Designation: D5392 – 14

Standard Test Method for Isolation and Enumeration of *Escherichia Coli* in Water by the Two-Step Membrane Filter Procedure[1]

This standard is issued under the fixed designation D5392; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method describes a membrane filter (MF) procedure for the detection and enumeration of *Escherichia coli*, a bacterium found exclusively in the feces of humans and other warm-blooded animals. The presence of these microorganisms in water is an indication of fecal pollution and the possible presence of enteric pathogens. These bacteria are found in water and wastewater in a wide range of densities. The detection limit of this procedure is one colony forming unit (CFU) per volume filtered.

1.2 This test method has been used successfully with temperate fresh and marine ambient waters, and wastewaters. It is the user's responsibility to ensure the validity of this test method for waters of other types.

1.3 The values stated in SI units are to be regarded as standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazard statements, see Section 9.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D1193 Specification for Reagent Water

D3370 Practices for Sampling Water from Closed Conduits

D3870 Practice for Establishing Performance Characteristics for Colony Counting Methods in Microbiology (Withdrawn 2000)[3]

D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this test method, refer to Terminology D1129.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *Escherichia coli (E. coli), n*—a species of bacteria that is a member of the total coliform group and known to originate in the feces of warm-blooded animals.

3.3 *Performance Characteristics (Practice D3870):*

3.3.1 *accuracy, n*—the proportion of the observed count to the true density of a sample.

3.3.2 *bias, n*—the persistent positive or negative deviation of the average value of the test method from the assumed or accepted true value.

3.3.3 *precision, n*—the degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95 % confidence limits of the mean computed from the results of a series of controlled determinations.

3.3.4 *specificity, n*— the ability of a test method to select or distinguish, or both, the target bacteria in the same water sample; the specificity characteristic of the method is usually reported as the percent of false positive and false negative results.

3.3.5 *upper counting limit (UCL), n*—that colony count above which there is an unacceptable counting error; the error may be due to overcrowding or antibiosis.

4. Summary of Test Method

4.1 This two-step test method[4] provides a direct count of bacterial colonies developing on the surface of the filter when placed on a selective nutrient medium. The water sample is passed through a membrane filter that retains the bacteria. After filtration, the membrane filter containing the bacterial cells is

[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

[4] Dufour, A., Strickland, E., and Cabelli, V., "Membrane Filter Method for Enumerating *Escherichi coli*," *Applied and Environmental Microbiology*, Vol 41, 1981, pp. 1152–1158.

placed on a selective, differential medium, mTEC. The membrane on the medium is first incubated at 35°C for 2 h so that injured or stressed bacteria can be resuscitated and then the medium is incubated at 44.5°C for 22 h. Following incubation the filter is transferred to a filter pad saturated with urea substrate. After 15 min all yellow or yellow-brown colonies are counted with the aid of 10 to 15× magnifier and a fluorescent lamp.

5. Significance and Use

5.1 This test method is useful for measuring recreational water quality and chlorinated wastewaters, although it can be used for any water suspected of contamination by fecal wastes of warm-blooded animals. The significance of finding *E. coli* in recreational water samples, especially samples obtained from fresh recreational waters, is that there is a risk of gastrointestinal illness, directly related to the *E. coli* density, associated with swimming.[5]

5.2 Since small or large volumes of water or dilutions thereof can be analyzed by the MF technique, a wider range of levels of *E. coli* in water can be detected and enumerated than with other methods.

6. Interferences

6.1 Water with high levels of colloidal or suspended materials can clog the membrane filter pores and prevent filtration. Also, suspended materials cause spreading colonies that could interfere with target colonies and thereby prevent accurate counting.

6.2 Smaller sample size or sample dilution can be used to minimize the interference of turbidity or high background (nontarget) bacterial densities. Replicates of sample volumes or dilutions of sample may be filtered and the results combined. However, the membrane filter techniques may not be applicable to high turbid waters with low bacterial densities.

6.3 In some samples, chemicals may have toxic effects on the target organism.

7. Apparatus

7.1 *Stereoscopic Microscope,* wide-field type with magnification of 10 to 15×.

7.2 *Microscope Lamp,* producing diffuse light from a cool, white fluorescent lamp adjusted to give maximum visibility.

7.3 *Counting Device,* hand tally or electronic.

7.4 *Pipet Container,* stainless steel, aluminum, or borosilicate glass, for glass pipets.

7.5 *Pipets,* sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 *Graduated Cylinders,* 100 to 1000 mL, covered with aluminum foil or kraft paper and sterile.

7.7 *Membrane Filtration Units* (filter base and funnel), glass, plastic, or stainless steel, wrapped in aluminum foil or kraft paper and sterilized.

7.8 *Ultraviolet Unit,* for sterilizing the filtration unit (optional).

7.9 *Line Vacuum, Electric Vacuum Pump, or Aspirator,* for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used.

7.10 *Flask,* filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 *Forceps,* straight or curved, with smooth tips to handle filters without damage.

7.12 *Thermometer,* checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one traceable to a NIST thermometer.

7.13 *Petri Dishes,* sterile, plastic, 50 by 12 mm, with tight-fitting lids.

7.14 *Bottles,* milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1 to 100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1 to 10 dilutions.

7.15 *Inoculation Loops,* at least 3-mm diameter, and needles, nichrome or platinum wire, 26 B & S gage, in suitable holders.

7.16 *Incubator,* air, maintained at 35 ± 0.5°C.

7.17 *Incubator, Waterbath,* maintained at 44 ± 0.2°C.

7.18 *Test Tubes,* 150 by 20 mm, borosilicate glass or plastic.

7.19 *Test Tubes,* 75 by 10 mm, borosilicate glass.

7.20 *Caps,* aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.21 *Test Tubes,* screw-cap, borosilicate glass, 125 by 16 mm or other appropriate size.

8. Reagents and Materials

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[6] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination. The agar used in preparation of culture media must be of microbiological grade. Whenever possible, use commercial culture media and reagents as means of quality control.

[5] Cabelli, V. J., Dufour, A. P., Levin, M. A., McCabe, L. J., and Haberman, P. W., "Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches," *American Journal of Public Health*, Vol 69, 1979, pp. 690–696.

[6] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

8.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water as defined by Type III of Specification D1193.

8.3 *Ethanol, Methanol, or Isopropanol,* denatured, in a small, wide-mouth container, for flame-sterilization or pipets.

8.4 *Membrane Filters,* sterile, white, grid marked, 47-mm diameter, with 0.45 ± 0.02 µm pore size or other pore sizes for which the manufacturer provides data demonstrating equivalency.

8.5 *Buffered Dilution Water/Buffered Rinse Water:*

8.5.1 *Composition Per Litre:*

Sodium Dihydrogen Phosphate (NaH_2PO_4)	0.58 g
Sodium Monohydrogen Phosphate (Na_2HPO_4)	2.50 g
Sodium Chloride	8.50 g

8.5.2 *Preparation*—Dissolve the ingredients in 1 L of water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C for 15 min. The final pH is 7.4.

8.6 *mTEC Agar:*

8.6.1 *Composition Per Litre:*

Proteose Peptone	5.0 g
Yeast Extract	3.0 g
Lactose	10.0 g
Sodium Chloride	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

8.6.2 Add 45.26 g of dehydrated mTEC medium to 1 L of water in a flask and heat to boiling, until ingredients dissolve. Autoclave at 121°C for 15 min and pour into 10 by 47 mm plates (4 mL/plate) after cooling. The pH of the medium is 7.3.

8.7 *Urea Substrate Medium:*

8.7.1 *Composition Per Litre:*

Ingredients	Grams/100 mL
Urea	2.01 g
Phenol Red	0.01 g

8.7.2 Dissolve the ingredients and bring the pH of the solution to between 4.5 and 5.2. The substrate should be a straw yellow color at this pH range.

8.8 *Cytochrome Oxidase Reagent* (1 g/L)—Dissolve 1 g of N,N,N',N'-tetramethyl-p-phenylenediamine-dihydrochloride in water and dilute to 100 mL. The cytochrome oxidase reagent is also commercially available.

8.9 *Kovac's Indole Reagent* (acid/alcohol solution)—Dissolve 10 g p-dimethylaminobenzaldehyde in 150 mL of amyl or isoamyl alcohol, then slowly add 50 mL of concentrated hydrochloric acid and mix. The indole reagent is also commercially available.

8.10 *Tryptic Soy Broth (TSB)*—It is recommended to purchase and not prepare from individual components.

8.10.1 *Composition per Litre:*

Casein Peptone (pancreatic)	17.0 g
Dipotassium Hydrogen Phosphate	2.5 g
Dextrose (glucose)	2.5 g
Sodium Chloride	5.0 g
Soy Peptone	3.0 g

8.10.2 *Procedure*—Follow vendor recommended procedure.

8.10.2.1 Add 30 g of TSB in 1 L of reagent water and mix to dissolve. If necessary, warm slightly to dissolve.

8.10.2.2 Dispense as required, such as 5 to 10 mL into tubes.

8.10.2.3 Sterilize in the autoclave at 121°C for 15 to 20 min at 15 lbs pressure.

8.10.2.4 Final pH should be 7.3 ± 0.2 at 25°C.

8.11 *Simmon's Citrate Agar*—It is recommend to purchase and not prepare from individual components.

8.11.1 *Composition per Litre:*

Magnesium Sulfate ($7H_2O$)	0.2 g
Ammonium Dihydrogen Phosphate	1.0 g
Dipotassium Hydrogen Phosphate	1.0 g
Sodium Citrate ($2H_2O$)	2.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Bromothymol Blue	0.08 g

8.11.2 Add 24.2 g in 1 L of reagent water and heat to boiling to dissolve completely while mixing.

8.11.3 Dispense into tubes.

8.11.4 Sterilize in the autoclave at 121°C for 15 to 20 min at 15 lbs pressure.

8.11.5 After autoclaving, allow media to solidify in a slanted position.

8.11.6 Final pH should be 6.8 ± 0.2 at 25°C.

8.12 *EC Broth*—It is recommend to purchase and not prepare from individual components.

8.12.1 *Composition per Litre:*

Tryptose or Trypticase	20.0 g
Lactose	5.0 g
Bile Salt Mixture	1.5 g
Dipotassium Hydrogen Phosphate	4.0 g
Potassium Dihydrogen Phosphate	1.5 g
Sodium Chloride	5.0 g

8.12.2 Add 37 g in 1 L of reagent water and mix to dissolve. If necessary, warm slightly to dissolve.

8.12.3 Dispense into tubes containing an inverted fermentation tube (Durham tube). Add sufficient volume to cover the inverted tube.

8.12.4 Place metal caps onto tubes or use screw cap tubes. If using screw cap tubes do not tighten completely. After autoclaving, the screw caps should be completely tightened.

8.12.5 Autoclave at 15 lbs pressure. Before opening the autoclave, allow temperature to drop below 5°C to avoid entrapping air bubbles in the inverted tubes.

8.12.6 Final pH should be 6.9 ± 0.2 at 25°C.

9. Hazards

9.1 The analyst/technician must have been trained in, and observe the normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents and materials and while operating sterilization and other equipment and instrumentation.

9.2 Mouth-pipetting is prohibited.

10. Sample Collection, Preservation, and Holding Times

10.1 Sampling procedures are described in detail in the USEPA microbiological methods manual [7] and in Practices D3370. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these rules should not be analyzed.

10.2 *Sample Storage Temperature and Handling Conditions:*

10.2.1 Ice or refrigerate water samples at a temperature of 1 to 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 during transit or storage.

10.3 *Holding Time Limitations:*

10.3.1 Examine samples as soon as possible after collection. Do not hold samples longer than 8 h between collection and initiation of analyses.

11. Quality Control Checks

11.1 Check temperatures in incubators daily to ensure operation within stated limits.

11.2 Check thermometers at least annually against a NIST-certified thermometer or one traceable to NIST. Check mercury columns for separation.

11.3 See recommendations on quality control for microbiological analyses in the USEPA's Microbiological Methods for Monitoring the Environment, Water, and Wastes, Part IV, C.[7]

12. Procedures

12.1 Prepare petri dishes containing mTEC agar and urea substrate as directed in 8.6 and 8.7. Mark the petri dishes containing the mTEC agar and prepare report forms with sample identification and sample volumes.

12.2 Place a sterile MF on the filter base, grid-side up and attach the funnel to the base; the MF is now held between the funnel and the base.

12.3 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly and measure the desired volume of sample or dilution into the funnel.

12.4 For ambient surface waters and wastewaters, select sample volumes based on previous knowledge to produce membrane filters with 20 to 80 colonies. Sample volumes are normally tested at half log intervals, for example 100, 30, 10, 3 mL, etc.

12.5 Use smaller sample size or sample dilution to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample or dilution of sample may be filtered and the results combined.

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

12.7 Use sterile forceps to aseptically remove the membrane from the filter base and roll it onto the mTEC medium to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and place it in a 35°C incubator for 2 h.

12.8 After two h incubation, place the plates in resealable water-proof plastic bags and transfer them to a 44.5°C incubator. Incubate the plates at 44.5 ± 0.2°C for 22 to 24 h.

12.9 After incubation, remove the plates from the waterbath and aseptically transfer the membrane to a filter pad saturated with urea substrate medium. After 15 to 20 min incubation at room temperature, examine the membrane for yellow to yellow-brown colonies. Count and record the number of typical colonies.

13. Calculation

13.1 Use the following rule to calculate the *E. coli* count per 100 mL of sample. If more than one sample volume contains colonies, select the membrane filter with 20 to 80 and calculate the count per 100 mL according to the general formula:

$$E.\ coli/100\ \text{mL} = \frac{\text{No. } E.\ coli \text{ colonies counted}}{\text{Volume in mL of sample filtered}} \times 100\ \text{mL}$$

13.2 See Practice D5465.

14. Verification Procedure

14.1 Yellow 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 QC procedure with initial use of the test and with changes in sample sites, lots of commercial media or major ingredients in media compounded in the laboratory. The verification procedure follows:

14.1.1 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 tryptic soy broth. Incubate the agar and broth cultures for 24 h at 35°C.

14.1.2 After incubation remove a generous portion of material from the nutrient agar with a platinum loop and deposit on the surface of filter paper that has been saturated with cytochrome oxidase reagent prepared fresh that day or use the commercial product. A positive test is indicated within 15 s by the development of a deep purple color where the bacteria were deposited.

14.1.3 Transfer growth from the tryptic soy broth to Simmons' citrate agar, tryptone broth, and EC broth in fermentation tubes. Incubate the Simmons' citrate agar for 24 h and tryptone broth for 48 h at 35°C. Incubate the EC broth at 44.5°C in a waterbath for 24 h. The water level must be above the level of the EC broth in the tube. Add one-half mL of Kovac's indole solution 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. *E. coli* is EC gas positive, indole positive, oxidase negative, and does not grow on citrate medium.

[7] Bordner, R. H., Winter, J. A., and Scarpino, P. V., Eds., *Microbiological Methods for Monitoring the Environment, Water, and Wastes*, EPA-600/8-78-017, U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory—Cincinnati, Cincinnati, Ohio, 1978.

14.1.4 Alternatively, use commercially available multi-test identification systems to verify colonies. Inoculate the colonies into an identification for Enterobacteriaceae that includes lactose fermentation or o-nitrophenyl-β-Dgalactopyrano-side (ONPG), or both, and cyto-chrome oxidase test tower case reactions.

15. Report

15.1 Adjust counts based on verification and report the results as *E. coli* per 100 mL of sample.

16. Precision and Bias

16.1 *Single Laboratory Data:*

16.1.1 *Precision*—The mTEC method precision was found to be fairly representative of what would be expected from counts with a Poisson distribution.

16.1.2 *Bias*—The bias of the *E. coli* MF with mTEC medium method has been reported to be −2 % of the true value.

16.1.3 *Other Statistics:*

16.1.3.1 *Specificity*—The specificity of mTEC medium as reported for various environmental samples was 9 % false positive and less than 1 % false negative.

16.1.3.2 *Upper Counting Limit*—The upper counting limit for *E. coli* on mTEC medium has been reported as 80 colonies per filter.

16.2 *Collaborative Study Data:*

16.2.1 A collective study was conducted among eleven volunteer laboratories, each with two analysts who independently tested reagent water, local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate.

16.2.2 The results of the study are shown in Fig. 1 where S_o equals the pooled standard deviation among replicate counts from a single analyst for three groupings (counts less than 30, counts from 30 to 50, and counts greater than 50) and S_B equals the pooled standard deviation between means of duplicates from analysts in the same laboratory for the same groupings. The precision estimates from this study did not differ among the water types analyzed.

16.2.3 By linear regression, the precision of the test method can be generalized as:

$$S_o = 0.028\,(X) + 6.11/\text{plate}$$

and

$$S_B = 0.223\,(X) + 0.82 \text{ count/plate}$$

where:

X = the count/plate or average count/plate.

16.2.3.1 To convert the count/plate to the count/100 mL, multiply this count by the dilution factor as follows:

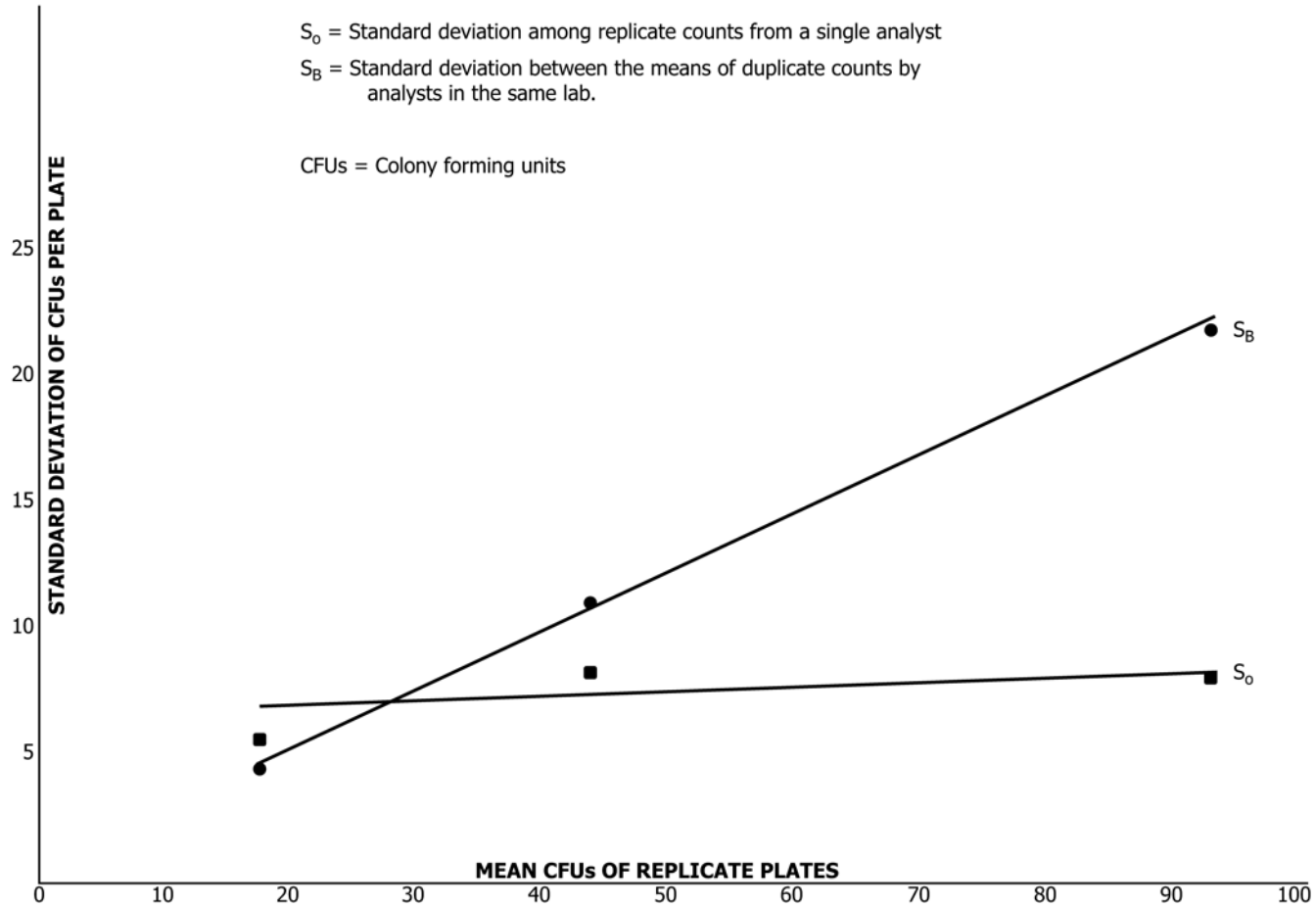

FIG. 1 Precision Estimates for *E. coli* in Water by the Membrane Filter/mTEC Procedure

$$\text{dilution factor} = \frac{100}{\text{volume of original sample filtered}}$$

16.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series and 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 (X) and the overall standard deviation of a single count (S_T) including the variability among laboratories for this standardized *E. coli* sample, were 31.6 colonies/membrane and $S_T = 7.61$ colonies/membrane, respectively.

17. Keywords

17.1 ambient waters; *Escherichia coli*; fecal pollution; mTEC agar; recreational waters; two-step membrane filter method; wastewaters