SOP-C-114

Determination of Fecal Coliform and *Escherichia coli* by Membrane Filtration

Revision 16

Approval:

_____	8-23-23
Laboratory Manager	Date
_____	8/14/2023
Concurrence	Date

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Texas Institute for Applied Environmental Research

i. **Identification of the method**
   a. EPA 1603, September 2014, EPA–821–R–14–010
   b. Additional quality control, Standard Methods online 9020 (approved 2017)

ii. **Applicable matrix or matrices**
   a. Nonpotable water: ambient waters and disinfected wastewaters.

iii. **Limits of detection and quantitation**
   a. The LOD is 1 colony/100 mL
   b. Plates that do not contain the ideal number of colonies (20-80) are calculated and listed as Estimates, though values may be used as valid for particular projects. Plates containing more than 200 colonies are not valid for estimates and are recorded as Too Numerous To Count (TNTC).

iv. **Scope and application, including parameters to be analyzed**
   a. Detection and enumeration of *Escherichia coli* bacteria

v. **Summary of the Method**
   a. Method 1603 provides a direct count of *E. coli* in ambient water or wastewater based on the development of colonies that grow on the surface of a membrane filter. A sample is filtered through the membrane, which retains the bacteria. After filtration, the membrane is placed on a selective and differential medium, modified mTEC agar, 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. The target colonies on a modified mTEC agar are red or magenta in color after the incubation period.
   b. Modifications from the method:
      i. Sterile IDEXX dilution bottles are used instead of milk bottles.
      ii. An insulated, low airspace and non-circulation incubator chamber by Millipore Corporation may be used instead of a water bath, if requested by the specific client.
      iii. Alcohol flaming of labware is used instead of UV light.

iv. Purchased sterile water and sample containers are used instead of Whirl-Pak® bags and autoclaved water. These items have certificates ensuring cleanliness and purity.
v. Holding time has been extended to 6+2 hours by the TCEQ on certain projects, or longer if requested by client.
vi. Preservation temperature is >0-≤6° C as set by TCEQ.
vii. Colony verification is done by DNA analysis at another laboratory as required by the project requiring these analyses.
viii. New filters may also be a different color than white.

vi. **Definitions**

a. *Escherichia coli* is a common inhabitant of the intestinal tract of warm-blooded animals, and its presence in water samples is an indication of fecal pollution and the possible presence of enteric pathogens.
b. The *E. coli* test is recommended as a measure of ambient recreational fresh water quality. In Method 1603, *E. coli* are those bacteria which produce red or magenta colonies on the modified mTEC agar.

vii. **Interferences**

a. Water samples containing colloidal or suspended particulate material can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with enumeration and identification of target colonies.
b. Growth from other colonies, if plentiful, can overtake *E. coli* colonies of interest resulting in unreadable results. These are recorded as Interference (INT) present and are invalid.

viii. **Safety**

a. Follow Tarleton safety regulations in accordance with QAM-S-101, “Laboratory Safety,” while preparing, using, and disposing of cultures, reagents, and materials and while operating sterilization equipment.
b. This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA and state regulations regarding the safe handling of the chemicals specified in this method. A reference file

containing material safety data sheets (MSDSs) is available to all personnel involved in these analyses.

c. Coliform bacteria are an indicator for the potential presence of pathogens; therefore, avoid all direct skin contact with sample.
d. All work areas are to be disinfected upon completion of work with a 10% bleach solution or 70% Isopropyl Alcohol.
e. Isopropyl and Ethyl alcohol are flammable. Analysts will take care to avoid igniting the alcohol when disinfecting the biosafety cabinet or other surfaces.
f. Gloves and lab coats are available for analysts. Safety glasses should be worn when in the laboratory.

ix. **Equipment and supplies**

a. Glass lens with magnification of 2-5X, or stereoscopic microscope
b. Lamp, with a cool, white fluorescent tube
c. Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume
d. Sterilized graduated cylinders, 100-1000 mL
e. Sterilized membrane filtration units (filter base and funnel), glass, plastic or stainless steel; the filter apparatus and other glassware are sterilized between samples. Coating with alcohol and igniting under flame may accomplish this or sanitize by UV light.
f. Ultraviolet unit for sanitization of the filter funnel between filtrations (optional); alcohol flaming of equipment also used.
g. 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)
h. Filter flask, vacuum, usually 1 L, with appropriate tubing
i. Filter manifold to hold a number of filter bases (optional)
j. Flask for safety trap placed between the filter flask and the vacuum source
k. Forceps, straight or curved, with smooth tips to handle filters without damage
l. Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps

m. Burner, Bunsen, propane or Fisher type, or electric incinerator unit for sterilizing loops and needles
n. 10% bleach
o. 70% Isopropyl Alcohol
p. Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer
q. Petri dishes, sterile, plastic, 9 × 50 mm, with tight-fitting lids; and 15 × 100 mm with loose fitting lids
r. Dilution bottles, IDEXX sterile bottles with lids, generally 100 mL capacity
s. Flasks, borosilicate glass, screw-cap or coverable, 250-2000 mL volume
t. Membrane filters, sterile, white, grid marked, 47 mm diameter, with pore size of 0.45 µm
u. Waterbath maintained at 44.5°C ± 0.2°C (specific bacterial incubator may be used if called for by client project)
v. Incubator maintained at 35°C ± 0.5°C
w. Waterbath maintained at 50°C ±2°C for tempering agar
x. Whirl-Pak® bags or equivalent
y. Autoclave or steam sterilizer capable of achieving 121°C [15 lb pressure per square inch (PSI)] for 15 minutes
z. Biosafety Cabinet
aa. Washed labware are tested at least once daily, each day of washing, for possible acid or alkaline residue by testing at least one piece of labware with 0.04% bromothymol blue (BTB-see below). To test clean glassware, add a few drops of BTB and observe the color reaction. BTB is blue-green in the acceptable neutral range.
bb. Labware that is washed and reused are tested for possible presence of residues which may inhibit or promote growth of microorganisms by performing the **Inhibitory Residue Test** annually, and each time the lab changes the lot of detergent or washing procedures.
    i. This test is based on the growth of *Enterobacter aerogenes* to determine if residues are present on glassware.
        1. Wash 6 Petri dishes according to usual laboratory practice and designate as group A.
        2. Wash 6 Petri dishes as above, rinse 12 times with Deionized water and designate as group B.

3. Rinse 6 Petri dishes with detergent wash water, air-dry without further rinsing and designate as group C.
4. Sterilize dishes in groups A, B, and C by the usual procedure.
5. Set up 6 plastic Petri dishes and designate as group D.
6. Prepare and sterilize 200 mL plate count agar and hold in a 44 to 46 °C water bath.
7. Prepare a culture of *Enterobacter aerogenes* known to contain 50-150 colony forming units (CFU)/mL. Preliminary testing may be necessary to achieve this count range.
8. Inoculate 3 dishes from each group with 0.1 mL and the other 3 dishes from each group with 1 mL culture. Do not let more than 20 minutes elapse between starting pipetting and pouring plates.
9. Add 10-12 mL liquefied agar medium to each Petri dish. (Plastic dishes may only hold 4-6 mL of agar.) As each plate is poured mix melted medium with test portions by rotating in one direction and then the other, or by rotating and tilting.
10. Let the plates solidify. Invert and incubate at 35 degrees C for 48 hours. Count plates with 30 to 300 colonies and record results as CFU/mL.
11. This test is usually performed by an outside laboratory.
12. Differences in averaged counts on plates in groups A through D are less than 15% if there are no toxic or inhibitory effects. The difference in averaged groups of less than 15% between groups A and B and greater than 15% between groups A and C indicate that the cleaning detergent has inhibitory properties that are eliminated during routine washing.
13. Differences between B and D greater than 15% indicate an inhibitory residue is present and plasticware must not be used for microbiological analyses.

x. **Reagents and standards**

a. BTB-To prepare 0.04% bromthymol blue indicator solution, add 16 mL 0.01*N* NaOH to 0.1 g BTB and dilute to 250 mL with DI water.

b. Purity- ACS grade or better. Whenever possible, use commercial culture media as a means of quality control. Purity of DI water: Type II ASTM, see QAM-Q-101, "Laboratory Quality Control." Laboratory produced DI water is not used for anything except labware cleaning. All water used for reagents, media and standard are made from water purchased and certified sterile by an outside vendor. The manufacturer also provides a certificate of analyses for metals, ammonia/organic nitrogen, organic carbon and heterotrophoic plate count. If TIAER laboratory water is used, the quality of the DI water used is monitored for total chlorine residual (<0.1 mg/L), specific conductance (<2 µmhos/cm at 25 degrees C), Total organic Carbon (<1.0 mg/L) and heterotrophic bacteria plate count (<500 CFU/mL) monthly (when in use), when maintenance is performed on the water system, or at startup after a period of disuse longer than one month. The heterotrophic plate count is a procedure for estimating the number of live culturable heterotrophic bacteria in water. Analysis for heavy metals (Cd, Cr, Cu, Ni, Pb and Zn) singly (<0.05mg/L) and total heavy metals (<0.10 mg/L) are performed at least annually on the TIAER DI water, if used to prepare dilution water, standards, media or reagents. Normally, this water is purchased from an outside vendor and comes with a certificate of metal concentration meeting this standard. The **Bacteriological Water Quality Test** (0.8-3.0 ratio) is performed annually on the TIAER DI water if used to prepare dilution water, standards, media or reagents. This test also is performed when the source of water is changed or when an analytical problem occurs. The Bacteriological Water Quality Test or water suitability test is based on the growth of *Enterobacter aerogenes* in a chemically defined minimal-growth medium to determine the presence of toxic agents or growth promoting substances. Again, this water is normally purchased from an outside vendor and comes with a certificate meeting this standard.

c. Phosphate buffered saline (PBS)
   i. Monosodium phosphate (NaH_2PO_4) 0.58 g
   ii. Disodium phosphate (Na_2HPO_4) 2.5 g

iii. Sodium chloride 8.5 g
iv. Dissolve the ingredients in 1 L of DI 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 is 7.4 ± 0.2.

d. Tryptic soy agar (TSA)- follow manufacturer's instructions for preparing tryptic soy broth. Add 15 g of agar per liter of DI and autoclave at 121°C (15 PSI) for 15 minutes, and cool in a 50°C water bath. Pipette the media using sterile equipment into 9x50 mm culture dishes to about a 4-5 mm depth (about 4-6 mL per plate) and allow to solidify. Store in a refrigerator.
e. Modified mTEC agar- use commercially available. Follow manufacturer's instruction on making up, or prepare as follows:
   i. Adjust pH to 7.3 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide
   ii. Autoclave at 121°C (15 PSI) for 15 minutes, and cool in a 50°C water bath.
   iii. Pour the medium into each 9 × 50 mm culture dish to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. Store in a refrigerator. If not available, refer to EPA 1603 (2009) for instructions on how to make.
f. Control cultures
   i. Positive control and/or spiking organism (either of the following are acceptable): Stock cultures of *Escherichia coli* (*E. coli*) ATCC #11775 or *E. coli* ATCC #11775 BioBalls (bioMérieux Inc., Durham NC). If not available refer to EPA 1603 (2014) for options using other *E. coli* sources.
   ii. Negative control organism (either of the following are acceptable): Stock cultures of *Enterococcus faecalis* (*E. faecalis*) ATCC #19433 or *E. faecalis* ATCC #19433 BioBalls (bioMérieux Inc., Durham NC) OR Stock cultures of *Enterobacter aerogenes* (*E. aerogenes*) ATCC #13048

xi. **Sample collection, preservation, shipment and storage**
   a. Sampling is not done by the TIAER Lab.

b. Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory in 6 hours, and samples are processed within 2 hours of receipt at the laboratory.
c. Ice or refrigerate water samples 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.

xii. **Quality control**

a. The initial and ongoing precision and recovery (IPR and OPR) performance criteria established for Method 1603 were determined using spiked PBS samples. The TIAER Lab uses PBS when performing IPR and OPR sample analyses. However, phosphate-buffered dilution water may be substituted for PBS as a sample diluent and filtration rinse buffer.
b. Refer to QAM-Q-100, "Quality Assurance Manual," and Q-101, "Laboratory Quality Control," for general quality control and quality systems guidelines including demonstration of competency. Additional requirements follow.
c. The minimum analytical QC requirements for the analysis of samples using Method 1603 include an initial demonstration of laboratory capability through performance of the initial precision and recovery **(IPR)** analyses, ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery **(OPR)** analysis and matrix spike (MS) analysis (normally for disinfected wastewater only), and the routine analysis of positive and negative controls, filter sterility checks, method blanks, and media sterility checks. For the IPR, OPR and MS analyses, it is necessary to spike samples with either laboratory-prepared spiking suspensions (EPA 1603) or BioBalls. TIAER normally uses the BioBalls only. Although the matrix spike recovery criteria in EPA 1603 pertain only to disinfected wastewaters, the IPR and OPR recovery criteria are valid method performance criteria that are met, regardless of the matrix being evaluated.

d. Initial precision and recovery **(IPR)**—The IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and are performed by each laboratory before the method is used for monitoring field samples. EPA recommends but does not require that an IPR be performed by each analyst. IPR samples are accompanied by an acceptable method blank and appropriate media sterility checks. The IPR analyses are performed as follows:
   i. Prepare four, 100-mL samples of PBS and spike each sample with *E. coli* ATCC #11775 according to the spiking procedure. Spiking with laboratory-prepared suspensions and spiking with BioBalls are described in this procedure. Filter and process each IPR sample as with all other samples. Calculate the number of *E. coli* per 100 mL.
   ii. Calculate the percent recovery (R) for each IPR sample using the appropriate equation for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.
   iii. 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.
   iv. 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 recovery 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 the IPR analyses.
   v. The IPR acceptance limits are: mean percent recovery detectable up to 144%, precision (as RSD) at 61% when using Bioballs.

e. Ongoing precision and recovery **(OPR)**- Percent recovery is acceptable from detectable up to 144%. The analyst tests 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 must be accompanied by an acceptable method blank and appropriate media sterility checks.
f. Negative controls**-**The analyst analyzes negative controls to ensure that the modified mTEC agar is performing properly. Negative controls are analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory performs a negative control every day that samples are analyzed. Negative controls are conducted by filtering a dilute suspension of viable *E. faecalis* (e.g., ATCC #19433) and analyzing as a sample. Viability of the negative controls is demonstrated using a non-selective media (e.g., nutrient agar or tryptic soy agar). Follow manufacturer's instructions on making these. 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.
g. Positive controls- The laboratory analyzes positive controls to ensure that the modified mTEC agar is performing properly. Positive controls are analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory performs a positive control every day that samples are analyzed. An OPR sample may take the place of a positive control. Positive controls are conducted by filtering a dilute suspension of viable *E. coli* (e.g., ATCC #11775) and analyzing. 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.
h. Filter sterility check—Place at least one membrane filter per lot of filters 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 performs a filter sterility check every day that samples are analyzed.

i. Method blank—Filter a 50-mL volume of sterile PBS or phosphate-buffered dilution water, place the filter on a modified mTEC agar plate and process. Absence of growth indicates freedom of contamination from the target organism. On an ongoing basis, the laboratory performs a method blank every day that samples are analyzed.
j. 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 PBS buffer and filtration assembly.
k. Media sterility check—test media sterility by incubating one unit (tube or plate) from each batch of medium (TSA, modified mTEC, and verification media) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, perform a media sterility check every day that samples are analyzed.
l. The pH of each batch of media is verified prior to first use.
m. Analyst colony counting variability—two or more analysts compare each analyst's colony counts from one positive field sample per month. Colony counts are within 10% between analysts. Laboratories with a single analyst, or where the second analyst is not available for a valid count, have that analyst perform duplicate colony counts of a single membrane filter each month. Duplicate colony counts are be within 5% for a single analyst. If no positive field samples are available, an OPR sample may be substituted for these determinations.
n. It is recommended that each new batch or lot of water, filters or reagents are compared to the old with 5 side by side replicates of positive samples or culture controls to assure quality. It is not always possible to compare if the old batch is no longer available.
o. Records of tests on water, glassware, reagents and equipment are maintained. Ensure all equipment is tested prior to use. Record all raw data associated with volume checks and sterility checks.
p. All microbiology incubators and waterbaths will have the temperature of at least the top and bottom shelves recorded

at least twice a day at least 4 hours apart on each day that samples are analyzed.

q. Media must be stored separately from samples, as are other reagents and standards.
r. Air quality is tested monthly with non-selective growth media. A settling plate is placed open on the workspace where sample analysis takes place for 15 minutes. The plate is incubated at 35˚C±0.5ºC for 48 hours. There can be no more than 15 CFUs. Record all raw data associated with the Air Quality Test. Log air test in the Maintenance Logbook.

xiii. **Calibration and standardization**

a. Check temperatures in incubators twice daily, when in use, with a minimum of 4 hours between each reading to ensure operation within stated limits.
b. Check thermometers at least annually against a NIST certified thermometer. Check mercury columns for breaks.
c. Refrigerators used to store media and reagents are monitored daily to ensure proper temperature control.

xiv. **Procedure**

a. Disinfect the work surface with 10% bleach <u>for 15 minutes or 70% Isopropyl alcohol for 5 minutes</u> immediately before beginning analysis.
b. Screen all samples for chlorine presence in accordance with SOP-C-121, “Determination of Chlorine.” Sodium thiosulfate has normally been added prior to sample collection. If chlorine is still detectable, the sample will be discarded and the client notified that resampling should be done. Some samples may have insufficient volume for chlorine testing. Initiate corrective action for such occurrence as the client may want to resample.
c. Prepare the modified mTEC agar.
d. Mark the petri dish with the sample identification and volume.
e. 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.

f. Shake the sample bottle vigorously at least 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.
g. Select sample volumes based on previous knowledge of the pollution level, 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 is obtained.
h. Smaller sample sizes 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 may be filtered.
i. When analyzing smaller sample volumes (e.g., <20 mL), 20-30 mL of PBS or phosphate buffered dilution water is added to the funnel or an aliquot of sample is dispensed into a dilution blank prior to filtration. This will allow even distribution of the sample on the membrane.
j. Filter the sample, and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum, and remove the funnel from the filter base.
k. Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the modified 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.
l. After a 2 ± 0.5 hour incubation at 35°C ± 0.5°C, transfer the plates to a 44.5°C ± 0.2°C water bath or incubator for 22 ± 2 hours.
m. After 22 ± 2 hours, remove the plates, count and record the number of red or magenta colonies with the aid of an illuminated lens with a 2-5X magnification or a stereoscopic microscope as needed. Colonies can be better counted using a fluorescent lamp with a magnifying lens. The fluorescent lamp is nearly perpendicular to the

membrane filter. Count colonies individually, even if they are in contact with each other. The analyst can demonstrate 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.

n. Red or magenta colonies are considered "typical" *E. coli*. Verification of typical and atypical colonies may be required in evidence gathering and may be required by a project as a means of quality control.

o. Matrix spikes (MS)—MS analyses are performed to determine the effect of a particular matrix on *E. coli* recoveries. The laboratory analyzes one MS sample when disinfected wastewater samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given disinfected wastewater source include an MS sample. MS samples are accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site, an acceptable method blank, and appropriate media sterility checks. When possible, MS analyses are also accompanied by an OPR sample, using the same spiking procedure (laboratory-prepared spiking suspension or BioBalls). The MS analysis is performed as follows:

    i. Prepare two, 100-mL field samples that were sequentially collected from the same site. One sample will remain unspiked and will be analyzed to determine the background or ambient concentration of *E. coli* for calculating MS recoveries. The other sample will serve as the MS sample and will be spiked with *E. coli* ATCC #11775 Select sample volumes based on previous analytical results or anticipated levels of *E. coli* in the field sample in order to achieve the recommended target range of

E. coli (20-80 CFU, including spike) per filter. If the laboratory is not familiar with the matrix being analyzed, it is recommended that a minimum of three dilutions be analyzed to ensure that a countable plate is obtained for the MS and associated unspiked sample. If possible, 100-mL of sample is analyzed. Spike the MS sample volume(s) with a laboratory-prepared suspension with BioBalls. Immediately filter and process the unspiked and spiked field samples.

ii. For the MS sample, calculate the number of *E. coli* (CFU/100 mL) and adjust the colony counts based on any background *E. coli* observed in the unspiked matrix sample. Only processed wastewater is spiked.

iii. Calculate the percent recovery (R) for the MS sample (adjusted based on ambient *E. coli* in the unspiked sample) for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

iv. The MS result (percent recovery) with the appropriate method performance criteria in Table 1 (17-117%). If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this disinfected wastewater source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, a matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data are flagged. Acceptance criteria for MS recovery (Table 1) are based on data from spiked disinfected wastewater matrices and are not appropriate for use with other matrices (e.g., ambient recreational waters).

v. BioBall™ Spiking Procedure: 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

with other samples. Normally the sample is disinfected wastewater chosen to be spiked.

vi. 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 2.

p. Disinfect the work surface with 10% bleach for 15 minutes or 70% isopropyl alcohol for 5 minutes immediately after analysis.

xv. **Data analysis and calculations;**

a. Calculate the *E. coli* count (CFU) per 100 mL of sample. If possible, select a membrane filter with 20-80 magenta or red colonies, and calculate the number of *E. coli* per 100 mL according to the following general formula:

i. *E. coli* / 100 mL = Number of *E. coli* colonies × 100 divided by the volume of sample filtered (mL)

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

b. 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 that follow assume that the acceptable range of counts is between 20-80 colonies per membrane.

i. 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 formula to calculate the count/100 mL.

ii. 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. Example, if the counts are 24 and 36 for replicate plates of 100 mL

each, then the arithmetic mean is calculated as follows:

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

iii. If there is more than one dilution having an acceptable range of counts, independently carry counts to final reporting units, then average for a final reported value. For example, if volumes of 100, 10, 1 and 0.1 mL produced colony counts of Too Numerous To Count (TNTC), 55, 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:

$$\frac{55}{10} \times 100 = 550 \text{ CFU /100 mL}$$

and

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

Calculate the arithmetic mean as in above:

$$\frac{550 \text{ CFU/100 mL} + 3000 \text{ CFU/100 mL}}{2} = 1775 \text{ CFU/100 mL}$$

Report this as 1775 CFU/100 mL.

iv. If all MF counts are below the lower acceptable count limit, select the most nearly acceptable count. 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* that 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”

v. 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 above.

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

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

vi. 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:

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

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

vii. 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:

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

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

viii. If typical colonies are too numerous to count (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:

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

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

ix. 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. 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* that 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” 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 above.

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

Report this as estimated count 180 CFU/100 mL.

x. 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.

xvi. **Method performance;** refer to SOP-Q-101, "Laboratory Quality Control"
   a. Uncertainty is determined at least annually (Table 3) and reported with all data.

xvii. **Pollution prevention;** refer to SOP-W-101, "Disposal of Laboratory Waste". The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly. Solutions and reagents are prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

xviii. **Data assessment and acceptance criteria for quality control measures Sample Spiking Procedure**
   a. Method 1603 QC requirements include the preparation and analysis of spiked reference (PBS) and matrix samples in order to monitor initial and ongoing method performance.
   b. For the IPR, OPR, and MS tests it is necessary to spike samples with either laboratory-prepared spiking suspensions or BioBalls.
   c. Each QC requirement has separate acceptance criteria mentioned previously in this SOP.

xiii. **Corrective actions for out-of-control data**- refer to QAM-Q-105, “Corrective Actions”.

xiv. **Contingencies for handling out-of-control or unacceptable data**- refer to QAM-Q-105, “Corrective Actions”.

xv. **Waste management;** for waste management, refer to SOP-W-101, "Disposal of Laboratory Waste".
   a. Samples, reference materials, and equipment known or suspected to have viable *E. coli* attached or contained must be sterilized prior to disposal.
   b. Samples preserved with HCl to pH <2 are hazardous and must be neutralized before being disposed, or must be handled as hazardous waste.

xvi. **References;**

a. Standard Methods for the Examination of Water and Wastewater, Latest online edition (approved 2017), Methods 9020, 9215B, 9222B, D and G.
b. National Environmental Laboratory Accreditation Conference, the NELAC Institute, TNI Standard 2016.
c. Method 1603: *Escherichia coli* (*E. coli*) in Water by Membrane Filtration Using Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC), USEPA, Sept. 2014.

xvii. **Any tables, diagrams, flowcharts and validation data;**

a. Table 1: Initial and Ongoing Precision and Recovery Acceptance Criteria
b. Table 2: Example Percent Recovery Calculations for Spikes and Acceptance Criteria
c. Table 3: Determination of Uncertainty

Table 1: Initial and Ongoing Precision and Recovery (IPR, OPR, MS) ***Acceptance Criteria***

Performance Test	**Lab-prepared spike acceptance criteria**	**BioBall acceptance criteria**
Initial precision and recovery (IPR) Mean percent recovery	46-119%	detect-144%
Precision (as maximum relative standard deviation)	36%	61%
Ongoing precision and recovery (OPR) as percent recovery	38-127%	detect-144%
Matrix spikes (MS)	12-149%	17-117%

Table 2. Example Percent Recovery Calculations for Spikes and Acceptance Criteria.

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

$$R = 100 \times (Ns - Nu) / T$$

Where,
R = Percent recovery
Ns = *E. coli* (CFU / 100 mL) in the spiked sample
Nu = *E. coli* (CFU / 100 mL) in the unspiked sample
T = True spiked *E. coli* (CFU / 100 mL) in spiked sample based on the lot mean value provided by manufacturer. The OPR analysis is performed by spiking a 100-mL PBS sample with an *E. coli* Bioball. Calculate the percent recovery (R) for the OPR sample.

Table 3. Determination of Uncertainty

Determine the square root of the variance of at least 7 replicates of BioBall® measurements on at least an annual basis. Report all measurements by this method with an uncertainty of plus or minus the value determined.

Example:

Replicate	Measurement
1	30
2	31
3	24
4	35
5	29
6	26
7	20
Variance (v)= 24.48	sq. root of v= 4.95

Replicate 1 reported as 30 ± 4.95
4.95 is the Standard Uncertainty