

STANDARD OPERATING PROCEDURE

For

U.S. EPA Method 1603

E. coli Membrane Filtration Procedure

SOP #: EPA 1603

REVISION #: 1.5

DATE: December 2006

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LIST OF REVISIONS

Rev. #	Date	Description of Revision	Page #
0	August 2001	None	
1.0	April 2003	Method number changed from EPA Modified 1103.1 to EPA 1603	Title page & header
1.2	September 2005	Table 2 added – QC elements, acceptance criteria, and corrective actions	12
1.5	December 2006	Replaced old DEP Logo with state seal + MassDEP Numerous revisions throughout document Table 2 – Updated/expanded QC Table	Title page & header 12



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1.0 SCOPE & APPLICATION

- 1.1 The membrane filtration technique is a single-step method which may be used to select for *E. coli* by incubating a filtered sample on modified m-TEC agar containing a chromogen (5-bromo-6-chloro-3-indolyl- β -D-glucuronide) which produces a red- or magenta-colored compound when broken down by *E. coli* when incubated for 22-24 hours at $44.5 \pm 0.2^\circ\text{C}$.

2.0 SUMMARY OF METHOD

- 2.1 A well-mixed sample is drawn through a $0.45\text{-}\mu\text{m}$ membrane filter which is then placed on a petri dish containing modified m-TEC agar, incubated for 2 hours at $35 \pm 0.5^\circ\text{C}$ followed by 22-24 hours incubation at $44.5 \pm 0.2^\circ\text{C}$, and then observed for red or magenta colonies.

3.0 DEFINITIONS

- 3.1 When using the modified membrane filtration method, *E. coli* is defined as bacteria that produce red or magenta colonies when incubated for 22-24 hours at $44.5 \pm 0.2^\circ\text{C}$ on modified m-TEC agar.

4.0 INTERFERENCES

- 4.1 The accuracy of the modified *E. coli* membrane filtration method is decreased when samples are highly turbid.

5.0 SAFETY

- 5.1 Samples (and positive controls) may contain microorganisms that are pathogenic to humans. All precautions are to be taken to minimize exposure. All personnel must wear lab coats, safety glasses, and protective latex gloves while working in the laboratory.

6.0 EQUIPMENT AND SUPPLIES

- 6.1 Sample bottles: Sterile wide-mouthed borosilicate glass or plastic. For sampling chlorinated wastewater effluent, a 120-mL sample bottle must contain 0.1 mL of a 10% solution of sodium thiosulfate. The WES Microbiology Laboratory uses sterile 120-mL Corning® disposable wide-mouthed plastic ready-to-use bottles containing a sodium thiosulfate tablet (note: provides a final concentration of 100 mg/L $\text{Na}_2\text{S}_2\text{O}_3$).
- 6.2 Dilution bottles: Sterile bottles of the appropriate size to prepare positive control cultures to run with each sample batch.
- 6.3 Containers for culture medium: Use clean borosilicate glass flasks or beakers for preparation of media.
- 6.3.1 Culture dishes: Use pre-sterilized 50- x 9-mm plastic petri dishes with tight-fitting lids. Reseal opened packages of disposable dishes for storage.
- 6.4 Filtration units: The WES Microbiology Laboratory has two stainless steel 6-funnel filtration manifolds and stainless steel as well as plastic filtration funnels. Discard plastic funnels with deep scratches on inner surface.

Stainless steel funnel assemblies are placed on a stainless steel perforated rack for sterilization in the autoclave (15 minutes). Immediately following sterilization, the stainless steel funnels are



to be placed in the UV light box for storage. The plastic funnels are wrapped, typically in a clear plastic autoclavable instrument bag, and autoclaved prior to use.

Filtration funnels shall be sterilized for the initial filtration of a sample series. Following the filtration of a sample, the funnel(s) shall be placed in the UV light box and exposed to UV for 3 minutes before re-using units between successive filtration series.

The filtration manifold is connected to two glass flasks in series with an electric vacuum pump securing a pressure differential of 138 to 207 kPa. The flask closest to the vacuum pump is for the protection of the pump from carryover water and must not be allowed to fill with water.

- 6.5 Membrane filter: Use 0.45- μ m pore size, 47-mm diameter pre-sterilized, gridded membrane filters. Use only those filter membranes that have been found, through adequate quality control testing and *certification by the manufacturer*, to exhibit: full retention of the organisms to be cultivated, stability in use, freedom from chemical extractables that may inhibit bacterial growth and development, a satisfactory speed of filtration (within 5 min), no significant influence on medium pH (beyond ± 0.2 units), and no increase in number of confluent colonies or spreaders compared to control membrane filters. Use membranes grid-marked in such a manner that bacterial growth is neither inhibited nor stimulated along the grid lines when the membranes with entrapped bacteria are incubated on a suitable medium. Preferably use fresh stocks of membrane filters and if necessary store them in an environment without extremes of temperature and humidity.
- 6.6 Forceps: Smooth flat forceps, without corrugations on the inner sides of the tips. Sterilize before use by dipping in 95% ethyl or absolute methyl alcohol and flaming.
- 6.7 Incubators: Use an incubator that can maintain a temperature of $35 \pm 0.5^{\circ}\text{C}$ and a water bath to provide a temperature of $44.5 \pm 0.2^{\circ}\text{C}$.
- 6.8 Microscope and light source: To determine colony counts on membrane filters, use a stereoscopic microscope at a magnification of 10x to 15x and a cool white fluorescent light source.

7.0 MEDIA

- 7.1 Modified m-TEC Agar (refer to the manufacturer's directions on the bottle for preparation)

Proteose peptone #3	5.0 g
Yeast extract	3.0 g
Lactose	10.0 g
Sodium chloride, NaCl	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
Chromogen (5-bromo-6-chloro-3-indolyl- β -D-glucoronide)	0.5 g
Agar	15.0 g
Reagent-grade water	1 L

- 7.1.1 Add 45.6 g dehydrated modified m-TEC medium to 1 L of reagent grade water in a 2-L flask. Heat to boiling (using a hot plate) until the ingredients dissolve; autoclave at 121°C for 15 min; cool to $45 - 50^{\circ}\text{C}$ and then measure the pH of the prepared medium to ensure that its final pH is 7.3 ± 0.2 . If necessary, adjust the pH of the medium to within the required range with sterile 1 N NaOH or 1 N HCl.



- 7.1.2 Dispense in 5- to 7-mL quantities into lower section of 50-mm plastic petri dishes and let solidify. Do not expose poured plates to direct sunlight; refrigerate in a lidded plastic container containing a card labeled with the name of the medium, stamped with the date of preparation, and labeled with the 14-day discard date. Discard unused medium after 2 weeks or sooner if there is evidence of moisture loss, medium contamination, or medium deterioration (darkening of the medium).

7.2 Preparation of Buffered Rinse Water

- 7.2.1 To prepare stock phosphate buffer solution, dissolve 34.0 grams of potassium dihydrogen phosphate (KH_2PO_4), in 500 mL of reagent grade water, adjust to pH 7.2 ± 0.5 with 1 N sodium hydroxide (NaOH) and dilute to 1 L with reagent-grade water. Store solution at 4°C in a one-liter screw cap bottle. Discard solution if it becomes turbid.
- 7.2.2 To prepare magnesium chloride solution, dissolve 81.1 grams of magnesium chloride ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$) in one liter of reagent water. Store solution at 4°C in a one-liter screw cap bottle.
- 7.2.3 In a large carboy, add 95 mL of magnesium chloride solution and 24 mL of phosphate buffer solution to 5 gallons of reagent-grade water. Dispense the buffer from the carboy into one-liter bottles. Place nine bottles of the buffer solution into the autoclave and autoclave at 121°C for 45 minutes.
- 7.2.4 Remove bottles of buffered water from the autoclave and store at room temperature.
- 7.2.5 A sterility check must be performed for each batch of buffered water prepared. Add 50 mL of buffered water and 50 mL of lauryl tryptose broth (2x) to a sterile glass jar. Incubate for 24 hours at 35°C . Discard any batch of water showing turbidity or growth after incubation. Record the results of the sterility check in the autoclave log.

8.0 **SAMPLE COLLECTION, PRESERVATION AND STORAGE**

- 8.1 Samples must be aseptically collected in a sterile disposable plastic bottle leaving at least 1" (2.5 cm) of headspace to allow for sufficient mixing of the sample prior to analysis. If the sample is chlorinated, make sure that the appropriate concentration of sodium thiosulfate is added (see 6.1)
- 8.2 Keep sample bottle closed until it is to be filled, fill the bottle without rinsing, replace cap immediately, and secure the top with the attached plastic "lock".
- 8.3 Samples must be kept at $\leq 10^\circ\text{C}$ from the time of collection to the time of analysis.
- 8.4 Non-potable water samples must be analyzed within 8 hours of collection (i.e., maximum of 6 hours in the field and two hours in the laboratory).
- 8.5 All samples must be accompanied by a sample tracking sheet/chain-of-custody form filled out by the collector.

9.0 **QUALITY CONTROL**

- 9.1 Test each new lot of medium against a previously acceptable lot for satisfactory performance by making dilutions of a thermotolerant *E. coli* culture and filtering the appropriate volume to yield plates with counts in the range of 20-80 colonies per filter.



9.2 Filter a sterile buffered water sample at the beginning and end of each filtration series. Incubate the rinse water control membrane culture under the same conditions as the sample.

9.3 Run one sample in duplicate for each batch of 10 or fewer samples.

9.4 For each filtration series, run a positive control culture organism (thermotolerant *E. coli*).

10.0 CALIBRATION AND STANDARDIZATION

10.1 The temperature of the incubator must be checked twice each day (with readings at least 4 hrs. apart). Readings must be $35 \pm 0.5^{\circ}\text{C}$.

10.2 The temperature of the waterbath must be checked twice each day (with readings at least 4 hrs. apart). Readings must be $44.5 \pm 0.2^{\circ}\text{C}$.

10.3 Refer to Laboratory Quality Assurance Plan for calibration and standardization procedures of laboratory equipment used for this analysis.

11.0 PROCEDURE

11.1 The sample volume filtered is based on the expected bacterial density (see Table 1).

11.1.1 An ideal sample volume will yield 20 to 80 *E. coli* colonies on a membrane filter. Filter three different volumes depending on the expected bacterial density. When less than 20 mL is to be filtered, add approximately 10 mL sterile dilution water to the funnel before filtration or pipet the sample volume into a sterile dilution bottle, and then filter the entire dilution. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

11.2 Sterilize/sanitize filtration units: Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 minutes or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all funnels and bases in use.

11.2.1 Filtration units are to be sanitized in the UV light box for three minutes before use for a new sample and/or if a higher dilution of the same sample is to be filtered. **Note:** It is unnecessary to expose the funnel(s) to UV between filtrations of dilutions of the same sample (for nonpotable samples) as long as the smallest dilution (volume) of the sample is filtered first and the funnel is rinsed thoroughly with sterile buffered water before proceeding to the next dilution.

11.3 Label petri dishes: Label the bottom (side containing the agar) of each petri dish with the: 1) LIMS sample number, and 2) dilution/volume filtered.

11.4 Filtration of sample: Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of filter base. Attach funnel to base and lock it in place. Turn on the vacuum and filter the sample completely. With filter still in place, rinse the interior surface of the funnel by filtering three 20- to 30-mL portions of sterile buffered rinse water. Rinsing between samples prevents carryover contamination. Turn off the vacuum, remove the funnel, and remove the membrane filter with sterile forceps (touching only within the outer 1/8" of the filter), and place it on the modified m-TEC agar surface with a rolling motion to avoid entrapment of air.



11.5 Incubation: Seal inverted petri dishes in Whirlpak bags and incubate at $35 \pm 0.5^{\circ}\text{C}$ for 2 hours. After the 2 hour incubation, incubate the dish, completely submerged, in a waterbath for 22 to 24 h at $44.5 \pm 0.2^{\circ}\text{C}$. Place WhirlPak bags in test tube racks and place lead donuts on top of rack to prevent bags from floating to the surface of the bath.

11.6 Counting: Using the low-power (10x to 15x) binocular dissecting microscope, count all red or magenta colonies as *E. coli*.

12.0 DATA ANALYSIS AND CALCULATIONS

12.1 Quantification: Compute the count, using membrane filters with 20 to 80 *E. coli* colonies by the following equation:

$$E. coli/100 \text{ mL} = \frac{E. coli \text{ colonies counted} \times 100}{\text{mL sample filtered}}$$

12.3 Out-of-Range Plates: If no filter has an *E. coli* count falling in the ideal range, total the *E. coli* counts on all filters and report as number per 100 mL. For example, if duplicate 50-mL portions were examined and the two membranes had five and three *E. coli* colonies, respectively, report the count as eight *E. coli* colonies per 100 mL.

12.4 Confluent growth: If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth".

12.4 Calculation of Precision QC Criterion: Determine the range of logs for the duplicate colony counts as follows (*Standard Methods for the Examination of Water and Wastewater*, 1998, Page 9-10):

$$\text{Range of Logs for a Duplicate Set} = \text{Log}[(\text{Count } 1) + 1] - \text{Log}[(\text{Count } 2) + 1]$$

$$\text{Precision QC Criterion} = 3.27 \text{ (Mean Range of Logs for 15 Most Recent Duplicate Sets)}$$

13.0 METHOD PERFORMANCE

13.1 The detection limit of this method is one colony-forming unit per sample volume or dilution tested.

14.0 POLLUTION PREVENTION

14.1 Refer to the WES Environmental Management System (EMS) policy and SOPs regarding pollution prevention.

14.2 The quantity of media and reagents purchased should be based on expected usage during its shelf life. Actual media and reagent preparation volumes should reflect anticipated usage and stability.

15.0 WASTE MANAGEMENT

15.1 WES laboratories fully comply with all applicable federal, state, and local environmental regulations. WES is also committed to protecting the air, water, and land by minimizing and controlling all chemical releases from fume hoods, biological safety cabinets, and bench operations. Refer to the WES EMS policy and SOPs regarding waste management.



- 15.2 All positive sample plates, including filter positive control plates, are placed in autoclave bags and autoclaved at 121°C for a minimum of 30 minutes. Decontaminated plastic petri plates are separated from agar for recycling; agar waste is disposed to the WES dumpster.

16.0 REFERENCES

- 16.1 *Standard Methods for the Examination of Water and Wastewater*, 20th Edition, 1998. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.
- 16.2 Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane – Thermotolerant *Escherichia coli* Agar (modified mTEC), Environmental Protection Agency, Office of Water, EPA-821-R-02-023, September 2002.



17.0 TABLES

TABLE 1. Recommended Filtration Volumes for *E. coli* Membrane Filtration Test

Sample Type	Recommended Filtration Volume (mL)							
	100	50	10	1	0.1	0.01	0.001	0.0001
Lakes, reservoirs	X	X						
Wells, springs	X	X						
Water supply intake		X	X	X				
Natural bathing waters		X	X	X				
Sewage treatment plant			X	X	X			
Farm ponds, rivers				X	X	X		
Stormwater runoff				X	X	X		
Raw municipal sewage					X	X	X	
Feedlot runoff					X	X	X	
Sewage sludge						X	X	X



TABLE 2. Quality Control Elements, Acceptance Limits, and Corrective Actions for the Analysis of *E. coli* by the Membrane Filtration Procedure – U.S. EPA Method 1603

QC Elements	Frequency	Acceptance Criteria	Corrective Action
Sample storage	Every sample	Samples are analyzed within a maximum of 8 hours from collection (i.e., maximum of 6 hours in the field + 2 hours in the laboratory) and are stored at $\leq 10^{\circ}\text{C}$ from time of collection to time of analysis	Qualify data (H or J) as estimated value (exceeded holding time or holding temperature, respectively) and contact sample collector to obtain new sample
Filter blank or negative control (equivalent to LRB; sterile buffered rinse water)	Beginning and end of batch	<i>E. coli</i> colonies absent	Qualify data (B) as estimated value (<i>E. coli</i> colonies present in sample, and in one or more of the filter blanks – laboratory contamination) and contact sample collector to obtain new sample
Filter positive control (equivalent to LCS and LFB; sterile buffered rinse water spiked with <i>E. coli</i>)	One per batch	<i>E. coli</i> colonies present	Qualify data (J) as estimated value (<i>E. coli</i> colonies absent in the filter positive control – media or other failure) and contact sample collector to obtain new sample
Laboratory duplicate	One per batch of 10 or fewer samples ($\geq 10\%$)	Range of Logs for Duplicate Counts = 0 - 0.40 (current laboratory calculated control limits for the ideal counting range of 20 - 80 colonies per plate)	Qualify data (J) as estimated value (precision criterion not met)
Field blanks	Not routinely included – only for special projects or if submitted by sample collector	<i>E. coli</i> colonies absent	If the sample is identified to the laboratory as a field blank, qualify data (J) as suspect due to possible sample contamination in the field (i.e., for all field samples collected in the same sampling event that have <i>E. coli</i> colonies)
Field duplicates	Not routinely included – only for special projects or if submitted by sample collector	Range of Logs for Duplicate Counts = 0 - 0.40 (current laboratory calculated control limits for the ideal counting range of 20 - 80 colonies per plate)	If the sample is identified to the laboratory as a field duplicate, qualify data (J) as not meeting the field precision criterion