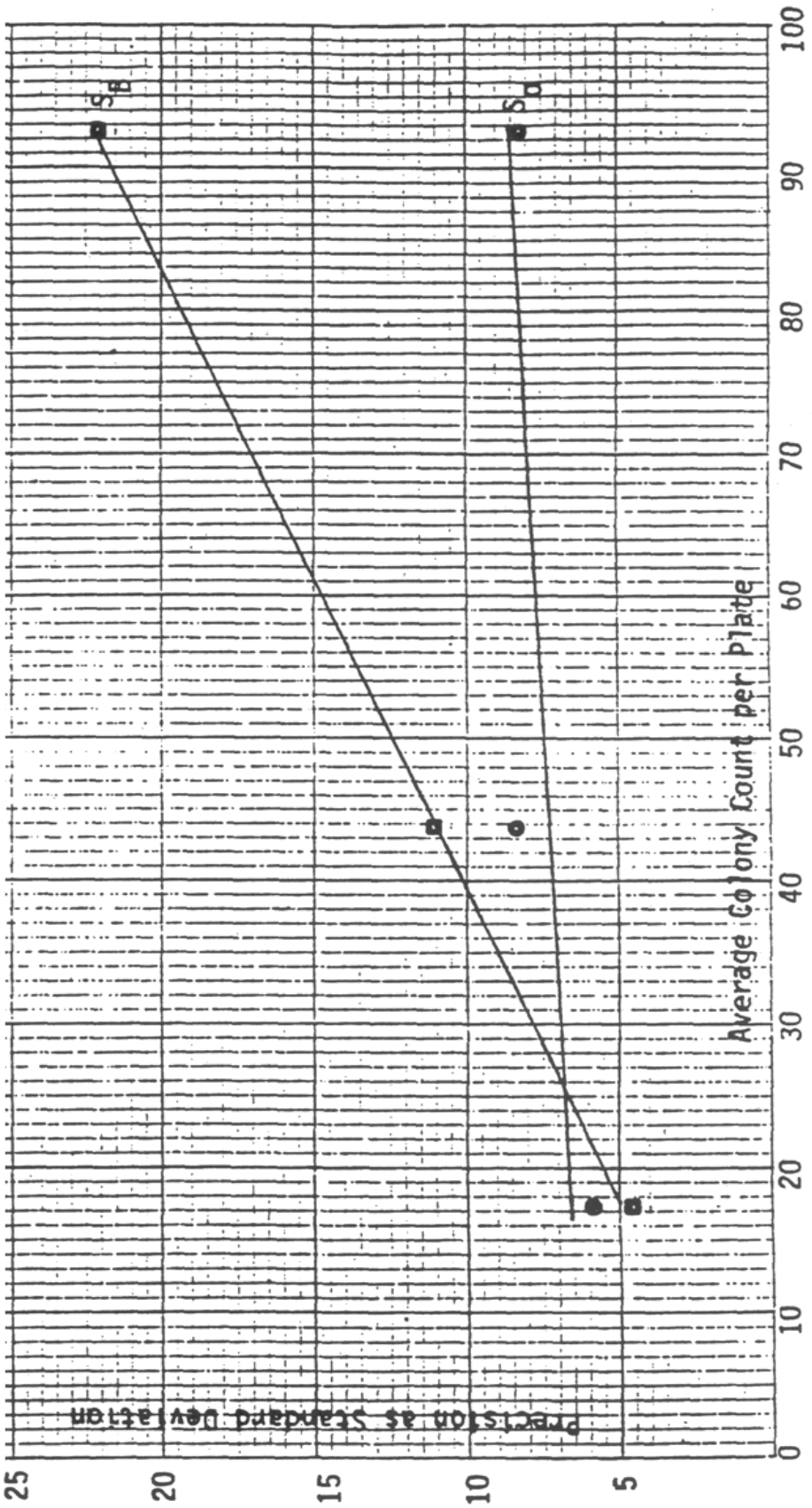

FIGURE 1. Precision Estimates for E. coli in Water by the Membrane Filter/mTEC Procedure.

15.3 Interlaboratory Validation Study Results for Wastewater

15.3.1 Ten volunteer participant laboratories, an *E. coli* verification laboratory, and two research laboratories participated in the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study of EPA Method 1103.1 for the analysis of *E. coli* in disinfected wastewater. The purposes of the Study were to characterize method performance across multiple laboratories and disinfected wastewater matrices and to develop quantitative QC acceptance criteria. The false positive and negative assessments are provided in **Tables 7** and **8**, respectively.

15.3.2 Method 1103.1 is not approved for the analysis of *E. coli* in disinfected wastewater because of the high false positive and false negative levels observed during the validation study compared to Method 1603. Laboratories wishing to test for *E. coli* in wastewater using a membrane filtration method are referred to EPA Method 1603.

Table 7. False Positive Assessment of Unspiked Disinfected and Unspiked Secondary Wastewater Effluents

Matrix	Total colonies		False positive (FP) assessment			
	Typical	Atypical	Typical colonies submitted	No. FP colonies	FP confirmation rate (%) [a]	Estimated % of total colonies that would have been a FP [b]
Disinfected	272	725	70	16	22.9%	6.2%
Secondary	1190	347	104	15	14.4%	11.2%
Disinfected + Secondary	1462	1072	174	31	17.8%	10.3%

[a] False positive confirmation rate = number of false positive colonies/number of typical colonies submitted

[b] Percent of total colonies estimated to be false positives = [(total typical colonies × FP confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(1190 × (15/104)) / (1190+347)] × 100 = 11.2%

Table 8. False Negative Assessment of Unspiked Disinfected and Unspiked Secondary Wastewater Effluents

Matrix	Total colonies		False negative (FN) assessment			
	Typical	Atypical	Atypical colonies submitted	No. FN colonies	FN confirmation rate (%) [a]	Estimated % of total colonies that would have been a FN [b]
Disinfected	272	725	89	15	16.9%	12.3%
Secondary	1190	347	45	4	8.9%	2.0%
Disinfected + Secondary	1462	1072	134	19	14.2%	6.0%

[a] False negative confirmation rate = number of false negative colonies/number of atypical colonies submitted

[b] Percent of total colonies estimated to be false negatives = [(total atypical colonies × FN confirmation rate) / (total number of typical and atypical colonies observed)] × 100; e.g., [(347 × (4/45)) / (1190+347)] × 100 = 2.0%

16.0 Pollution Prevention

16.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

16.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

17.0 Waste Management

17.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

17.2 Samples, reference materials, and equipment known or suspected to have viable *E. coli* attached or contained must be sterilized prior to disposal.

17.3 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

18.0 References

18.1 Dufour, A.P. *Health Effects Criteria for Fresh Recreational Waters*, EPA-600/1-84-004. Research Triangle Park, NC: U.S. Environmental Protection Agency, 1984.

18.2 Dufour, A.P., E.R. Strickland, V.J. Cabelli. 1981. Membrane filter method for enumerating *Escherichia coli*. Appl. Environ. Microbiol. 41:1152-1158.

18.3 ACS. 2000. *Reagent Chemicals, American Chemical Society Specifications*. American Chemical Society, New York. For suggestions of the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals*, BDH, Poole, Dorset, UK and the United States Pharmacopeia.

18.4 APHA. 2017. *Standard Methods for the Examination of Water and Wastewater*. 23rd Edition. American Public Health Association, Washington D.C.

18.5 Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). *Microbiological Methods for Monitoring the Environment. Water and Wastes*. EPA-600/8-78-017. Cincinnati, OH: U.S. Environmental Protection Agency, 1978.

Appendix A:
Part II (General Operations), Section A (Sample Collection, Preservation, and Storage)

Sample Collection[1]

1.0 Sample Containers

1.1 Sample Bottles: bottles must be resistant to sterilizing conditions and the solvent action of water. Wide-mouth borosilicate glass bottles with screw cap or ground-glass stopper or heat-resistant plastic bottles may be used if they can be sterilized without producing toxic materials (see examples A and C in Figure 1). Screw caps must not produce bacteriostatic or nutritive compounds upon sterilization.

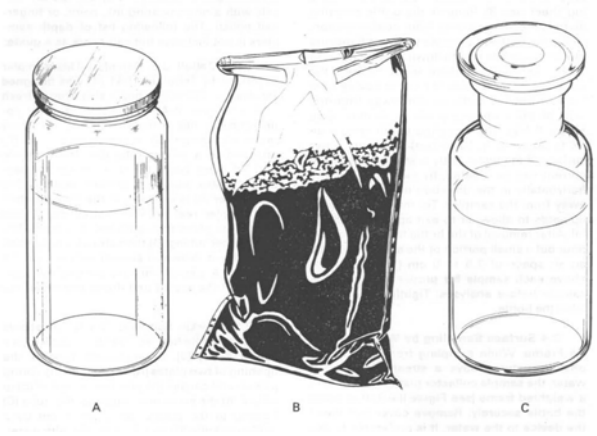

Figure 1. Suggested sample containers.

1.2 Selection and Cleaning of Bottles: Samples bottles should be at least 125 mL in volume for adequate sampling and for good mixing. Bottles of 250 mL, 500 mL, and 1000 mL volume are often used for multiple analyses. Discard bottles which have chips, cracks, and etched surfaces. Bottle closures must be water-tight. Before use, thoroughly cleanse bottles and closures with laboratory-grade detergent and hot water, followed by a hot water rinse to remove all trace of detergent. Then rinse them three times with laboratory-pure water.

1.3 Dechlorinating Agent: The agent must be placed in the bottle when water and wastewater samples containing residual chlorine are anticipated. Add sodium thiosulfate to the bottle before sterilization at a concentration of 0.1 mL of a 10% solution for each 125 mL sample volume. This concentration will neutralize approximately 15 mg/L of residue chlorine.

1.4 Chelating Agent: A chelating agent should be added to sample bottles used to collect samples suspected of containing >0.01 mg/L concentrations of heavy metals, such as copper, nickel or zinc, etc. Add 0.3 mL of a 15% solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, for each 125 mL sample volume prior, to sterilization.

[1]The text is largely taken from Part II, Section A, of the EPA publication *Microbiological Methods for Monitoring the Environment. Water and Wastes.* EPA-600/8-78-017, December 1978.

1.5 Wrapping Bottles: Protect the tops and necks of glass stoppered bottles from contamination by covering them before sterilization with aluminum foil or kraft paper.

1.6 Sterilization of Bottles: Autoclave glass or heat-resistant plastic bottles at 121°C (15 PSI) for 15 minutes. Alternatively, dry glassware may be sterilized in a hot oven at 170°C for not less than two hours. Ethylene oxide gas sterilization is acceptable for plastic containers that are not heat-resistant. Sample bottles sterilized by gas should be stored overnight before being used to allow the last traces of gas to dissipate.

1.7 Plastic Bags: The commercially available bags (Whirl-pak®) (see example B in Figure 1) are a practical substitute for plastic or glass samples bottles in sampling soil, sediment, or biosolids. The bags are sealed in manufacture and opened only at time of sampling. The manufacturer states that such bags are sterilized.

2.0 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access, such as a bridge or bank adjacent to a surface water.

2.1 Chlorinated Samples: When samples such as treated waters or chlorinated wastewaters or recreational waters are collected, the sample bottle must contain a dechlorinating agent (see Section 1.3 above).

2.2 Composite Sampling: In no case should a composite sample be collected for bacteriologic examination. Data from individual samples show a range of values. A composite sample will not display this range. Individual results will give information about industrial process variations in flow and composition. Also, one or more portions that make up a composite sample may contain toxic or nutritive materials and cause erroneous results.

2.3 Surface Sampling by Hand: A grab sample is obtained using a sample bottle prepared as described in (1) above. Identify the sampling site on the bottle label and on a field log sheet. Remove the bottle covering and closure and protect from contamination. Grasp the bottle at the base with one hand and plunge the bottle mouth down into the water to avoid introducing surface scum (Figure 2). Position the mouth of the bottle into the current away from the hand of the collector and, if applicable, away from the side of the sampling platform. The sampling depth should be 15-30 cm (6-12 inches) below the water surface. If the water body is static, an artificial current can be created, by moving the bottle horizontally in the direction it is pointed and away from the sampler. Tip the bottle slightly upwards to allow air to exit and the bottle to fill. After removal of the bottle from the stream, pour out a small portion of the sample to allow an air space of 2.5-5 cm (1-2 inches) above each sample for proper mixing of the sample before analyses. Tightly stopper the bottle and place on ice (do not freeze) for transport to the laboratory.

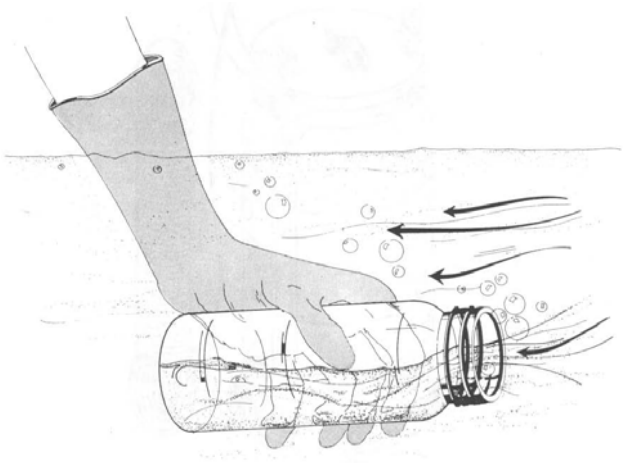

Figure 2. Grab sampling technique for surface waters.

3.0 Selection of Sampling Sites and Frequency

These sites will be described for streams, rivers, estuarine, marine, and recreational waters as well as domestic and industrial wastewaters.

3.1 Stream Sampling: The objectives of the initial survey dictate the location, frequency and number of samples to be collected.

3.1.1 Selection of Sampling Sites: A typical stream sampling program includes sampling locations upstream of the area of concern, upstream and downstream of waste discharges, upstream and downstream from tributary entrances to the river and upstream of the mouth of the tributary. For more complex situations, where several waste discharges are involved, sampling includes sites upstream and downstream from the combined discharge area and samples taken directly from each industrial or municipal waste discharge. Using available bacteriological, chemical and discharge rate data, the contribution of each pollution source can be determined.

3.1.2 Small Streams: Small streams should be sampled at background stations upstream of the pollution sources and at stations downstream from pollution sources. Additional sampling sites should be located downstream to delineate the zones of pollution. Avoid sampling areas where stagnation may occur (e.g., backwater of a tributary) and areas located near the inside bank of a curve in the stream which may not be representative of the main channel.

3.1.3 Large Streams and Rivers: Large streams are usually not well mixed laterally for long distances downstream from the pollution sources. Sampling sites below point source pollution should be established to provide desired downstream travel time and dispersal as determined by flow rate measurements. Particular care must be taken to establish the proper sampling points. Occasionally, depth samples are necessary to determine vertical mixing patterns.

3.2 Estuarine and Marine Sampling: Sampling estuarine and marine waters requires the consideration of other factors in addition to those usually recognized in fresh water

sampling. They include tidal cycles, current patterns, bottom currents and counter-currents, stratification, seasonal fluctuations, dispersion of discharges and multi-depth samplings.

The frequency of sampling varies with the objectives. When a sampling program is started, it may be necessary to sample every hour around the clock to establish pollution loads and dispersion patterns. The sewage discharges may occur continuously or intermittently.

When the sampling strategy for a survey is planned, data may be available from previous hydrological studies done by the Coast Guard, Corps of Engineers, National Oceanic and Atmospheric Administration (NOAA), U.S. Geological Survey, or university and private research investigations. In a survey, float studies and dye studies are often carried out to determine surface and undercurrents. Initially depth samples are taken on the bottom and at five feet increments between surface and bottom. A random grid pattern for selecting sampling sites is established statistically.

3.2.1 Estuarine Sampling: When a survey is made on an estuary, samples are often taken from a boat, usually making an end to end traverse of the estuary. Another method involves taking samples throughout a tidal cycle, every hour or two hours from a bridge or from an anchored boat at a number of fixed points.

In a large bay or estuary where many square miles of area are involved, a grid or series of stations may be necessary. Two sets of samples are usually taken from an area on a given day, one at ebb or flood slack water, and the other three hours earlier, or later, at the half tidal interval. Sampling is scheduled so that the mid-sampling time of each run coincides with the calculated occurrence of the tidal condition.

In location sampling sites, one must consider points at which tributary waters enter the main stream or estuary, location of shellfish beds and bathing beaches. The sampling stations can be adjusted as data accumulate. For example, if a series of stations half mile apart consistently show similar values, some of these stations may be dropped and other stations added in areas where data shows more variability.

Considerable stratification can occur between the salt water from the sea and the fresh water supplied by a river. It is essential when starting a survey of an unknown estuary to find out whether there is any marked stratification. This can be done by chloride determinations at different locations and depths. It is possible for stratification to occur in one part of an estuary and not in another.

On a flood tide, the more dense salt water pushing up into the less dense fresh river water will cause an overlapping with the fresh water flowing on top. A phenomenon called a salt water wedge can form. As a result, stratification occurs. If the discharge of pollution is in the salt water layer, the contamination will be concentrated near the bottom at the flood tide. The flow or velocity of the fresh water will influence the degree of stratification which occurs. If one is sampling only at the surface, it is possible that the data will not show the polluted underflowing water which was contaminated at the point below the fresh water river. Therefore, where stratification is suspected, samples at different depths will be needed to measure vertical distribution.

3.2.2 Marine Sampling: In ocean studies, the environmental conditions are most diverse along the coast where shore, atmosphere and the surf are strong influences. The shallow coastal waters are particularly susceptible to daily fluctuations in temperature and seasonal changes.

Sampling during the entire tidal cycle or during a half cycle may be required. Many ocean studies such as sampling over the continental shelf involve huge areas and no two areas of water are the same.

Selection of sampling sites and depths are most critical in marine waters. In winter, cooling of coastal waters can result in water layers which approach 0°C. In summer, the shallow waters warm much faster than the deeper waters. Despite the higher temperature, oxygen concentrations are higher in shallow than in deeper waters due to greater water movement, surf action and photosynthetic activity from macrophytes and the plankton.

Moving from the shallow waters to the intermediate depths, one observes a moderation of these shallow water characteristics. In the deeper waters, there is a marked stabilization of conditions. Water temperatures are lower and more stable. There is limited turbulence, little penetration of light, sparse vegetation and the ocean floor is covered with a layer of silts and sediments.

3.3 Recreational Waters (Bathing Beaches): Sampling sites at bathing beaches or other recreational areas should include upstream or peripheral areas and locations adjacent to natural drains that would discharge stormwater, or run-off areas draining septic wastes from restaurants, boat marinas, or garbage collection areas. Samples of bathing beach water should be collected at locations and times of heaviest use. Daily sampling is the optimum frequency during the season. Weekends and holidays which are periods of highest use must be included in the sampling program. Samples of estuarine bathing waters should be obtained at high tide, ebb tide and low tide in order to determine the cyclic water quality and deterioration that must be monitored during the swimming season.

3.4 Domestic and Industrial Waste Discharges: It is often necessary to sample secondary and tertiary wastes from municipal waste treatment plants and various industrial waste treatment operations. In situations where the plant treatment efficiency varies considerably, grab samples are collected around the clock at selected intervals for a three to five day period. If it is known that the process displays little variation, fewer samples are needed. In no case should a composite sample be collected for bacteriological examination. The National Pollution Discharge Elimination System (NPDES) has established wastewater treatment plant effluent limits for all dischargers. These are often based on maximum and mean values. A sufficient number of samples must be collected to satisfy the permit and/or to provide statistically sound data and give a fair representation of the bacteriological quality of the discharge.

Appendix B:
Part II (General Operations), Sections C.3.5 (Counting Colonies) and C.3.6 (Calculation of Results)

Counting Colonies [1]

1.0 Counting Colonies

Colonies should be counted using a fluorescent lamp with a magnifying lens. The fluorescent lamp should be nearly perpendicular to the membrane filter. Count colonies individually, even if they are in contact with each other. The analyst must learn to recognize the difference between two or more colonies which have grown into contact with each other and single, irregularly shaped colonies which sometimes develop on membrane filters. The latter colonies are usually associated with a fiber or particulate material and the colonies conform to the shape and size of the fiber or particulates. Colonies which have grown together almost invariably show a very fine line of contact.

2.0 Calculation of Results

2.1 Select the membrane filter with the number of colonies in the acceptable range and calculate count per 100 mL according to the general formula:

Count per 100 mL = (No. of colonies counted/Volume of sample filtered, in mL) × 100

2.2 Counts Within the Acceptable Limits

The acceptable range of colonies that are countable on a membrane is a function of the method. Different methods may have varying acceptable count ranges. All examples in this appendix assume that the acceptable range of counts is between 20-80 colonies per membrane.

For example, assume that filtration of volumes of 50, 15, 5, 1.5, and 0.5 mL produced colony counts of 200, 110, 40, 10, and 5, respectively.

An analyst would not actually count the colonies on all filters. By inspection the analyst would select the membrane filter with the acceptable range of target colonies, as defined by the method, and then limit the actual counting to such membranes.

After selecting the best membrane filter for counting, the analyst counts colonies and applies the general formula as in Section 2.1 above to calculate the count/100 mL.

2.3 More Than One Acceptable Count

2.3.1 If there are acceptable counts on replicate plates, carry counts independently to final reporting units, then calculate the arithmetic mean of these counts to obtain the final reporting value.

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[1]The text is largely taken from Part II, Section C, of the EPA publication *Microbiological Methods for Monitoring the Environment. Water and Wastes.* EPA-600/8-78-017, December 1978.

Some examples were kindly provided by Kristen Brenner, US EPA.

Example, if the counts are 24 and 36 for replicate plates of 100 mL each, then the arithmetic mean is calculated as follows:

$$\frac{24\ CFU/100\ mL + \ 36\ CFU/100\ \ mL}{2} = \ 30\ \ CFU/100\ \ mL$$

2.3.2 If there is more than one dilution having an acceptable range of counts, independently carry counts to final reporting units, and then average for final reported value.

For example, if volumes of 100, 10, 1 and 0.1 mL produced colony counts of too numerous to count (TNTC), 75, 30, and 1, respectively, then two volumes, 10 mL and 1 mL, produced colonies in the acceptable counting range.

Independently carry each MF count to a count per 100 mL:

$$\frac{75}{10}\ x\ 100 = 750\ \ CFU/100\ mL$$

and

$$\frac{30}{1}\ x\ 100 = \ 3000\ \ CFU/100\ mL$$

Calculate the arithmetic mean as in Section 2.3.1 above:

$$\frac{750\ CFU/100\ \ mL\ +\ 3000\ CFU/100\ \ mL}{2} = 1875\ CFU/100\ mL$$

Report this as 1875 CFU/100 mL.

2.4 If all MF counts are below the lower acceptable count limit, select the most nearly acceptable count.

2.4.1 For example, sample volumes of 100, 10 and 1 mL produced colony counts of 17, 1 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 17, and report as 17 CFU/100 mL.

***Note**: In this case, because no calculations were done (i.e., this is the count for 100 mL), the count is reported as 17 CFU/100 mL rather than an "estimated count of 17 CFU/100 mL"*

2.4.2 As a second example, assume a count in which sample volumes of 10 and 1 mL produced colony counts of 18 and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in Section 2.3.2 above.

$$\frac{18}{10}\ x\ 100 = 180\ CFU/100\ mL$$

Report this as an estimated count of 180 CFU/100 mL.

2.5 If counts from all membranes are zero, calculate using count from largest filtration volume.

For example, sample volumes of 25, 10, and 2 mL produced colony counts of 0, 0, and 0, respectively, and no actual calculation is possible, even as an estimated report. Calculate the number of colonies per 100 mL that would have been reported if there had been one colony on the filter representing the largest filtration volume. In this example, the largest volume filtered was 25 mL and thus the calculation would be:

$$\frac{1}{25}\ x\ 100 = 4\ CFU/100\ mL$$

Report this as < (less than) 4 CFU/100 mL.

2.6 If all membrane counts are above the upper acceptable limit, calculate count using the smallest volume filtered.

For example, assume that the volumes 1, 0.3, and 0.01 mL produced colony counts of TNTC, 150, and 110 colonies, respectively. Since all colony counts are above the acceptable limit, use the colony count from the smallest sample volume filtered and estimate the count as:

$$\frac{110}{0.01}\ x\ 100 =\ 1{,}100{,}000\ CFU/100\ mL$$

Report this as estimated count 1.1×10^6 CFU/100 mL

2.7 If typical colonies are TNTC, use upper limit count with smallest filtration volume.

For example, assume that the volumes 1, 0.3, and 0.01 mL all produced too many typical colonies, and that the laboratory bench record indicated TNTC.

Use the upper acceptable count for the method (80 colonies in this example) as the basis of calculation with the smallest filtration volume and estimate the count as:

$$\frac{80}{0.01}\ x\ 100 = 800{,}000\ CFU/100\ mL$$

Report this as > (greater than) 8×10^5 CFU/100 mL

2.8 If colonies are both above and below the upper and lower acceptable limits (i.e., no counts are within the acceptable limits), select the most nearly acceptable count.

2.8.1 For example, sample volumes of 100, 10 and 1 mL produced colony counts of 84, 8, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 84, and report as 84 CFU/100 mL.

***Note**: In this case, because no calculations were done (i.e., this is the count for 100 mL), the count is reported as 84 CFU/100 mL rather than an "estimated count of 84 CFU/100 mL"*

2.8.2 As a second example, assume a count in which sample volumes of 100, 10 and 1 mL produced colony counts of 98, 18, and 0, respectively.

Here, no colony count falls within recommended limits. Calculate on the basis of the most nearly acceptable plate count, 18, and calculate as in Section 2.3.2 above.

$$\frac{18}{10}\ x\ 100 = 180\ CFU/100\ mL$$

Report this as estimated count 180 CFU/100 mL.

2.9 If there is no result because of a confluent growth, > 200 atypical colonies (TNTC), lab accident, etc., report as No Data and specify the reason.