

STANDARD OPERATING PROCEDURE

For

SM 9222D

Standard Fecal Coliform Membrane Filtration Procedure

SOP #: SM 9222D SOP REVISION #: 2.0 DATE: September 2010

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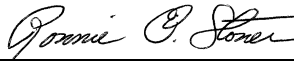

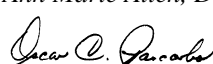
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LIST OF REVISIONS

Rev. #	Date	Description of Revision	Page #
0	August 2000	None	
1.0	February 2003	Numerous edits and additions	Throughout document
1.1	September 2005	Table 2 added – QC elements, acceptance criteria, and corrective actions	12
1.2	December 2006	Replaced old DEP Logo with state seal + MassDEP	Title page & header
1.3	January 2008	Revised Section 7.2.5 – Sterility check of buffered rinse water changed from just at 24 hours to both at 24 and 48 hours.	7
		Added Section 9.5	8
2.0	September 2010	Section 1.2 added – Sample matrices tested in the WES Microbiology Laboratory using this method	5
		Sections 6.9 - 6.11 added	6
		Section 7.2 revised	7
		Section 8.4: $\leq 10^{\circ}\text{C}$ changed to $1-8^{\circ}\text{C}$	8
		Section 12.4 – Revised duplicate range of logs calculation.	10
		Table 2 revised	13
		Other minor edits	Throughout document



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1.0 SCOPE & APPLICATION

- 1.1 This membrane filtration procedure may be used to selectively enumerate fecal coliforms in raw drinking water source, ambient water, and wastewater samples using an enriched lactose medium and an incubation temperature of $44.5 \pm 0.2^{\circ}\text{C}$. This is a highly reproducible method for analyzing relatively large water sample volumes assuming that the samples are not highly turbid.
- 1.2 In the WES Microbiology Laboratory, this method is used primarily for the enumeration of fecal coliforms in raw drinking water sources. For this reason, our laboratory participates in proficiency test studies with lower fecal coliform densities representative of source water samples. On special request, our laboratory may also use this method to analyze ambient water and wastewater samples with higher fecal coliform densities.

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 M-FC agar and incubated for 24 ± 2 hours at $44.5 \pm 0.2^{\circ}\text{C}$; all blue colonies (any shade of blue) are then counted.

3.0 DEFINITIONS

- 3.1 When using this membrane filtration method, fecal coliforms are defined as bacteria that produce blue colonies (any shade of blue) on M-FC agar within 24 ± 2 hours at $44.5 \pm 0.2^{\circ}\text{C}$.

4.0 INTERFERENCES

- 4.1 The accuracy of this fecal coliform 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 or nitrile 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 Media – 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 uses two stainless steel 6-funnel filtration manifolds and stainless steel as well as plastic filtration funnels. Discard plastic funnels if they develop deep scratches on the inner surface.
- 6.4.1 Stainless steel funnel assemblies are wrapped in Kraft paper and placed on a stainless steel perforated rack for sterilization in the autoclave (15 minutes). Immediately following sterilization, the stainless steel funnels are placed in a UV light sterilizer for storage. The plastic funnels are wrapped in a clear plastic autoclavable instrument bag and autoclaved (15 minutes) prior to use.
- 6.4.2 Filtration funnels are sterilized by autoclaving for the initial filtration of a sample batch. Following the filtration of a 6-sample series, the 6 funnels are placed in a UV light sterilizer and exposed to UV for 3 minutes before re-using the funnels for successive sample filtration series.
- 6.4.3 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 Filters – Use 0.45- μ m pore size, 47-mm diameter pre-sterilized, gridded membrane filters. Use only those membrane filters that have been found, through adequate quality control testing and ***certification by the manufacturer***, to exhibit, as compared to control membrane filters: 1) Full retention of the organisms to be cultivated; 2) Stability in use; 3) Freedom from chemical extractables that may inhibit bacterial growth and development; 4) Satisfactory speed of filtration (within 5 min); 5) No significant influence on medium pH (beyond ± 0.2 pH units); and 6) No increase in the number of confluent colonies or spreaders. 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 then flaming.
- 6.7 Incubator – Use a water bath than provides 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 10-15x and a cool white fluorescent light source.
- 6.9 UV Light Sterilizer – Millipore Catalog # XX6370000.
- 6.10 Whirl-Pak® Bags – To protect plates when incubating in water bath.
- 6.11 Water Bath – Capable of maintaining 50°C for tempering media.
- 7.0 MEDIA AND BUFFERED RINSE WATER**
- 7.1 The need for uniformity dictates the use of commercial dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions for re-hydration. Store opened supplies of dehydrated media in a desiccator for up to one year:



M-FC Agar – Refer to the manufacturer's directions on the bottle for preparation.

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride, NaCl	5.0 g
Lactose	12.5 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline Blue	0.1 g
Agar	15.0 g
Reagent water (ASTM Type I)	1 L

- 7.1.1 In a sterile 2-L Erlenmeyer flask, re-hydrate 52 g of product in 1 L of reagent water containing 10 mL of 1% rosolic acid in 0.2 N sodium hydroxide (NaOH) (note: rosolic acid reagent will decompose if sterilized by autoclaving; stock solution must be refrigerated in the dark and discarded after 2 weeks or sooner if its color changes from dark red to muddy brown). Bring to a near boil on a hot plate with constant stirring to dissolve the agar, then promptly remove from the heat and cool to 50°C – **do not sterilize by autoclaving**. The final pH must be 7.4 ± 0.2 .
- 7.1.2 Dispense the M-FC agar in 5- to 7-mL quantities into the lower section of 50-mm plastic Petri dishes and let solidify. Do not expose poured plates to direct sunlight. Refrigerate plates inverted in a lidded plastic container containing a card labeled with the name of the medium, date of preparation, and expiration date. Discard unused medium after 2 weeks or sooner if there is evidence of moisture loss, medium contamination, or medium deterioration (i.e., darkening of the medium).

7.2 Preparation of Buffered Rinse Water

- 7.2.1 To prepare the stock phosphate buffer solution, dissolve 34.0 grams of potassium dihydrogen phosphate (KH_2PO_4), in 500 mL of ASTM Type I reagent water, adjust to pH 7.2 ± 0.5 with 1 N NaOH, and dilute to 1 L with reagent water. Autoclave for 15 min @ 121° C or filter sterilize through a 0.22- μm pore size filter. Store solution at 4°C in a 1-L screw cap bottle. Discard solution if it becomes turbid.
- 7.2.2 To prepare the magnesium chloride solution, dissolve 81.1 grams of magnesium chloride ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$) in 1 L of ASTM Type I reagent water. Autoclave for 15 min @ 121° C or filter sterilize through a 0.22- μm pore size filter. Store solution at 4°C in a 1-L screw cap bottle.
- 7.2.3 In a large 5-gallon carboy, add 95 mL of magnesium chloride solution and 24 mL of phosphate buffer solution to 5 gallons of ASTM Type I reagent water. Dispense the buffer from the carboy into 1-L bottles. Place the bottles of buffer solution into the autoclave (up to 15 Liters) and autoclave at 121°C for 45 minutes.
- 7.2.4 Remove the bottles of buffered rinse water from the autoclave and store at room temperature.
- 7.2.5 A sterility check must be performed for each batch of buffered rinse water prepared. Add 50 mL of buffered rinse water and 50 mL of tryptic soy broth (TSB) (2X) into a sterile 250-



mL Erlenmeyer flask. Incubate for 48 hours at $35^{\circ} \pm 0.5^{\circ} \text{C}$, checking the sterility at both 24 and 48 hours. 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 Section 6.1)
- 8.2 Remove all attachments from drinking source water taps (screens, etc.). Open tap and let run to waste for 2-3 minutes. Reduce the water flow to allow for filling of the bottle without splashing.
- 8.3 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".
- 8.4 Samples must be kept at 1-8°C from the time of collection to the time of analysis.
- 8.5 All water samples must be transported to the laboratory as quickly as possible, received at the laboratory no later than 6 hours from collection, and analyzed within 2 hours of receipt at the laboratory – maximum holding time of 8 hours from collection.
- 8.6 All samples must be accompanied by the *WES Sample Tracking/Chain-of-Custody Form* filled out by the collector, including all applicable chain-of-custody signatures.

9.0 QUALITY CONTROL

- 9.1 Each new lot of medium is tested against a previously acceptable lot for satisfactory performance by using a known positive fecal coliform culture (*E. coli*), a negative control organism (*Pseudomonas aeruginosa*), and a sterility blank (an uninoculated plate).
- 9.2 Filter a sterile buffered rinse water sample at the beginning and end of each filtration series to check for possible sample cross-contamination or contamination of the rinse water (note: the filter blank is equivalent to an LRB – i.e., a laboratory reagent blank). The membrane filters for the filter blank controls are cultured under the same conditions as the samples.
- 9.3 One sample is tested in duplicate (i.e., laboratory duplicate) for each batch of 10 or fewer samples.
- 9.4 For each filtration series, a positive control culture (*E. coli*) is run.
- 9.5 A colony count comparison between analysts on a single field sample must be performed for this and other quantification methods used to test field samples each month. Quantification methods not used to test field samples during a calendar month are exempt from this requirement for that month – e.g., if there were no requests to test samples by SM 9222D during December, a colony count comparison between analysts for this methodology would not have to be performed for this month. The colony counts between analysts for a single field sample must be within 10% and documented in the laboratory QC records.



10.0 CALIBRATION AND STANDARDIZATION

- 10.1 Refer to the 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 will depend on the expected fecal coliform density for the sample type to be tested (see Table 1).

11.1.1 The ideal sample volume will yield 20 to 60 fecal coliform colonies on a membrane filter. Filter three different volumes depending on the expected fecal coliform density. When less than 20 mL is to be filtered, add approximately 10 mL of sterile buffered rinse water to the funnel before adding and filtering the sample volume, or add the sample volume into a sterile dilution bottle containing sterile buffered rinse water and then filter the entire bottle content. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective membrane filter surface. For volumes of 20-100 mL, use a sterile graduated cylinder to measure the sample volume. For sample volumes less than 20 mL, use a sterile wide-tip pipette.

Note: Bacteria must not be suspended in dilution water for more than 20 minutes at room temperature as death or multiplication could occur.

- 11.2 Sterilize 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 sterilized in the UV light sterilizer for three minutes before use for a new sample or if a smaller volume of the same sample is to be filtered. **Note:** It is unnecessary to expose the funnel(s) to UV light between filtrations of dilution volumes of the same sample as long as the dilutions are filtered in series from the smallest volume (first) to the largest volume (last) and the funnel is rinsed thoroughly with sterile buffered rinse water between dilutions.

- 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 (flamed) forceps, place a sterile membrane filter (grid side up) over the porous plate of the filter base. Attach funnel to the base and lock it in place. Shake the sample vigorously at least 25 times, and take sample volume and place in the filter funnel. Turn on the vacuum and filter the sample volume 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 3 times between samples prevents carryover contamination. Turn off the vacuum, remove the funnel, and remove the membrane filter with sterile (flamed) forceps (touching only within the outer 1/8" of the filter), and place it on the M-FC agar surface with a rolling motion to avoid entrapment of air. Invert the Petri dish, place the dish into a Whirl-Pak® bag, seal the bag, and incubate the dish, **completely submerged**, in the water bath for 22 to 24 h at $44.5 \pm 0.2^\circ\text{C}$. Place Whirl-Pak® bags in test tube racks and place lead donuts on top of rack to prevent bags from floating to the surface of the bath.

Note: All prepared plates must be placed in the water bath within 30 minutes after filtration. The bath must be at the required temperature prior to placing the plates in it.



- 11.5 Counting – Using the low-power (10x to 15x) binocular dissecting microscope, count all blue colonies (may exhibit various shades of blue) as fecal coliform colonies. Non-fecal coliform colonies are gray to cream colored.
- 11.6 Fecal Coliform Verification – At least 10 blue colonies should be picked from one positive sample monthly and verified in lauryl tryptose broth (LTB) and EC broth (see SM 9222B SOP, Section 11.8, and SM 9221E SOP, respectively).

12.0 DATA ANALYSIS AND CALCULATIONS

- 12.1 Quantification – Using membrane filters with 20 to 60 fecal coliform colonies, compute the fecal coliform concentration in each sample by the following equation:

$$\text{Fecal coliforms} / 100 \text{ mL} = \frac{\text{fecal coliform colonies counted} \times 100}{\text{mL sample filtered}}$$

- 12.2 Out-of-Range Plates – If no filter has a fecal coliform count falling in the ideal range, total the fecal coliform 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 fecal coliform colonies, respectively, report the count as eight fecal coliform colonies per 100 mL.
- 12.3 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):

Range of Logs for a Duplicate Set = Log (Count 1) - Log (Count 2)

If either result of a duplicate set is < 1, add 1 to both values before calculating the logarithms as follows:

Range of Logs for a Duplicate Set = Log [(Count 1) + 1] - Log [(Count 2) + 1]

Precision QC Criterion = 3.27 (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 (CFU) per sample volume 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.



17.0 TABLES

TABLE 1. Recommended Filtration Volumes for the Fecal Coliform 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 Fecal Coliforms by the Membrane Filtration Procedure - SM 9222D

QC Elements	Frequency	Acceptance Criteria	Corrective Action
Sample storage	Every sample	Samples must be analyzed within a maximum of 8 hours from collection (i.e., maximum of 6 hours in the field + 2 hours in the laboratory) and must be stored at 1-8°C from time of collection to the 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., filtration series)	No fecal coliform or other colonies present	Qualify data (B) as estimated value (fecal coliform colonies present in sample, and in one or more of the filter blanks – laboratory contamination); determine and eliminate the contamination source, and contact sample collector to obtain new sample
Filter positive culture control (equivalent to LCS and LFB; sterile buffered rinse water spike with <i>E. coli</i>)	One per batch (i.e., filtration series)	Fecal coliform colonies present	Qualify data (J) as estimated value (fecal coliform colonies absent in the filter positive control – media or other failure); determine cause of and correct the problem, 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 - 0.33 (current laboratory calculated control limits for the ideal counting range of 20 – 60 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	Fecal coliform 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 fecal coliform colonies)
Field duplicates	Not routinely included – only for special projects or if submitted by sample collector	Range of Logs for Duplicate Counts = 0 – 0.33 (current laboratory calculated control limits for the ideal range of 20 – 60 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