EPA United States Environmental Protection Agency
Office of Science and Technology
Washington DC 20460
EPA/821/R-97/004
March 2000

Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and *Escherichia coli*

Contents

PHOTOGRAPHS

ABSTRACT

In 1986, the U.S. Environmental Protection Agency (USEPA) recommended two new indicator organisms for recreational water quality assessment. They were enterococci (for both marine and fresh waters) and Escherichia coli (E. coli, for fresh waters only). These organisms were chosen based on epidemiological studies conducted at various beaches in the United States that showed a strong positive correlation between the organisms and the occurrence of swimming-associated gastroenteritis. The two new target organisms required the use of media designed specifically for enumeration of them from ambient waters. Since then, these two media (mE Agar for enterococci and mTEC Agar for E. coli) have been improved, resulting in faster and easier enumeration. The modified media are mEI Agar for enterococci and modified mTEC Agar for E. coli.

The purpose of this manual is to provide specific step-by-step instructions for both the original and the revised test methods (mE, mEI, mTEC, and modified mTEC) used for the new USEPA recommended recreational water quality indicators. This manual is intended to supplement the 1999 USEPA video entitled "Improved Enumeration Media for the Recreational Water Quality Indicators Enterococci and Escherichia coli," although it may be used without the video.

1. INTRODUCTION

Epidemiological studies of marine and fresh water bathing beaches have established a direct relationship between the density of enterococci and *E. coli* in water and the occurrence of swimming-associated gastroenteritis. Recognition of this relationship has led to the development of criteria that can be used to establish recreational water standards (USEPA, 1986a).

In 1976, the U.S. Environmental Protection Agency recommended fecal coliforms as indicators of recreational water quality (USEPA, 1976). The guidelines were based on a 1968 recommendation from the National Technical Advisory Committee of the Department of the Interior to the Federal Water Pollution Control Administration. Since then, USEPA has conducted multi-site epidemiological studies that found that enterococci have a much higher correlation with swimming-associated gastroenteritis in both

fresh and marine water environments than fecal coliforms. *E. coli* was found to have a high correlation with gastroenteritis in fresh water environments only (USEPA, 1986a).

In 1986, USEPA recommended that these two indicators be used as bacterial water quality indicators to monitor recreational waters (USEPA, 1986b). This change in indicators was based on the development of two new media (Dufour *et al*., 1981; Levin *et al*., 1975; USEPA, 1985) for ambient water, namely, mE Agar for enterococci and mTEC Agar for *E. coli*. Since then, these media have been improved, allowing faster (24-hour) and easier (one-step) enumeration of the target organisms. The improved media (Messer and Dufour, 1998; USEPA, 1997) are mEI Agar for enterococci and modified mTEC Agar for *E. coli*. These media are recommended for enumeration of the target organisms from ambient waters and are not intended for enumeration from other water sources, such as drinking water.

Four test methods for measuring bacteriological densities in ambient waters are described in this manual: the original and a revised method for detecting enterococci, and the original and a revised method for detecting *E. coli*. All four methods use a membrane filter procedure.

The original test method (Levin *et al*., 1975; USEPA, 1985) for enterococci was introduced in 1986 (USEPA, 1986b). It uses two media: a primary isolation medium, mE Agar, and Esculin Iron Agar (EIA) for the confirmation of colonies on the transferred filter. The revised method, introduced in 1997 (USEPA, 1997), uses a single medium, reduces analysis time from 48 hours to 24 hours, and improves analytical quality. For the revised method (Messer and Dufour, 1998; USEPA, 1997), the original mE Agar medium was modified by reducing the concentration of triphenyltetrazolium chloride and adding a chromogenic cellobiose analogue, indoxyl-ß-D-glucoside.

The mTEC method (Dufour *et al*., 1981; USEPA, 1985), originally developed in 1981, is a two-step method utilizing the fermentation of lactose at 44.5°C to detect thermotolerant coliforms. A second substrate medium

containing urea is used to distinguish urease-negative *E. coli* from other thermotolerant coliforms that can hydrolyze urea. Many laboratories are reluctant to use the mTEC procedure because of the need to transfer the membrane to a substrate medium after incubation on the primary medium.

The modified mTEC method is a single-step method that uses one medium and does not require the transfer of the membrane filter to another substrate. The modified medium contains a chromogen, 5-bromo-6-chloro-3-indolyl-ß-D-glucuronide, which is catabolized to glucuronic acid and a red- or magenta-colored compound by *E. coli* that produce the enzyme ß-D-glucuronidase.

2 SAFETY PRECAUTIONS

The analyst/technician must know and observe safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials and while operating sterilization equipment. Mouth-pipetting is prohibited.

3 SAMPLE COLLECTION, PRESERVATION, AND STORAGE

Sampling procedures are described in detail in the USEPA microbiology methods manual (Bordner *et al.*, 1978, Section II, A). Briefly, samples should be collected in sterile containers and stored on ice until analyzed. Samples should not be held longer than 6 h prior to analysis, and analyses should be completed within 8 h after collection of the samples (Bordner *et al.*, 1978; CFR, 1999). Adherence to sample preservation procedures and holding time limits is critical to the production of valid results. Samples must not be analyzed if these conditions are not met.

3.1 Storage Temperature and Handling Conditions

Refrigerate bacteriological samples at a temperature of 1–4°C during transit to the laboratory. Use insulated containers to ensure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water from melting ice during transit or storage.

3.2 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 h between collection and initiation of analyses (Bordner *et al.*, 1978; CFR, 1999).

4 CALIBRATION AND STANDARDIZATION

Check temperatures in incubators daily to ensure operation within recommended limits.

Check thermometers at least annually against a National Institute of Standards and Technology (NIST)-certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check mercury columns for breaks (APHA, 1998).

5 PURITY OF REAGENTS

Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (1981). For suggestions on testing reagents not listed by the American Chemical Society, see Rosin (1967) and U.S. Pharmacopeia (1974).

Agar used in the preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media as a means of quality control.

6 PURITY OF WATER

Reagent-grade distilled water should conform to Specification D1193-91, Type II water, as specified by the American Society for Testing and Materials (1993).

7 QUALITY CONTROL

See recommendations on quality control for microbiological analyses in the USEPA microbiology methods manual, Part IV, A–C (Bordner *et al.*, 1978).

8 METHOD PERFORMANCE CHARACTERISTICS—DEFINITIONS (APHA, 1998; ASTM, 1993)

Precision—The degree of agreement of repeated measurements of the same sample, usually reported as the standard deviation.

Bias—Consistent deviation of measured values from the true value, caused by systematic errors in a procedure.

Specificity—The ability of a method to select and distinguish the target bacteria from all others in the same sample. The specificity of a method is usually reported as the percentage of false-positive and false-negative results.

False-Positive Rate—The percentage of test results that are read as positive when they are really negative.

False-Negative Rate—The percentage of test results that are read as negative when they are really positive.

9 TEST METHODS FOR ENTEROCOCCI

9.1 Summary

Two test methods to detect and enumerate enterococci in water are presented here: the original method and a revised method, both using a membrane filter (MF) procedure. The two methods provide direct counts of enterococci in the water based on the number of colonies that develop on the surface of a membrane filter.

In the original method (Levin *et al.*, 1975; USEPA, 1985), a water sample is filtered through the membrane, which retains the bacteria. Following filtration, the membrane containing the bacteria is incubated on a selective medium, mE Agar, for 48 h at 41±0.5°C. The filter is transferred to EIA and incubated at 41±0.5°C for 20–30 min. Pink to red enterococci colonies on mE Agar will develop a black or reddish-brown precipitate on the underside of the filter on EIA. These colonies are counted under a fluorescent

lamp and a glass lens (2–5x magnification), or a stereoscopic microscope may be used.

In the modified method (Messer and Dufour, 1998; USEPA, 1997), the membrane filter containing the bacterial cells is placed on mEI Agar and incubated for 24 h at 41±0.5°C. All colonies with a blue halo, regardless of colony color, are recorded as enterococci colonies. A stereoscopic microscope provides maximum visibility of colonies when counting. The revised method reduces analysis time from 48 h to 24 h and improves analytical quality.

9.2 Original Enterococci Method (Method 1106.1)

9.2.1 Equipment and Supplies

- Stereoscopic microscope or glass lens (2–5x magnification).
- Lamp with a cool, white fluorescent bulb and diffuser.
- Hand tally or electronic counting device.
- Pipets, sterile, To Deliver (T.D.) bacteriological or Mohr, glass or plastic, of appropriate volume.
- Graduated cylinders, 100–1000 mL, sterile, covered with aluminum foil or kraft paper.
- Membrane filtration units (filter base and funnel), sterile; glass, plastic, or stainless steel; wrapped with aluminum foil or kraft paper to maintain sterility.
- Ultraviolet unit for sanitizing the filter funnel between filtrations (optional).
- Line vacuum, electric vacuum pump, or aspirator. (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.)
- Filter flask, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

- Vacuum flask for safety trap, placed between the filter flask and the vacuum source.
- Forceps, straight or curved, with smooth tips to handle filters without damage.
- Ethanol, methanol, or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles.
- Thermometer, checked against a National Institute of Standards and Technology (NIST)-certified thermometer, or one traceable to a NIST thermometer.
- Petri dishes, sterile, plastic, 9x50 mm, with tight-fitting lids.
- Bottles, milk dilution, borosilicate glass, screwcap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.
- Flasks, borosilicate glass, screwcap, 250–2000 mL volume.
- Membrane filters, sterile, white, grid-marked, 47-mm diameter, with 0.45±0.02 µm pore size.
- Inoculation loops, at least 3-mm diameter, and needles, nichrome and platinum wire, 26 B&S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops.
- Incubator maintained at 41±0.5°C.
- Waterbath maintained at 50°C for tempering agar.
- Test tubes, 20x150 mm, borosilicate glass or plastic.
- Test tube caps, aluminum or autoclavable plastic, for 20-mm diameter test tubes.
- Test tubes, borosilicate glass, 16x125 mm or other appropriate size, with screwcaps.
- Whirl-Pak ® bags

9.2.2 Reagents and Media

Preparation of the following reagents and media used in the original enterococci test are described below:

- Phosphate buffered saline or phosphate buffered dilution water
- mE Agar
- Esculin Iron Agar (EIA)
- Brain Heart Infusion Broth (BHIB)
- Brain Heart Infusion Broth (BHIB) with 6.5% NaCl
- Brain Heart Infusion Agar (BHIA)
- Bile Esculin Agar (BEA)

9.2.2.1 Phosphate Buffered Saline

Ingredients:

sodium dihydrogen phosphate	0.58 g
sodium monohydrogen phosphate	2.5 g
sodium chloride	8.5 g
reagent-grade distilled water	1.0 L

Preparation: Dissolve the ingredients in 1 L of reagent-grade distilled water in a flask, and dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4±0.2.

9.2.2.2 Phosphate Buffered Dilution Water (APHA, 1998; Bordner *et al.*, 1978)

Stock phosphate buffer solution:

phosphate dihydrogen phosphate	34.0 g
reagent-grade distilled water	500 mL

Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring the volume to 1 L with reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Stock magnesium chloride solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$ to 1 L reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Storage of stock solutions: After sterilization, store the stock solutions in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.

Working phosphate buffered dilution water: Mix 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade distilled water. Dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.0±0.2.

9.2.2.3 mE Agar (Difco 0333-17)

Basal medium ingredients:

peptone	10.0 g
sodium chloride	15.0 g
yeast extract	30.0 g
esculin	1.0 g
actidione (cycloheximide)	0.05 g
sodium azide	0.15 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Prepare basal medium: Add 71.2 g dehydrated mE basal medium to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients dissolve using a magnetic stirrer. Autoclave at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath.

Reagents added after sterilization: Mix 0.24 g nalidixic acid in 5 mL of reagent-grade distilled water, add 0.2 mL of 10 N NaOH. Allow the mixture to dissolve, and add the mixture

to the basal medium. Add 0.15 g triphenyltetrazolium chloride to the basal medium and mix.

Alternately, the following solutions may be used:

(a) *Nalidixic acid:* Add 0.48 g of nalidixic acid and 0.4 mL 10 N NaOH to 10 mL of reagent-grade distilled water and mix. Filter-sterilize the solution, and add 5.2 mL per liter of medium.

(b) *Triphenyltetrazolium chloride (TTC):* Add 0.25 g of TTC to 25 mL of reagent-grade distilled water, and warm to dissolve. Filter-sterilize the solution, and add 15 mL per liter of medium.

Prepare mE Agar: Pour the mE Agar into 9x50 mm petri dishes to a 4–5 mm depth (approximately 4–6 mL), and allow to solidify. Final pH of medium should be 7.1±0.2. Store in a refrigerator.

9.2.2.4 Esculin Iron Agar (EIA) (Difco 0488-15-4)

Ingredients:

esculin	1.0 g
ferric citrate	0.5 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 16.5 g dehydrated EIA to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients are dissolved. Autoclave the medium at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath. After cooling, pour the medium into 9x50 mm petri dishes to a depth of 4–5 mm (approximately 4–6 mL), and allow to solidify. Final pH should be 7.1±0.2. Store in a refrigerator.

9.2.2.5 Brain Heart Infusion Broth (BHIB) (Difco 0037-17, BD 4311059)

Ingredients:

calf brain infusion	200.0 g
beef heart infusion	250.0 g
proteose peptone	10.0 g
sodium chloride	5.0 g
disodium phosphate	2.5 g
dextrose	2.0 g
reagent-grade distilled water	1.0 L

Preparation: Dissolve 37 g dehydrated BHIB in 1 L of reagent-grade distilled water. Dispense in 10-mL volumes in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4±0.2.

9.2.2.6 Brain Heart Infusion Broth (BHIB) with 6.5% NaCl

Ingredients:

BHIB with 6.5% NaCl is the same as BHIB broth above, but with additional NaCl.

Preparation: Add 60.0 g NaCl per liter of medium. Since most commercially available dehydrated media already contain 5 g of sodium chloride, this amount is subtracted from the 65 g per liter required to make a final concentration of 6.5% NaCl.

9.2.2.7 Brain Heart Infusion Agar (BHIA) (Difco 0418-17-7, BD 4311065)

Ingredients:

BHIA contains the same components as BHIB (See above.) with the addition of 15.0 g agar per liter of BHIB.

Preparation: Suspend 52 g dehydrated BHIA in 1 L of reagent-grade distilled water. Heat to boiling until the ingredients are dissolved. Dispense 10 mL of medium in screwcap test tubes, and sterilize for 15 min at 121°C (15 lb

pressure). After sterilization, slant until solid. Final pH should be 7.4±0.2.

9.2.2.8 Bile Esculin Agar (BEA) (Difco 0879-02-6)

Ingredients:

Bacto beef extract	3.0 g
Bacto peptone	5.0 g
Bacto oxgall	40.0 g
Bacto esculin	1.0 g
ferric citrate	0.5 g
Bacto agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 64.0 g dehydrated BEA to 1 L reagent-grade distilled water, and heat to boiling to dissolve completely. Dispense 10-mL volumes in tubes for slants or larger volumes into flasks for subsequent plating. Autoclave at 121°C (15 lb pressure) for 15 min. Overheating may cause darkening of the medium. Cool in a 50°C waterbath, and dispense into sterile petri dishes. Final pH should be 6.6±0.2. Store in a refrigerator.

9.2.3 Original Enterococci Test Procedure

- Prepare the mE Agar as directed under "Reagents and Media" above.
- Mark the petri dishes and report form with the sample identification and volume.
- 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.
- 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.
- Select sample volumes based on previous knowledge of the pollution level, to produce 20–60 enterococci colonies on the membranes. Sample volumes of 1–100 mL are normally tested at half-log intervals (*e.g.*, 100, 30, 10, 3 mL).

» 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 dilutions of sample may be filtered, and the results may be combined.

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

» Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the mE Agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the filter if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 41±0.5°C for 48 h. (See photo 1.)

» After incubation, transfer the membranes to EIA plates that have been warmed up at room temperature for 20–30 min, and incubate at 41±0.5°C for an additional 20–30 min. (See photo 2.)

» After the second incubation, count and record colonies on those membrane filters containing, if practical, 20–60 pink-to-red colonies with black or reddish-brown precipitate on the underside of the membrane. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

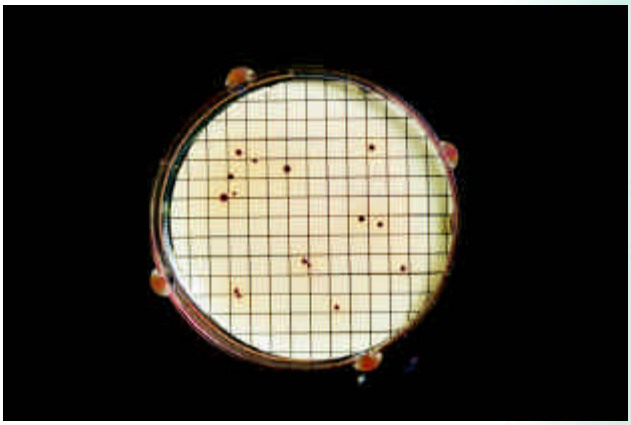

Photo 1. Enterococci on mE Agar. Colonies that are pink to dark red are considered to be presumptive enterococci.

9.2.4 *Calculation of Results*

Use the following general rules to calculate the enterococci per 100 mL of sample.

Select and count membranes ideally containing 20–60 pink-to-red colonies that form a black or reddish-brown precipitate on the underside of the filter when placed on EIA. Calculate the final value using the following formula:

$$\text{Enterococci}/100\text{ mL} = \frac{100\text{ (number of enterococci colonies)}}{\text{(volume of sample filtered, in mL)}}$$

See the USEPA microbiology methods manual, Part II, Section C, 3.5, for general counting rules (Bordner *et al*., 1978).

9.2.5 *Reporting Results*

There should be at least three volumes tested per sample. Report the results as enterococci per 100 mL of sample.

9.2.6 *Verification Procedure*

Pink-to-red colonies on mE Agar that produce a black or reddish-brown precipitate after incubation on EIA can be verified as enterococci. Verification of colonies may be required in evidence gathering. It is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

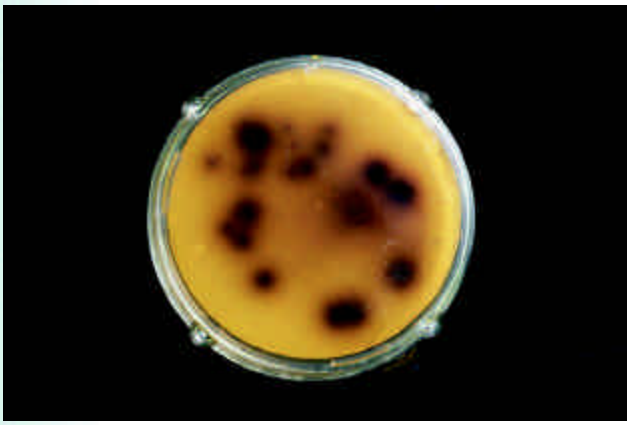

Photo 2. Enterococci on Esculin Iron Agar (EIA). Colonies that are pink to dark red on mE Agar and have a reddish brown to black precipitate on the underside of the filter when placed on EIA are confirmed as enterococci.

» Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a BHIB tube and onto a BHIA slant. Incubate broth tubes for 24 h and agar slants for 48 h at 35±0.5°C.

» After a 24-h incubation, transfer a loopful of material from each BHIB tube to each of the following media:

- BEA and incubate at 35±0.5°C for 48 h.
- BHIB and incubate at 45±0.5°C for 48 h.
- BHIB with 6.5% NaCl and incubate at 35±0.5° for 48 h.

» Observe for growth on all media.

» After 48-h incubation, apply a Gram stain to growth from each BHIA slant.

» Gram-positive cocci that grow and hydrolyze esculin on BEA (*i.e.*, produce a black or brown precipitate), and grow in BHIB at 45±0.5°C and BHIB with 6.5% NaCl at 35±0.5°C are verified as enterococci.

9.2.7 Method Performance (USEPA, 1985)

The specificity for this medium, as reported for various environmental water samples, was 10% false-positive and 11.7% false-negative results.

9.3 Modified Enterococci Method (Method 1600)

9.3.1 Equipment and Supplies

» Stereoscopic microscope or glass lens (2–5x magnification).

» Lamp with a cool, white fluorescent bulb and diffuser.

» Hand tally or electronic counting device.

» Pipets, sterile, To Deliver (T.D.) bacteriological or Mohr, glass or plastic, of appropriate volume.

» Graduated cylinders, 100–1000 mL, sterile,

covered with aluminum foil or kraft paper.

- Membrane filtration units (filter base and funnel), sterile; glass, plastic, or stainless steel; wrapped with aluminum foil or kraft paper to maintain sterility.
- Ultraviolet unit for sanitizing the filter funnel between filtrations (optional).
- Line vacuum, electric vacuum pump, or aspirator. (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.)
- Filter flask, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.
- Vacuum flask for safety trap, placed between the filter flask and the vacuum source.
- Forceps, straight or curved, with smooth tips to handle filters without damage.
- Ethanol, methanol, or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles.
- Thermometer, checked against a National Institute of Standards and Technology (NIST)-certified thermometer, or one traceable to a NIST thermometer.
- Petri dishes, sterile, plastic, 9x50 mm, with tight-fitting lids.
- Bottles, milk dilution, borosilicate glass, screwcap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.
- Flasks, borosilicate glass, screwcap, 250–2000 mL volume.
- Membrane filters, sterile, white, grid-marked, 47-mm diameter, with 0.45±0.02 µm pore size.
- Inoculation loops, at least 3-µm diameter, and

needles, nichrome and platinum wire, 26 B&S gauge, in suitable holders. Sterile disposable applicator sticks or plastic loops are alternatives to inoculation loops.

- Incubator maintained at 41±0.5°C.
- Waterbath maintained at 50°C for tempering agar.
- Test tubes, 20x150 mm, borosilicate glass or plastic.
- Test tube caps, aluminum or autoclavable plastic, for 20-mm diameter test tubes.
- Test tubes, borosilicate glass, 16x125 mm or other appropriate size, with screwcaps.
- Whirl-Pak® bags.

9.3.2 Reagents and Media

Preparation of the following reagents and media used in the revised enterococci test are described below:

- Phosphate buffered saline or phosphate buffered dilution water
- mEI Agar
- Brain Heart Infusion Broth (BHIB)
- Brain Heart Infusion Broth (BHIB) with 6.5% NaCl
- Brain Heart Infusion Agar (BHIA)
- Bile Esculin Agar (BEA)

9.3.2.1 Phosphate Buffered Saline

Ingredients:

sodium dihydrogen phosphate	0.58 g
sodium monohydrogen phosphate	2.5 g
sodium chloride	8.5 g
reagent-grade distilled water	1.0 L

Preparation: Dissolve ingredients in 1 L of reagent-grade distilled water in a flask, and dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4±0.2.

9.3.2.2 Phosphate Buffered Dilution Water (APHA, 1998; Bordner *et al*., 1978)

Stock phosphate buffer solution:

phosphate dihydrogen phosphate	34.0 g
reagent-grade distilled water	500 mL

Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring the volume to 1 L with reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Stock magnesium chloride solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$ to 1 L reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Storage of stock solutions: After sterilization, store the stock solution in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.

Working phosphate buffered dilution water: Mix 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade distilled water. Dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.0±0.2.

9.3.2.3 mEI Agar

Ingredients of basal medium (mE Agar, Difco 0333-17):

peptone	10.0 g
sodium chloride	15.0 g
yeast extract	30.0 g
esculin	1.0 g
actidione (cycloheximide)	0.05 g
sodium azide	0.15 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation of mEI medium: Add 71.2 g dehydrated mE basal medium plus 0.75 g indoxyl-ß-D-glucoside to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients dissolve. Autoclave at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath.

Reagents added after sterilization: Mix 0.24 g nalidixic acid in 5 mL of reagent-grade distilled water; add a few drops of 0.1 N NaOH to dissolve; add to the mEI medium. Add 0.02 g triphenyltetrazolium chloride separately to the mEI medium and mix.

Alternately, the following solutions may be used:

(a) *Nalidixic acid:* Add 0.48 g of nalidixic acid and 0.4 mL 10 N NaOH to 10 mL of reagent-grade distilled water and mix. Filter-sterilize the solution, and add 5.2 mL per liter of medium.

(b) *Triphenyltetrazolium chloride (TTC):* Add 0.1 g of TTC to 10 mL of reagent-grade distilled water, and warm to dissolve. Filter-sterilize the solution, and add 2 mL per liter of medium.

Preparation of mEI agar plates: Pour the mEI agar into 9x50 mm petri dishes to a 4–5 mm depth (approximately 4–6 mL), and allow to solidify. Final pH of medium should be 7.1±0.2. Store in a refrigerator.

9.3.2.4 Brain Heart Infusion Broth (BHIB) (Difco 0037-17, BD 4311059)

Ingredients:

calf brain infusion	200.0 g
beef heart infusion	250.0 g
proteose peptone	10.0 g
sodium chloride	5.0 g
disodium phosphate	2.5 g
dextrose	2.0 g
reagent-grade distilled water	1.0 L

Preparation: Dissolve 37 g dehydrated BHIB in 1 L of reagent-grade distilled water. Dispense in 10-mL volumes in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4±0.2.

9.3.2.5 Brain Heart Infusion Broth (BHIB) with 6.5% NaCl

Ingredients: BHIB with 6.5% NaCl is the same as BHIB described above, but with additional NaCl.

Preparation: Add 60.0 g NaCl per liter of medium. Since most commercially available dehydrated media already contain 5 g of sodium chloride, this amount is deducted from the 65 g per liter required to make a final concentration of 6.5% NaCl.

9.3.2.6 Brain Heart Infusion Agar (BHIA) (Difco 0418-17-7, BD 4311065)

Ingredients: BHIA contains the same components as BHIB (See above.) with the addition of 15.0 g agar per liter of BHIB.

Preparation: Suspend 52 g dehydrated BHIA in 1 L of reagent-grade distilled water. Heat to boiling until the ingredients are dissolved. Dispense 10 mL of medium in screwcap test tubes, and sterilize for 15 min at 121°C (15 lb pressure). After sterilization, slant until solid. Final pH should be 7.4±0.2.

9.3.2.7 Bile Esculin Agar (BEA) (Difco 0879-02-6)

Ingredients:

Bacto beef extract	3.0 g
Bacto peptone	5.0 g
Bacto oxgall	40.0 g
Bacto esculin	1.0 g
ferric citrate	0.5 g
Bacto agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 64.0 g dehydrated BEA to 1 L of reagent-grade distilled water, and heat to boiling to dissolve completely. Dispense in 10-mL volumes in tubes for slants or

larger volumes into flasks for subsequent plating. Autoclave at 121°C (15 lb pressure) for 15 min. Overheating may cause darkening of the medium. Cool in a 50°C waterbath, and dispense into sterile petri dishes. Final pH should be 6.6±0.2. Store in a refrigerator.

9.3.3 Modified Enterococci Test Procedure

- Prepare the mEI Agar as directed above.
- Mark the petri dishes and report form with the sample identification and volume.
- 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.
- 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.
- Select sample volumes based on previous knowledge of the pollution level, to produce 20–60 enterococci colonies on membranes. Sample volumes of 1–100 mL are normally tested at half-log intervals (*e.g.*, 100, 30, 10, 3 mL).
- 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 dilutions of sample may be filtered, and the results may be combined.
- 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.
- Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the mEI 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 to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 41±0.5°C for 24 h. (See photo 3.)

» After incubation, count and record as enterococci any colonies (regardless of color) with a blue halo on the membranes, ideally containing 20–60 colonies. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

9.3.4 *Calculation of Results*

To calculate the number of enterococci per 100 mL of sample:

» Select and count as enterococci any colonies (regardless of color) with a blue halo on the membranes, ideally containing 20–60 colonies.

» Calculate the final value using the following formula:

$$\text{Enterococci/100 mL} = \frac{100\ (\text{number of enterococci colonies})}{(\text{volume of sample filtered, in mL})}$$

See the USEPA microbiology methods manual, Part II, Section C, 3.5, for general counting rules (Bordner *et al.*, 1978).

9.3.5 *Reporting Results*

There should be at least three volumes tested per sample. Report the results as enterococci per 100 mL of sample.

9.3.6 *Verification Procedure*

Colonies with a blue halo, regardless of color, can be verified as enterococci. Verification of colonies may be

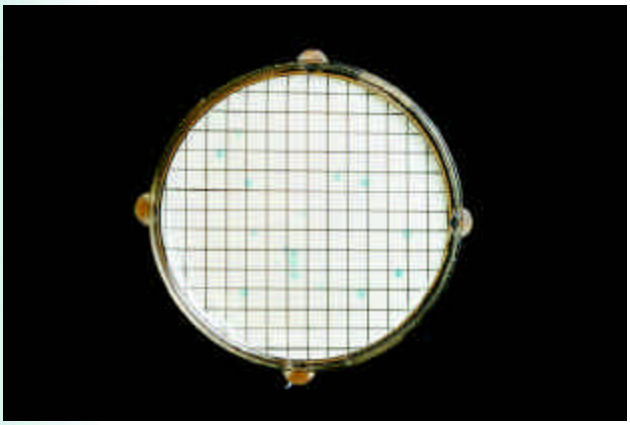

Photo 3. Enterococci on mEI Agar. Colonies with a blue halo are considered to be enterococci.

required in evidence gathering. It is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

» Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a BHIB tube and onto a BHIA slant. Incubate broth tubes for 24 h and agar slants for 48 h at 35±0.5°C.

» After a 24-h incubation, transfer a loopful of material from each BHIB tube to each of the following media:

- BEA and incubate at 35±0.5°C for 48 h.
- BHIB and incubate at 45±0.5°C for 48 h.
- BHIB with 6.5% NaCl and incubate at 35±0.5°C for 48 h.

» Observe for growth on all media.

» After 48-h incubation, apply a Gram stain to growth from each BHIA slant.

» Gram-positive cocci that grow and hydrolyze esculin on BEA (*i.e.*, produce a black or brown precipitate) and grow in BHIB at 45±0.5°C and BHIB with 6.5% NaCl at 35±0.5°C are verified as enterococci.

9.3.7 Method Performance (Messer and Dufour, 1998; USEPA, 1997)

The false-positive and false-negative rates, reported for various environmental water samples, were 6.0% and 6.5%, respectively. The precision among laboratories for marine water and surface water was 2.2% and 18.9%, respectively, and the bias was not significant.

10 TEST METHODS FOR *E. COLI*

10.1 Summary

Two test methods for the detection and enumeration of *Escherichia coli* in water are presented here. The original mTEC Agar enumeration method (Dufour *et al.*, 1981) for

E. coli was introduced by EPA in 1986 (USEPA, 1986b). The revised method was developed in 1998 by the Agency. Both the mTEC and modified mTEC Agar methods use the membrane filter procedure. The two membrane filter methods provide a direct count of *E. coli* in water based on the development of colonies that grow on the surface of a membrane filter.

In both methods, a water sample is filtered through the membrane, which retains the bacteria. After filtration, the membrane containing the bacteria is placed on a selective and differential medium, either mTEC (Dufour *et al.*, 1981; USEPA, 1985), or modified mTEC Agar, incubated at 35±0.5°C for 2 h to resuscitate the injured or stressed bacteria, and then incubated at 44.5±0.2°C for 22 h. With the original method, the filter is transferred from mTEC Agar to a filter pad saturated with Urea Substrate Medium. After 15–20 min, yellow, yellow-green, or yellow-brown colonies on mTEC are counted with the aid of a fluorescent lamp and a glass lens (2–5x magnification) or stereoscopic microscope. The modified method eliminates the transfer of the membrane filter to another substrate. The target colonies on modified mTEC Agar are red in color after the incubation period.

10.2 Original *E. coli* Method (Method 1103.1)

10.2.1 Equipment and Supplies

- Glass lens, 2–5x magnification, or stereoscopic microscope.
- Lamp with cool, white fluorescent bulb and diffuser.
- Hand tally or electronic counting device.
- Pipets, sterile, To Deliver (T.D.) bacteriological or Mohr, glass or plastic, of appropriate volume.
- Graduated cylinders, 100–1000 mL, sterile, covered with aluminum foil or kraft paper.
- Membrane filtration units (filter base and funnel), sterile; glass, plastic, or stainless steel; wrapped with aluminum foil or kraft paper to maintain sterility.
- Ultraviolet unit for sanitizing the filter funnel between filtrations (optional).

- Line vacuum, electric vacuum pump, or aspirator. (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.)
- Filter flask, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.
- Vacuum flask for safety trap, placed between the filter flask and the vacuum source.
- Forceps, straight or curved, with smooth tips to handle filters without damage.
- Ethanol, methanol, or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.
- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing inoculation loops.
- Thermometer, checked against a National Institute of Standards and Technology (NIST)-certified thermometer, or one traceable to a NIST thermometer.
- Petri dishes, sterile, plastic, 9x50 mm, with tight-fitting lids; or 15x60 mm, glass or plastic, with loose-fitting lids; or 15x100 mm.
- Bottles, milk dilution, borosilicate glass, screwcap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.
- Flasks, borosilicate glass, screwcap, 250–2000 mL volume.
- Membrane filters, sterile, white, grid-marked, 47-mm diameter, with 0.45±0.02-µm pore size.
- Absorbent pads, sterile, 47-mm diameter (usually supplied with membrane filters).
- Inoculation loops, at least 3-mm diameter, and needles, nichrome and platinum wire, 26 B&S gauge, in suitable holders. Disposable applicator sticks or plastic loops are alternatives to inoculation loops. *Note:* A platinum loop is required for the cytochrome oxidase test in the verification procedure.

- Incubator maintained at 35±0.5°C, with approximately 90% humidity if loose-lidded petri dishes are used.
- Waterbath maintained at 44.5±0.2°C.
- Waterbath maintained at 50°C for tempering agar.
- Test tubes, 20x150 mm, borosilicate glass or plastic.
- Test tubes, 10x75 mm, borosilicate glass.
- Test tube caps, aluminum or autoclavable plastic, for 20-mm diameter test tubes.
- Test tubes, 16x125 mm or other appropriate size, with screwcaps.
- Filter paper.
- Whirl-Pak® bags.

10.2.2 Reagents and Media

Preparation of the following reagents and media used in the original *E. coli* test are described below:

- Phosphate buffered saline or phosphate buffered dilution water
- mTEC Agar
- Urea Substrate Medium
- Nutrient Agar
- Tryptic Soy Broth or Trypticase Soy Broth
- Simmons Citrate Agar
- Tryptone 1% or Tryptophane Broth
- EC Broth
- Oxidase Reagent
- Kovacs Indole Reagent

10.2.2.1 Phosphate Buffered Saline

Ingredients:

sodium dihydrogen phosphate	0.58 g
sodium monohydrogen phosphate	2.5 g
sodium chloride	8.5 g
reagent-grade distilled water	1.0 L

Preparation: Dissolve the ingredients above in 1 L of reagent-grade distilled water in a flask, and dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4±0.2.

10.2.2.2 Phosphate Buffered Dilution Water (APHA, 1998; Bordner *et al.*, 1978)

Stock phosphate buffer solution:

phosphate dihydrogen phosphate	34.0 g
reagent-grade distilled water	500 mL

Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring the volume to 1 L with reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Stock magnesium chloride solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$ to 1 L reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Storage of stock solutions: After sterilization, store the stock solutions in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.

Working phosphate buffered dilution water: Mix 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade distilled water. Dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.0±0.2.

10.2.2.3 mTEC Agar (Difco 0334-15-0)

Ingredients:

proteose peptone #3	5.0 g
yeast extract	3.0 g
lactose	10.0 g
NaCl	7.5 g
dipotassium phosphate	3.3 g
monopotassium phosphate	1.0 g
sodium lauryl sulfate	0.2 g
sodium desoxycholate	0.1 g
brom cresol purple	0.08 g
brom phenol red	0.08 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 45.3 g dehydrated mTEC Agar to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients dissolve. Autoclave at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath. Pour the medium into each 9x50 mm culture dish to a 4–5 mm depth (approximately 4–6 mL), and allow to solidify. Final pH should be 7.3±0.2. Store in a refrigerator.

10.2.2.4 Urea Substrate Medium

Ingredients:

urea	2.0 g
phenol red	0.01 g
reagent-grade distilled water	100.0 mL

Preparation: Add dry ingredients to 100 mL reagent-grade distilled water in a flask. Stir to dissolve, and adjust to pH 3–4 with 1 N HCl. The substrate solution should be a straw-yellow color at this pH. (See photo 4.)

10.2.2.5 Nutrient Agar (Difco 0001-17-0, BD 4311472)

Ingredients:

peptone	5.0 g
beef extract	3.0 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 23 g of dehydrated Nutrient Agar to 1 L of reagent-grade distilled water, and mix well. Heat to boiling to dissolve the agar completely. Dispense in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Remove the tubes and slant. Final pH should be 6.8±0.2.

Photo 4. Urea Substrate Medium. After adjusting the pH of the medium to 3–4, the Urea Substrate Medium should be straw-yellow in color.

10.2.2.6 Tryptic Soy Broth (Difco 0370-17); Trypticase Soy Broth (BD 99071)

Ingredients:

tryptone or trypticase	17.0 g
soytone or phytone	3.0 g
sodium chloride	5.0 g
dextrose	2.5 g
dipotassium phosphate	2.5 g
reagent-grade distilled water	1.0 L

Preparation: Add 30 g of dehydrated Tryptic/Trypticase Soy Broth to 1 L of reagent-grade distilled water. Warm the broth, and mix gently to dissolve the medium completely. Dispense in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.3±0.2.

10.2.2.7 Simmons Citrate Agar (BD 4311620, Difco 0091-17-1)

Ingredients:

magnesium sulfate	0.2 g
monoammonium phosphate	1.0 g
dipotassium phosphate	1.0 g
sodium citrate	2.0 g
sodium chloride	5.0 g
brom thymol blue	0.08 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 24.2 g Simmons Citrate Agar to 1 L of reagent-grade distilled water. Heat to boiling to dissolve completely. Dispense into screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Cool the tubes and slant. Final pH should be 6.8±0.2.

10.2.2.8 Tryptone 1% (Difco 0123-01); Tryptophane Broth (BD 4321717 and 4321718)

Ingredients:

tryptone or trypticase peptone	10.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 10 g tryptone or trypticase peptone to 1 L of reagent-grade distilled water, and heat, mixing until dissolved. Dispense in 5-mL volumes into tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.2±0.2.

10.2.2.9 EC Broth (Difco 0314-01-0, BD 4311187)

Ingredients:

tryptose or trypticase peptone	20.0 g
lactose	5.0 g
bile salts no. 3 or bile salts mixture	1.5 g
dipotassium phosphate	4.0 g
monopotassium phosphate	1.5 g
sodium chloride	5.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 37 g dehydrated EC Broth to 1 L of reagent-grade distilled water, and warm to dissolve completely. Dispense into fermentation tubes (20x150 mm tubes containing inverted 10x75 mm vials). Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 6.9±0.2.

10.2.2.10 Oxidase Reagent

Ingredients:

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

10.2.2.11 Kovacs Indole Reagent

Ingredients:

ρ-dimethylaminobenzaldehyde	10.0 g
amyl or isoamyl alcohol	150.0 mL
concentrated (12 M) hydrochloric acid	50.0 mL

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

10.2.3 Original E. coli Test Procedure

» Prepare mTEC Agar and Urea Substrate Medium as directed above in the "Reagents and Media" section.

- Mark the petri dish and report form with the sample identification and volume.
- 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.
- 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.
- Select sample volumes based on previous knowledge of the pollution level, to produce 20–80 *E. coli* colonies on the membranes. Sample volumes of 1–100 mL are normally tested at half-log intervals (*e.g.*, 100, 30, 10, 3 mL).
- 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, and the results may be combined.
- 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.
- Use sterile forceps to aseptically remove the membrane filter from the filter base, and roll it onto the mTEC Agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 35±0.5°C for 2 h.
- After a 2-h incubation at 35±0.5°C, transfer the plate to a Whirl-Pak® bag, seal the bag, place the bag with the plate inverted in a test-tube rack, and put the rack in a 44.5±0.2°C waterbath for 22–24 h.
- After 22–24 h, remove the plate from the waterbath. Place an absorbent pad in a new petri dish or the lid of the same petri dish, and saturate the pad with Urea Substrate Medium. Aseptically transfer the

membranes from mTEC Agar to the absorbent pads saturated with Urea Substrate Medium, and allow to sit at room temperature for 15–20 min. (See photo 5.)

» After incubation on the urea substrate at room temperature, count and record the number of yellow, yellow-green, or yellow-brown colonies on the membrane filters, ideally containing 20–80 colonies. (See photo 6.)

10.2.4 Calculation of Results

Select the membrane filter with an acceptable number of yellow, yellow-green, or yellow-brown colonies (20–80) on the urea substrate, and calculate the number of *E. coli* per 100 mL according to the following general formula:

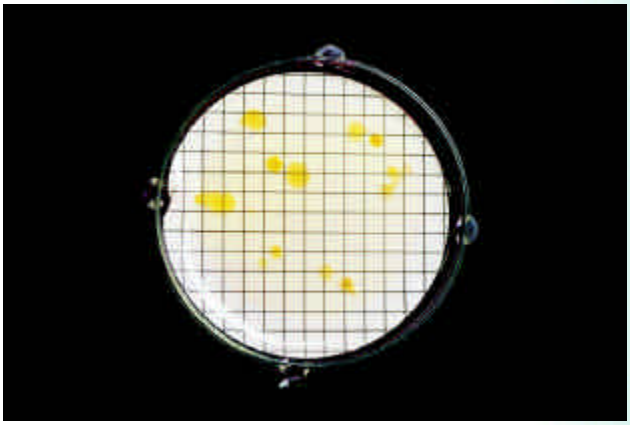

Photo 5. *Escherichia coli* colonies on mTEC Agar. Colonies that are yellow, yellow-green, or yellow-brown are *E. coli*.

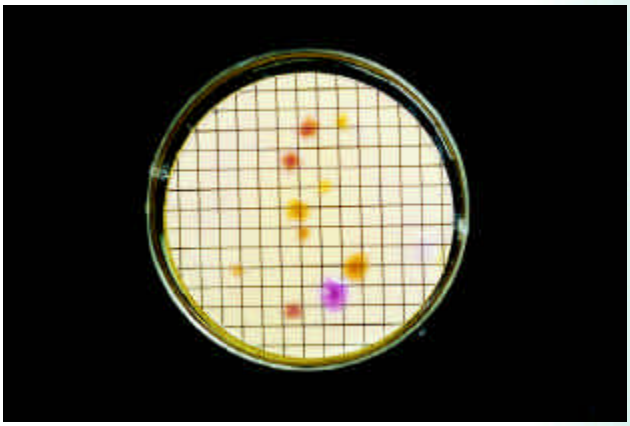

Photo 6. *Escherichia coli* colonies on an absorbent pad saturated with Urea Substrate Medium. *E. coli* colonies remain yellow, yellow-green, or yellow-brown when the filter is placed on the Urea Substrate Medium, while nontarget colonies turn pink or purple.

$$E.\ coli\ /\ 100\ \text{mL} = \frac{100\ (\text{number of } E.\ coli \text{ colonies counted})}{(\text{volume of sample filtered, in mL})}$$

See the USEPA microbiology methods manual, Part II, Section C, 3.5, for general counting rules (Bordner *et al.*, 1978).

10.2.5 Reporting Results

There should be at least three volumes filtered per sample. Report the results as *E. coli* per 100 mL of sample.

10.2.6 Verification Procedure

Yellow, yellow-green, or yellow-brown colonies from the urease test can be verified as *E. coli*. Verification of colonies may be required in evidence gathering and is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

» Using a sterile inoculation loop, transfer growth from the centers of at least 10 well-isolated colonies to Nutrient Agar plates or slants and to Trypticase Soy Broth. Incubate the agar and broth cultures for 24 h at 35±0.5°C.

» After incubation, remove a loopful of growth from the Nutrient Agar slant *with a platinum loop*, and deposit it on the surface of a piece of filter paper that has been saturated with freshly prepared Cytochrome Oxidase Reagent. If the spot where the bacteria were deposited turns deep purple within 15 seconds, the test is positive.

» Transfer growth from the Trypticase Soy Broth tube to Simmons Citrate Agar, Tryptone Broth, and an EC Broth fermentation tube.

- Incubate the Simmons Citrate Agar and Tryptone Broth for 48 h at 35±0.5°C.
- Incubate the EC Broth at 44.5±0.2°C in a waterbath for 24 h. The water level must be above the level of the EC Broth in the tube.
- Add 0.5 mL of Kovacs Indole Reagent to the 48-h Tryptone Broth culture, and shake the tube

gently. A positive test for indole is indicated by a deep red color which develops in the alcohol layer on top of the broth.

- *E. coli* is EC gas-positive, indole-positive, and oxidase-negative, and does not utilize citrate (*i.e.*, the medium remains green).

» Alternately, commercially available multi-test identification systems may be used to verify colonies. Inoculate the colonies into an identification system for *Enterobacteriaceae* that includes lactose fermentation, σ-nitrophenyl-ß-D-galactopyranoside (ONPG), and cytochrome oxidase test reactions.

10.2.7 Method Performance (Dufour et al., 1981; USEPA, 1985)

Using multilaboratory testing, the precision of the mTEC method was found to be fairly representative of what would be expected from counts with a Poisson distribution. The bias of the mTEC method has been reported to be –2% of the true value. Because of the instability of microbial populations in water, each laboratory analyzed its own samples. Therefore, no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of *E. coli*. The mean count ($\bar{X}$) and the overall standard deviation of the counts (S_T) (including the variability among laboratories for this standardized *E. coli* sample) were 31.6 colonies/membrane and 7.61 colonies/membrane, respectively.

The false-positive rate reported for mTEC medium averaged 9% for marine and fresh water samples. Less than 1% of the *E. coli* colonies observed gave a false-negative reaction.

The upper counting limit (*i.e.*, the number of colonies above which unacceptable counting errors occur) for *E. coli* on mTEC Agar has been reported as 80 colonies per filter.

10.3 Modified *E. coli* Method

The revised *Escherichia coli* method is a single-step method that uses one medium, modified mTEC Agar, and

does not require the transfer of the membrane filter to another medium or other substrate. The modified medium contains a chromogen (5-bromo-6-chloro-3-indolyl-ß-D-glucuronide), which is catabolized to glucuronic acid and a red- or magenta-colored compound by *E. coli* that produce the enzyme ß-D-glucuronidase.

The apparatus and equipment, and sampling, filtration, and verification procedures for this modified mTEC method are identical to those of the original mTEC method.

10.3.1 Equipment and Supplies

- Glass lens, 2–5x magnification, or stereoscopic microscope.
- Lamp with cool, white fluorescent bulb and diffuser.
- Hand tally or electronic counting device.
- Pipets, sterile, To Deliver (T.D.) bacteriological or Mohr, glass or plastic, of appropriate volume.
- Graduated cylinders, 100–1000 mL, sterile, covered with aluminum foil or kraft paper.
- Membrane filtration units (filter base and funnel), sterile, glass, plastic, or stainless steel, wrapped with aluminum foil or kraft paper to maintain sterility.
- Ultraviolet unit for sanitizing the filter funnel between filtrations (optional).
- Line vacuum, electric vacuum pump, or aspirator. (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.)
- Filter flask, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.
- Vacuum flask for safety trap, placed between the filter flask and the vacuum source.
- Forceps, straight or curved, with smooth tips to handle filters without damage.
- Ethanol, methanol, or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.

- Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing inoculation loops.
- Thermometer, checked against a National Institute of Standards and Technology (NIST)-certified thermometer, or one traceable to a NIST thermometer.
- Petri dishes, sterile, plastic, 9x50 mm, with tight-fitting lids; or 15x60 mm, glass or plastic, with loose-fitting lids; or 15x100 mm.
- Bottles, milk dilution, borosilicate glass, screwcap with neoprene liners, marked at 99 mL for 1:100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1:10 dilutions.
- Flasks, borosilicate glass, screwcap, 250–2000 mL volume.
- Membrane filters, sterile, white, grid-marked, 47-mm diameter, with 0.45±0.02 µm pore size.
- Inoculation loops, at least 3-mm diameter, and needles, nichrome and platinum wire, 26 B&S gauge, in suitable holders. Disposable applicator sticks or plastic loops are alternatives to inoculation loops. *Note:* A platinum loop is required for the cytochrome oxidase test in the verification procedure.
- Incubator maintained at 35±0.5°C, with approximately 90% humidity if loose-lidded petri dishes are used.
- Waterbath maintained at 44.5±0.2°C.
- Waterbath maintained at 50°C for tempering agar.
- Test tubes, 20x150 mm, borosilicate glass or plastic.
- Test tubes, 10x75 mm, borosilicate glass.
- Test tube caps, aluminum or autoclavable plastic, for 20-mm diameter test tubes.
- Test tubes, 16x125 mm or other appropriate size, with screwcaps.
- Filter paper.
- Whirl-Pak® bags.

10.3.2 Reagents and Media

Preparation of the following reagents and media used in the revised *E. coli* test are presented below:

- Phosphate buffered saline or phosphate buffered dilution water
- Modified mTEC Agar
- Nutrient Agar
- Tryptic Soy Broth or Trypticase Soy Broth
- Simmons Citrate Agar
- Tryptone 1% or Tryptophane Broth
- EC Broth
- Oxidase Reagent
- Kovacs Indole Reagent

10.3.2.1 Phosphate Buffered Saline

Ingredients:

sodium dihydrogen phosphate	0.58 g
sodium monohydrogen phosphate	2.5 g
sodium chloride	8.5 g
reagent-grade distilled water	1.0 L

Preparation: Dissolve the ingredients above in 1 L of reagent-grade distilled water in a flask, and dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4±0.2.

10.3.2.2 Phosphate Buffered Dilution Water (APHA, 1998; Bordner *et al.*, 1978)

Stock phosphate buffer solution:

phosphate dihydrogen phosphate	34.0 g
reagent-grade distilled water	500 mL

Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring the volume to 1 L with reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Stock magnesium chloride solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g $MgCl_2 \cdot 6H_2O$ to 1 L reagent-grade distilled water. Sterilize by filtration or autoclave at 121°C (15 lb pressure) for 15 min.

Storage of stock solutions: After sterilization, store the stock solutions in the refrigerator until used. Handle aseptically. If evidence of mold or other contamination appears, the affected stock solution should be discarded and a fresh solution should be prepared.

Working phosphate buffered dilution water: Mix 1.25 mL of the stock phosphate buffer and 5 mL of the $MgCl_2$ stock per liter of reagent-grade distilled water. Dispense in appropriate amounts for dilutions in screwcap bottles or culture tubes and/or into containers for use as rinse water. Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.0±0.2.

10.3.2.3 Modified mTEC Agar

Ingredients:

proteose peptone #3	5.0 g
yeast extract	3.0 g
lactose	10.0 g
NaCl	7.5 g
dipotassium phosphate	3.3 g
monopotassium phosphate	1.0 g
sodium lauryl sulfate	0.2 g
sodium desoxycholate	0.1 g
chromogen (5-bromo-6-chloro-3-indolyl-ß-D-glucuronide)	0.5 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 45.6 g dehydrated modified mTEC medium to 1 L of reagent-grade distilled water in a flask, and heat to boiling until the ingredients dissolve. Autoclave at 121°C (15 lb pressure) for 15 min, and cool in a 50°C waterbath. Pour the medium into each 9x50 mm culture dish

to a 4–5 mm depth (approximately 4–6 mL), and allow to solidify. Final pH should be 7.3±0.2. Store in a refrigerator.

10.3.2.4 Nutrient Agar (Difco 0001-17-0, BD 4311472)

Ingredients:

peptone	5.0 g
beef extract	3.0 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 23 g dehydrated Nutrient Agar to 1 L of reagent-grade distilled water, and mix well. Heat to boiling to dissolve the agar completely. Dispense in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Remove the tubes and slant. Final pH should be 6.8±0.2.

10.3.2.5 Tryptic Soy Broth (Difco 0370-17); Trypticase Soy Broth (BD 99071)

Ingredients:

tryptone or trypticase	17.0 g
soytone or phytone	3.0 g
sodium chloride	5.0 g
dextrose	2.5 g
dipotassium phosphate	2.5 g
reagent-grade distilled water	1.0 L

Preparation: Add 30 g dehydrated Tryptic/Trypticase Soy Broth to 1 L of reagent-grade distilled water. Warm the broth, and mix gently to dissolve the medium completely. Dispense in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.3±0.2.

10.3.2.6 Simmons Citrate Agar (BD 4311620, Difco 0091-17-1)

Ingredients:

magnesium sulfate	0.2 g
monoammonium phosphate	1.0 g
dipotassium phosphate	1.0 g
sodium citrate	2.0 g
sodium chloride	5.0 g
brom thymol blue	0.08 g
agar	15.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 24.2 g Simmons Citrate Agar to 1 L of reagent-grade distilled water. Heat to boiling to dissolve completely. Dispense in screwcap tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Cool the tubes in a 50°C waterbath and slant. Final pH should be 6.8±0.2.

10.3.2.7 Tryptone 1% (Difco 0123-01); Tryptophane Broth (BD 4321717 and 4321718)

Ingredients:

tryptone or trypticase peptone	10.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 10 g tryptone or trypticase peptone to 1 L of reagent-grade distilled water, and heat, mixing until dissolved. Dispense in 5-mL volumes in tubes, and autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 7.2±0.2.

10.3.2.8 EC Broth (Difco 0314-01-0, BD 4311187)

Ingredients:

tryptose or trypticase peptone	20.0 g
lactose	5.0 g
bile salts #3 or bile salts mixture	1.5 g
dipotassium phosphate	4.0 g
monopotassium phosphate	1.5 g
sodium chloride	5.0 g
reagent-grade distilled water	1.0 L

Preparation: Add 37 g dehydrated EC medium to 1 L of reagent-grade distilled water, and warm to dissolve completely. Dispense into fermentation tubes (20x150 mm tubes containing inverted 10x75 mm vials). Autoclave at 121°C (15 lb pressure) for 15 min. Final pH should be 6.9±0.2.

10.3.2.9 Oxidase Reagent

Ingredients:

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

10.3.2.10 Kovacs Indole Reagent

Ingredients:

ρ-dimethylaminobenzaldehyde	10 g
amyl or isoamyl alcohol	150 mL
concentrated (12 M) hydrochloric acid	50 mL

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

10.3.3 Modified E. coli Test Procedure

- Prepare the modified mTEC Agar as directed above in the "Reagents and Media" section.
- Mark the petri dish and report form with sample identification and volume.
- 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.
- 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.
- Select sample volumes based on previous knowledge of the pollution level, to produce 20–80 *E. coli* colonies on the membranes. Sample volumes of 1–100 mL are normally tested at half-log intervals (*e.g.*, 100, 30, 10, 3 mL).

- 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, and the results may be combined.
- 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.
- 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 filter if bubbles occur. Run the forceps around the edge of the filter to be sure that the filter is properly seated on the agar. Close the dish, invert, and incubate at 35±0.5°C for 2 h.
- After a 2-h incubation at 35±0.5°C, transfer the plate to a Whirl-Pak® bag, seal the bag, place the bag with the plate inverted in a test-tube rack, and put the rack in a 44.5±0.2°C waterbath for 22–24 h.
- After 22–24 h, remove the plate from the waterbath, and 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. (See photo 7.)

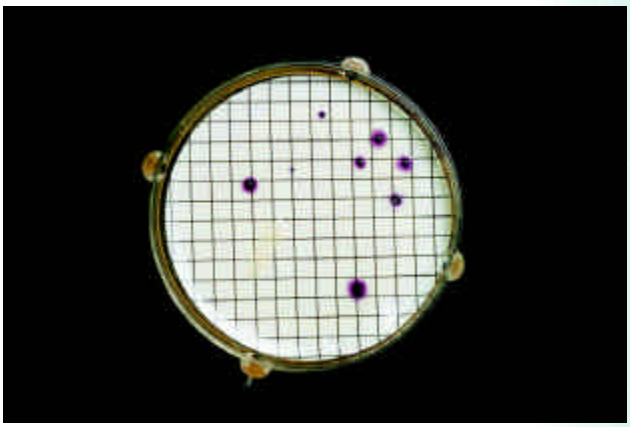

Photo 7. *Escherichia coli* colonies on modified mTEC Agar. *E. coli* colonies are red to magenta.

10.3.4 Calculation of Results

Select the membrane filter with an acceptable number of magenta or red colonies (20–80), and calculate the number of *E. coli* per 100 mL according to the following general formula:

$$E.\ coli\ /\ 100\ \text{mL} = \frac{100\ (\text{number of } E.\ coli \text{ colonies counted})}{(\text{volume of sample filtered, in mL})}$$

See the USEPA microbiology methods manual, Part II, Section C, 3.5 for general counting rules (Bordner *et al.*, 1978).

10.3.5 Reporting Results

There should be at least three volumes filtered per sample. Report the results as *E. coli* per 100 mL of sample.

10.3.6 Verification Procedure

Red or magenta colonies can be verified as *E. coli*. Verification of colonies may be required in evidence gathering and is also recommended as a means of quality control for the initial use of the test and for changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows.

- Using a sterile inoculation loop, transfer growth from the centers of at least 10 well-isolated typical colonies to Nutrient Agar plates or slants and to Trypticase Soy Broth. Incubate the agar and broth cultures for 24 h at 35±0.5°C.
- After incubation, remove a loopful of growth from the Nutrient Agar *with a platinum loop* and deposit it on the surface of a piece of filter paper that has been saturated with freshly prepared Cytochrome Oxidase Reagent. If the spot where the bacteria were deposited turns deep purple within 15 seconds, the test is positive.
- Transfer growth from the Trypticase Soy Broth to Simmons Citrate Agar, Tryptone Broth, and an EC Broth fermentation tube.
  - Incubate the Simmons Citrate Agar and Tryptone Broth for 48 h at 35±0.5°C.

- Incubate the EC Broth at 44.5±0.2°C in a waterbath for 24 h. The water level must be above the level of the EC Broth in the tube.
- Add 0.5 mL of Kovacs Indole Reagent to the 48-h Tryptone Broth culture, and shake the tube gently. A positive test for indole is indicated by a deep red color that develops in the alcohol layer on top of the broth.

» *E. coli* is EC gas-positive, indole-positive, and oxidase-negative, and does not utilize citrate (*i.e.*, the medium remains green).

» Alternately, commercially available multi-test identification systems may be used to verify colonies. Inoculate the colonies into an identification system for *Enterobacteriaceae* that includes lactose fermentation, σ-nitrophenyl-β-D-galactopyranoside (ONPG), and cytochrome oxidase test reactions.

10.3.7 Method Performance

The false-positive and false-negative rates, reported for various environmental water samples, were <1% and 4%, respectively.

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Disclaimer

This manual has been reviewed by the USEPA Office of Water and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Acknowledgments

This laboratory manual was prepared under the direction of Latisha Parker, Health and Ecological Criteria Division, U.S. Environmental Protection Agency (USEPA), Office of Water, Washington, DC. This manual was prepared under EPA Contract No. 68-C-98-141 by The CDM Group, Inc., Chevy Chase, Maryland.

For their technical contributions, special thanks are extended to: Alfred Dufour and Kristen Brenner, Microbiological and Chemical Exposure Assessment Research Division, National Exposure Research Laboratory, Cincinnati, Ohio; and Robin Oshiro, Health and Ecological Criteria Division, Office of Water, Washington, DC.

In preparation of the two original methods, the major contributions are acknowledged of Alfred Dufour and Theodore Erickson of the Toxicology and Microbiology Division (TMD), Health Effects Research Laboratory (HERL), and their assistance and that of Robert Bordner, Biological Methods Branch, and John Winter and Paul Britton, Quality Assurance Branch, Environmental Monitoring and Support Laboratory–Cincinnati (EMSL–Cincinnati), U.S. Environmental Protection Agency (USEPA), in preparing the final protocol and in completing the formal method validation studies.

The revised enterococci method was developed under the direction of James W. Messer and Alfred P. Dufour of the USEPA Microbiological and Chemical Exposure Assessment Research Division, National Exposure Research Laboratory, Cincinnati, Ohio. The method document was prepared under EPA Contract No. 68-C3-0337 by the DynCorp Environmental Programs Division, Washington, DC. The revised *E. coli* method was developed by Bennett G. Smith of the USEPA Microbiological and Chemical Exposure Assessment Research Division, National Exposure Research Laboratory, Cincinnati, Ohio.

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Requests for additional copies of this manual (doc. no. EPA/821/R-97/004) or videotape (doc. no. EPA/822/V-99/001) should be directed to:

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