Method 1681: Fecal Coliforms in Sewage Sludge (Biosolids) by Multiple-Tube Fermentation using A-1 medium

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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* (*E. 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 method 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 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 Parts 136.4 and 136.5.

1.9 Method 1681 was submitted to interlaboratory validation in Class A and Class B biosolid matrices. A summary of method performance results from this validation study are provided in Section 16.0. A comprehensive evaluation of the study results is presented in the validation study report (Reference 19.2). For method application please refer to Title 40 Code of Federal Regulations Part 136 (40 CFR Part 136).

Note: Based on the high false positive rates observed for Method 1681 in some matrices, EPA recommends that laboratories conduct their own matrix-specific comparisons to determine the most appropriate method (1680 or 1681).

2.0 Summary of Method

2.1 Fecal coliform densities of biosolids may be determined by the MPN procedure.

2.2 MPN procedure (Class A and B)

Method 1681 provides for the enumeration of fecal coliforms in Class A and Class B biosolids using the most probable number (MPN) procedure. In Method 1681, A-1 medium is used as a direct, single step test.

2.2.1 Summary of the A-1 procedure (see **Figure 1** in Section 20.0) [see Sections 11.0 and 12.0 for explanation]

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 A-1 medium and inverted vials.

2.2.1.2 Sample tubes are incubated in a waterbath or jacketed incubator at 35°C ± 0.5°C for 3 hours, then transferred to a waterbath 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.

2.2.1.3 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).

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 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.

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

6.1 Sterile plastic bags, 1-gallon

6.2 Sterile plastic or glass jars with lids, 1-L

6.3 Sterile auger

6.4 Sterile scoops (do not use curved scoops)

6.5 Ice chest

6.6 Wet ice

6.7 Ice packs, blue ice

6.8 Bubble wrap

6.9 Sterile trowels

6.10 Sterile aluminum foil or kraft paper

6.11 Sterile container, such as a stainless steel or plastic bucket suitable for sample collection

6.12 Flat shovel

6.13 Dilution bottles, borosilicate glass, screw cap, marked at 99 mL or screw cap, borosilicate glass or plastic tubes marked at 9 mL

6.14 Tubes, 16 × 150 mm, borosilicate glass, with loose-fitting aluminum, stainless steel or autoclavable caps

6.15 Durham tubes or vials, 10 × 75 mm, borosilicate glass

6.16 Tubes, 16 × 100 mm, screw cap, borosilicate glass, with autoclavable plastic caps

6.17 Test tube racks to hold sterile culture tubes

6.18 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets

6.19 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, wide-tip of appropriate volume

6.20 Pipet bulbs, or automatic pipettor

6.21 Platinum wire inoculation loops, at least 3 mm diameter in suitable holders; or sterile plastic loops

6.22 Sterile disposable applicator sticks

6.23 Bunsen burner or alcohol burner

6.24 Cornwall syringe, sterile, to deliver at least 5 mL

6.25 Media dispensing pump

6.26 Incubator, water- or air-jacketed, humidity-controlled, microbiological type to hold temperature at 35.0°C ± 0.5°C

6.27 Gable covered waterbath, with circulating system to maintain temperature of 44.5°C ± 0.2°C Water level should be maintained above the media in immersed tubes

6.28 Plastic sterile petri dishes, microbiological grade, 15 mm × 100 mm

6.29 Erlenmeyer flasks, 1-L and 2-L

6.30 Stir bar

6.31 Stir plate

6.32 Sterile blender jars and base

6.33 Waterbath maintained at 50°C for tempering agar

6.34 Balance, analytical balance capable of weighing 0.1 mg

6.35 Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23

6.36 Latex gloves for handling samples

6.37 pH meter

6.38 Vortex mixer

6.39 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume

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

6.41 Beakers, glass or plastic, assorted sizes

6.42 Steel pan of water, 30" × 26" × 10"

6.43 Autoclave or steam sterilizer capable of achieving 121°C [15 lb pressure per square inch (PSI)] for 15 minutes

6.44 Crucible or aluminum evaporating dish

6.45 Drying oven maintained at 103°C - 105°C for tempering agar

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 19.3). 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 reagent water: Reagent-grade water conforming to specifications in *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 136 or 141, as applicable), Section 9020 (Reference 19.1).

7.4 Phosphate buffered dilution water

7.4.1 Composition of stock phosphate buffer solution:

Monopotassium phosphate (KH_2PO_4)	34.0 g
Reagent-grade water	500.0 mL

Preparation: Dissolve KH_2PO_4 in 500 mL reagent-grade water. Adjust the pH of the solution to 7.2 with 1 N NaOH, and bring the volume to 1 L with reagent-grade water. Sterilize by filtration or autoclave at 121°C (15 PSI) for 15 minutes.

7.4.2 Preparation of stock magnesium chloride ($MgCl_2$) solution: Add 38 g anhydrous $MgCl_2$ or 81.1 g magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) to 1 L reagent-grade water. Sterilize by filtration or autoclave at 121°C (15 PSI) for 15 minutes.

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

7.4.4 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 water. Dispense in appropriate amounts for dilutions and/or for use as rinse buffer. Autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.0 ± 0.2. 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 agar (HIA)

7.5.1 Composition:

Beef heart, infusion from 500 g	10.0 g
Bacto tryptose	10.0 g
Sodium chloride	5.0 g
Bacto agar	15.0 g
Reagent-grade water	1.0 L

7.5.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve. Adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Stir well and autoclave at 121°C (15 PSI) for 15 minutes. Pour into 15 × 100 mm sterile petri plates. Let the media warm to room temperature prior to inoculation. Other general growth media may be used for quality assurance (QA) (Section 9.0) purposes.

7.6 A-1 medium

7.6.1 Composition:

Lactose	5.0 g
Tryptone	20.0 g
Sodium chloride	5.0 g
Salicin	0.5 g
Triton® X-100	1.0 mL
Reagent-grade water	1.0 L

7.6.2 For single strength (1X) A-1, add reagents to 1 L of reagent-grade water, mix thoroughly, heat to dissolve and add 1.0 mL of Triton® X-100. 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 (15 PSI) for ***10 minutes***. Ignore formation of precipitate. Media should fill inverted tubes leaving no air spaces.

7.6.3 For double strength (2X) A-1, prepare as in Section 7.6.2 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 LTB medium

7.7.1 Composition:

Tryptose	20.0 g
Lactose	5.0 g
Dipotassium phosphate (K_2HPO_4)	2.75 g
Monopotassium phosphate (KH_2PO_4)	2.75 g
Sodium chloride (NaCl)	5.0 g
Sodium lauryl sulfate	0.1 g
Reagent-grade water	1.0 L

7.7.2 Add reagents 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 medium into appropriate tubes/bottles. Close tubes with metal or autoclavable plastic caps and autoclave at 121°C (15 PSI) for 15 minutes. After cooling, the medium should fill the inverted vials completely, leaving no air space.

7.8 Positive controls

7.8.1 Obtain a stock culture of *E. coli* (e.g., ATCC # 25922) as a positive control for A-1. *Note:* ATCC recommends that no more than 5 transfers be made before returning to the original culture. This will minimize the chance of contamination during transfers and genetic shift of the culture. One suggestion is to make your own frozen seed stock upon receipt of the organism that can be used for future work. For additional information go to http://www.atcc.org.

7.9 Negative controls

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

7.10 The storage times for prepared media used in this method are provided in **Table 1**.

Table 1. Storage Times for Prepared Media

Media	Storage Time
Agar in loose-cap tubes	2 weeks
Agar 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

(*Note*: If media is refrigerated, remove from refrigerator 1-1.5 hours prior to inoculation, so that it reaches room temperature prior to use.)

7.11 Milorganite® (CAS 8049-99-8) or equivalent

Milorganite® (heat-dried Class A biosolid) is produced by Milwaukee Metropolitan Sewerage District. It is available in many home gardening centers.

Obtain Milorganite® as the reference matrix for initial precision and recovery (IPR) and ongoing precision and recovery (OPR) analyses. Milorganite® is used as the reference matrix because it is easily accessible, inexpensive, generally does not contain the analyte of interest, and is of consistent quality.

8.0 Sample Collection, Handling, 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 discharge pipe.

8.4.2 Purge the discharge pipe of old biosolids and warm to the digester temperature by allowing biosolids to flow through the pipe into a container or waste collection device.

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 sterile 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 set it aside. Divide the underlying 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 container.

8.6.4 After all the samples have been taken, pour the contents of the container out onto a sterile surface and mix by folding the sample back onto itself several times.

8.6.5 Reduce the sample size by "coning and quartering". Divide the container 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 contents of the container that has been placed on a sterile surface 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 Sampler name

8.8.4 Sample location

8.8.5 Parameters (e.g., type of analysis, field measurements- pH and temperature)

8.8.6 Volume

8.8.7 Observations

8.9 Ensure that the chain-of-custody form is filled out.

8.10 Sample handling: Maintain bacteriological samples at <10°C during transit to the laboratory. Do not allow the sample to freeze. 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 Holding time and temperature limitations: For fecal coliform samples for sewage sludge (biosolids) only, the holding time is extended to 24 hours for the following sample types using either EPA Method 1680 (LTB-EC) or 1681 (A-1): Class A composted, Class B aerobically digested, and Class B anaerobically digested. All other matrices should be analyzed within 8 hours of sample collection, 6 hour maximum transport and 2 hours for sample processing.

Note: Adherence to sample handling 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 (Sections 9.6 and 9.7). Laboratory performance is compared to the performance criteria specified in Section 16.0 to determine whether the results of the analyses meet the performance characteristics of the method. Specific quality control (QC) requirements for Method 1681 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 Reference 19.4.

9.2 The minimum analytical QC requirements for the analysis of samples using Method 1681 include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.3), ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery (OPR) analysis (Section 9.4) and matrix spike (MS) analysis (Section 9.5, disinfected wastewater only), and the routine analysis of positive and negative controls (Section 9.6), method blanks (Section 9.7), and media sterility checks (Section 9.8). For the IPR, OPR and MS analyses, it is necessary to spike samples with laboratory-prepared spiking suspensions as described in Section 15.0.

9.3 **Initial precision and recovery (IPR)**: The IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and should be performed by each laboratory before the method is used for monitoring field samples. EPA recommends but does not require that an IPR be performed by each analyst. IPR samples should be accompanied by an acceptable method blank (Section 9.7) and appropriate media sterility checks (Section 9.8). The IPR analyses are performed as follows:

9.3.1 Prepare four, 30-g samples of Milorganite® and spike each sample with *E. coli* ATCC # 25922 according to the spiking procedure in Section 15.0. Process and analyze each IPR sample according to the procedures in Sections 11.0 and 12.0 and calculate the fecal coliform MPN/g dry weight according to Section 14.0.

9.3.2 Calculate the percent recovery (R) for each IPR sample using the appropriate equations in Section 15.7.

9.3.3 Using the percent recoveries of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.

9.3.4 Compare the mean recovery and RSD with the corresponding IPR criteria in **Table 2**, below. If the mean and RSD for recovery of fecal coliforms meet acceptance criteria, system performance is acceptable and analysis of field samples may begin. If the mean recovery or the RSD fall outside of the required range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the IPR analyses.

Table 2. Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria

Performance test	A-1 acceptance criteria
Initial precision and recovery (IPR)	
• Mean percent recovery	1% - 312%
• Precision (as maximum relative standard deviation)	96%
Ongoing precision and recovery (OPR) as percent recovery	1% - 371%

9.4 **Ongoing precision and recovery (OPR)**: To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked Milorganite® samples. The laboratory should analyze one OPR sample after every 20 field and matrix spike samples or one per week that samples are analyzed, whichever occurs more frequently. OPR samples must be accompanied by an acceptable method blank (Section 9.7) and appropriate media sterility checks (Section 9.8). The OPR analysis is performed as follows:

9.4.1 Spike a 30-g sample of Milorganite® with *E. coli* ATCC # 25922 according to the spiking procedure in Section 15.0. Process and analyze each OPR sample according to the procedures in Sections 11.0 and 12.0 and calculate the number of fecal coliform MPN/g dry weight according to Section 14.0.

9.4.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equations in Section 15.7.

9.4.3 Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 2, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process (media, reagents, and controls), correct the problem and repeat the OPR analysis.

9.4.4 As part of the laboratory QA program, results for OPR and IPR samples should be charted and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1681 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the accuracy as a recovery interval from R - $2s_r$ to R + $2s_r$.

9.5 **Matrix spikes (MS)**: MS analysis are performed to determine the effect of a particular matrix on fecal coliform recoveries. The laboratory should analyze one MS sample when biosolid samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given biosolids source should include a MS sample. MS samples must be accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site, an acceptable method blank (Section 9.7), and appropriate media sterility checks (Section 9.8). When possible, MS analyses should also be accompanied by an OPR sample (Section 9.4). The MS analysis is performed as follows:

9.5.1 Prepare two, 30-g field samples that were sequentially collected from the same site. One sample will remain unspiked and will be analyzed to determine the background or ambient concentration of fecal coliforms for calculating MS recoveries (Section 9.5.3). The other sample will serve as the MS sample and will be spiked with *E. coli* ATCC # 25922 according to the spiking procedure in Section 15.0.

9.5.2 Select dilutions based on previous analytical results or anticipated levels of fecal coliforms in the field sample in order to accurately estimate fecal coliform density. Neither above or below the detection limit of the method. Section 12.0 includes possible dilution schemes for both Class A and Class B biosolids.

9.5.3 Spike the MS sample with a laboratory-prepared suspension as described in Section 15.0. Process and analyze the unspiked and spiked field samples according to the procedures in Sections 11.0 and 12.0.

9.5.4 For the MS sample, calculate the fecal coliform MPN/g dry weight according to Section 14.0 and adjust the density (MPN/g dry weight) based on the ambient concentration of fecal coliforms observed in the unspiked matrix sample.

9.5.5 Calculate the percent recovery (R) for the MS sample (adjusted based on ambient fecal coliform in the unspiked sample) using the appropriate equations in Section 15.7.

9.5.6 Compare the MS result (percent recovery) with the appropriate method performance criteria in **Table 3**. If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this biosolid source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, a matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data should be flagged.

Table 3. Matrix Spike Precision and Recovery Acceptance Criteria

Performance test	A-1 acceptance criteria
Class A Biosolids: Matrix spike (MS)	
MS percent recovery	2% - 541%
Class B Biosolids: Matrix spike (MS)	
MS percent recovery	>0% - 6172%
Class A Biosolids: Matrix spike, matrix spike duplicate (MS/MSD)	
• Percent recovery for MS/MSD	2% - 541%
• Precision (as maximum relative percent difference of MS/MSD)	182%
Class B Biosolids: Matrix spike, matrix spike duplicate (MS/MSD)	
• Percent recovery for MS/MSD	>0% - 6172%
• Precision (as maximum relative percent difference of MS/MSD)	184%

9.5.7 Laboratories should record and maintain a control chart comparing MS recoveries for all matrices to batch-specific and cumulative OPR sample results analyzed using Method 1681. These comparisons should help laboratories recognize matrix effects on method recovery and may also help to recognize inconsistent or sporadic matrix effects from a particular source.

9.6 Culture Controls

9.6.1 Negative controls: The laboratory should analyze negative controls to ensure that the A-1 is performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a negative control every day that samples are analyzed.

9.6.1.1 Negative controls are conducted by inoculating A-1 with a known negative fecal coliform species (e.g., *Enterobacter aerogenes* ATCC # 13048) and analyzing as described in Section 12.0. Viability of the negative controls should be demonstrated using a non-selective media (e.g., nutrient agar or tryptic soy agar).

9.6.1.2 If a negative control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the negative control, and reanalyze the appropriate negative control.

9.6.2 Positive controls: The laboratory should analyze positive controls to ensure that the A-1 is performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should perform a positive control every day that samples are analyzed. An OPR sample (Section 9.4) may take the place of a positive control.

9.6.2.1 Positive controls are conducted by inoculating A-1 with a known positive fecal coliform species (e.g., *E. coli* ATCC # 25922) and analyzing as described in Section 12.0.

9.6.2.2 If the positive control fails to exhibit the appropriate response, check and/or replace the associated media or reagents, and/or the positive control, and reanalyze the appropriate positive control.

9.7 **Method blank.** Test a 20-mL sterile dilution water sample in the analytical scheme to verify the sterility of equipment, materials, and supplies. Absence of growth indicates freedom of contamination from the target organism. On an ongoing basis, the laboratory should perform a method blank every day that samples are analyzed.

9.8 **Media sterility check.** To test sterility of media, subject a representative portion of each batch to incubation at 44.5°C ± 0.2°C for 24 ± 2 hours 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. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check every day that samples are analyzed.

10.0 Equipment Calibration and Standardization

10.1 Check temperatures in incubators/waterbaths 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 Check temperatures in refrigerators/freezers at least once daily to ensure operation is within stated limits of the method. Record daily measurements in refrigerator/freezer log book.

10.3 Calibrate thermometers and incubators at least annually against an NIST certified thermometer or one that meets requirements of NIST Monograph SP 250-23 (Reference 19.1). Check mercury columns for breaks.

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.

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

11.0 Sample Preparation

11.1 Homogenization

Sample homogenization procedures are based on whether the sample is a liquid or a solid. If sample is alkaline-stabilized (liquid or solid), adjust the pH as described in Section 11.1.3. Liquid samples are generally defined as samples containing ≤7% 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 or 1.0 N sodium hydroxide, if necessary. This is the "homogenized" sample. When adjusting the pH do not exceed the homogenized sample volume by greater than 5% (15 mL).

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 or 1.0 N sodium hydroxide, if necessary.

Note: Do not suspend bacteria in dilution water for more than 30 minutes at room temperature. Chill on wet ice or at 4°C ± 1°C to slow replication between spiking samples.

11.1.3 Alkaline-stabilized: The alkaline-stabilized biosolid samples generally have a pH of approximately 12. Prior to analysis, the alkaline-stabilized biosolid sample must be neutralized to a pH of approximately 7.5. Do not add lab-prepared spikes to the samples prior to pH adjustment.

11.1.3.1 Adjustment of pH should be done in a fume hood. Prior to adjusting the pH of the sample, calibrate/standardize the pH meter with pH buffers 7.0 and 10.0. Weigh out 30 g of sample into a sterile 600 mL beaker, add 250 mL of sterile buffered dilution water and a sterile magnetic stir bar. Place beaker on a mixing plate, insert pH probe into mixture, begin stirring, and take an initial pH reading. To minimize the amount of volume added to each sample, pH should be adjusted using 10 N HCl.

Note: The addition of the 10 N HCl will produce fumes, do not be alarmed. The addition of the acid should be done incrementally to ensure that the pH does not drop instantaneously below 5.0. It is recommended that the pH adjustment be completed within 10-15 minutes and monitored for an additional 15 minutes to ensure that the sample is able to maintain a constant pH of around 7.5. Pour pH adjusted sample into blender jar, use the remaining sterile buffered dilution water (15 mL) to rinse the beaker twice and pour rinse water into the blender jar.

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 fecal coliform concentrations in undiluted samples could easily exceed the analytical range of this procedure, the laboratory must follow the dilution and inoculation schemes in Section 11.2.1 (liquid) or 11.2.2 (solid), if necessary additional dilutions may be analyzed to ensure results obtained are not censored (less-than or greater-than) values. 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 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 2** in Section 20.0 for an overview of this dilution and inoculation scheme. *Note:* 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.

11.2.1.1 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 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." One mL of dilution "B" is 10^{-2} mL of the original sample.

(C) 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." One 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 dilution "C" to 99 mL of sterile dilution water, and mix as before. This is dilution "D." One mL 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." One mL of dilution "E" is 10^{-5} 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 the original "homogenized" sample per tube (unspiked samples only).

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

11.2.1.3 Repeat steps 11.2.3.1 and 11.2.3.2 for the remaining Class A samples. When inoculations are complete, go to Section 12.3.4 to continue the A-1 method.

11.2.2 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** in Section 20.0 for a summary of this dilution and inoculation scheme. *Note:* 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.

11.2.2.1 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." One 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." One 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." One 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." One mL of dilution "D" contains 10^{-5} g of the original sample.

11.2.2.2 Inoculation

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

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

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

11.2.2.3 Repeat Section 11.2.4.1 and 11.2.4.2 for remaining Class A samples. When inoculations are complete, go to Section 12.3.4 to continue the A-1 method.

11.2.3 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** in Section 20.0 for a summary of this dilution and inoculation scheme. *Note:* 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.

11.2.3.1 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." One mL 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." One mL of dilution "B" is 10^{-2} mL of the original sample.

(C) 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." One mL of dilution "C" is 10^{-3} mL of the original sample.

(D) 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." One mL 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." One mL 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." One mL 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." One mL 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." One mL of dilution "H" is 10^{-8} mL of the original sample.
- Use a sterile pipette to transfer 11.0 mL of dilution "H" to 99 mL of sterile dilution water, and mix as before. This is dilution "I." One mL of dilution "I" is 10^{-9} 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 dilution "C" (unspiked samples only). This is 10^{-3} mL of the original sample.

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

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

(G) Use a sterile pipette to inoculate each of five tubes with 1.0 mL of dilution "I" (spiked samples). This is 10^{-9} mL of the original sample.

11.2.3.3 Repeat Section 11.2.1.1 and 11.2.1.2 for each remaining Class B sample. When inoculations are complete, proceed to Section 12.3.4 to continue the A-1 method.

11.2.4 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** in Section 20.0 for a summary of this dilution and inoculation scheme. *Note:* 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.

11.2.4.1 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 (Section 7.4) and shake vigorously a minimum of 25 times. This is dilution "A." One 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." One 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." One 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." One 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." One 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." One mL of dilution "F" is 10^{-7} g 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." One mL of dilution "G" is 10^{-8} g 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." One mL of dilution "H" is 10^{-9} g of the original sample.

11.2.4.2 Inoculation

(A) Use a sterile pipette to 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) Use a sterile pipette to inoculate each of five tubes with 1.0 mL of dilution "C" (unspiked samples only). This is 10^{-4} g of the original sample.

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

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

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

(G) Use a sterile pipette to inoculate each of five tubes with 1.0 mL of dilution "H" (spiked samples). This is 10^{-9} g of the original sample.

11.2.4.3 When inoculations are complete, go to Section 12.3.4 to continue the A-1 method.

12.0 A-1 Procedure

12.1 In this protocol, A-1 medium is used to determine fecal coliform densities in Class A and 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. In Method 1681, A-1 is used as a direct, single step test. Precision of the test increases with increasing numbers of replicates per sample tested. For an overview of the MPN procedure, refer to Figure 1 in Section 20.0.

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 A-1 procedure

12.3.1 Prepare A-1 broth tubes as directed in Section 7.6. *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 For each sample, arrange test tubes in four or five 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.

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

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

12.3.5 Transfer A-1 tubes to a waterbath 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.

12.3.6 After incubation, remove tubes from the waterbath, 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 (See **Photo 1**). Collection of gas in the durham tube is not necessary. *Note*: The presence of gas in the absence of growth is usually due to mishandling or improper shaking of the tubes after inoculation.

12.3.7 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 Section 14.0.

Photo 1. Fecal coliforms produce gas in A-1 medium.

12.4 Total solids determination

12.4.1 Determination of percent dry weight - When sample results are to be calculated on a dry weight basis, a second portion of sample should be weighed at the same time as the portion used for analytical determination.

WARNING: The drying oven should be contained in a hood or be vented. Significant laboratory contamination may result from drying a heavily contaminated sample.

12.4.2 Immediately after weighing the sample for microbiological examination, weigh 10-30 g of the sample into a tarred crucible or aluminum evaporating dish. Dry this aliquot overnight at 103°C to 105°C. Allow to cool in a desiccator before weighing. Calculate the % dry weight as follows:

$$\%\ dry\ weight = \frac{g\ dry\ sample}{g\ sample} \times 100$$

13.0 Verification

13.1 Individual biochemical tests including oxidase, citrate, lactose fermentation, indole, ONPG hydroloysis, methyl red, and Voges-Proskauer may be used to verify positive and negative results.

13.2 Alternately, commercially available multi-test identification systems may be used to verify positive and negative results. Such identification systems for Enterobacteriaceae must include lactose fermentation, σ-nitrophenyl-β-D-galactopyranoside (ONPG), and cytochrome oxidase test reactions.

Note: Due to the high false positive and negative rates, it is recommended that analysts be required to submit all positive and negative tubes with growth to verification for at least one biosolid sample for each type of biosolid that the analyst normally evaluates on a monthly basis. This should increase analyst proficiency in using these procedures.

14.0 Data Analysis and Calculations

The estimated density of fecal coliform bacteria, based on 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 14.1, 14.2, and 14.3):

- 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 14.4.

14.1 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 solid samples (Section 11.2.4), four, five-tube dilutions are used, with 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} g 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 5**, 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.

14.1.1 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 Table 5, Example A, 10^{-4} is higher/more dilute than 10^{-3}.)

14.1.2 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 5, Examples B and C).

14.1.3 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 5, Example D).

14.1.4 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 5, Example E).

14.1.5 If there were not enough higher dilutions tested to select three dilutions, then select the next lower dilution (Table 5, Example F).

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

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

Note: The example calculated numbers provided in the tables below have been rounded at the end of each step. If your laboratory recalculates the examples using a spreadsheet and rounds only after the final calculation, values may be slightly different.

$$\text{MPN / mL} = \frac{\text{MPN Index from Table 4}}{\text{Largest volume tested in the dilution series used for MPN determination}}$$

14.2.2 When using MPN tables other than those provided in this method (e.g. Table 9221:IV, *Standard Methods for Water and Wastewater,* Reference 19.1), additional steps/calculations are required to determine final reporting value of MPN/g dry weight. For example, Table 9221:IV MPN index is per 100 mL (MPN/100 mL); which will need to be converted to MPN/mL . In addition, the MPN index must be multiplied by a factor of 10 when using the largest volume tested in the dilution series used for MPN determination.

$$\text{MPN / 100 mL} = \frac{10 \times \text{MPN Index from Table 9221:VI}}{\text{Largest volume tested in the dilution series used for MPN determination}}$$

$$\text{MPN / mL} = \frac{\text{MPN/100 mL}}{100}$$

Table 4. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Dilution [a]

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.1803			1-3-0	0.83	0.12	1.96
0-0-1	0.18	0.03	0.63	1-3-1	1.04	0.20	2.43
0-0-2	0.36	0.03	1.01	1-3-2	1.25	0.29	2.96
0-0-3	0.54	0.03	1.37	1-3-3	1.47	0.38	3.64
0-0-4	0.72	0.08	1.74	1-3-4	1.69	0.48	4.60
0-0-5	0.91	0.15	2.12	1-3-5	1.91	0.57	5.66
0-1-0	0.18	0.03	0.63	1-4-0	1.05	0.21	2.45
0-1-1	0.36	0.03	1.01	1-4-1	1.27	0.30	3.00
0-1-2	0.55	0.03	1.38	1-4-2	1.48	0.39	3.70
0-1-3	0.73	0.08	1.75	1-4-3	1.70	0.48	4.68
0-1-4	0.91	0.15	2.14	1-4-4	1.93	0.58	5.75
0-1-5	1.10	0.23	2.56	1-4-5	2.15	0.67	6.57
0-2-0	0.37	0.03	1.02	1-5-0	1.28	0.30	3.03
0-2-1	0.55	0.03	1.39	1-5-1	1.50	0.40	3.75
0-2-2	0.74	0.08	1.76	1-5-2	1.72	0.49	4.77
0-2-3	0.92	0.15	2.15	1-5-3	1.95	0.58	5.83
0-2-4	1.11	0.23	2.58	1-5-4	2.17	0.68	6.64
0-2-5	1.29	0.31	3.07	1-5-5	2.40	0.77	7.31
0-3-0	0.56	0.03	1.40	2-0-0	0.45	0.03	1.19
0-3-1	0.74	0.09	1.77	2-0-1	0.68	0.06	1.64
0-3-2	0.93	0.16	2.17	2-0-2	0.91	0.15	2.13
0-3-3	1.12	0.23	2.60	2-0-3	1.15	0.25	2.69
0-3-4	1.30	0.31	3.10	2-0-4	1.39	0.35	3.38
0-3-5	1.49	0.39	3.72	2-0-5	1.64	0.46	4.37
0-4-0	0.75	0.09	1.79	2-1-0	0.68	0.06	1.66
0-4-1	0.94	0.16	2.19	2-1-1	0.92	0.15	2.16
0-4-2	1.12	0.24	2.63	2-1-2	1.16	0.25	2.72
0-4-3	1.31	0.32	3.13	2-1-3	1.41	0.36	3.43
0-4-4	1.50	0.40	3.77	2-1-4	1.66	0.46	4.47
0-4-5	1.69	0.48	4.62	2-1-5	1.92	0.57	5.71
0-5-0	0.94	0.16	2.21	2-2-0	0.93	0.16	2.18
0-5-1	1.13	0.24	2.65	2-2-1	1.18	0.26	2.76
0-5-2	1.33	0.32	3.17	2-2-2	1.43	0.36	3.49
0-5-3	1.52	0.40	3.82	2-2-3	1.68	0.47	4.56
0-5-4	1.71	0.48	4.70	2-2-4	1.94	0.58	5.81
0-5-5	1.90	0.56	5.63	2-2-5	2.21	0.69	6.75
1-0-0	0.20	0.03	0.68	2-3-0	1.19	0.26	2.79
1-0-1	0.40	0.03	1.08	2-3-1	1.44	0.37	3.55
1-0-2	0.60	0.03	1.49	2-3-2	1.70	0.48	4.67
1-0-3	0.81	0.11	1.91	2-3-3	1.97	0.59	5.91
1-0-4	1.01	0.19	2.36	2-3-4	2.23	0.70	6.83
1-0-5	1.22	0.28	2.87	2-3-5	2.51	0.82	7.59
1-1-0	0.40	0.03	1.09	2-4-0	1.46	0.38	3.61
1-1-1	0.61	0.03	1.50	2-4-1	1.72	0.49	4.77
1-1-2	0.81	0.11	1.92	2-4-2	1.99	0.60	6.00
1-1-3	1.02	0.19	2.38	2-4-3	2.26	0.72	6.92
1-1-4	1.23	0.28	2.90	2-4-4	2.54	0.83	7.68
1-1-5	1.44	0.37	3.54	2-4-5	2.82	0.94	8.36
1-2-0	0.61	0.03	1.51	2-5-0	1.74	0.50	4.88
1-2-1	0.82	0.12	1.94	2-5-1	2.01	0.61	6.10
1-2-2	1.03	0.20	2.40	2-5-2	2.29	0.73	7.00
1-2-3	1.24	0.29	2.93	2-5-3	2.57	0.84	7.76
1-2-4	1.46	0.38	3.59	2-5-4	2.86	0.95	8.45
1-2-5	1.67	0.47	4.51	2-5-5	3.15	1.07	9.10

[a] Table was developed using the MPN calculator developed by Albert Klee (Reference 19.5)

Table 4. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Dilution (cont.) [a]

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.79	0.10	1.88	4-3-0	2.71	0.90	8.09
3-0-1	1.06	0.21	2.46	4-3-1	3.26	1.11	9.34
3-0-2	1.35	0.33	3.23	4-3-2	3.86	1.32	10.60
3-0-3	1.65	0.46	4.40	4-3-3	4.51	1.54	11.92
3-0-4	1.96	0.59	5.89	4-3-4	5.21	1.76	13.31
3-0-5	2.29	0.73	6.99	4-3-5	5.93	1.96	14.77
3-1-0	1.07	0.22	2.50	4-4-0	3.35	1.14	9.53
3-1-1	1.37	0.34	3.29	4-4-1	3.98	1.37	10.84
3-1-2	1.67	0.47	4.52	4-4-2	4.66	1.59	12.23
3-1-3	1.99	0.60	6.01	4-4-3	5.39	1.81	13.68
3-1-4	2.32	0.74	7.10	4-4-4	6.15	2.02	15.21
3-1-5	2.67	0.88	8.00	4-4-5	6.93	2.23	16.81
3-2-0	1.38	0.35	3.35	4-5-0	4.11	1.41	11.11
3-2-1	1.70	0.48	4.64	4-5-1	4.83	1.64	12.56
3-2-2	2.02	0.62	6.13	4-5-2	5.59	1.87	14.09
3-2-3	2.36	0.76	7.20	4-5-3	6.39	2.09	15.70
3-2-4	2.71	0.90	8.10	4-5-4	7.22	2.30	17.39
3-2-5	3.08	1.04	8.94	4-5-5	8.06	2.50	19.16
3-3-0	1.72	0.49	4.77	5-0-0	2.40	0.76	7.63
3-3-1	2.05	0.63	6.24	5-0-1	3.14	1.06	9.08
3-3-2	2.40	0.77	7.31	5-0-2	4.27	1.46	11.42
3-3-3	2.76	0.92	8.21	5-0-3	5.78	1.92	14.46
3-3-4	3.13	1.06	9.06	5-0-4	7.59	2.39	18.16
3-3-5	3.52	1.20	9.89	5-0-5	9.53	1.65	22.34
3-4-0	2.09	0.64	6.36	5-1-0	3.29	1.12	9.40
3-4-1	2.44	0.79	7.42	5-1-1	4.56	1.56	12.02
3-4-2	2.81	0.93	8.33	5-1-2	6.31	2.07	15.53
3-4-3	3.19	1.08	9.18	5-1-3	8.39	2.57	19.85
3-4-4	3.58	1.23	10.02	5-1-4	10.62	3.04	24.85
3-4-5	3.99	1.37	10.86	5-1-5	12.93	3.04	30.90
3-5-0	2.48	0.80	7.53	5-2-0	4.93	1.67	12.76
3-5-1	2.86	0.95	8.44	5-2-1	7.00	2.24	16.94
3-5-2	3.25	1.10	9.31	5-2-2	9.44	2.80	22.13
3-5-3	3.65	1.25	10.17	5-2-3	12.05	3.31	28.43
3-5-4	4.07	1.40	11.03	5-2-4	14.79	3.81	37.14
3-5-5	4.50	1.54	11.89	5-2-5	17.67	5.03	52.30
4-0-0	1.30	0.31	3.11	5-3-0	7.92	2.47	18.86
4-0-1	1.66	0.46	4.45	5-3-1	10.86	3.08	25.44
4-0-2	2.07	0.64	6.31	5-3-2	14.06	3.68	34.45
4-0-3	2.53	0.82	7.64	5-3-3	17.50	4.34	51.31
4-0-4	3.02	1.02	8.81	5-3-4	21.22	5.29	67.98
4-0-5	3.55	1.21	9.96	5-3-5	25.27	8.14	79.71
4-1-0	1.69	0.48	4.60	5-4-0	12.99	3.48	31.08
4-1-1	2.12	0.66	6.46	5-4-1	17.24	4.29	49.75
4-1-2	2.58	0.85	7.79	5-4-2	22.12	5.63	70.87
4-1-3	3.10	1.05	8.98	5-4-3	27.81	8.82	86.00
4-1-4	3.65	1.25	10.16	5-4-4	34.54	11.59	101.10
4-1-5	4.25	1.45	11.38	5-4-5	42.56	14.37	118.00
4-2-0	2.16	0.67	6.61	5-5-0	23.98	7.62	76.29
4-2-1	2.64	0.87	7.94	5-5-1	34.77	11.72	101.60
4-2-2	3.17	1.08	9.15	5-5-2	54.22	17.91	141.90
4-2-3	3.75	1.29	10.37	5-5-3	91.78	26.72	220.10
4-2-4	4.38	1.50	11.64	5-5-4	160.90	38.37	410.30
4-2-5	5.04	1.71	12.97	5-5-5	>160.90		

[a] Table was developed using the MPN calculator developed by Albert Klee (Reference 19.5)

Examples of MPN / mL calculations are provided in Table 5.

Table 5. Examples of Significant Dilution Selection and Calculation of MPN / mL [a]

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	$(7.92 / 10^{-4})$ = 79,200 MPN / mL 79,000 MPN / mL
B	4/5	5/5	1/5	0/5	4-5-1	$(4.8\ 3/ 10^{-3})$ = 4830 MPN / mL 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	$(1.37 / 10^{-4})$ = 13,700 MPN / mL 14,000 MPN / mL
E	4/5	4/5	0/5	1/5	4-4-1	$(3.98 / 10^{-3})$ = 3980 MPN / mL 4000 MPN / mL
F	5/5	5/5	5/5	2/5	5-5-2	$(54.22 / 10^{-4})$ = 542,200 MPN / mL 540,000 MPN / mL

[a] Significant dilutions are underlined and largest significant dilutions highlighted

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

For analysis and calculation of percent total solids, see Section 12.4.

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 6**.

Table 6. Examples of Conversion to MPN / g Total Solids (Dry Weight), Continuing From Step 2 in Table 5.

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

14.4 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 7**.

Table 7. 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)		

15.0 Sample Spiking Procedure

15.1 Method 1681 QC requirements (Section 9.0) include the preparation and analysis of spiked reference (Milorganite®) samples in order to monitor initial and ongoing method performance. For the IPR (Section 9.3) and OPR (Section 9.4) analyses it is necessary to spike samples with laboratory-prepared spiking suspensions. Section 15.0 is arranged in the following order preparation of the *E. coli* spiking suspension (Section 15.2), spiking suspension dilution (Section 15.3), spiking suspension enumeration (Section 15.4), Class A sample spiking (Section 15.5), Class B sample spiking (Section 15.6), and calculation of spiked *E. coli* percent recovery (Section 15.7).

15.2 Preparation of *E. coli* Spiking Suspension (Class A or B)

15.2.1 Stock Culture. Prepare a stock culture by inoculating a heart infusion agar (HIA) slant [or other non-selective media (e.g., Tryptic Soy Agar)] with *Escherichia coli* ATCC # 25922 and incubating at 35°C ± 3°C for 20 ± 4 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

15.2.2 1% Lauryl Tryptose Broth (LTB). Prepare a 1% solution of LTB by combining 99 mL of sterile phosphate buffered dilution water and 1 mL of sterile single strength lauryl tryptose broth in a sterile screw cap bottle or re-sealable dilution water container. Shake to mix.

15.2.3 Spiking Suspension (Undiluted). From the stock culture of *E. coli* ATCC # 25922, transfer a small loopful of growth to the 1% LTB solution and vigorously shake a minimum of 25 times. Incubate at 35°C ± 3°C for 20 ± 4 hours. The resulting spiking suspension contains approximately 1.0 x 10^7 to 1.0 x 10^8 *E. coli* colony forming units (CFU) per mL. This is referred to as the "undiluted spiking suspension."

15.3 Spiking Suspension Dilution

15.3.1 Mix the spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted spiking suspension 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 spiking suspension dilution "A." A 1.0-mL volume of dilution "A" is 10^{-2} mL of the original undiluted spiking suspension.

15.3.2 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "A" to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "B." A 1.0-mL volume of dilution "B" is 10^{-3} mL of the original undiluted spiking suspension.

15.3.3 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "B" to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "C." A 1.0-mL volume of dilution "C" is 10^{-4} mL of the original undiluted spiking suspension.

15.3.4 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "C" to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "D." A 1.0-mL volume of dilution "D" is 10^{-5} mL of the original undiluted spiking suspension.

15.3.5 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "D" to 99 mL of sterile dilution water, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution "E." A 1.0-mL volume of dilution "E" is 10^{-6} mL of the original undiluted spiking suspension.

15.4 Spiking Suspension Enumeration

15.4.1 Prepare heart infusion agar (HIA) (Section 7.5), add 10 - 15 mL of HIA per 100 × 15 mm petri dish, and allow to solidify. Ensure that agar surface is dry. *Note*: To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

15.4.2 Each of the following will be conducted in triplicate, resulting in the evaluation of nine spread plates:

- Pipet 0.1 mL of dilution "C" onto surface of pre-dried HIA plate [10^{-5} mL (0.00001) of the original spiking suspension].
- Pipet 0.1 mL of dilution "D" onto surface of pre-dried HIA plate [10^{-6} mL (0.000001) of the original spiking suspension].
- Pipet 0.1 mL of dilution "E" onto surface of pre-dried HIA plate [10^{-7} mL (0.0000001) of the original spiking suspension].

15.4.3 For each spread plate, using a sterile bent glass rod or spreader, distribute inoculum over surface of medium by rotating the dish by hand or on a turntable.

15.4.4 Allow inoculum to absorb into the medium completely.

15.4.5 Invert plates and incubate at 35°C ± 0.5°C for 24 ± 4 hours.

15.4.6 Count and record number of colonies per plate. Refer to Section 15.7 for calculation of spiking suspension concentration.

15.5 Class A Biosolid Sample Spiking

Homogenize the unspiked Class A biosolid sample (Section 11.1). For the enumeration of fecal coliforms in the unspiked sample, dilute and inoculate according to Section 11.2. After the unspiked sample has been diluted and media inoculated, spike the biosolid sample as indicated below.

Since the objective of spiking the biosolid sample is to establish percent recovery, it is necessary to determine the number of *E. coli* in the undiluted spiking suspension. Instructions for spiking suspension enumeration are provided below.

15.5.1 Liquid Samples: Since the unspiked, homogenized sample was analyzed by the A-1 procedure and a dilution series was prepared (Section 11.2), 284 mL of the original 300 mL of unspiked, homogenized sample remains. To spike the sample, add 1.0 mL of spiking suspension dilution "B" (from Section 15.3.2) for every 100-mL of unspiked homogenized sample remaining, cover, and blend on high speed for 1 - 2 minutes. This is the "spiked, homogenized" sample. The volume (mL) of undiluted spiking suspension added to each mL of the spiked biosolid sample is 1.0×10^{-5} mL per mL [(2.8 mL × 10^{-3}

mL) / 284 mL of biosolid], which is referred to as $V_{spiked\ per\ unit\ biosolids}$ below. Proceed to Section 11.2.3 (dilution and inoculation).

15.5.2 Solid Samples: Since the unspiked, homogenized sample was analyzed by the A-1 procedure and a dilution series was prepared (Section 11.2), 234 mL of the original 300 mL of unspiked, homogenized sample remains. To spike the sample, add 1.0 mL of spiking suspension dilution "B" (from Section 15.3.2) for every 100-mL of unspiked homogenized sample remaining, cover, and blend on high speed for 1 - 2 minutes. This is the "spiked, homogenized" sample. The volume (mL) of undiluted spiking suspension added to each g (wet weight) of the spiked biosolid sample is 1.0×10^{-4} mL per g [(2.3 mL $\times 10^{-3}$ mL) / 23.4 g of biosolid], which is referred to as $V_{spiked\ per\ unit\ biosolids}$ below. Proceed to Section 11.2.4 (dilution and inoculation).

15.6 Class B Biosolid Sample Spiking

Homogenize the unspiked Class B biosolid sample (Section 11.1). For the enumeration of fecal coliforms in the unspiked sample, dilute and inoculate according to Section 11.2. After the unspiked sample has been diluted and media inoculated, spike the biosolid sample as indicated below.

Since the objective of spiking the biosolid sample is to establish percent recovery, it is necessary to determine the number of *E. coli* in the undiluted spiking suspension. Instructions for spiking suspension enumeration are provided below.

Since a dilution series was prepared from the unspiked, homogenized sample (Section 11.2); 289 mL of the original 300 mL of unspiked, homogenized sample remains. To spike the sample, add 1.0 mL of well-mixed undiluted spiking suspension, for every 100-mL of unspiked homogenized sample remaining after unspiked sample evaluation and dilution preparation, cover, and blend on high speed for 1 - 2 minutes. This is the "spiked, homogenized" sample.

Note: The volumes of undiluted spiking suspensions added per mL or g (wet weight) of the spiked Class B biosolids are different from those for the Class A biosolids, since different volumes of the unspiked, homogenized sample remains.

15.6.1 Liquid Samples: The volume (mL) of undiluted spiking suspension added to each mL of the spiked biosolid sample is 1.0×10^{-2} mL [(2.9 mL spiking suspension) / 289 mL of biosolid], which is referred to as $V_{spiked\ per\ unit\ biosolids}$ below. Proceed to Section 11.2.1 (dilution and inoculation).

15.6.2 Solid Samples: The volume (mL) of undiluted spiking suspension added to each g (wet weight) of the spiked biosolid sample is 1.0×10^{-1} mL per g [(2.9 mL spiking suspension) / 28.9 g of biosolids], which is referred to as $V_{spiked\ per\ unit\ biosolids}$ below. Proceed to Section 11.2.2 (dilution and inoculation).

15.7 Calculation of Spiked *E. coli* Percent Recovery

Spiked *E. coli* percent recovery will be conducted in four steps as indicated below.

Note: The example calculated numbers provided in the tables below have been rounded at the end of each step. If your laboratory recalculates the examples using a spreadsheet and rounds only after the final calculation (Step 4), the percent recoveries may be slightly different.

15.7.1 Step 1: Calculate Concentration of *E. coli* (CFU / mL) in Undiluted Spiking Suspension

15.7.1.1 The number of *E. coli* CFU / mL in the spiking suspension will be calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate.

15.7.1.2 If the number of colonies exceeds the upper range (i.e. >300) or if the colonies are not discrete, results should be recorded as "too numerous to count" (TNTC).

15.7.1.3 Calculate the concentration of *E. coli* (CFU / mL) in the undiluted spiking suspension according to the following equation. Example calculations are provided in **Table 8**, below.

$$EC_{\text{undiluted spike}} = \frac{CFU_1 + CFU_2 + \ldots + CFU_n}{V_1 + V_2 + \ldots + V_n}$$

Where:

$EC_{\text{undiluted spike}}$	=	*E. coli* CFU / mL in undiluted spiking suspension
CFU	=	number of colony forming units from HIA plates yielding counts within the ideal range of 30 to 300 CFU per plate
V	=	volume of undiluted sample in each HIA plate yielding counts within the ideal range of 30 to 300 CFU per plate
n	=	number of plates with counts within the ideal range of 30 to 300 CFU per plate

Table 8. Example Calculations of *E. coli* Spiking Suspension Concentration

Examples	CFU / plate (triplicate analyses) from HIA plates			*E. coli* CFU / mL in undiluted spiking suspension ($EC_{\text{undiluted spike}}$) [a]
	10^{-5} mL plates	10^{-6} mL plates	10^{-7} mL plates	
Example 1	275, 250, 301	30, 10, 5	0, 0, 0	$(275+250+30) / (10^{-5}+10^{-5}+10^{-6}) =$ $555 / (2.1 \times 10^{-5}) = 26{,}428{,}571 =$ **2.6×10^{7} CFU / mL**
Example 2	TNTC, TNTC, TNTC	TNTC, 299, TNTC	12, 109, 32	$(299+109+32) / (10^{-6}+10^{-7}+10^{-7}) =$ $440 / (1.2 \times 10^{-6}) = 366{,}666{,}667 =$ **3.7×10^{8} CFU / mL**

[a] $EC_{\text{undiluted spike}}$ is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

15.7.2 Step 2: Calculate Spiked *E. coli* [CFU / mL or g (wet weight)]

15.7.2.1 The volume of undiluted spiking suspension per unit (mL or g) of spiked biosolid samples ($V_{spiked\ per\ unit\ biosolids}$) is provided in **Table 9**.

Table 9. Volume of Undiluted Spiking Suspension per Unit (mL or g) of Spiked Biosolid Samples ($V_{spiked\ per\ unit\ biosolids}$)

Description of spiked sample	$V_{spiked\ per\ unit\ biosolids}$
Class A liquid	1.0×10^{-5} mL per mL of biosolids
Class A solid	1.0×10^{-4} mL per g of biosolids (wet weight)
Class B liquid	1.0×10^{-2} mL per mL of biosolids
Class B solid	1.0×10^{-1} mL per g of biosolids (wet weight)

15.7.2.2 Calculate concentration of spiked *E. coli* (wet weight) in biosolid sample according to the following equation.

$$\text{Spiked } EC_{wet\ weight} = (EC_{undiluted\ spike}) \times (V_{spiked\ per\ unit\ biosolids})$$

Where:

Spiked $EC_{wet\ weight}$	=	Number of spiked *E. coli* CFU per mL or g of biosolid (wet weight)
$EC_{undiluted\ spike}$	=	*E. coli* CFU / mL in undiluted spiking suspension
$V_{spiked\ per\ unit\ biosolids}$	=	mL of undiluted spiking suspension per mL or g of spiked biosolid

Examples are provided in **Table 10**.

Table 10. Example Calculations of Spiked $EC_{wet\ weight}$

$EC_{undiluted\ spike}$	V_{spiked}	Spiked $EC_{wet\ weight}$
2.6×10^7 CFU / mL	Class A liquid: 1.0×10^{-5} mL per mL of biosolids	$(2.6 \times 10^7$ CFU / mL) x $(1.0 \times 10^{-5}$ mL / mL) = 2.6×10^2 CFU / mL
	Class A solid: 1.0×10^{-4} mL per g of biosolids (wet weight)	$(2.6 \times 10^7$ CFU / mL) x $(1.0 \times 10^{-4}$ mL / g) = 2.6×10^3 CFU / g (wet weight)
	Class B liquid: 1.0×10^{-2} mL per mL of biosolids	$(2.6 \times 10^7$ CFU / mL) x $(1.0 \times 10^{-2}$ mL / mL) = 2.6×10^5 CFU / mL
	Class B solid: 1.0×10^{-1} mL per g of biosolids (wet weight)	$(2.6 \times 10^7$ CFU / mL) x $(1.0 \times 10^{-1}$ mL / g) = 2.6×10^6 CFU / g (wet weight)
3.7×10^8 CFU / mL	Class A liquid: 1.0×10^{-5} mL per mL of biosolids	$(3.7 \times 10^8$ CFU / mL) x $(1.0 \times 10^{-5}$ mL / mL) = 3.7×10^3 CFU / mL
	Class A solid: 1.0×10^{-4} mL per g of biosolids (wet weight)	$(3.7 \times 10^8$ CFU / mL) x $(1.0 \times 10^{-4}$ mL / g) = 3.7×10^4 CFU / g (wet weight)
	Class B liquid: 1.0×10^{-2} mL per mL of biosolids	$(3.7 \times 10^8$ CFU / mL) x $(1.0 \times 10^{-2}$ mL / mL) = $3.7x\ 10^6$ CFU / mL
	Class B solid: 1.0×10^{-1} mL per g of biosolids (wet weight)	$(3.7 \times 10^8$ CFU / mL) x $(1.0 \times 10^{-1}$ mL / g) = 3.7×10^7 CFU / g (wet weight)

15.7.3 Step 3: Convert to "True" Spiked *E. coli* CFU / g Total Solids (dry weight)

Convert to "true" spiked CFU / g total solids (dry weight) as indicated in Section 14.3 using the *E. coli* mL or g (wet weight) from Section 15.7.2 as the numerator in the equation. Examples are provided in **Table 11**.

Table 11. Examples of Conversion to "True" Spiked *E. coli* CFU / g Total Solids (Dry Weight)

Example Total Solids	(CFU / mL or g) / percent total solids =True spiked *E. coli* CFU / g dry weight
Class A liquid: 9%	$2.6 \times 10^2 / 0.09 = 2889 = 2.9 \times 10^3$ CFU / g dry weight
Class A solid: 82%	$2.6 \times 10^3 / 0.82 = 3171 = 3.2 \times 10^3$ CFU / g dry weight
Class B liquid: 4%	$2.6 \times 10^5 / 0.04 = 6{,}500{,}000 = 6.5 \times 10^6$ CFU / g dry weight
Class B solid: 23%	$2.6 \times 10^6 / 0.23 = 11{,}304{,}348 = 1.1 \times 10^7$ CFU / g dry weight
Class A liquid: 7%	$3.7 \times 10^3 / 0.07 = 52{,}857 = 5.3 \times 10^4$ CFU / g dry weight
Class A solid: 88%	$3.7 \times 10^4 / 0.88 = 42{,}045 = 4.2 \times 10^4$ CFU / g dry weight
Class B liquid: 3%	$3.7 \times 10^6 / 0.03 = 123{,}333{,}333 = 1.2 \times 10^8$ CFU / g dry
Class B solid: 40%	$3.7 \times 10^7 / 0.40 = 92{,}500{,}000 = 9.3 \times 10^7$ CFU / g dry weight

15.7.4 Step 4: Calculate Percent Recovery

15.7.4.1 Calculate percent recovery (R) using the following equation:

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where:

R	=	Percent recovery
N_s	=	Fecal coliform MPN / g (dry weight) in the spiked sample
N_u	=	Fecal coliform MPN / g (dry weight) in the unspiked sample
T	=	True spiked *E. coli* CFU / g (dry weight) in spiked sample

15.7.4.2 Example percent recovery calculations are provided in **Table 12**.

Table 12. Example Percent Recovery Calculations

Matrix	N_s	N_u	T	Percent recovery (R)
Class A liquid	2.5×10^3	1.5×10^1	2.9×10^3	$100 \times [(2.5 \times 10^3) - (1.5 \times 10^1)] / 2.9 \times 10^3 =$ 86%
Class A solid	3.9×10^3	5.0×10^2	3.2×10^3	$100 \times [(3.9 \times 10^3) - (5.0 \times 10^2)] / 3.2 \times 10^3 =$ 107%
Class B liquid	1.4×10^8	1.7×10^6	1.2×10^8	$100 \times [(1.4 \times 10^8) - (1.7 \times 10^6)] / 1.2 \times 10^8 =$ 115%
Class B solid	8.3×10^6	8.0×10^5	1.1×10^7	$100 \times [(8.3 \times 10^6) - (8.0 \times 10^5)] / 1.1 \times 10^7 =$ 68%

16.0 Method Performance

16.1 Interlaboratory validation of Method 1681

16.1.1 Twelve volunteer laboratories and a referee laboratory participated in the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study of EPA Method 1681. The purposes of the study were to characterize method performance across multiple laboratories and multiple biosolid matrices and to develop quantitative quality control (QC) acceptance criteria. A detailed description of the of the study and results are provided in the validation study report (Reference 19.2). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with study-specific instructions and the October 2002 Draft version of EPA Method 1681.

16.1.2 Recovery - Method 1681 mean recoveries of fecal coliforms from Class A matrices, compost, and thermophilically digested biosolids, spiked with laboratory-prepared spikes were 91% and 71%, respectively. Median recoveries of fecal coliforms from compost, thermophilically digested biosolids, spiked with laboratory-prepared spikes were 46% and 52% , respectively. For Milorganite® (a heat-dried, Class A biosolid that was used

as the reference matrix) samples spiked with laboratory spiking suspensions, the mean recovery was 53%, with a median percent recovery of 29%. Mean recoveries of fecal coliforms from Class B matrices, aerobically and anaerobically digested biosolids, spiked with laboratory-prepared spikes were 660% and 140%, respectively. Median recoveries of fecal coliforms from aerobically and anaerobically digested biosolids, spiked with laboratory-prepared spikes were 64% and 22%, respectively.

16.1.3 Precision - Method 1681 was characterized by high variability. For percent recovery, the overall relative standard deviations (RSDs) ranged from 81% to 250% for Class A matrices and from 210% to 340% for Class B matrices for EPA-prepared spikes. For ambient (unspiked) fecal coliform results, the overall RSDs ranged from 37% to 300% for Class A matrices and from 100% to 170% for Class B matrices.

16.1.4 False positive rates - Method 1681 false positive rates were relatively high for both thermophilically digested (54%) and composted (73%) matrices. False positive rates for Milorganite® could not be accurately assessed during the study because no positive tubes were observed during the study. False positive rates for aerobically digested and anaerobically digested samples were relatively low compared to Class A rates and ranged from 5% to 9%.

16.1.5 False negative rates - Method 1681 false negative rates for Class A were relatively low compared to Class B rates and ranged from 0% to 9%. False negative rates for aerobically digested and anaerobically digested matrices were 23% and 18%, respectively.

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.

17.2 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 USEPA. 2004. *Results of the Interlaboratory Validation of EPA Method 1681 (A-1) for Fecal Coliforms in Biosolids*. EPA-821-R-04-009. December 2004.

19.3 American Chemical Society (ACS). 2000. *Reagent Chemicals, American Chemical Society Specifications.* American Chemical Society, New York. For suggestions of the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals*, BDH, Poole, Dorset, UK and the United States Pharmacopeia.

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 Klee, A. J. 1993. A computer program for the determination of the most probable number and its confidence limits. *Journal of Microbiological Methods.* 18:91-98.

20.0 Figures

20.1 The following pages contain flow charts of dilution and inoculation schemes (Section 11.0) and for the procedures (Section 12.0). Schemes for dilution and inoculation are dependent on Class (A or B) and matrix (solid or liquid).

FIGURE 1. MULTIPLE TUBE FERMENTATION PROCEDURE

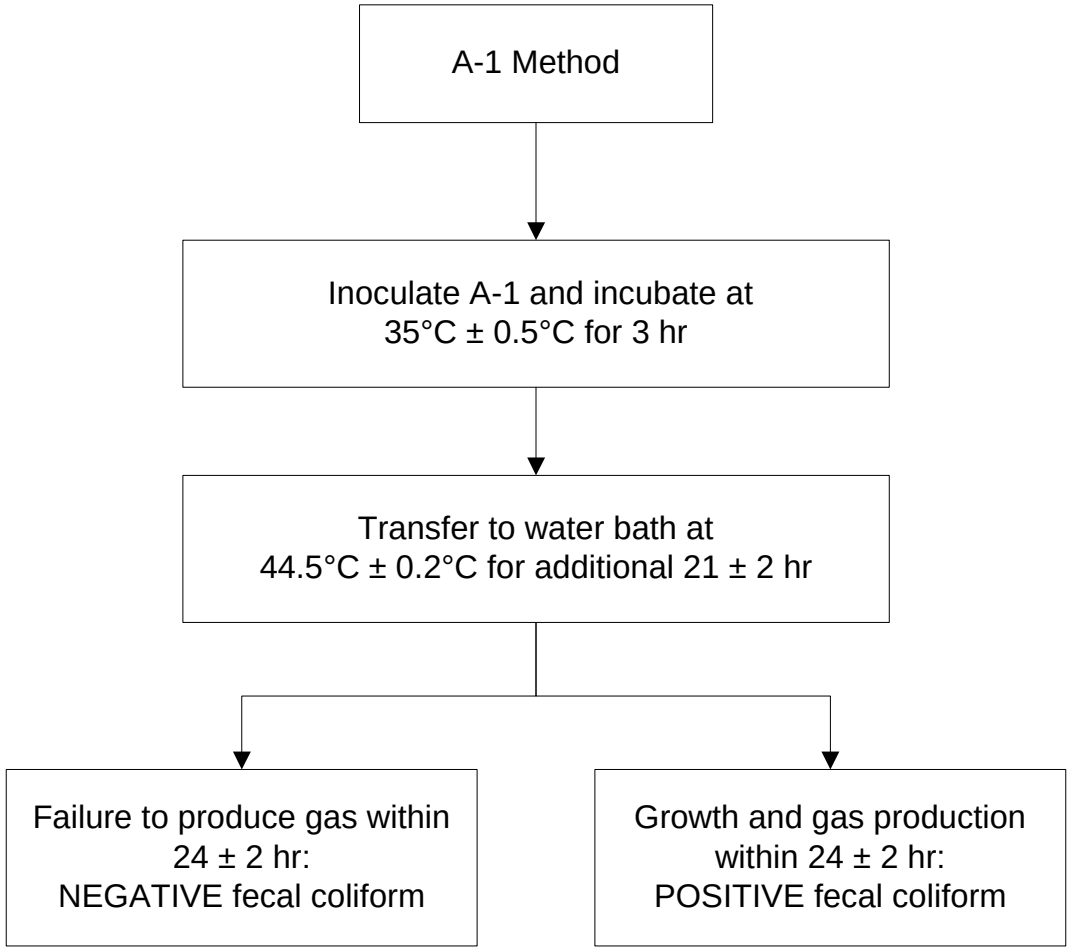


FIGURE 2. CLASS A LIQUID SAMPLE DILUTION AND INOCULATION SCHEME

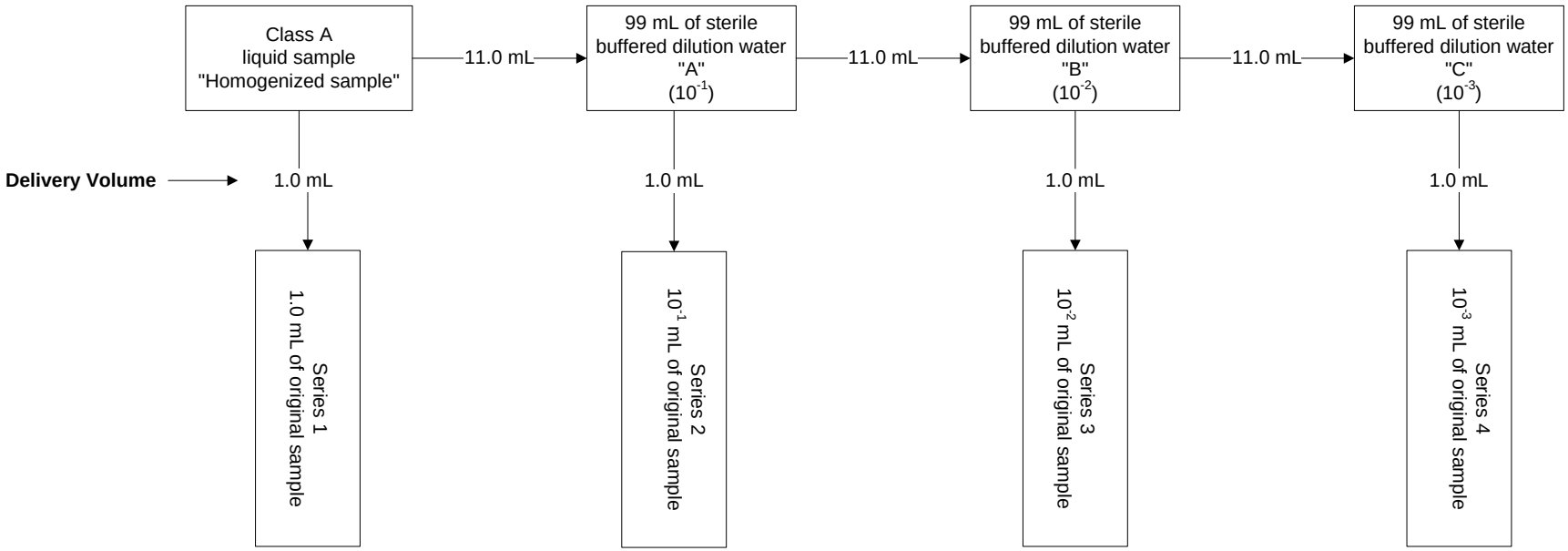


FIGURE 3. CLASS A SOLID SAMPLE DILUTION AND INOCULATION SCHEME

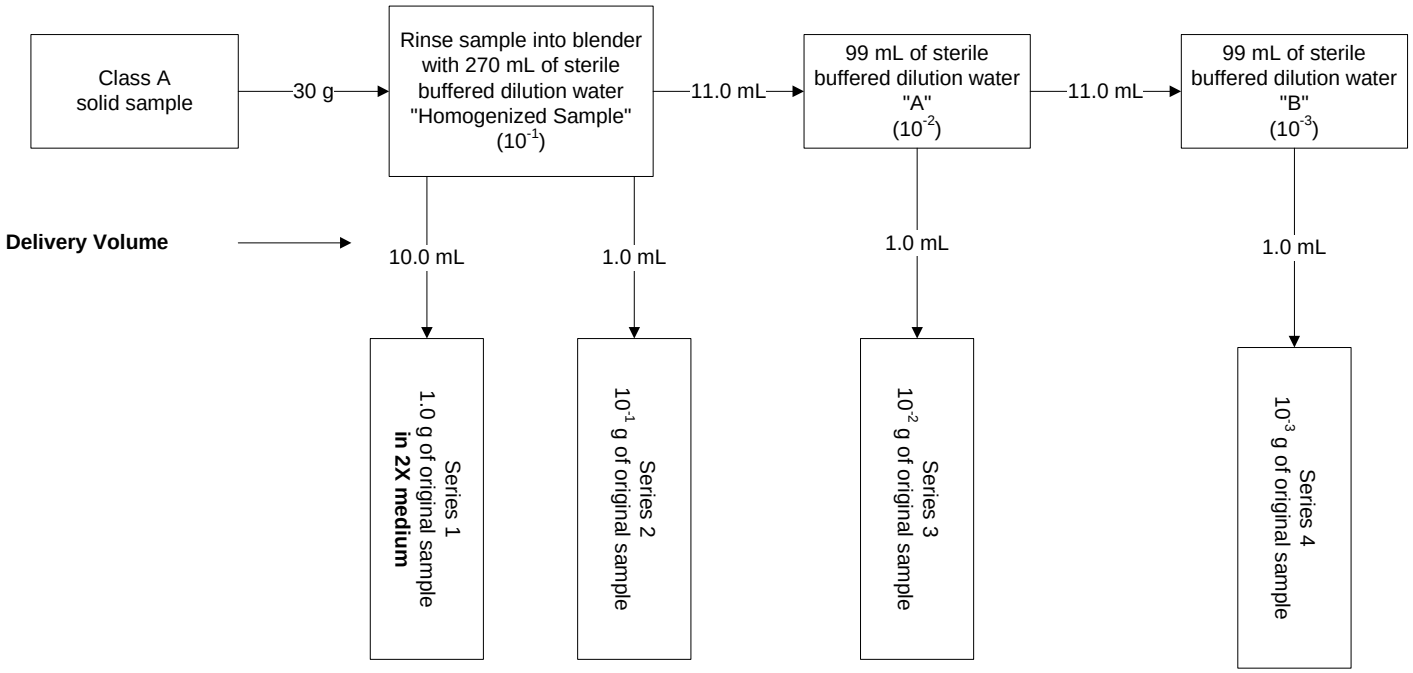


FIGURE 4. CLASS B LIQUID SAMPLE DILUTION AND INOCULATION SCHEME

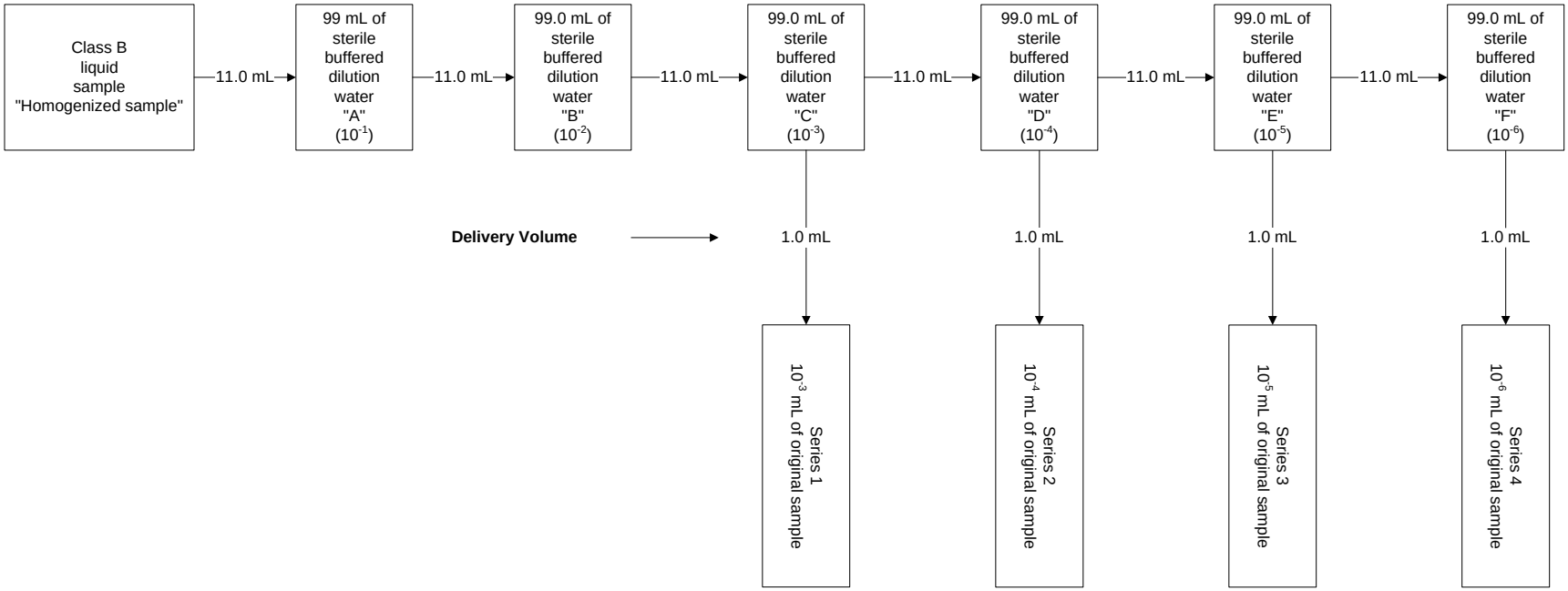
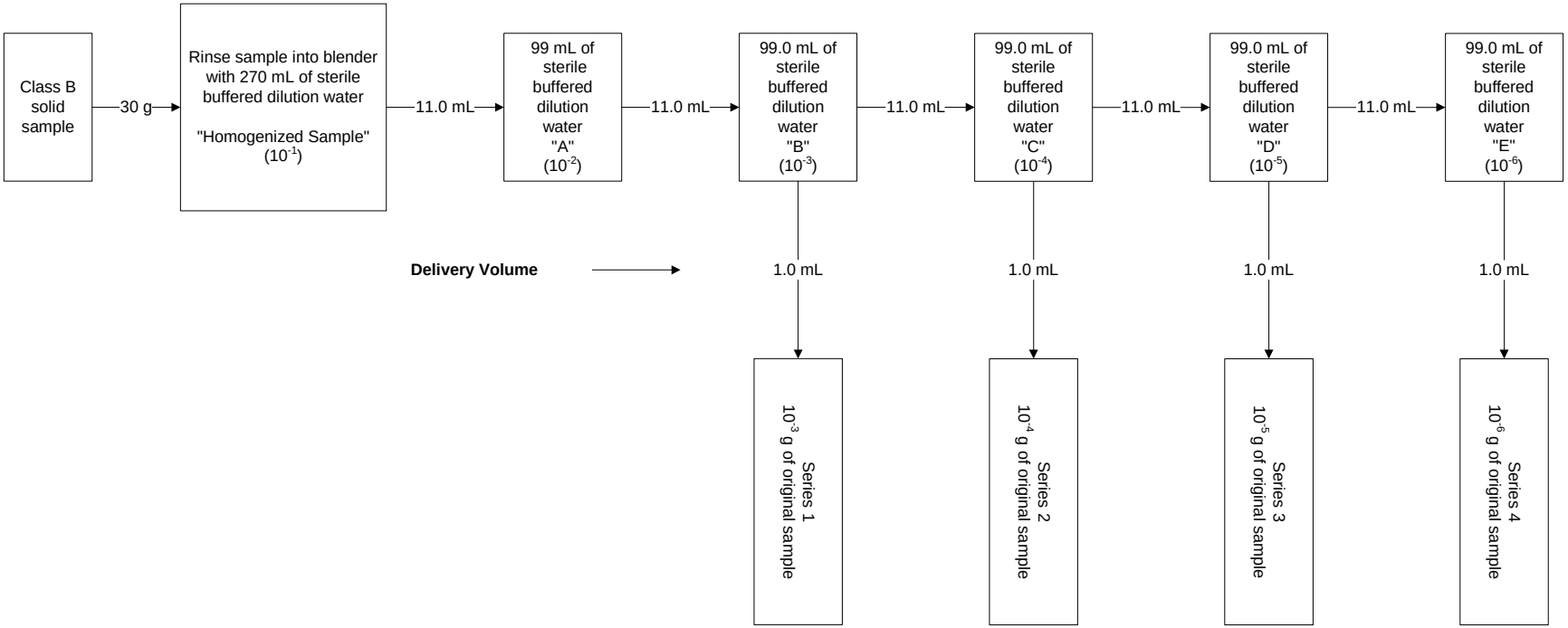


FIGURE 5. CLASS B SOLID SAMPLE DILUTION AND INOCULATION SCHEME



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

EPA	United States Environmental Protection Agency
EC	*Escherichia coli*
g	gram
L	liter
LTB	lauryl tryptose broth
mg	milligram
mL	milliliter
mm	millimeter
MPN	most probable number
NIST	National Institute of Standards and Technology
QA	quality assurance
QC	quality control
TD	to deliver

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

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

Enrichment—A non-selective culture media for enhanced growth.

Liquid samples—Generally defined as samples containing ≤7% 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.

Method blank—An aliquot of sterile reagent water that is treated exactly as a sample including exposure to all glassware, equipment, media, procedures that are used with samples. The method blank is used to verify the sterility of equipment, materials, and supplies.

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.

Negative control—A control culture that, when analyzed exactly like a field sample, will produce a known negative result for a given type of media.

Positive control—A control culture that, when analyzed exactly like a field sample, will produce a known positive result for a given type of media.

Preparation blank—See Method blank.

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, samples containing >7 % total solids (dry weight).