Standard Operating Procedure

Kwik*Count* ® MF EC

Membrane Filtration
Rapid Detection of *E. coli* in Ambient Waters

Roth Bioscience, LLC
1303 Eisenhower Drive S.
Goshen, IN 46526
(574) 533-3351
hello@rothbioscience.com

1. Scope and Application

1.1. This method describes a membrane filter (MF) procedure for rapid detection and enumeration of *Escherichia coli (E. coli).* Because these bacteria are natural inhabitants of the intestinal tract of warm-blooded animals, their presence in water samples are an indication of fecal pollution and the possible presence of other enteric pathogens. This test for *E. coli* can be applied to fresh water, or other waters matrices. Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of *E. coli* levels in water can be detected and enumerated. The KwikCount® MF EC medium (Roth Bioscience LLC, Goshen, Indiana) can be used to detect and enumerate E. coli within 8 to 10 hrs.

1.2. The method allows for the detection and enumeration of *E. coli* within 8-10 hrs and does not require a confirmation step. It is also useful in the analysis of other waters, such as treated-wastewater effluent testing.

1.3. The detection limit is one colony forming unit (CFU) per sample.

2. Summary of Method

2.1. A water sample is filtered through a 0.45 µm pore size, 47 mm diameter membrane filter using standard equipment and methodology. The filter is then placed into a 50 mm plate containing a pad holding a measured amount of KwikCount® MF EC broth. Plates are incubated with membrane filters at 41±0.5°C for 8 -10 hrs. The plates are then viewed in a dark place using a long wave UV light (366nm preferred) and any fluorescent colony is counted as *E.coli* .

2.2. KwikCount® MF EC broth contains nutrients that promote the growth of the target organisms and buffers to maintain appropriate pH. It also contains a fluorogenic/chromogenic enzyme substrate mixture that is used to cause Coliform bacteria to fluoresce and is detectable as early as 6 hrs incubation time (8-10 hrs incubation is the peak fluorescent intensity.) The fluorescent light will gradually decrease until about 24 hrs when it will be totally dissipated. Beginning about 14-16 hrs incubation, the *E.coli* colonies will not only fluoresce in UV light, but will also appear as blue/purple colonies in ambient light. This phenomenon of a coloring compound having two unique properties simultaneously has been trademarked as DUOGEN®.

2.3 The KwikCount® method therefore incorporates and includes the advantages of utilizing a Duogen®. When the blue fluorescence has dissipated, the *E.coli* colonies will continue to be present as blue/purple colonies that may continue in growth and intensify of color. The blue/purple colonies should not be counted after 48 hrs incubation.

3. Method Definition

3.1. In the KwikCount® method, *E. coli* are those bacteria which produce fluorescent colonies on the medium between 8-20 hrs incubation when using a long wave UV light source (366nm preferred).

3.2. KwikCount® MF EC medium is ready-to-use with a sterile 47 mm, 0.45µm pore size membrane and pad.

4. Interferences

4.1. Water samples containing colloidal or suspended particulate materials may interfere with filtration efficiency by clogging filter pores, and they may cause some spreading of bacterial colonies as they grow on the filter surface during incubation. These materials could interfere with accurate enumeration of colonies but rarely prevent the accurate detection of target colonies.

4.2. In the case that excessive crowding is observed on the plates, samples should be recollected and rerun at a higher dilution. Small colored colonies of this nature should not be counted unless they are isolated as individual colonies with densities below 80 CFU per plate.

5. Safety

5.1. Analyst/technician 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.

6. Equipment and Supplies

6.1. Sterile pipettes (1 to 25 mL) or graduated cylinders (100 mL)

6.2. Petri dishes: 60 x 15 mm, 50 x 9 mm, or other appropriate size dish with sterile absorbent pads.

6.3. Filter membranes: sterile 47 mm, 0.45 µm pore size membranes and pads may be supplied with the KwikCount® MF EC as a kit for optimum performance.

6.4. Filtration units: Sterile filtration unit must be used for membrane filtration procedures. The filter-holding assembly may consist of a seamless funnel fastened to a base by a locking device or by magnetic force (glass, plastic, or stainless steel). Funnels may be reusable or single-use disposable units. Reusable funnel units are sterilized by autoclave at 121°C for 15 min (reusable non autoclavable membrane filtration device is available at www.rothbioscience.com).

6.5. Vacuum pump or equivalent.

6.6. Forceps: smooth, flat, sterilizable metal forceps.

6.7. Microscope: A 10 to 15 X magnification binocular wide-field dissecting microscope.

6.8. Light box with a UV lamp (366nm) and a camera for taking pictures (optional)

6.9. Bunsen burner or alcohol lamp for sterilizing forceps.

6.10. Sterile dilution and rinse bottles.

6.11. Biohazard bag

7. Reagents and Standards

7.1. Buffered water

7.1.1. Stock phosphate buffer solution. 10X phosphate saline buffer, pH 7.0— EMD Chemicals Cat. No. 6508 or equivalent. Empty content of one bottle of PBS to a beaker and add reagent water to one liter. Mix to dissolve the content and transfer to a 1L bottle. One bottle makes 1L of 10X PBS. Autoclave at 121ºC for 15 min and store at room temperature.

7.1.2. Working solution. Add 100 mL 10x phosphate buffer solution and 900 mL reagent water. Autoclave at 121ºC for 15 min.

7.2. KwikCount® MF EC medium with filter membranes and pads (Roth Bioscience, LLC)

7.2.1. KwikCount® MF EC medium is provided in a 20 mL per bottle package. The medium should be kept frozen and has an expiration time of 1 year.

7.2.2. The broth medium should be thawed and added to dishes (1.8 mL per dish or according to the kit instructions). Aseptic technique should be practiced to keep the absorbent pad and the leftover medium sterile.

8. Sample Collection, Dechlorination, Preservation and Storage

8.1. Collection containers: Any sterile 120-mL shrink-banded vessels are used as standard sample collection containers. Clients may also collect samples in a sterile, clean glass or heat-resistant plastic bottle with a leak-proof closure. Collect a minimum sample volume of 100 mL.

8.2. Sampling procedures

8.2.1. During sample collection, leave sample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking.

8.2.2. 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.

8.3. Samples should be held at <10ºC during transit to the laboratory and tested as soon as possible after collection. If processing is not done within 1 hour, the sample should be stored at <10ºC in the laboratory before analysis.

8.4. Holding times

8.4.1. Sample analysis should begin immediately, preferably within 2 hours of collection. The maximum transport time to the laboratory is 6 hours, and samples should be processed within 2 hours of receipt at the laboratory.

9. Quality Assurance/Quality Control

9.1. Quality control

9.1.1. Each lot of KwikCount® MF EC medium should be evaluated by the laboratory by preparing three plates of the medium (one to serve as an uninoculated control, one to serve as a negative growth control, and one to serve as positive control).

9.1.1.1. *E. coli* ATCC #11775 may be used as positive control. *E. faecalis (e.g., ATCC #19433)* is used as a negative growth control microorganism. Filter 100 mL of sterile buffered water and place the filter on the surface of the first plate of KiwkCount® MF EC medium as an uninoculated control. Prepare serial dilutions of the *E. faecalis* so that the inoculum will include 20-80 CFU/100 mL and filter. After filtration, place the filter on the surface of the second plate of KiwkCount® MF EC medium as a negative control. Prepare serial dilutions of the *E.coli* so that the inoculum will result in
20-80 CFU/100 mL. After filtration, place the filter on the surface of the third plate of KiwkCount® MF EC medium as a positive control. Flag any sample results that accompany QC failure and recollect samples.

9.1.1.2. Incubate the plates at 41±0.5°C for 8-20 hrs. The *E. coli* control should appear as blue fluorescent colonies under a 366nm UV lamp, the *E. faecalis* control should not have any blue fluorescent colonies, and the buffered water should have no colonies.

9.1.2. Each batch of filtered samples should include at least one blank control plate (sterile buffered water), one negative growth control plate (*E. faecalis* or equivalent) and one positive growth control plate (*E. coli* or equivalent). These control plates can be the same as the lot control as in section 8.1.1 or a regular control set up in parallel with the filtration batch resulting in correct blank, negative and positive control results. Flag any samples that accompany failed QC plates and recollect samples for retesting.

10. 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 Monograph SP 250-23. Check mercury columns for breaks.

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

11. Procedure

11.1 Prepare Petri dishes and absorbent pads by adding 1.8 mL of medium onto the pad. This should wet the entire pad surface without any excessive overflow of the liquid medium. Dishes may be checked by tilting the dish to determine if excessive medium collects on one side. If so, that excessive medium can be removed (with a sterile fine tip dropper or pipette) so that the pad has no excessive medium running over the pad top. (This is a precaution so that when the membrane filter holding the filtered microbes will not be too wet, so that individual CFUs will grow as smooth edged circular colonies instead of irregular spreading colonies.)

11.2. Using sterile forceps, 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.

11.3. 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.

11.4. Select sample volumes based on previous knowledge of the pollution level, to produce 20-80 *E. coli* colonies on the membranes (Table 1). Sample volumes of 1-100 mL are normally tested at half-$\log_{10}$ intervals (*e.g.* , 100, 30, 10, 3 mL).

11.5. 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 can be filtered, and the results can be combined.

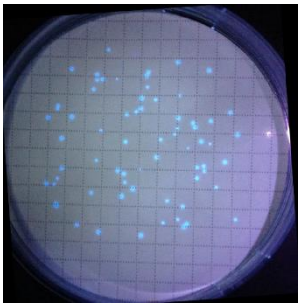
11.6. Filter the sample, and rinse the sides of the funnel twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum, and remove the funnel from the filter base.

11.7. Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the absorbent pad containing the KwikCount® MF EC medium. Avoid the formation of bubbles between the membrane and the pad surface when applying the membranes. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the absorbent pad. Cap the dish and incubate at 41±0.5°C for 8-20 hrs.

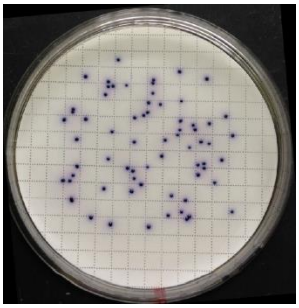
Table 1. Suggested sample volumes to test for Coliforms by membrane filtration								
Volume (X) to be filtered (mL)								
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Lake, Reservoirs	X	X						
Wells, Springs	X	X						
Water Supply Intake		X	X	X				
Natural Bathing Waters		X	X	X				
Sewage Treatment Plant			X	X	X			
Farm Ponds, Rivers				X	X	X	X	
Storm Water Runoff				X	X	X	X	
Raw Municipal Sewage					X	X	X	
Feedlot Runoff					X	X	X	
Sewage Sludge						X	X	X

12. Data Analysis and Calculations

12.1. Count the number of colonies detected by blue fluorescence (Figure 1) present on the membrane filter between 8-20 hr incubation and record as the number of *E. coli* / volume of sample filtered for that test.



UV light 8 hr
Blue fluorescence (Duogen® effect) *E.coli*



Ambient light at 16 hr
Blue-purple (Chromogenic color) *E.coli*

12.2. Use the following general formula to calculate the *E. coli* count per 100 mL of sample.

Coliforms/100 mL = [Number of Coliform colonies / volume of sample filtered (mL)] x 100

For example, choose the plate with counts between 20-80 colonies and if the volume of sample was 10 mL and there were 25 blue fluorescent colonies, record as 25 CFU per 10 mL. According to the above formula, *Coliforms/* 100 mL = 25 CFU/10 mL x 100 = 250 cfu/100 mL

12.3. If the observed range does not fall between 20 and 80 colonies in any membrane,

E. coli CFU/100 mL = (Sum of colonies counted / total volume filtered (mL) x 100

12.4. Report <1 cfu/100 mL if no *E. coli* colonies are observed.

13. Method Performance

A detail method performance will be included after ATP study

14. Pollution Prevention and Waste Management

All biohazardous waste should be sterilized at 121ºC for 30 min prior to disposal. Laboratory personnel should use pollution control techniques to minimize waste generation wherever possible.

15. References

1. Guidance for Preparing Standard Operating Procedures (SOPs), 2001. EPA/240/B-01/004

2. Standard Methods for the Examination of Water and Wastewater, 2005, 21st Edition, APHA, AWWA, WEF.

3. Method 1103.1: *Coliform* (*E. coli*) in Water by Membrane Filtration Using membrane-Thermotolerant *Coliform* Agar (mTEC). 2002. EPA 821-R-02-020. United States Office of Water Environmental Protection Agency, Washington, DC.

4. Method 1603: *Coliform* (*E. coli*) in Water by Membrane Filtration Using Modified membrane-Thermo-tolerant *Coliform* Agar (Modified mTEC). 2002. EPA 821-R-02-023. United States Office of Water, Environmental Protection Agency, Washington DC.

5. FederAL Register/Vol.88, No:95/Wednesday, May 19,2021/Rules and Regulations 27235 I. Changes to 49 CFR 136.3 To Include Alternate Test Procedures (EPA Approval of KwikCount® for “Rapid Detection and enumerations of E.coli in Ambient Waters”).
6. Federal Register/Vol.86. Vol No:95/----Table IH---- List of Approved Microbiological Methods for Ambient Water (lists KwikCount® MF EC)