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Method 1680: Fecal Coliforms in Biosolids by Multiple-Tube Fermentation Procedures

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Introduction

Application of treated biosolids to land is helpful as a crop nutrient and soil conditioner, but may pose the risk of releasing pathogens into the environment if disinfection and use criteria are not met. The density of fecal coliforms in biosolids is used as an indicator of the average density of bacterial and viral pathogens. Under 40 CFR Part 503, a biosolid sample is classified as Class A if it contains a fecal coliform density below 1,000 most probable number (MPN) / g of total solids (dry weight basis). A biosolid sample is classified as Class B if the geometric mean fecal coliform density is less than 2×10^6 MPN / g of total solids (dry weight basis).

Method 1680 is adapted from *Standard Methods* 9221E (Reference 19.1). Although these methods currently are approved for the detection of fecal coliform bacteria in biosolids, they were designed for use in water matrices. Method 1680 was developed to determine the presence of fecal coliforms reliably in biosolid matrices.

The multiple tube fermentation procedures in Method 1680 are used to detect fecal coliform bacteria. Although Method 1680 is a performance-based method, fecal coliforms are considered “method-defined analytes,” and requests to modify the procedures are limited, and handled on a case-by-case basis.

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Method 1680: Fecal Coliforms in Biosolids by Multiple-Tube Fermentation Procedures

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1.0 Scope and Application

1.1 This method describes multiple-tube fermentation procedures [also called the most probable number (MPN) procedure] for the detection and enumeration of fecal coliform bacteria in biosolids. These methods use culture-specific media and elevated temperature to isolate and enumerate fecal coliform organisms. Fecal coliform bacteria, including *Escherichia coli,* are commonly found in the feces of humans and other warm-blooded animals, and indicate the potential presence of other bacterial and viral pathogens.

1.2 This method is adapted from methods 9221E in *Standard Methods for the Examination of Water and Wastewater*, 20th Edition, for the determination of fecal coliform bacteria in a variety of matrices (Reference 19.1).

1.3 This method is designed to meet the survey and monitoring requirements of the U.S. Environmental Protection Agency (EPA) in regulating the use and disposal of biosolids under 40 CFR Part 503. Subpart D of the 503 regulations protects public health and the environment through requirements designed to reduce the potential for contact with disease-bearing microorganisms (pathogens) in biosolids applied to land or placed on a surface disposal site.

1.4 Fecal coliform density is expected to correlate with the probability of pathogens present and document process performance (vector attraction reduction).

1.5 This method may be used to determine the density of fecal coliform bacteria in biosolids. This method also may be applied specifically to determine the density of fecal coliform bacteria in Class A and Class B biosolids to satisfy the pathogen reduction requirements of Subpart D of Part 503. A biosolid sample is classified as Class A if it contains a fecal coliform density below 1,000 MPN / g of total solids (dry weight basis). A biosolid sample is classified as Class B if the geometric mean fecal coliform density is less than 2×10^6 MPN / g of total solids (dry weight basis).

1.6 To satisfy the pathogen reduction monitoring alternatives for Class B biosolids, seven samples of treated biosolids are collected at the time of use or disposal and the geometric mean fecal coliform bacterial density of these samples is confirmed not to exceed 2×10^6 MPN / g of total solids (dry weight basis). Although the Part 503 regulation does not specify the total number of samples for Class A biosolids, it is recommended that a sampling event extend over two weeks, and that at least seven samples be tested to confirm that the mean bacterial density of the samples is below 1,000 MPN / g of total solids (dry weight basis). The analysis of seven samples increases the method precision by reducing the standard error caused by inherent variations in biosolid quality.

1.7 The presence of fecal coliforms may be determined in both Class A and Class B biosolids using the MPN procedure.

1.8 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternative test procedures under 40 CFR Part 136.4 and 136.5.

2.0 Summary of Method

2.1 Fecal coliform densities of biosolids may be determined by the MPN procedure using two media options.

2.2 MPN procedure (Class A and B)

Two method options are provided in Method 1680 for the MPN procedure: (1) A presumptive step using lauryl tryptose broth (LTB) plus a confirmation step using EC broth. (EC broth must not be used for direct fecal coliform isolation from a biosolid sample because prior enrichment is required in LTB medium for optimum recovery of fecal coliforms). (2) A direct, single step test using A-1 medium. The precision of both tests increases with increasing numbers of replicates per sample tested.

2.2.1 Summary of the LTB/EC procedure

2.2.1.1 A minimum of four sample dilutions are required, while five or more are preferred. Each sample dilution is inoculated into five test tubes, containing sterile LTB and an inverted vial (gas production).

2.2.1.2 LTB sample tubes are incubated in a water bath or jacketed incubator at 35°C ± 0.5°C. After 24 ± 2 hours, the tubes are examined for presumptive growth and gas production. Gas production is indicated by gas bubble formation within the inverted-vial. Negative tubes are reincubated for an additional 24 hours and reassessed. Failure to produce gas in LTB medium within 48 ± 3 hours is a negative presumptive test. EC tubes are incubated in a water bath at 44.5° ± 0.2°C for 24 ± 2 hours. Gas production in EC broth within 24 ± 2 hours is considered a positive fecal coliform reaction. Failure to produce gas is a negative reaction and indicates fecal coliform bacteria are not present.

2.2.1.3 Results of the MPN procedure using LTB/EC media are reported in terms of MPN / g calculated from the number of positive EC tubes and percent total solids (dry weight basis, see Draft Method 1684, Section 11 for the determination of total solids).

2.2.2 Summary of the A-1 procedure

2.2.2.1 A minimum of four sample dilutions are required, while five or more are preferred. Each sample dilution is inoculated into five test tubes containing A-1 medium and inverted vials.

2.2.2.2 Sample tubes are incubated in a water bath or jacketed incubator at 35°C ± 0.5 °C for 3 hours, then transferred to a water bath at 44.5°C ± 0.2°C. After 21 ± 2 hours, tubes are examined for growth and gas production. Gas production in 24 ± 2 hours or less is a positive reaction indicating the presence of fecal coliforms.

2.2.2.3 Results of the MPN procedure using A-1 medium are reported in terms of the most probable number (MPN)/g calculated from the number of positive A-1 culture tubes and percent total solids (dry weight basis, see Draft Method 1684 for determination of total solids).

3.0 Definitions

3.1 Fecal coliform bacteria are gram-negative, non-spore-forming rods that are found in the intestines and feces of humans and other warm-blooded animals. The predominant fecal coliform is *E. coli*. In this method, fecal coliforms are those bacteria that ferment lactose and produce gas within 24 ± 2 hours in EC or A-1 broth after incubation at 44.5°C ± 0.2°C. Since coliforms from other sources often cannot produce gas under these conditions, this criterion is used to define the fecal component of the coliform group.

3.2 Class A biosolids contain a fecal coliform density below 1,000 MPN / g of total solids (dry weight basis).

3.3 Class B biosolids contain a geometric mean fecal coliform density of less than 2×10^6 MPN / g of total solids (dry weight basis).

3.4 Definitions for other terms are given in the glossary at the end of the method.

4.0 Interferences

4.1 MPN procedure: Since the MPN tables are based on a Poisson distribution, if the sample is not adequately mixed to ensure equal bacterial cell distribution before portions are removed, the MPN value will be a misrepresentation of the bacterial density.

4.2 Percent total solids interferences: see Draft Method 1684.

5.0 Safety

5.1 The analyst must observe normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of media, cultures, reagents, and materials, and while operating sterilization equipment.

5.2 Field and laboratory staff collecting and analyzing environmental samples are under some risk of exposure to pathogenic microorganisms. Staff should apply safety procedures used for pathogens to handle all samples.

5.3 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

6.1 Sample bottles—Autoclavable, ground-glass, or plastic wide-mouthed (stoppered or screw cap), minimum of 125 mL capacity

6.2 Sterile waterproof plastic bags—Whirl-Pak® or equivalent (may be used for collection of solid samples)

6.3 Dilution containers

6.3.1 Sterile, borosilicate glass, screw cap, marked at 99 mL

6.3.2 Sterile, screw cap, borosilicate glass or plastic tubes marked at 9 mL

6.4 Pipette container—Stainless steel, aluminum or borosilicate glass, for glass pipettes

6.5 Pipettes

6.5.1 Sterile, to deliver (TD) bacteriological or Mohr, glass or plastic, of appropriate volume

6.5.2 Sterile, wide-mouth

6.6 Volumetric flasks—Borosilicate glass, screw-cap, 250- to 2000-mL volume

6.7 Graduated cylinders—100- to 1000-mL, covered with aluminum foil or kraft paper and sterilized

6.8 Thermometers—0°C to 50°C with 0.2°C graduations checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23

6.9 Burner—Bunsen or Fisher type, or electric incinerator unit for sterilizing loops

6.10 pH meter

6.11 Blender and sterile blender jar

6.12 Equipment for MPN procedure

6.12.1 Covered water bath—With circulating system to maintain temperature of 44.5°C ± 0.2°C. Water level should be maintained above the media in immersed tubes.

6.12.2 Autoclave capable of 121°C at 15 psi

6.12.3 Covered water bath or water- or air-jacketed incubator at 35°C ± 0.5°C

6.12.4 Inoculation loops—Nichrome or platinum wire, disposable sterile plastic loops or sterile wooden applicator, at least 3 mm in diameter

6.12.5 Sterile culture tubes—16 x 150 mm, borosilicate glass

6.12.6 Inverted tubes or vials—10 × 75 mm

6.12.7 Balance—Analytical balance capable of weighing 0.1 mg

6.12.8 Caps—Loose-fitting aluminum, stainless steel, or autoclavable plastic, for 16 mm diameter test tubes

6.12.9 Test tube racks

6.13 Equipment for percent total solids—see Draft Method 1684

7.0 Reagents and Standards

7.1 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 (Reference 19.2). The agar used in preparation of culture media must be of microbiological grade.

7.2 Whenever possible, use commercial dehydrated culture media.

7.3 Purity of water—Reagent water conforming to Specification D1193, Annual Book of ASTM Standards (Reference 19.3).

7.4 Phosphate buffered dilution water

7.4.1 Prepare stock phosphate buffer solution by dissolving 34.0 g potassium dihydrogen phosphate (KH_2PO_4), in 500 mL of reagent-grade water, adjust to pH 7.2 ± 0.5 with 1 N sodium hydroxide (NaOH), and dilute to 1 L with reagent-grade water.

7.4.2 Prepare stock magnesium chloride solution by dissolving 81.1 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) to 1 L of reagent-grade water.

7.4.3 To prepare the buffered dilution water, add 1.25 mL stock phosphate buffer solution and 5.0 mL of the magnesium chloride solution to 1 L reagent-grade water. Dispense in appropriate amounts for dilutions in bottles or culture tubes. After preparation, autoclave at 121°C (15 psi) for 15 minutes. The amount of time in the autoclave must be adjusted for the volume of buffer in the containers and the size of the load. *Note: When test tube racks containing 9.0 mL sterile dilution water are prepared, they are*

placed into an autoclavable pan with a small amount of water to contain breakage and minimize evaporation from the tubes.

7.5 **Heart infusion (HI) broth** (DIFCO# 0038-15, BBL# 238300, or equivalent) **or agar** (DIFCO# 0044-15, BBL# 244300, or equivalent)—For preparation follow procedure as specified on bottle of media. If dehydrated media is not available, see below for directions

7.5.1 50 g Beef heart, infusion from

7.5.2 10 g Bacto tryptose

7.5.3 5.0 g sodium chloride (NaCl)

7.5.4 15 g Bacto agar

7.5.5 For HI agar, add reagents in Sections 7.5.1 through 7.5.4 to 1-L of reagent-grade water, mix thoroughly, and heat to dissolve. For HI broth, add reagents in Sections 7.5.1 through 7.5.3 to 1-L of reagent-grade water, mix thoroughly, and heat to dissolve. Stir well and autoclave at 121°C for 15 minutes. Other general growth media may be used for QA (Section 9.0) purposes.

7.6 Media for the MPN procedure:

7.6.1 **LTB medium** (DIFCO# 0241-17, BBL# 224150, or equivalent)—For preparation follow procedure as specified on bottle of media. If dehydrated media is not available, see below for directions

7.6.1.1 20.0 g tryptose

7.6.1.2 5.0 g lactose

7.6.1.3 2.75 g dipotassium hydrogen phosphate (K_2HPO_4)

7.6.1.4 2.75 g potassium dihydrogen phosphate (KH_2PO_4)

7.6.1.5 5.0 g sodium chloride (NaCl)

7.6.1.6 0.1 g sodium lauryl sulfate

7.6.1.7 For single strength LTB, add reagents in Sections 7.6.1.1 through 7.6.1.6 to 1-L of reagent-grade water, mix thoroughly, and heat to dissolve. Adjust pH to 6.8 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Prior to sterilization, dispense 10 mL into 16 × 150 mm test tubes with inverted vials. Close tubes with metal or autoclavable plastic caps and autoclave at 121°C at 15 psi for 15 minutes. After cooling, the medium should fill the inverted vials completely, leaving no air space.

7.6.1.8 For double strength (2X) LTB, prepare as in Section 7.6.1.7 but use 500 mL of reagent-grade water instead of 1 L. *Note: 2X LTB is necessary for 10-mL inoculations, to ensure that the 10-mL inoculation volume does not excessively dilute the media.*

7.6.2 **EC medium** (DIFCO# 0314-17, BBL# 231430, or equivalent)—Follow procedure as specified on bottle of media for preparation. If dehydrated media is not available, see below for directions

7.6.2.1 20.0 g tryptose or trypticase

7.6.2.2 5.0 g lactose

7.6.2.3 1.5 g bile salts mixture or bile salts No.3

7.6.2.4 4.0 g dipotassium hydrogen phosphate (K_2HPO_4)

7.6.2.5 1.5 g potassium dihydrogen phosphate (KH_2PO_4)

7.6.2.6 5.0 g sodium chloride (NaCl)

7.6.2.7 Add reagents in Sections 7.6.2.1 through 7.6.2.6 to 1-L of reagent-grade water, mix thoroughly, and heat to dissolve. Adjust pH to 6.9 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Prior to sterilization, dispense 10 mL per 16 × 150 mm test tubes, each with an inverted vial, and sufficient medium to cover the inverted vial half way after sterilization. Close tubes with metal or heat-resistant plastic caps. Autoclave at 121°C at 15 psi for 15 minutes. Medium should fill inverted tubes leaving no air spaces.

7.6.3 **A-1 medium** (DIFCO# 1823-17, BBL# 218231, or equivalent)—Follow procedure as specified on bottle of media for preparation. If dehydrated media is not available, see below for directions.

7.6.3.1 5.0 g lactose

7.6.3.2 20.0 g tryptone

7.6.3.3 5.0 g sodium chloride (NaCl)

7.6.3.4 0.5 g salicin

7.6.3.5 For single strength A-1, add reagents in Sections 7.6.3.1 through 7.6.3.4 to 1-L of reagent-grade water, mix thoroughly, heat to dissolve, and add 1.0 mL polyethylene glycol p-isooctylphenyl ether. Adjust pH to 6.9 ± 0.1 by addition of 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Prior to sterilization, dispense 10 mL into 16 × 150 mm test tubes with inverted vials. Make sure there is enough medium to cover the inverted vial at least half way after sterilization. Close with metal or autoclavable plastic caps. Sterilize by autoclaving at 121°C at 15 psi for ***10 minutes***. Ignore formation of precipitate. Media should fill inverted tubes leaving no air spaces.

7.6.3.6 For double strength (2X) A-1, prepare as in Section 7.6.3.5 but use 500 mL of reagent-grade water instead of 1 L. *Note: 2X A-1 is necessary for 10-mL inoculations, to ensure that the 10-mL inoculation volume does not excessively dilute the media.*

7.7 **Positive control**—Obtain a stock culture of *E. coli* (e.g. ATCC # 25922) as a positive control for LTB, EC, and A-1.

7.8 **Negative controls**

7.8.1 Obtain a stock culture of *Enterobacter aerogenes* (e.g. ATCC # 13048) as a negative control for EC and A-1.

7.8.2 Obtain a stock culture of *Pseudomonas* (e.g. ATCC # 27853) as a negative control for LTB.

7.9 The storage times for prepared media used in this method are provided in Table 1 below:

TABLE 1. STORAGE TIMES FOR PREPARED MEDIA (Note: If media is refrigerated, remove from refrigerator 1-1.5 hours prior to inoculation, so that it reaches room temperature prior to use.)

Media	Storage Time
Agar or broth (EC, LTB, and HI) in loose-cap tubes	2 weeks
Agar or broth (EC, LTB, and HI) in tightly closed screw-cap tubes	3 months
Broth (A-1)	7 days
Poured agar plates (should be stored inverted)	2 weeks
Large volume of agar in tightly closed screw-cap flask or bottle	3 months

8.0 Sample Collection, Preservation, and Storage

8.1 The most appropriate location for biosolid sample collection is the point prior to leaving the wastewater treatment plant. Samples may be taken from pipes, conveyor belts, bins, compost heaps, drying beds and stockpiles.

8.2 Collect samples in sterile, non-toxic glass or plastic containers with leak-proof lids. All sampling containers and equipment must be clean and sterile.

8.3 Equipment and container cleaning procedure

8.3.1 Wash apparatus with laboratory-grade detergent and water

8.3.2 Rinse with tap water

8.3.3 Rinse with 10% HCl acid wash

8.3.4 Rinse with distilled water

8.3.5 Allow to air dry

8.3.6 Cover with foil and autoclave for 15 minutes at 121°C (15 psi)

8.4 Digester biosolids sampling procedure

8.4.1 Collect digester biosolids sample from the outlet pipe used to fill the truck.

8.4.2 Purge the pipe of old biosolids and warm to the digester temperature by allowing biosolids to flow through the pipe into a bucket.

8.4.3 Position a 1-gal. sterile bag under the flow so that only the sample touches the inside of the bag. Fill the bag, leaving 0.5 inches of head space in the bag for gas production. Leaving head room is extremely important when taking samples of biosolids that have been anaerobically digested.

8.5 Procedure for sampling conveyor belt biosolid output

8.5.1 Using a sterile scoop, transfer the pressed biosolids directly from the conveyer into a sterile container, without mixing or transferring to another area.

8.5.2 Pack sample into container. Leaving additional head space is not as important as in Section 8.4 because there is less gas formation.

8.6 Procedure for sampling from a bin, drying bed, truck bed, or similar container

8.6.1 Remove surface material (upper six inches) and divide material to be sampled into four quadrants.

8.6.2 Use a scoop or core the sample if material is deep.

8.6.3 Take a sample from each of the quadrants and combine in a sterile stainless steel or plastic bucket.

8.6.4 After all the samples have been taken, pour the bucket out onto a sterile plastic sheet

and mix by folding the sample back onto itself several times.

8.6.5 Reduce the sample size by "coning and quartering." Divide the bucket contents into four even piles. If sample size is still too large, divide each quarter into quarters and discard half. Put into a glass or plastic sampling container.

8.6.6 An alternate method to "coning and quartering" is to randomly take a flat shovel full of biosolids from the bucket that has been dumped onto a sterile plastic sheet and put samples into a sampling container. (Curved scoops have been shown to favor a certain size particle and should not be used.).

8.7 Record the following in your log book:

8.7.1 Facility name and location

8.7.2 Date

8.7.3 Arrival time

8.7.4 Name of facility and contact

8.8 Record the following onto sample container and in log book when known:

8.8.1 Sample number

8.8.2 Date and time

8.8.3 Sample name

8.8.4 Sample location

8.8.5 Parameters

8.8.6 Volume

8.8.7 Observations

8.9 Ensure that the chain of custody form is filled out

8.10 Sample preservation and handling—Ice or refrigerate bacteriological samples at a temperature of 1°C to 4°C during transit to the laboratory. Do not freeze the sample. Use insulated containers to ensure proper maintenance of storage temperature. Sample bottles should be placed inside waterproof bags, excess air purged, and bags sealed to ensure that bottles remain dry during transit or storage. Refrigerate samples upon arrival in the laboratory and analyze as soon as possible after collection. Bring samples to room temperature before analysis.

8.11 Chlorinated samples—Add a reducing agent to containers intended for the collection of biosolids containing residual chlorine or other halogen. Sodium thiosulfate ($Na_2S_2O_3$) is a satisfactory dechlorinating agent that neutralizes any residual halogen and prevents continuation of bactericidal action during sample transport. [If $Na_2S_2O_3$ is used, add a sufficient volume of $Na_2S_2O_3$ to a clean sample bottle, to give a concentration of 100 mg/L in the sample.] In a 120-mL sample bottle, a volume of 0.1 mL of a 10% solution of $Na_2S_2O_3$ will neutralize a sample containing about 15 mg/L residual chlorine.

8.12 Holding time and temperature limitations—Analyses should begin immediately, preferably, within 2 hours of collection. If it is impossible to examine samples within 2 hours, samples must be maintained at a temperature of 1°C to 10°C until analysis. Samples must not be frozen. Sample analysis must begin within 24 hours. *Note: Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Sample results will be considered invalid if these conditions are not met.*

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program. The minimum requirements of this program consist of an initial demonstration of laboratory capability through the analysis of positive and negative control samples and blanks (Section 9.3). Laboratory performance is compared to the performance criteria specified in Section 14 to determine whether the results of the analyses meet the performance characteristics of the method. Specific quality control (QC) requirements for Method 1680 are provided below. General recommendations on QA and QC for facilities, personnel, and laboratory equipment, instrumentation, and supplies used in microbiological analyses are provided in the USEPA Microbiology Methods Manual, Part IV, C (Reference 19.4).

9.2 General analytical QC procedures

9.2.1 **Dilution water sterility check.** Check each batch of dilution water for sterility by adding 20 mL water to 100 mL of a non-selective broth such as HI broth (Section 7.5). Incubate at 35°C ± 0.5°C for 48 to 72 hours and observe for growth. If any contamination is indicated, reject analytical data from samples tested with these materials.

9.2.2 **Media sterility check.** To test sterility of media, subject a representative portion of each batch to incubation at 35°C ± 0.5°C (LTB) or 44.5°C ± 0.2°C (A-1 and EC) for 48 ± 3 or 24 ± 2 hours respectively and observe for growth. With respect to media, a batch is defined as one tube/plate out of 50 in each lot or one tube/plate, if the lot contains less than 50 tubes/plates.

9.2.3 Perform duplicate analyses on 10% of samples or one sample per test run, whichever is greater.

9.2.4 In laboratories with more than one analyst, have each analyst conduct parallel tests on at least one positive sample monthly.

9.2.5 Obtain reference cultures from qualified outside sources and use them to establish pure cultures that are maintained for the laboratory. Use these in routine analytical runs, quarterly. Review results to correct causes of improper responses and document actions.

9.2.6 Participate in available interlaboratory performance studies conducted by local, state, and federal agencies or commercial organizations. Review results to correct unsatisfactory performance and record corrective actions.

9.3 Analytical QC procedures for MPN procedure—These procedures are required with each new lot of media, and in conjunction with each batch of samples.

9.3.1 **LTB, EC, and A-1 positive controls**—Inoculate LTB, EC, or A-1 (correlating with procedure being used by the lab) with a known positive (e.g. *E. coli* ATCC # 25922). Examine for appropriate responses, record results, and pursue causes of irregularities.

9.3.2 **EC and A-1 negative controls**—Inoculate EC or A-1 (correlating with procedure being used by the lab) with a known negative fecal coliform species (e.g. *Enterobacter aerogenes* ATCC # 13048). Examine for appropriate responses, record results, and pursue causes of irregularities.

9.3.3 **LTB negative control**—Inoculate LTB (if LTB/EC procedure is being used by the lab) with a known negative total coliform species (e.g. *Pseudomonas* ATCC# 27853). Examine for appropriate responses, record results and pursue causes of irregularities.

9.3.4 **Method Blank**—Test a 20 mL sterile dilution water sample in the analytical scheme to verify the sterility of equipment, materials, and supplies.

10.0 Equipment Calibration and Standardization

10.1 Check temperatures in incubators twice daily, with the readings separated by at least 4 hours, to ensure operation is within stated limits of the method and record daily measurements in the incubator log book.

10.2 Calibrate thermometers and incubators at least annually against an NIST certified thermometer or one that meets requirements of NIST Monograph SP 250-23. Check mercury columns for breaks.

10.3 Calibrate top-loading balances monthly with reference weights of ASTM Class 2.

10.4 Calibrate the pH meter prior to each use period with the two standards (pH 4.0, 7.0, and 10.0) closest to the range being tested.

11.0 Sample Preparation

11.1 Homogenization

Liquid samples are generally defined as samples containing <10% total solids (dry weight).

11.1.1 Liquid samples—Homogenize 300 mL of sample in a sterile blender on high speed for one to two minutes. Adjust the pH to 7.0-7.5 by adding 1.0 N hydrochloric acid or1.0 N sodium hydroxide, if necessary. This is the "homogenized" sample.

11.1.2 Solid samples—Weigh out 30.0 ± 0.1 g of well-mixed sample in a sterile dish. Whenever possible, the sample tested should contain all materials that will be included in the biosolid. For example, if wood chips are part of the biosolid compost, some mixing or grinding may be needed to achieve homogeneity before testing. Large pieces of wood that are not easily ground may be discarded before homogenizing. Transfer the sample to a sterile blender. Alternatively, the sample may be weighed directly into the sterile blender jar. Use 270 mL of sterile dilution water (Section 7.4) to rinse any remaining sample into the blender. Cover and blend on high speed for one minute. This is the "homogenized" sample. A volume of 1.0-mL of the "homogenized" sample contains 10^{-1} g of the original sample. Adjust the pH to 7.0-7.5 by adding 1.0 N hydrochloric acid or1.0 N sodium hydroxide, if necessary.

11.2 Dilution and inoculation

Biosolid samples analyzed for fecal coliforms using this method may require dilution prior to analysis. An ideal sample volume will yield results that accurately estimate fecal coliform density. Because the number of fecal coliform bacteria in undiluted samples could easily exceed the detection limits of these procedures, the laboratory must follow the dilution and inoculation scheme in Section 11.2 or a scheme that results in analysis of the same sample volumes for each dilution series, at a minimum (additional dilutions may be analyzed as necessary). ***Please note:*** Although other dilution and inoculation schemes may be used, the first transfer from the "homogenized" sample should always be 11 mL of homogenized sample to 99 mL dilution water or 10 mL of homogenized sample to 90 mL dilution water. This will ensure that a sufficient amount of the original biosolid sample is transferred at the beginning of the dilution scheme.

Note: Do not suspend bacteria in dilution water for more than 30 minutes at room temperature. For some transfers, it may be convenient to use a sterile, wide-mouth pipette, capable of transferring particulate matter. If samples are being spiked, a maximum of 1 hour may elapse between initial unspiked sample homogenization and analysis of spiked samples.

11.2.1 Class B liquid samples—For unspiked samples, four series of five tubes each will be used for the analysis with 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mL of the original sample (additional dilutions may be analyzed as necessary). See Figure 2 for a summary of this dilution and inoculation scheme. (For spiked samples, five series of five tubes each will be used for the analysis with 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} mL of the original sample.)

11.2.1.1 Dilution

(A) Use a sterile pipette to transfer 11.0 mL of homogenized sample (from Section 11.1.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is dilution "A." A 1.0-mL volume of dilution "A" is 10^{-1} mL of the original sample.

(B) Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is 10^{-2} mL of the original sample.

(C) Use a sterile pipette to transfer 11.0 mL of well-mixed dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A 1.0-mL volume of dilution "C" is 10^{-3} mL of the original sample.

(D) Use a sterile pipette to transfer 11.0 mL of well mixed dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A 1.0-mL volume of dilution "D" is 10^{-4} mL of the original sample.

(E) Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water, and mix as before. This is dilution "E." A 1.0-mL volume of dilution "E" is 10^{-5} mL of the original sample.

(F) Use a sterile pipette to transfer 11.0 mL of dilution "E" to 99 mL of sterile dilution water, and mix as before. This is dilution "F." A 1.0-mL volume of dilution "F" is 10^{-6} mL of the original sample.

(G) **Additional dilutions for analysis of spiked samples.**

- Use a sterile pipette to transfer 11.0 mL of dilution "F" to 99 mL of sterile dilution water, and mix as before. This is dilution "G." A 1.0-mL volume of dilution "G" is 10^{-7} mL of the original sample.
- Use a sterile pipette to transfer 11.0 mL of dilution "G" to 99 mL of sterile dilution water, and mix as before. This is dilution "H." A 1.0-mL volume of dilution "H" is 10^{-8} mL of the original sample.

11.2.1.2 Inoculation

(A) Use a sterile pipette to inoculate each of the first series of five tubes with 1.0 mL of dilution "C" (unspiked samples only). This is 10^{-3} mL of the original sample.

(B) Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "D" (unspiked samples only). This is 10^{-4} mL of the original sample.

(C) Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of "E" (unspiked or spiked samples). This is 10^{-5} mL of the original sample.

(D) Use a sterile pipette to inoculate each of the fourth series of five tubes each with 1.0 mL of dilution "F" (unspiked or spiked samples) This is 10^{-6} mL of the original sample.

(E) Use a sterile pipette to inoculate each of the five tubes each with 1.0 mL of dilution "G"(spiked samples). This is 10^{-7} mL of the original sample.

(F) Use a sterile pipette to inoculate each of the five tubes each with 1.0 mL of dilution "H"(spiked samples). This is 10^{-8} mL of the original sample.

11.2.1.3 Repeat Section 11.2.1.1 and 11.2.1.2 for each remaining Class B sample. When inoculations are complete, proceed to Section 12.3.1.4 to continue the LTB/EC method or to Section 12.4.4 to continue the A-1 method.

11.2.2 Class B solid samples—For unspiked samples, four series of five tubes each will contain 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} g of the original sample (additional dilutions may be analyzed as necessary). See Figure 3 for a summary of this dilution and inoculation scheme. (For spiked samples, five series of five tubes each will be used for the analysis with 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} g of the original sample.)

11.2.2.1 Dilution

(A) A volume of 1.0-mL of the "homogenized" sample (Section 11.1.2) contains 10^{-1} g of the original sample.

(B) Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains 10^{-2} g of the original sample.

(C) Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B"contains 10^{-3} g of the original sample.

(D) Use a sterile pipette to transfer 11.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" contains 10^{-4} g of the original sample.

(E) Use a sterile pipette to transfer 11.0 mL of dilution "C" to 99 mL of sterile dilution water and mix as before. This is dilution "D." A volume of 1.0-mL of dilution "D" contains 10^{-5} g of the original sample.

(F) Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water and mix as before. This is dilution "E." A volume of 1.0-mL of dilution "E" contains 10^{-6} g of the original sample.

(G) **Additional dilutions for analysis of spiked samples.**

- Use a sterile pipette to transfer 11.0 mL of dilution "E" to 99 mL of sterile dilution water, and mix as before. This is dilution "F." A 1.0-mL volume of dilution "F" is 10^{-7} mL of the original sample.
- Use a sterile pipette to transfer 11.0 mL of dilution "F" to 99 mL of sterile dilution water, and mix as before. This is dilution "G." A 1.0-mL volume of dilution "G" is 10^{-8} mL of the original sample.

11.2.2.2 Inoculation

(A) Inoculate each of the first series of five tubes with 1.0 mL of dilution "B"(unspiked samples only). This is 10^{-3} g of the original sample.

(B) Inoculate each of the second series of tubes with 1.0 mL of dilution "C"(unspiked samples only). This is 10^{-4} g of the original sample.

(C) Inoculate each of the third series of tubes with 1.0 mL of "D" (unspiked or spiked samples) This is 10^{-5} g of the original sample.

(D) Inoculate each of the fourth series of five tubes each with 1.0 mL of dilution "E" (unspiked or spiked samples) This is 10^{-6} g of the original sample.

(E) Use a sterile pipette to inoculate each of the five tubes each with 1.0 mL of dilution "F" (spiked samples). This is 10^{-7} mL of the original sample.

(F) Use a sterile pipette to inoculate each of the five tubes each with 1.0 mL of dilution "G" (spiked samples). This is 10^{-8} mL of the original sample.

11.2.2.3 When inoculations are complete, go to Section 12.3.1.4 to continue the LTB/EC method or to Section 12.4.4 to continue the A-1 method.

11.2.3 Class A liquid samples—Four series of five tubes each will contain 1.0, 10^{-1}, 10^{-2}, and 10^{-3} mL of the original sample. See Figure 4 for an overview of this dilution and inoculation scheme. (For spiked samples, four series of five tubes each will be used for the analysis with 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} mL of the original sample.)

11.2.3.1 Dilution

(A) Use a sterile pipette to transfer 11.0 mL of "homogenized" sample (Section 11.1.1) to 99 mL of sterile dilution water (Section 7.4), cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains 10^{-1} mL of the original sample.

(B) Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water, and mix as before. This is dilution "B." A 1.0-mL volume of dilution "B" is 10^{-2} mL of the original sample.

(C) Use a sterile pipette to transfer 11.0 mL of well mixed dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" is 10^{-3} mL of the original sample.

(D) **Additional dilutions for analysis of spiked samples.**

- Use a sterile pipette to transfer 11.0 mL of well mixed dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." A 1.0-mL volume of dilution "D" is 10^{-4} mL of the original sample.
- Use a sterile pipette to transfer 11.0 mL of dilution "D" to 99 mL of sterile dilution water, and mix as before. This is dilution "E." A 1.0-mL volume of dilution "E" is 10^{-5} mL of the original sample.

11.2.3.2 Inoculation

(A) Use a sterile pipette to inoculate each of the first series of five tubes with 1.0 mL of the original "homogenized" sample per tube (unspiked samples only).

(B) Use a sterile pipette to inoculate each of the second series of tubes with 1.0 mL of dilution "A" (unspiked samples only). This is 10^{-1} mL of the original sample.

(C) Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of dilution "B" (unspiked or spiked samples). This is 10^{-2} mL of the original sample.

(D) Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "C" (unspiked or spiked samples). This is 10^{-3} mL of the original sample.

(E) Use a sterile pipette to inoculate each of the five tubes with 1.0 mL of dilution "D" (spiked samples). This is 10^{-4} mL of the original sample.

(F) Use a sterile pipette to inoculate each of the five tubes with 1.0 mL of "E" (spiked samples). This is 10^{-5} mL of the original sample.

11.2.3.3 Repeat steps 11.2.3.1 and 11.2.3.2 for the remaining Class A samples. When inoculations are complete, go to Section 12.3.1.4 to continue the LTB/EC method or to Section 12.4.4 to continue the A-1 method.

11.2.4 Class A solid samples—For unspiked samples, four series of five tubes will be used for the analysis with 1.0, 10^{-1}, 10^{-2} and 10^{-3} g of the original sample. The first series of tubes must contain 2X media. See Figure 5 for a summary of this dilution and inoculation scheme. (For spiked samples, four series of five tubes each will be used for the analysis with 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} g of the original sample.)

11.2.4.1 Dilution

(A) A 1.0-mL volume of the "homogenized" sample (Section 11.1.2) contains 10^{-1} g of the original sample.

(B) Use a sterile pipette to transfer 11.0 mL of the blender contents to 99 mL of sterile dilution water (Section 7.4) and shake vigorously a minimum of 25 times. This is dilution "A." A volume of 1.0-mL of dilution "A" contains 10^{-2} g of the original sample.

(C) Use a sterile pipette to transfer 11.0 mL of dilution "A" to 99 mL of sterile dilution water and mix as before. This is dilution "B." A volume of 1.0-mL of dilution "B" contains 10^{-3} g of the original sample.

(D) **Additional dilutions for analysis of spiked samples.**

- Use a sterile pipette to transfer 11.0 mL of dilution "B" to 99 mL of sterile dilution water, and mix as before. This is dilution "C." A volume of 1.0-mL of dilution "C" contains 10^{-4} g of the original sample.
- Use a sterile pipette to transfer 11.0 mL of dilution "C" to 99 mL of sterile dilution water and mix as before. This is dilution "D." A volume of 1.0-mL of dilution "D" contains 10^{-5} g of the original sample.

11.2.4.2 Inoculation

(A) Use a sterile pipette to inoculate each of the first series of tubes with 10.0 mL of the "homogenized" sample (unspiked samples only). ***This series of tubes must contain 2X media.*** This is 1.0 g of the original sample. Since test tubes with inverted vials are being used, shaking is not practical. Solids that will not separate easily and/or may float should be submerged into the broth with a sterile loop.

(B) Use a sterile pipette to inoculate each of the second series of tubes with 1 mL of the "homogenized" mixture (unspiked samples only). This is 10^{-1} g of the original sample.

(C) Use a sterile pipette to inoculate each of the third series of tubes with 1.0 mL of dilution "A" (unspiked or spiked samples) This is 10^{-2} g of the original sample.

(D) Use a sterile pipette to inoculate each of the fourth series of tubes with 1.0 mL of dilution "B" (unspiked or spiked samples). This is 10^{-3} g of the original sample.

(E) Inoculate each of the five tubes with 1.0 mL of dilution "C" (spiked samples). This is 10^{-4} g of the original sample.

(F) Inoculate each of the five tubes with 1.0 mL of "D" (spiked samples). This is 10^{-5} g of the original sample.

11.2.4.3 Repeat Section 11.2.4.1 and 11.2.4.2 for remaining Class A solid samples. When inoculations are complete, go to Section 12.3.1.4 to continue the LTB/EC method or to Section 12.4.4 to continue the A-1 method.

12.0 Multiple-Tube Fermentation Procedures

12.1 The MPN procedure may be used to determine fecal coliform densities in unknown, Class A, and Class B biosolid samples. Analysis of seven samples collected at the time of disposal using this procedure will satisfy the requirements of the monitoring alternative for demonstrating pathogen reduction in both Class A and Class B biosolids. There are two options included in Method 1680. For the first option, LTB is used as a presumptive medium followed by EC as confirmation of fecal coliforms. EC may not be used for direct isolation from a biosolid sample because prior enrichment in presumptive medium (LTB) is required for optimum recovery of fecal coliforms. The second option is a direct test using A-1 medium in a single step procedure (not requiring a presumptive phase). The precision of both tests increases with increasing numbers of replicates per sample tested. For an overview of the MPN procedure, refer to Figure 1.

12.2 Since sample fecal coliform densities are expected to be variable, it is recommended that at least seven biosolid samples be analyzed using this method. The geometric mean fecal coliform density of the seven biosolids samples should not exceed 2×10^6 MPN / g of total solids (dry weight basis) to qualify as Class B biosolids. Although there is not a specific number of samples required for Class A biosolids, it is recommended that a sampling event extend over two weeks and that at least seven samples be collected and determined to be below 1,000 MPN / g of total solids (dry weight basis) to qualify as Class A biosolids.

12.3 LTB/EC procedure

12.3.1 Presumptive phase with LTB medium

12.3.1.1 Prepare LTB media and dispense into tubes as directed in Section 7.6.1. *Note: If media is refrigerated, remove from refrigerator 1-1.5 hours prior to inoculation, so that it reaches room temperature prior to use.*

12.3.1.2 For each sample, arrange test tubes in four rows of five tubes each (Section 11.2). When 10 mL of sample or dilution is used, tubes should contain 10 mL of 2X LTB media. Clearly label each row of tubes to identify the sample and dilution to be inoculated. *Note: 2X LTB is needed for 10-mL inoculations, to ensure that the 10-mL inoculation volume does not excessively dilute the LTB.*

12.3.1.3 Based on the matrix (i.e. Class A solid, Class B liquid), dilute and inoculate samples according to Section 11.2.

12.3.1.4 Incubate inoculated tubes at 35°C ± 0.5°C. After 24 ± 2 hours, swirl each tube gently and examine it for growth and gas production. If no gas has formed, reincubate for an additional 24 ± 2 hours and reassess. Final assessment should be within a total of 48 ± 3 hours.

12.3.1.5 For tubes with growth, the presence of gas in inverted vials within 48 ± 3 hours signifies a positive presumptive reaction. *Note: The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.*

12.3.1.6 For tubes with a positive presumptive reaction, proceed to the confirmation phase (Section 12.3.2).

12.3.2 Confirmation phase for fecal coliforms using EC medium

12.3.2.1 Prepare EC broth tubes as described in Section 7.6.2. For each positive LTB tube, one EC tube will be inoculated. *Note: If media is refrigerated, remove from refrigerator 1-1.5 hours prior to inoculation, so that it reaches room temperature prior to use.*

12.3.2.2 Gently shake tubes from presumptive test showing

positive reaction.

12.3.2.3 Using a sterile 3- to 3.5-mm-diameter loop or sterile wooden applicator stick, transfer growth from each positively presumptive LTB tube to corresponding tubes containing EC broth.

12.3.2.4 Place all EC tubes in a 44.5°C ± 0.2°C water bath within 30 minutes of inoculation and incubate for 24 ± 2 hours. Maintain water level above the media in immersed tubes.

12.3.2.5 After incubation, examine each tube for growth and gas production. Gas production with growth in EC broth at 24 ± 2 hours is considered a positive fecal coliform reaction. Failure to produce gas constitutes a negative reaction. *Note: The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.*

12.3.2.6 Record positive and negative reactions for the EC tubes. Calculate MPN / g of total solids (dry weight) from the number of positive EC tubes as described in Section 13.1.

12.4 A-1 procedure

12.4.1 Prepare A-1 broth tubes as directed in Section 7.6.3. *Note: If media is refrigerated, remove from refrigerator 1-1.5 hours prior to inoculation, so that it reaches room temperature prior to use.*

12.4.2 For each sample, arrange test tubes in four rows of five tubes each (Section 11.2). Use 10 mL of 2X A-1 broth for 10-mL inoculations. Clearly label each row of tubes to identify the sample and dilution volume to be inoculated. *Note: 2X A-1 is needed for 10-mL inoculations, to ensure that the 10-mL inoculation volume does not excessively dilute the A-1.*

12.4.3 Dilute and inoculate samples depending on the matrix (i.e. Class A solid, Class B liquid), as described in Section 11.2.

12.4.4 Incubate inoculated A-1 tubes at 35°C ± 0.5°C for 3 hours ± 15 minutes.

12.4.5 Transfer A-1 tubes to a water bath at 44.5°C ± 0.2°C and incubate for an additional 21 ± 2 hours. Maintain water level above the media in immersed tubes. Total incubation time should not exceed 24 ± 2 hours.

12.4.6 After incubation, remove tubes from the water bath, swirl each tube gently, and examine for growth and gas production. Gas production with growth is considered a positive fecal coliform reaction. Please note that for the A-1 procedure, any evolution of gas is considered a positive result. Collection of gas in the durham tube is not necessary. The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.

12.4.7 Record positive and negative A-1 results and calculate MPN / g total solids (dry weight) from the number of positive A-1 broth tubes as described in 14.1.

13.0 Data Analysis and Calculations

The estimated density of fecal coliform bacteria, based on the confirmation test using EC or the direct test with A-1, is calculated in terms of most probable number (MPN). Due to the extreme variability in the solid content of biosolids, fecal coliform results from biosolid samples are reported as MPN / g total solids (dry weight basis). MPN / g total solids (dry weight) is calculated in three steps (Sections 13.1, 13.2, and 13.3):

- Selection of significant dilutions
- Calculation of MPN / mL (wet weight)
- Conversion to MPN / g total solids (dry weight)

The calculation of geometric means is provided in Section 13.4.

13.1 Step 1: Select Significant Dilutions

A dilution refers to the mL (liquid samples) or g (solid samples) of original sample that was inoculated into each series of tubes. For example, with Class B liquid samples (Section 11.2.1), four, five-tube dilutions are used, with 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mL of the original sample in each tube. Only three of the four dilution series will be used to estimate the MPN. The three selected dilutions are called significant dilutions and are selected according to the following criteria. Examples of significant dilution selections are provided in Table 3, below. For these examples, the numerator represents the number of positive tubes per sample dilution series and the denominator represents the total number of tubes inoculated per dilution series.

13.1.1 Choose the highest dilution (the most dilute, with the least amount of sample) giving positive results in all five tubes inoculated and the two succeeding higher (more dilute) dilutions. (For Example A from Table 3, 10^{-4} is higher/more dilute than 10^{-3}.)

13.1.2 If the lowest dilution (least dilute) tested has less than five tubes with positive results, select it and the two next succeeding higher dilutions (Table 3, Examples B and C).

13.1.3 When a positive result occurs in a dilution higher (more dilute) than the three significant dilutions selected according to the rules above, change the selection to the lowest dilution (least dilute) that has less than five positive results and the next two higher dilutions (more dilute) (Table 3, Example D).

13.1.4 When the selection rules above have left unselected any higher dilutions (more dilute) with positive results, add those higher-dilution positive results to the results for the highest selected dilution (Table 3, Example E).

13.1.5 If there were not enough higher dilutions tested to select three dilutions, then select the next lower dilution (Table 3, Example F).

13.2 Step 2: Calculate MPN / mL (wet weight)

Obtain the MPN index value from Table 2 using the number of positive tubes in the three significant dilutions series and calculate MPN / mL using the following equation. (95% confidence limits may also be obtained from Table 4.)

$$\text{MPN / mL} = \frac{\text{MPN index value from Table 2}}{\text{largest volume tested in dilution series used for MPN determination}}$$

MPN / mL for significant dilution combinations not appearing in Table 4 may be estimated by the following derivation of Thomas' formula (Equation 2, Reference 19.6). If such unlikely tube combinations occur in more than 1% of the samples, it is an indication that the procedure is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled.

$$\text{MPN / mL} = \frac{\text{number of positive test tubes}}{\sqrt{(\text{mL sample in negative tubes X mL sample in all tubes})}}$$

Table 2. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Dilution

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	< 0.18	---	0.68	4-0-3	2.5	0.98	7.0
0-0-1	0.18	0.009	0.68	4-1-0	1.7	0.60	4.0
0-1-0	0.18	0.009	0.69	4-1-1	2.1	0.68	4.2
0-1-1	0.36	0.07	1.0	4-1-2	2.6	0.98	7.0
0-2-0	0.37	0.07	1.0	4-1-3	3.1	1.0	7.0
0-2-1	0.55	0.18	1.5	4-2-0	2.2	0.68	5.0
0-3-0	0.56	0.18	1.5	4-2-1	2.6	0.98	7.0
1-0-0	0.20	0.01	1.0	4-2-2	3.2	1.0	7.0
1-0-1	0.40	0.07	1.0	4-2-3	3.8	1.4	10
1-0-2	0.60	0.18	1.5	4-3-0	2.7	0.99	7.0
1-1-0	0.40	0.071	1.2	4-3-1	3.3	1.0	7.0
1-1-1	0.61	0.18	1.5	4-3-2	3.9	1.4	10
1-1-2	0.81	0.34	2.2	4-4-0	3.4	1.4	10
1-2-0	0.61	0.18	1.5	4-4-1	4.0	1.4	10
1-2-1	0.82	0.34	2.2	4-4-2	4.7	1.5	12
1-3-0	0.83	0.34	2.2	4-5-0	4.1	1.4	10
1-3-1	1.0	0.35	2.2	4-5-1	4.8	1.5	12
1-4-0	1.0	0.35	2.2	5-0-0	2.3	0.68	7.0
2-0-0	0.45	0.079	1.5	5-0-1	3.1	1.0	7.0
2-0-1	0.68	0.18	1.5	5-0-2	4.3	1.4	10
2-0-2	0.91	0.34	2.2	5-0-3	5.8	2.2	15
2-1-0	0.68	0.18	1.7	5-1-0	3.3	1.0	10
2-1-1	0.92	0.34	2.2	5-1-1	4.6	1.4	12
2-1-2	1.2	0.41	2.6	5-1-2	6.3	2.2	15
2-2-0	0.93	0.34	2.2	5-1-3	8.4	3.4	22
2-2-1	1.2	0.41	2.6	5-2-0	4.9	1.5	15
2-2-2	1.4	0.59	3.6	5-2-1	7.0	2.2	17
2-3-0	1.2	0.41	2.6	5-2-2	9.4	3.4	23
2-3-1	1.4	0.59	3.6	5-2-3	12	3.6	25
2-4-0	1.5	0.59	3.6	5-2-4	15	5.8	40
3-0-0	0.78	0.21	2.2	5-3-0	7.9	2.2	22
3-0-1	1.1	0.35	2.3	5-3-1	11	3.4	25
3-0-2	1.3	0.56	3.5	5-3-2	14	5.2	40
3-1-0	1.1	0.35	2.6	5-3-3	17	7.0	40
3-1-1	1.4	0.56	3.6	5-3-4	21	7.0	40
3-1-2	1.7	0.60	3.6	5-4-0	13	3.6	40
3-2-0	1.4	0.57	3.6	5-4-1	17	5.8	40
3-2-1	1.7	0.68	4.0	5-4-2	22	7.0	44
3-2-2	2.0	0.68	4.0	5-4-3	28	10	71
3-3-0	1.7	0.68	4.0	5-4-4	35	10	71
3-3-1	2.1	0.68	4.0	5-4-5	43	15	110
3-3-2	2.4	0.98	7.0	5-5-0	24	7.0	71
3-4-0	2.1	0.68	4.0	5-5-1	35	10	110
3-4-1	2.4	0.98	7.0	5-5-2	54	15	170
3-5-0	2.5	0.98	7.0	5-5-3	92	22	260
4-0-0	1.3	0.41	3.5	5-5-4	160	40	460
4-0-1	1.7	0.59	3.6	5-5-5	>160	70	—
4-0-2	2.1	0.68	4.0				

Examples of MPN / mL calculations are provided in Table 3, below.

TABLE 3. EXAMPLES OF SIGNIFICANT DILUTION SELECTION AND CALCULATION OF MPN / mL (SIGNIFICANT DILUTIONS ARE UNDERLINED AND LARGEST SIGNIFICANT DILUTIONS HIGHLIGHTED)

Example (liquid or solid)	10^{-3} mL or g	10^{-4} mL or g	10^{-5} mL or g	10^{-6} mL or g	**Step 1:** Significant Dilutions	**Step 2:** (MPN from Table 2 / largest sig. dilution) = MPN / mL wet weight
A	5/5	5/5	3/5	0/5	5-3-0	$(7.9 / 10^{-4})$ = 79,000 MPN / mL
B	4/5	5/5	1/5	0/5	4-5-1	$(4.8 / 10^{-3})$ = 4800 MPN / mL
C	0/5	1/5	0/5	0/5	0-1-0	$(0.18 / 10^{-3})$ = 180 MPN / mL
D	5/5	3/5	1/5	1/5	3-1-1	$(1.4 / 10^{-4})$ = 14,000 MPN / mL
E	4/5	4/5	0/5	1/5	4-4-1	$(4.0 / 10^{-3})$ = 4,000MPN / mL
F	5/5	5/5	5/5	2/5	5-5-2	$(54 / 10^{-4})$ = 540,000 MPN / mL

13.3 Step 3: Convert to MPN / g total solids (dry weight)

For analysis and calculation of percent total solids, refer to Draft Method 1684 (Section 11).

For the conversion to MPN / g total solids (dry weight), we assume that,

MPN / mL wet weight = MPN / g wet weight.

Therefore, we may convert to MPN / g total solids (dry weight) using the following equation:

$$\text{MPN / g (dry weight)} = \frac{\text{MPN / mL (wet weight) from step 2}}{\text{percent total solids (expressed as a decimal)}}$$

Examples of the conversion to MPN / g (dry weight) are provided in Table 4.

TABLE 4. EXAMPLES OF CONVERSION TO MPN / g TOTAL SOLIDS (DRY WEIGHT), CONTINUING FROM STEP 2 IN TABLE 3.

Example (liquid or solid)	Total Solids	**Step 3:** (MPN / mL wet weight from step 2) / percent total solids = MPN /g dry weight
A	4%	79,000 / 0.04 = 1,975,000 = 2.0×10^6 MPN / g dry weight
B	60%	4800 / 0.6 = 8000 = 8.0×10^3 MPN / g dry weight
C	56%	180 / 0.56 = 321 = 3.2×10^2 MPN / g dry weight
D	22%	14,000 / 0.22 = 63,636 = 6.4×10^4 MPN / g dry weight
E	18%	4,000 / 0.18 = 22,222 = 2.2×10^4 MPN / g dry weight
F	43%	540,000 / 0.43 = 1,255,814 = 1.3×10^6 MPN / g dry weight

13.4 Calculation of geometric mean

To satisfy pathogen reduction requirements for Class B biosolids in Subpart D of Part 503, seven biosolid samples are collected and the geometric mean density of fecal coliforms is calculated. The geometric mean is calculated by

- converting each sample's MPN fecal coliforms / g (dry weight) to the $\log_{10}$ value,
- averaging the $\log_{10}$ values, and
- taking the antilog of the mean $\log_{10}$ value.

An example is provided in Table 5 below.

TABLE 5. CALCULATION OF GEOMETRIC MEAN FECAL COLIFORM DENSITY FOR BIOSOLID SAMPLES

Sample No.	MPN Fecal coliforms / g (dry weight)	$\log_{10}$
1	600,000 = 6.0×10^5	5.78
2	4,200,000 = 4.2×10^6	6.62
3	1,700,000 = 1.7×10^6	6.23
4	1,400,000 = 1.4×10^6	6.15
5	400,000 = 4.0×10^5	5.60
6	1,100,000 = 1.1×10^6	6.04
7	510,000 = 5.1×10^5	5.71
Mean of $\log_{10}$ values = (5.78 + 6.62 + 6.23 + 6.15 + 5.60 + 6.04 + 5.71) / 7 = 6.02		
Antilog of 6.02 = 1,047,128 = 1.0×10^6 geometric mean MPN of fecal coliforms / g (dry weight)		

14.0 Method Performance

[This section will be updated based on validation study results]

15.0 Reporting Results

15.1 Report sample results as MPN fecal coliforms / g of total solids when using the multiple-tube fermentation procedures. See Draft Method 1684 for determination of total solids.

16.0 Verification Procedures

[Section will be updated after the validation study]

17.0 Pollution Prevention

17.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly. Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

18.0 Waste Management

18.1 The laboratory is responsible for complying with all Federal, State, and local regulations governing waste management, particularly hazardous waste identification rules and land disposal restrictions, and for protecting the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required. An overview of requirements can be found in *Environmental Management Guide for Small Laboratories* (EPA 233-B-98-001).

18.2 Samples, reference materials, and equipment known or suspected to have viable bacteria or viral contamination must be sterilized prior to disposal.

18.3 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* and *Less Is Better: Laboratory Chemical Management for Waste Reduction,* both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

19.0 References

19.1 American Public Health Association, American Water Works Association, and Water Environment Federation. 1995. *Standard Methods for Water and Wastewater*. 20th Edition. Sections: 9020, 9221, 9222.

19.2 American Society for Testing and Materials. Reagent Chemicals, American Chemical Society Specifications. American Chemical Society. Washington, DC. For suggestions of the testing of reagents not listed by the American Chemical Society, see Reference 19.7.

19.3 *Annual Book of ASTM Standards*. Vol. 11.01. American Society for Testing and Materials. Philadelphia, PA 19103.

19.4 Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA-600/8-78-017. Office of Research and Development. USEPA.

19.5 *Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in*

Biosolids. 1992. EPA/625/R-92/013. Office of Research and Development. USEPA.

19.6 Thomas, H. A. Jr. 1942. Bacterial densities from fermenation tubes. *Journal of the American Water Works Association.* 34:572.

19.7 *Analytical Standards for Laboratory Chemicals*. BDH Ltd. Poole, Dorset, UK, and the United States Pharmacopeia.

19.8 Bordner, R. H., C. F. Frith and J. A. Winter (eds.). 1977. *Proceedings of the Symposium on the Recovery of Indicator Organisms Employing Membrane Filters.* EPA600/9-77-024. USEPA, Environmental Monitoring and Support Laboratory, Cincinnati, OH.

19.9 Lin, S. D. 1973. Evaluation of coliform tests for chlorinated secondary effluents. *Journal of the Water and Pollution Control Federation.* 45:498.

20.0 Flowcharts and Validation Data

20.1 The following pages contain flow charts of dilution and inoculation schemes (Section 11) and for the procedures (Section 12). Schemes for dilution and inoculation are dependent on Class (A or B) and matrix (solid) or (liquid).

FIGURE 1. MULTIPLE TUBE FERMENTATION PROCEDURE

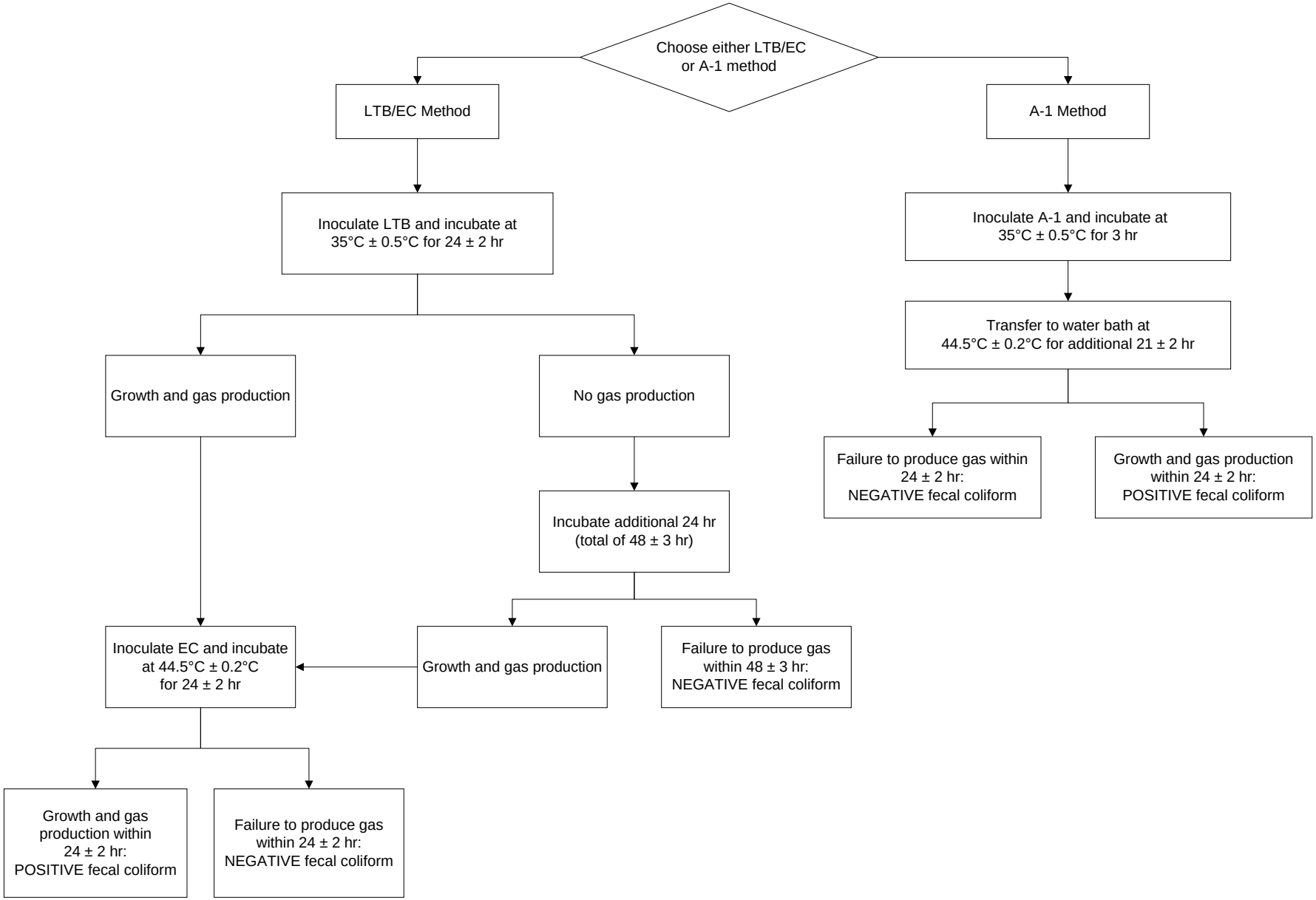


FIGURE 2. CLASS B LIQUID SAMPLE DILUTION AND INOCULATION SCHEME

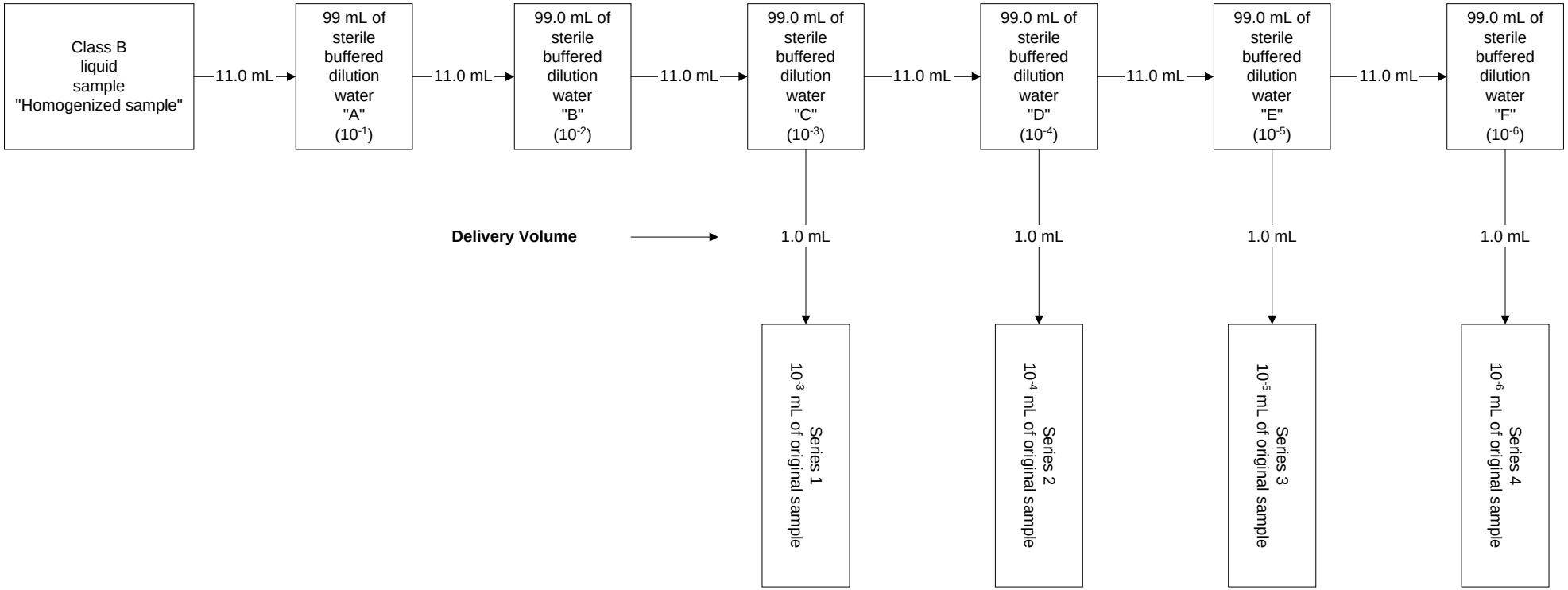


FIGURE 3. CLASS B SOLID SAMPLE DILUTION AND INOCULATION SCHEME

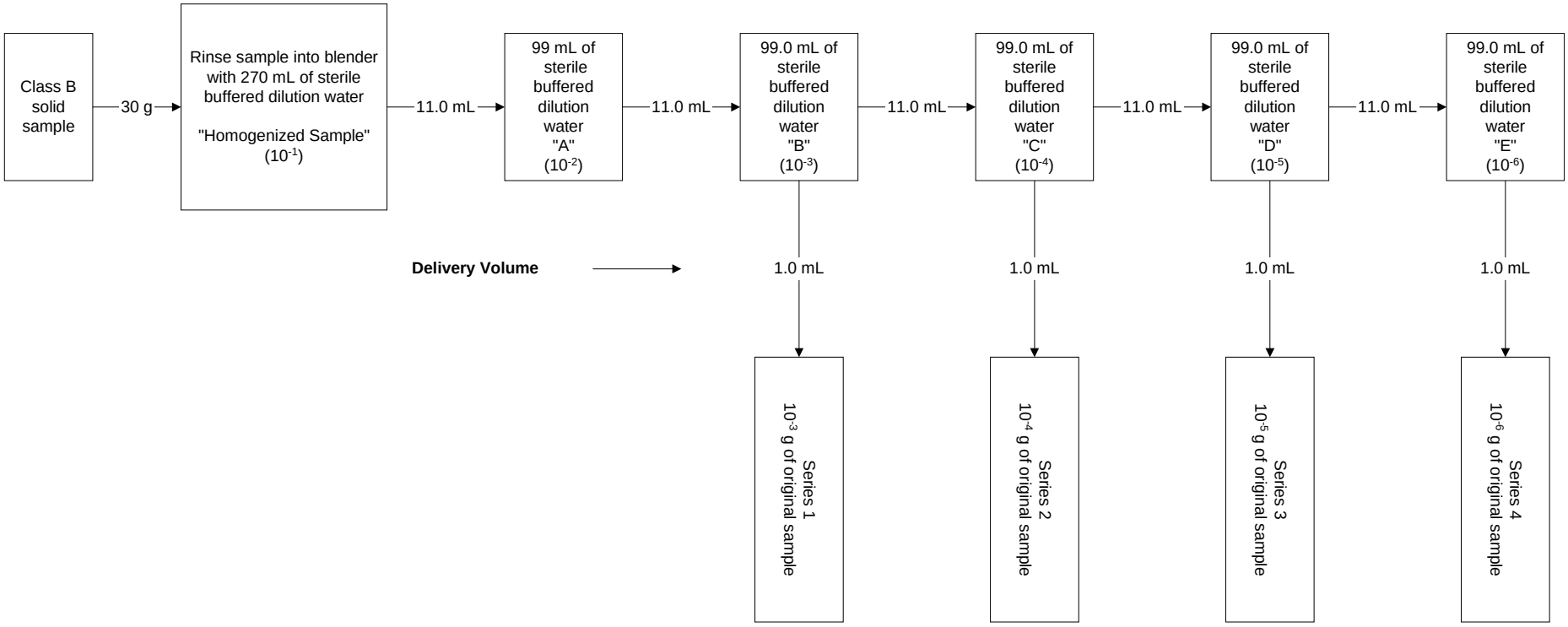


FIGURE 4. CLASS A LIQUID SAMPLE DILUTION AND INOCULATION SCHEME

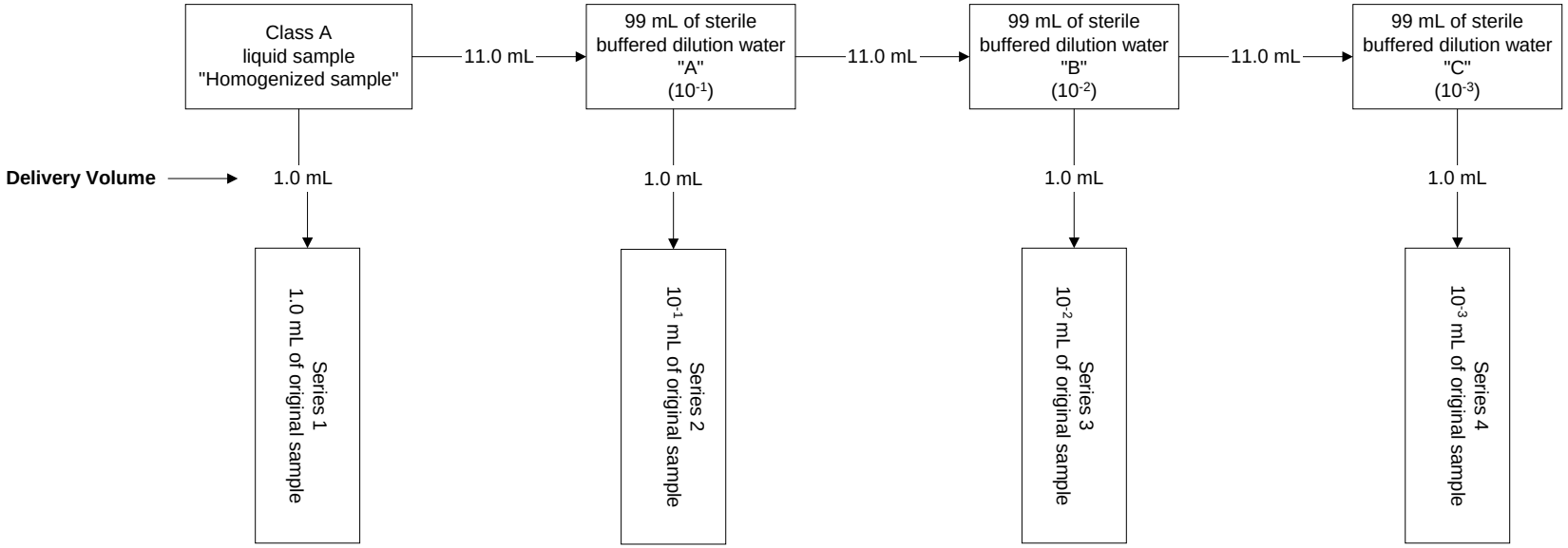
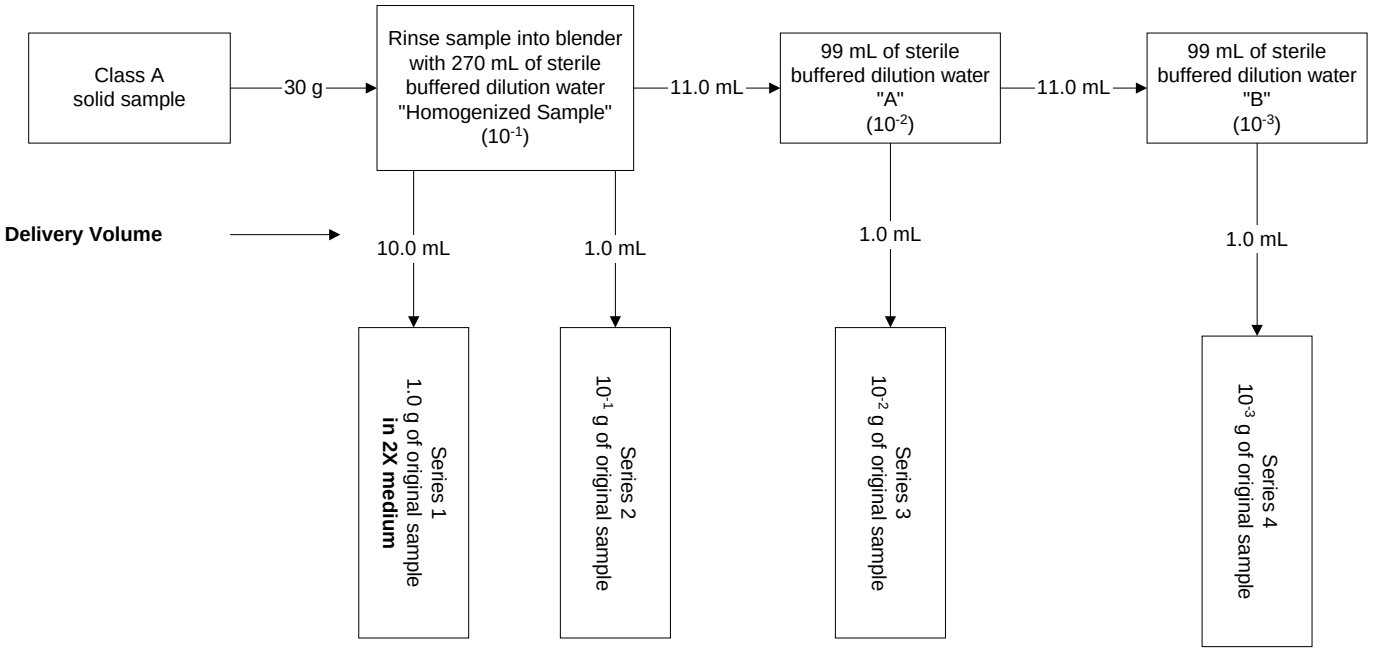


FIGURE 5. CLASS A SOLID SAMPLE DILUTION AND INOCULATION SCHEME



21.0 Glossary

The definitions and purposes are specific to this method but have been conformed to common usage as much as possible.

21.1 Units of weight and measure and their abbreviations

21.1.1 Symbols

°C	degrees Celsius
<	less than
>	greater than
%	percent
±	plus or minus

21.1.2 Alphabetical characters

EC	*Escherichia coli*
EPA	Environmental Protection Agency
g	gram
L	liter
LTB	lauryl tryptose broth
mg	milligram
mL	milliliter
mm	millimeter
MPN	most probable number (in this method, multiple tube fermentation)
NIST	National Institute of Standards and Technology
TD	to deliver
QC	quality control

21.2 Definitions, acronyms, and abbreviations (in alphabetical order).

Analyst—The analyst must have two years of college lecture and laboratory course work in microbiology or a closely related field. The analyst also must have at least 6 months bench experience, must have at least 3 months experience with plating procedures, and must have successfully analyzed at least 50 biosolid samples for fecal coliforms. Six months of additional experience in the above area may be substituted for two years of college. The analyst must also demonstrate acceptable performance during an on-site evaluation.

Analyte—The microorganism tested for by this method. The analytes in this method are fecal coliforms.

Enrichment—Using a culture media for preliminary isolation that favors the growth of a particular kind of organism.

Liquid samples—Generally defined as samples containing <10% total solids (dry weight).

May—This action, activity, or procedural step is neither required nor prohibited.

May not—This action, activity, or procedural step is prohibited.

Most probable number method (MPN)—A statistical determination of the number of bacteria per weight or volume of sample. It is based on the fact that the greater the number of bacteria in a sample, the more dilution is needed to reduce the density to the point at which no bacteria are left to grow in a dilution series.

Must—This action, activity, or procedural step is required.

Preferred—Optional

Preparation blank—See Method blank.

Quantitative transfer—the process of transferring a solution from one container to another using a pipette in which as much solution as possible is transferred, followed by rinsing of the walls of the source container with a small volume of rinsing solution (e.g., PBS), followed by transfer of the rinsing solution, followed by a second rinse and transfer.

Selective media—A culture media designed to suppress the growth of unwanted microorganisms and encourage the growth of desired ones.

Should—This action, activity, or procedural step is suggested but not required.

Solid samples—Generally defined as samples containing >10% total solids (dry weight).

Technician—See Analyst.