Method 1682: *Salmonella* in Sewage Sludge (Biosolids) by Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium

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Introduction

Application of treated biosolids to land can be helpful as a crop nutrient and soil conditioner but may pose the risk of releasing pathogens into the environment if proper disinfection and use criteria are not met. Among these organisms are *Salmonella*, which are pathogenic enteric bacteria that can cause salmonellosis in animals and humans, if concentrations able to give rise to infections are present.

The density of *Salmonella* in Class A biosolids for unrestricted use is to be less than three most probable number (MPN) per four grams of total solids (dry weight basis) at the time the biosolids are used or disposed.

Method 1682 is a performance-based method for detecting *Salmonella* in biosolids. Method 1682 requires calculation of the MPN via enrichment, with selection and biochemical confirmation for determination of *Salmonella*. The enrichment step utilizes tryptic soy broth (TSB). After incubation, TSB is spotted onto selective modified semisolid Rappaport-Vassiliadis (MSRV) medium. Presumptively identified colonies are isolated on xylose-lysine desoxycholate agar (XLD). Biochemical confirmation includes lysine-iron agar (LIA), triple sugar iron agar (TSI), and urea broth, followed by serological typing using polyvalent O antisera. Calculations for concentration are based on dry weight.

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1.0 Scope and Application

1.1 This method is for the detection and enumeration of *Salmonella* (CAS registry number 68583-35-7) in treated biosolids by enrichment, selection, and characterization. It is intended to enumerate *Salmonella* to help determine the suitability of biosolids for land application in compliance with 40 Code of Federal Regulations (CFR) Part 503. Although Method 1682 is similar to existing recognized procedures using separate media for enrichment, selection, and confirmation of the organism, it is intended to be more specific and have greater recovery.

1.2 This method is designed to meet monitoring requirements for *Salmonella* under 40 CFR Part 503 Subpart D. Subpart D of the Part 503 regulation defines the requirements for biosolids to be classified as either Class A or B with respect to pathogens. Classification of biosolids prior to land application provides a means to protect public health and the environment. Following appropriate treatment, a biosolid sample is classified as Class A if *Salmonella* densities are below 3 MPN / 4 grams of total solids (dry weight basis).

1.3 Although the Part 503 regulation does not specify the total number of samples for Class A biosolids, it suggests 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 3 MPN / 4 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.

1.4 Although Method 1682 is selective for *Salmonella* bacteria, it does not differentiate among *Salmonella* species.

Note: H2S negative *Salmonella* will be missed if translucent pink to red colonies are not submitted to biochemical and serological confirmation.

1.5 Method 1682 was submitted to interlaboratory validation in Class A biosolid matrices. A comprehensive evaluation of the study results is presented in the validation study report (Reference 18.2). For method application please refer to Title 40 Code of Federal Regulations Part 136 (40 CFR Part 136).

1.6 This method is not intended for use in water samples or as a test for microorganisms other than *Salmonella*. Use of this method and appropriate validation for matrices other than Class A biosolids is the responsibility of the user.

1.7 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.

2.0 Summary of Method

2.1 The modified semisolid Rappaport-Vassiliadis (MSRV) medium protocol presented in Method 1682 provides enumeration of *Salmonella* in biosolids based on the most probable number (MPN) technique. The determination of *Salmonella* involves inoculating the enrichment medium, tryptic soy broth (TSB), with a measured amount of sample and incubating for 24 hours. After incubation, TSB is spotted onto the selective MSRV medium. The MSRV medium uses novobiocin and malachite green to inhibit non-*Salmonella* species, while allowing most *Salmonella* species to grow. Presumptively identified colonies are isolated on xylose-lysine desoxycholate agar (XLD), and confirmed using lysine-iron agar (LIA), triple sugar iron agar (TSI), and urea broth, followed by positive serological typing using polyvalent O antisera. A total solids (% dry weight) determination is performed on a representative biosolids sample and is used to calculate MPN / g dry weight. *Salmonella* density is reported as MPN / 4 g dry weight.

3.0 Definitions

3.1 *Salmonella* are gram-negative, predominately motile, facultatively-anaerobic, rod-shaped bacteria that comprise about 2,000 serovars.

3.2 Class A biosolids are biosolids that meet bacteriological and treatment requirements stipulated in the 40 CFR 503 Subpart D.

3.3 Definitions for other terms are provided in the glossary at the end of the method (Section 20.0).

4.0 Interferences

4.1 Low estimates of *Salmonella* may be caused by the presence of high numbers of competing or inhibitory organisms, or toxic substances such as metals or organic compounds.

5.0 Safety

5.1 The analyst must know and observe normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of media, cultures, reagents, and materials, including operation of 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 This method does not address all safety issues associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in Method 1682 analyses.

5.4 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 Tubes, 25 × 150 mm, borosilicate glass, with loose-fitting aluminum, stainless steel or autoclavable caps

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

6.15 Test tube racks to hold sterile culture tubes

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

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

6.18 Pipet bulbs, or automatic pipettor

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

6.20 Sterile disposable applicator sticks

6.21 Bunsen burner or alcohol burner

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

6.23 Media dispensing pump

6.24 Incubators, water- or air-jacketed, humidity-controlled, microbiological type to hold temperatures at 36°C ± 1.5°C and 42°C ± 0.5°C

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

6.26 Glass slides for agglutination test

6.27 Erlenmeyer flasks, 1-L and 2-L

6.28 Stir bar

6.29 Stir plate

6.30 Sterile blender jars and base

6.31 Waterbath maintained at 50°C for tempering agar

6.32 Media filtration equipment, sterile, 0.22-µm pore size syringe filters

6.33 Magnifying glass or dissection scope

6.34 Latex gloves for handling samples and extraction equipment

6.35 pH meter

6.36 Vortex mixer

6.37 Micro pipettor

6.38 Pipet tips to deliver 30 µL

6.39 Autoclave

6.40 Drying oven, maintained at 103°C to 105°C for tempering agar

6.41 Beakers, glass or plastic, assorted sizes

6.42 Lint-free tissues

6.43 Steel pan of water, 30" x 26" x 10"

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

6.45 Crucible or aluminum evaporating dish

7.0 Reagents and Standards

7.1 Purity of reagents: Reagent-grade chemicals must 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 18.4). The agar used for preparation of culture media must be microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control. Storage temperatures and times for prepared media and reagents are provided in **Table 1** in Section 7.16 below.

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 18.1).

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 Sterile physiological saline (0.85% w/v)

7.5.1 Dissolve 8.5 g NaCl in 1 L reagent-grade water. Dispense 5-10 mL into 16 × 100 mm screw cap test tubes, cap and autoclave for 15 minutes at 121°C (15 PSI). Store at room temperature.

7.6 Tryptic soy broth (TSB)

7.6.1 Composition:

Pancreatic digest of casein	17.0 g
Enzymatic digest of soybean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate (K_2HPO_4)	2.5 g
Dextrose	2.5 g
Reagent-grade water	1.0 L

7.6.2 For single strength (1X) TSB, add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve. Adjust pH to 7.3 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Dispense 10 mL volumes into 25 × 150 mm culture tubes. The 1X TSB will be used for inoculation volumes ≤1 mL. Autoclave for 15 minutes at 121°C (15 PSI).

7.6.3 For triple strength (3X) TSB, prepare as in Section 7.6.2 but use 333 mL of reagent-grade water instead of 1 L. Dispense 10 mL and 5 mL volumes into 25 × 150 mm culture tubes. The 3X TSB tubes containing 10 mL of media will be inoculated with 20 mL of homogenized sample. The 3X TSB tubes containing 5 mL of media will be inoculated with 10 mL of homogenized sample. Autoclave for 15 minutes at 121°C (15 PSI). Let the media warm to room temperature prior to analysis.

Note: 3X TSB is necessary for 20- and 10-mL inoculations to ensure that the inoculation volume does not excessively dilute the media.

7.7 Modified Semisolid Rappaport-Vassiliadis (MSRV) medium

7.7.1 Basal medium composition:

Tryptose	4.59 g
Casein hydrolysate (acid)	4.59 g
Sodium chloride	7.34 g
Monopotassium phosphate (KH_2PO_4)	1.47 g
Magnesium chloride, anhydrous ($MgCl_2$)	10.93 g
Malachite green oxalate	0.037 g (37 mg)
Agar	2.7 g
Reagent-grade water	1.0 L

7.7.2 Novobiocin (2%) stock solution:

Sodium novobiocin	500 mg
Reagent-grade water	25 mL

7.7.2.1 Dissolve 500 mg of sodium novobiocin into 25 mL of reagent-grade water and filter sterilize by passing solution through a sterile, 0.22µm pore-size filter into a sterile container. Aliquot 1.1 mL of the stock solution into 2.0 mL cryovials and freeze at -20°C.

7.7.3 Add reagents for basal medium to 1 L of reagent-grade water, mix thoroughly, and heat to boiling to dissolve completely (*do not autoclave*). Adjust pH to 5.2 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, cool to 50°C, and add 1.0 mL of a 2% stock solution of novobiocin per liter of medium. Mix well by swirling the medium. Immediately pour approximately 25 mL into 15 × 100 mm petri plates. Do not invert plates to store.

Note: If using a commercially prepared novobiocin antimicrobic supplement add sufficient volume to achieve a concentration of 0.002% per liter.

7.8 Xylose-lysine desoxycholate agar (XLD)

7.8.1 Composition:

Yeast extract	3.0 g
L-lysine	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Saccharose	7.5 g
Sodium desoxycholate	2.5 g
Ferric ammonium citrate	0.8 g
Sodium thiosulfate	6.8 g
Sodium chloride	5.0 g
Agar	15.0 g
Phenol red	0.08 g
Reagent-grade water	1.0 L

7.8.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to boiling to dissolve completely, avoid overheating (*do not autoclave*). Adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Cool to 45°C - 50°C and immediately pour approximately 12 mL into 15 × 100 mm sterile petri plates. Let the media warm to room temperature prior to inoculation.

Note: Heating media to boiling sterilizes the media, overheating or autoclaving may cause precipitation.

7.9 Triple sugar iron agar (TSI)

7.9.1 Composition:

Beef extract	3.0 g
Yeast extract	3.0 g
Pancreatic digest of casein	15.0 g
Proteose peptone No. 3	5.0 g
Dextrose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
Ferrous sulfate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulfate	0.3 g
Agar	12.0 g
Phenol red	0.024 g
Reagent-grade water	1.0 L

7.9.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Dispense 5-7 mL aliquots into 16 × 100 mm screw cap test tubes, cap and autoclave at 121°C (15 PSI) for 15 minutes. Allow medium to solidify in a slant rack or rack that is tilted in such a manner that the surface area is equally divided between the slant and butt. Let the media warm to room temperature prior to inoculation.

7.10 Lysine iron agar (LIA)

7.10.1 Composition:

Peptone	5.0 g
Yeast extract	3.0 g
Dextrose	1.0 g
L-lysine hydrochloride	10.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.04 g
Bromcresol purple	0.02 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.10.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Adjust pH to 6.7 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Dispense 5-7 mL aliquots into 16 × 100 mm screw cap test tubes, cap and autoclave at 121°C (15 PSI) for 12 minutes. Allow medium to solidify in a slant rack or rack that is tilted in such a manner that the surface area is equally divided between the slant and butt. Let the media warm to room temperature prior to inoculation.

7.11 Urea broth

7.11.1 Composition:

Yeast extract	0.1 g
Monopotassium phosphate (KH_2PO_4)	9.1 g
Dipotassium phosphate (K_2HPO_4)	9.5 g
Urea	20.0 g
Phenol red	0.01 g
Reagent-grade water	1.0 L

7.11.2 Add reagents to 1 L of reagent-grade water, mix thoroughly to dissolve (*do not boil or autoclave*). Adjust pH to 6.8 ± 0.1 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide. Filter sterilize by passing solution through a sterile, 0.22 µm filter into a sterile flask. Aseptically dispense 3 mL into sterile 16 × 100 mm screw cap test tubes using a sterile pipet or sterile dispensing syringe. Let the media warm to room temperature prior to inoculation.

7.12 Heart infusion agar (HIA)

7.12.1 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

7.12.2 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 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.

7.13 *Salmonella* O antiserum Polyvalent Groups A-I and Vi

7.14 Positive controls

7.14.1 Obtain a stock culture of *Salmonella typhimurium* ATCC # 14028 as a positive control for MSRV, XLD, TSI, LIA, and polyvalent O antiserum. *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.

7.14.2 Obtain a stock culture of *Proteus vulgaris* ATCC # 13315 as a positive control for urease.

7.15 Negative controls

7.15.1 Obtain a stock culture of *Escherichia coli* ATCC # 25922 as a negative control for MSRV, XLD, TSI, LIA, and polyvalent O antiserum.

7.15.2 Obtain a stock culture of *Salmonella typhimurium* ATCC # 14028 as a negative control for urease.

7.16 Storage temperatures and times for prepared media and reagents are provided in Table 1, below:

Table 1. Storage Temperatures and Times for Prepared Media and Reagents [1]

Media	Storage Temperature	Storage Time
Sterile physiological saline (0.85% w/v)	room temperature	≤3 months
TSB: loose-capped tubes	room temperature	≤2 weeks
MSRV: poured-plates (do not store inverted)	room temperature	≤48 hours
XLD: poured plates (store inverted)	1°C to 5°C	≤2 weeks
TSI, LIA, Urea broth: tight-capped tubes	1°C to 5°C	≤3 months
HIA: poured plates (store inverted)	1°C to 5°C	≤2 weeks
2% novobiocin	-20°C to -10°C	≤1 year
Polyvalent O antiserum	2° to 8°C lyophilized	≤3 years

[1] If media is refrigerated, remove from refrigerator 1-1.5 hours prior to inoculation to ensure that it reaches room temperature prior to use.

7.17 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 sample collection 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-gallon 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 with the auger 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: 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 <10°C until analysis. Samples must not be frozen. Sample analysis must begin within 6 hours unless otherwise specified in the Code of Federal Regulations Part 503.

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 Method 1682 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. General requirements and recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 18.3.

9.2 The minimum analytical QC requirements for the analysis of samples using Method 1682 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), 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 either laboratory-prepared spiking suspensions or BioBalls as described in Section 14.0.

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 *Salmonella typhimurium* ATCC # 14028 according to the spiking procedure in Section 14.0. Spiking with laboratory-prepared suspensions is described in Section 14.3 and spiking with BioBalls is described in Section 14.6. Process and analyze each IPR sample according to the procedures in Sections 11 and 12 and calculate the *Salmonella* MPN / 4 g dry weight according to Section 13.0.

9.3.2 Calculate the percent recovery (R) for each IPR sample using the appropriate equation in Sections 14.5 or 14.8 for laboratory-prepared and BioBall™ spikes, respectively.

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**. If the mean and RSD for recover of *Salmonella* 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	BioBall™ acceptance criteria	Lab-prepared spike acceptance criteria
Initial precision and recovery (IPR)		
• Mean percent recovery	22% - 126%	0% - 254%
• Precision (as maximum relative standard deviation)	69%	92%
Ongoing precision and recovery (OPR) as percent recovery	1% - 147%	0% - 287%

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 *Salmonella typhimurium* ATCC # 14028 according to the spiking procedure in Section 14.0. Spiking with laboratory-prepared suspensions is described in Section 14.3 and spiking with BioBalls is described in Section 14.6. Process and analyze each OPR sample according to the procedures in Sections 11.0 and 12.0 and calculate the number of *Salmonella* MPN / 4 g dry weight according to Section 13.0.

9.4.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equation in Section 14.5 or 14.8 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

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 1682 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 *Salmonella* 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), using the same spiking procedure (laboratory-prepared spiking suspension or BioBalls). 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 *Salmonella* for calculating MS recoveries. The other sample will serve as the MS sample and will be spiked with *Salmonella typhimurium* ATCC # 14028 according to the spiking procedure in Section 14.0.

9.5.2 Select dilutions based on previous analytical results or anticipated levels of *Salmonella* in the field sample in order to accurately estimate *Salmonella* density. Neither above or below the detection limit of the method.

9.5.3 Spike the MS sample with a laboratory-prepared suspension as described in Section 14.3 or with BioBalls as described in Section 14.6. 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 *Salmonella* MPN / 4 g dry weight according to Section 13.0 and adjust the density (MPN / 4 g dry weight) based on the ambient concentration of *Salmonella* observed in the unspiked matrix sample.

9.5.5 Calculate the percent recovery (R) for the MS sample (adjust based on ambient *Salmonella* in the unspiked sample) using the appropriate equations Section 14.5 or 14.8 for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.

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	BioBall™ acceptance criteria	Lab-prepared acceptance criteria
Percent recovery for MS or MS/MSD	0% - 158%	0% - 246%
Precision (as maximum relative percent difference of MS/MSD)	177%	172%

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 1682. 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 MSRV, XLD, TSI, LIA, urea broth, and polyvalent O antiserum are performing properly. Negative controls should be analyzed whenever a new batch of media or reagent is used. On an ongoing basis, the laboratory should perform a negative control every day that samples are analyzed. Positive and negative control results are provided in **Table 4**.

9.6.1.1 Negative controls are conducted by inoculating MSRV, XLD, TSI, LIA, and polyvalent O antiserum with a known negative control (e.g., *E. coli* ATCC # 25922) and analyzing as described in Section 12.0.

9.6.1.2 Negative controls are conducted by inoculating urea broth with a known negative control (e.g., *Salmonella typhimurium* ATCC # 14028) and analyzing as described in Section 12.0.

9.6.1.3 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 MSRV, XLD, TSI, LIA, urea broth, and polyvalent O antiserum are performing properly. Positive controls should be analyzed whenever a new batch of media or reagent 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. Positive and negative control results are provided in Table 4.

9.6.2.1 Positive controls are conducted by inoculating MSRV, XLD, TSI, LIA, and polyvalent O antiserum with a known positive culture (e.g., *Salmonella typhimurium* ATCC # 14028) and analyzing as described in Section 12.0.

9.6.2.2 Positive controls are conducted by inoculating urea broth with a known positive culture (e.g., *Proteus vulgaris* ATCC # 13315) and analyzing as described in Section 12.0.

9.6.2.3 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, incubate a representative portion of each batch at 36°C ± 1.5°C (TSB, XLD, TSI, LIA, HIA, and urea broth) or 42°C ± 0.5°C (MSRV) for 24 ± 2 hours and observe for growth. 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.

Table 4. Positive and Negative Control Results

Medium	*Salmonella* result	Positive Method 1682 reaction	Negative Method 1682 reaction
Tryptic Soy Broth (TSB)	Positive	Turbidity	No turbidity
Modified Semisolid Rappaport-Vassiliadis (MSRV) medium	Positive	Migrated cells visible as a gray-white turbid zone (halo) extending out from inoculations	Medium remains blue-green around inoculations with no gray-white turbid zone (halo) (*E. coli* has marked inhibition)
Xylose lysine desoxycholate agar (XLD)	Positive	Pink to red colonies with black centers	Other colors with or without black centers (e.g. *E. coli* is yellow without black center)
Triple sugar iron agar (TSI)	Positive	Good growth with alkaline slant (red) with acid butt (yellow) with or without H_2S production (which may result in a black butt)	Other color combinations (e.g., *E. coli* is yellow slant and butt)
Lysine iron agar (LIA)	Positive	Alkaline slant (purple) with alkaline butt (purple) with or without H_2S production (which may result in a black butt)	Other color combinatiions (e.g., *E. coli* is red to red/purple slant and butt without H_2S production)
Urea broth	Negative	Pink	No color change (*Salmonella* is urease negative)
Polyvalent O	Positive	Agglutination	No agglutination

10.0 Equipment Calibration and Standardization

10.1 Check temperatures in incubators/waterbaths twice daily, a minimum of four hours apart, to ensure operation is within stated limits of the method and record daily measurements in 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 semiannually against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23 (Reference 18.1). Check mercury columns for breaks.

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

10.5 Calibrate top-loading balances once per month 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 pH do not exceed the homogenized sample volume by greater than 5% (15 mL).

11.1.2 Solid samples: Weigh out 30.0 g ± 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. Use 270 mL of sterile buffered dilution water (Section 7.4) to rinse any remaining sample into the blender. Alternatively, the sample may be directly weighed in the sterile blender jar. Cover and blend on high speed for one to two minutes. This is the "homogenized" sample. A volume of 10-mL of the "homogenized" sample contains 1.0 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. When adjusting pH do not exceed the homogenized sample volume by greater than 5% (15 mL).

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 or BioBalls 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 Inoculation

Media inoculation procedures are based on whether the original sample was a liquid or a solid. 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 Liquid samples: For unspiked and spiked samples, three series of five tubes will be used for the analysis with 20-, 10-, and 1.0-mL of the original sample. See **Figure 1** in Section 19.0 for an overview of the inoculation scheme.

11.2.1.1 Inoculation

(A) Use a sterile pipette to inoculate each of the first series of five tubes (containing 10 mL of 3X TSB) with 20.0 mL of the original "homogenized" sample per tube.

(B) Use a sterile pipette to inoculate each of the second series of tubes (containing 5 mL of 3X TSB) with 10.0 mL of the original "homogenized" sample per tube.

(C) Use a sterile pipette to inoculate each of the third series of tubes (containing 10 mL of 1X TSB) with 1.0 mL of the original "homogenized" sample per tube.

11.2.1.2 Repeat Section 11.2.1.1 for the remaining liquid samples. When inoculations are complete, go to Section 12.0 to continue the analyses.

11.2.2 Solid samples: For unspiked and spiked samples, three series of five tubes will be used for the analysis with 2.0-, 1.0-, and 0.1-g of the original sample (20.0, 10.0, and 1.0 mL of the homogenized sample). The first two series of tubes must contain 3X TSB. See **Figure 2** in Section 19.0 for an overview of the inoculation scheme.

11.2.2.1 Inoculation

(A) Use a sterile pipette to inoculate each of the first series of tubes (containing 10 mL of 3X TSB) with 20.0 mL of the "homogenized" sample per tube. This is 2.0 g of the original sample. 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 (containing 5 mL of 3X TSB) with 10.0 mL of the "homogenized" sample per tube. This is 1.0 g of the original sample. Solids that will not separate easily and/or may float should be submerged into the broth with a sterile loop.

(C) Use a sterile pipette to inoculate each of the third series of tubes (containing 10 mL of 1X TSB) with 1.0 mL of the "homogenized" sample per tube. This is 0.1 g of the original sample.

11.2.2.2 Repeat Section 11.2.2.1 for remaining solid samples. When inoculations are complete, go to Section 12.0 to continue the analysis. *Note*: For regulatory purposes, *Salmonella* monitoring is required only for Class A biosolids in 40 CFR Part 503. If this procedure is being used to enumerate *Salmonella* in samples other than Class A biosolid samples, additional dilutions may be required prior to analysis. When attempting to quantify samples containing higher *Salmonella* concentrations than can be evaluated using the 20-10-1 scheme, it may be necessary to evaluate additional dilution volumes of 0.1-, 0.01-, and 0.001-mL. The MPN table in *Standard Methods* would then be used, instead of **Table 5** in Section 13.0.

Although other dilution and inoculation schemes may be used for the analysis of samples with higher *Salmonella* concentrations, 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.

12.0 Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium Procedure

12.1 In this protocol, the modified semisolid Rappaport-Vassiliadis (MSRV) medium MPN technique is used to determine *Salmonella* densities in Class A biosolid samples. Although the Part 503 regulation does not specify the total number of samples for Class A biosolids, it suggests 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 3 MPN / 4 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. The precision of the test increases with increasing numbers of replicates per sample tested. For an overview of the MPN procedure refer to **Figure 3** in Section 19.0.

12.2 Enrichment phase

12.2.1 Prepare TSB media and dispense into 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.

12.2.2 For each sample, arrange test tubes in three rows of five tubes each. When 20 mL of homogenized sample is inoculated, tubes should contain 10 mL of 3X TSB media, when 10 mL of homogenized sample is inoculated, tubes should contain 5 mL of 3X TSB media, and when 1.0 mL of homogenized sample is inoculated, tubes should contain 10 mL of 1X TSB media. *Note:* 3X TSB is necessary for 20- and 10-mL inoculation volumes, to ensure that the TSB is not excessively diluted.

12.2.3 Inoculate samples according to Section 11.2, based on whether the original sample was liquid or solid.

12.2.4 Incubate the TSB tubes and controls for 24 ± 2 hours at 36°C ± 1.5°C.

12.2.5 Record all turbid tubes as positive. Because of the non-inhibitory nature of the enrichment medium, all tubes will be positive in most instances. If none of the tubes appear to be positive, then this may indicate the presence of a toxic substance or that the tubes were not inoculated.

12.3 Selection phase

12.3.1 Apply six discrete, 30-µL drops from each TSB tube onto a corresponding MSRV plate that has been labeled with sample ID, date, and original inoculation volume (e.g., 20.0, 10.0, or 1.0 mL). Space the drops evenly over the entire plate. In addition, inoculate an MSRV plate with positive and negative controls. **Do not invert the plates**. Allow the drops to absorb into the agar for approximately 1 hour at room temperature and incubate plates at 42°C ± 0.5°C for 16 to 18 hours in a humidity-controlled hot air incubator. If a humidity-controlled hot air incubator is not available, an open pan of water placed in the bottom of the incubator will suffice.

12.3.2 Examine plates for the appearance of motility surrounding the inoculations, as evidenced by a "whitish halo" of growth approximately 2 cm from the center of the spot.

12.3.3 Using a sterile inoculating loop, stab into a halo from the outer edge of a target colony on the MSRV plate and streak onto an XLD plate. Since *Salmonella* are predominately located within the MSRV media, the loop should penetrate the MSRV at least half-way. Repeat this step using another target colony from the MSRV plate. In addition, inoculate XLD positive and negative controls. (See **Photo 1**)

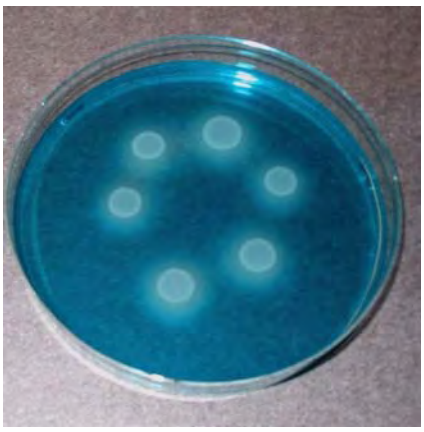


Photo 1. *Salmonella spp*. produce halos indicating motility on MSRV plates.

12.3.4 Incubate XLD plates for 18 to 24 hours at 36°C ± 1.5°C. After incubation, refrigerate one of the XLD plates at 1°C to 5°C and submit the other plate to biochemical confirmation. (If issues arise in subsequent steps of the method, the laboratory may wish to return to the refrigerated XLD plate.) *Note:* XLD plates may be refrigerated over the weekend prior to submitting to biochemical confirmation.

12.3.5 Black and pink to red colonies with black centers are considered *Salmonella.* (See **Photo 2**)

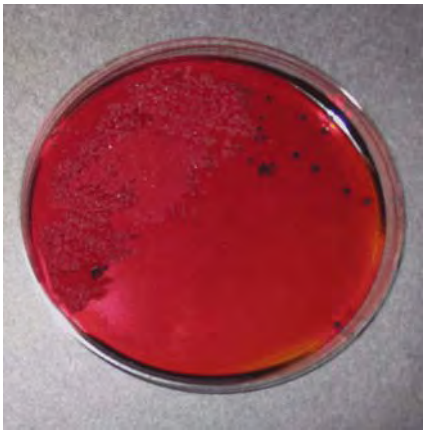


Photo 2. *Salmonella spp*. produce pink to red colonies with black centers on XLD plates.

12.4 Biochemical confirmation phase

12.4.1 Label all tubes with inoculation date, sample identification, and original inoculation volume (e.g., 20.0, 10.0, or 1.0 mL). Pick isolated colonies exhibiting *Salmonella* morphology (pink to red colonies with black centers) and inoculate triple sugar iron agar (TSI) slants, lysine iron agar (LIA) slants, and urea broth. Inoculate slants by stabbing the butt and streaking the slant. Use the same XLD colony to inoculate all three media. This will require going back to the same XLD colony multiple times to ensure sufficient inoculum for each medium. In addition, inoculate each medium with the appropriate positive and negative controls. Incubate for 24 ± 2 hours at 36°C ± 1.5°C. If only atypical colony morphology is observed on XLD plates, pick from an atypical colony and inoculate TSI slants, LIA slants, and urea broth and incubate as described above.

12.4.1.1 TSI: A positive TSI reaction is an acid butt (yellow in color) and an alkaline slant (red in color) with or without H_2S gas production. When H_2S gas production is present, the butts of both the LIA and TSI may be black, which would be considered a positive reaction for *Salmonella*. H_2S is more likely but acid butt is also possible (but rare).

12.4.1.2 LIA: A positive LIA reaction is an alkaline butt (purple in color) and an alkaline slant (purple in color) with or without H_2S gas production. When H_2S gas production is present, the butts of both the LIA and TSI may be black, which would be considered a positive reaction for *Salmonella*.

12.4.1.3 Urea broth: Urea is an orange medium and will change to pink or deep purplish-red if positive. A negative urease test is one that exhibits no color change after inoculation. *Salmonella* are negative for urease.

Note: H_2S negative *Salmonella* will be missed if translucent pink to red colonies are not submitted to biochemical and serological confirmation.

12.4.2 To confirm cultures via polyvalent O antiserum:

Emulsify growth on the slant portion of TSI (regardless of whether TSI is positive or negative) using sterile physiological saline (Section 7.5). Place two discrete drops of emulsified growth onto slide. To the first drop of emulsified growth, add one drop of polyvalent O antiserum. To the second drop of emulsified growth, add one drop of sterile saline (as a visual comparison). Observe under magnification for an agglutination reaction which indicates a positive result. Appropriate positive and negative controls from TSI must be analyzed for each batch of samples.

12.4.3 In order for the original TSB tube to be considered positive for *Salmonella*, the associated inoculations must be MSRV positive, XLD positive, either TSI or LIA positive, urease negative, and polyvalent-O positive (Table 4). Correlate all positive plates and tubes to original TSB tube and record results. Determine the MPN from this information (see Section 13.0). Record all data clearly into a laboratory notebook.

12.5 Total solids determination

12.5.1 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.

12.5.2 Immediately after weighing the sample for microbiological examination, weigh 10-30 g of 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 Data Analysis and Calculations

The estimated density of *Salmonella* bacteria is calculated as the most probable number (MPN). *Salmonella* results from biosolid samples are reported as MPN / 4 g total solids (dry weight basis), which is calculated according to the following steps:

1. Selection of MPN / mL (wet weight)
2. Conversion to MPN / g (dry weight) and calculation of MPN / 4 g total solids (dry weight)

13.1 Step 1: Obtain MPN / mL (wet weight)

Obtain the MPN index value from Table 5 using the number of positive tubes in the three significant dilutions series. Since Table 5 assumes that 20.0, 10.0, and 1.0 mL of homogenized sample were inoculated into TSB and because liquid samples are not diluted in the homogenization step (Section 11.0), the MPN index = MPN / mL for liquid samples.

Since solid samples were diluted in the homogenization step (Section 11.1), the dilution must be taken into account when calculating MPN / mL (wet weight). As a result the MPN index value from Table 5 is divided by 0.1 to account for diluting the sample during the homogenization step.

Table 5. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are used per 20.0, 10.0, and 1.0 mL Homogenized Sample Inoculation Volumes [a]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.006473	----	0.0223	1-3-0	0.0312	0.0055	0.0678
0-0-1	0.0065	0.0012	0.0223	1-3-1	0.0393	0.0092	0.0821
0-0-2	0.0130	0.0012	0.0352	1-3-2	0.0475	0.0132	0.0967
0-0-3	0.0195	0.0012	0.0472	1-3-3	0.0559	0.0173	0.1119
0-0-4	0.0262	0.0033	0.0589	1-3-4	0.0644	0.0216	0.1277
0-0-5	0.0328	0.0062	0.0706	1-3-5	0.0730	0.0260	0.1444
0-1-0	0.0067	0.0012	0.0228	1-4-0	0.0409	0.0099	0.0849
0-1-1	0.0134	0.0012	0.0360	1-4-1	0.0495	0.0141	0.1002
0-1-2	0.0202	0.0012	0.0483	1-4-2	0.0583	0.0185	0.1163
0-1-3	0.0270	0.0037	0.0604	1-4-3	0.0672	0.0231	0.1331
0-1-4	0.0339	0.0067	0.0725	1-4-4	0.0763	0.0277	0.1509
0-1-5	0.0408	0.0099	0.0847	1-4-5	0.0855	0.0324	0.1700
0-2-0	0.0138	0.0012	0.0367	1-5-0	0.0517	0.0152	0.1042
0-2-1	0.0208	0.0012	0.0495	1-5-1	0.0609	0.0199	0.1212
0-2-2	0.0279	0.0040	0.0619	1-5-2	0.0703	0.0247	0.1391
0-2-3	0.0350	0.0072	0.0745	1-5-3	0.0799	0.0296	0.1583
0-2-4	0.0422	0.0106	0.0871	1-5-4	0.0897	0.0346	0.1790
0-2-5	0.0494	0.0141	0.1001	1-5-5	0.0998	0.0397	0.2015
0-3-0	0.0215	0.0012	0.0507	2-0-0	0.0155	0.0012	0.0404
0-3-1	0.0288	0.0044	0.0636	2-0-1	0.0226	0.0018	0.0526
0-3-2	0.0362	0.0077	0.0766	2-0-2	0.0303	0.0051	0.0662
0-3-3	0.0437	0.0113	0.0898	2-0-3	0.0382	0.0087	0.0801
0-3-4	0.0512	0.0051	0.1243	2-0-4	0.0462	0.0125	0.0943
0-3-5	0.0588	0.0095	0.1428	2-0-5	0.0543	0.0165	0.1090
0-4-0	0.0299	0.0049	0.0654	2-1-0	0.0234	0.0022	0.0540
0-4-1	0.0375	0.0084	0.0789	2-1-1	0.0315	0.0056	0.0683
0-4-2	0.0453	0.0121	0.0927	2-1-2	0.0397	0.0094	0.0827
0-4-3	0.0531	0.0160	0.1069	2-1-3	0.0480	0.0134	0.0976
0-4-4	0.0611	0.0200	0.1216	2-1-4	0.0565	0.0177	0.1131
0-4-5	0.0691	0.0241	0.1369	2-1-5	0.0652	0.0221	0.1293
0-5-0	0.0390	0.0090	0.0814	2-2-0	0.0327	0.0062	0.0705
0-5-1	0.0470	0.0129	0.0958	2-2-1	0.0413	0.0101	0.0856
0-5-2	0.0553	0.0170	0.1107	2-2-2	0.0501	0.0144	0.1013
0-5-3	0.0636	0.0212	0.1262	2-2-3	0.0590	0.0189	0.1176
0-5-4	0.0720	0.0255	0.1425	2-2-4	0.0681	0.0236	0.1349
0-5-5	0.0806	0.0299	0.1596	2-2-5	0.0774	0.0283	0.1533
1-0-0	0.0072	0.0012	0.0241	2-3-0	0.0431	0.0110	0.0887
1-0-1	0.0139	0.0012	0.0369	2-3-1	0.0523	0.0155	0.1053
1-0-2	0.0209	0.0012	0.0497	2-3-2	0.0617	0.0203	0.1227
1-0-3	0.0281	0.0041	0.0623	2-3-3	0.0714	0.0252	0.1412
1-0-4	0.0353	0.0073	0.0749	2-3-4	0.0813	0.0303	0.1611
1-0-5	0.0425	0.0107	0.0878	2-3-5	0.0914	0.0354	0.1826
1-1-0	0.0144	0.0012	0.0377	2-4-0	0.0547	0.0168	0.1098
1-1-1	0.0217	0.0013	0.0509	2-4-1	0.0647	0.0218	0.1284
1-1-2	0.0290	0.0045	0.0640	2-4-2	0.0750	0.0271	0.1484
1-1-3	0.0365	0.0079	0.0771	2-4-3	0.0855	0.0325	0.1700
1-1-4	0.0441	0.0115	0.0905	2-4-4	0.0964	0.0380	0.1937
1-1-5	0.0517	0.0153	0.1043	2-4-5	0.1076	0.0436	0.2201
1-2-0	0.0224	0.0017	0.0523	2-5-0	0.0681	0.0235	0.1349
1-2-1	0.0301	0.0050	0.0658	2-5-1	0.0791	0.0292	0.1566
1-2-2	0.0379	0.0085	0.0795	2-5-2	0.0904	0.0349	0.1805
1-2-3	0.0457	0.0123	0.0935	2-5-3	0.1021	0.0409	0.2070
1-2-4	0.0537	0.0162	0.1079	2-5-4	0.1143	0.0469	0.2372
1-2-5	0.0618	0.0203	0.1229	2-5-5	0.1268	0.0531	0.2725

[a] Table was developed using the MPN calculator developed by Albert Klee (Reference 18.5)

Table 5. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are used per 20.0, 10.0, and 1.0 mL Homogenized Sample Inoculation Volumes (cont.)

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.0255	0.0028	0.0585	4-3-0	0.0797	0.0295	0.1579
3-0-1	0.0330	0.0063	0.0710	4-3-1	0.0937	0.0366	0.1877
3-0-2	0.0417	0.0103	0.0863	4-3-2	0.1086	0.0441	0.2228
3-0-3	0.0506	0.0147	0.1023	4-3-3	0.1245	0.0520	0.2656
3-0-4	0.0598	0.0193	0.1191	4-3-4	0.1414	0.0602	0.3218
3-0-5	0.0691	0.0241	0.1368	4-3-5	0.1595	0.0686	0.4067
3-1-0	0.0344	0.0069	0.0734	4-4-0	0.1012	0.0404	0.2049
3-1-1	0.0435	0.0112	0.0896	4-4-1	0.1181	0.0489	0.2476
3-1-2	0.0529	0.0159	0.1065	4-4-2	0.1364	0.0578	0.3038
3-1-3	0.0626	0.0207	0.1244	4-4-3	0.1563	0.0672	0.3890
3-1-4	0.0725	0.0258	0.1434	4-4-4	0.1780	0.0770	0.5273
3-1-5	0.0827	0.0310	0.1640	4-4-5	0.2015	0.0873	0.6411
3-2-0	0.0456	0.0122	0.0932	4-5-0	0.1304	0.0549	0.2836
3-2-1	0.0555	0.0171	0.1112	4-5-1	0.1524	0.0653	0.3687
3-2-2	0.0657	0.0223	0.1303	4-5-2	0.1769	0.0766	0.5210
3-2-3	0.0763	0.0277	0.1510	4-5-3	0.2046	0.0886	0.6528
3-2-4	0.0872	0.0333	0.1735	4-5-4	0.2357	0.1015	0.7516
3-2-5	0.0984	0.0390	0.1984	4-5-5	0.2708	0.1150	0.8426
3-3-0	0.0583	0.0186	0.1164	5-0-0	0.0549	0.0162	0.1116
3-3-1	0.0693	0.0241	0.1371	5-0-1	0.0637	0.0213	0.1265
3-3-2	0.0806	0.0299	0.1597	5-0-2	0.0763	0.0277	0.1510
3-3-3	0.0924	0.0359	0.1847	5-0-3	0.0896	0.0345	0.1787
3-3-4	0.1046	0.0421	0.2128	5-0-4	0.1037	0.0417	0.2107
3-3-5	0.1173	0.0484	0.2452	5-0-5	0.0953	0.0165	0.2234
3-4-0	0.0733	0.0262	0.1450	5-1-0	0.0678	0.0234	0.1344
3-4-1	0.0856	0.0325	0.1700	5-1-1	0.0816	0.0304	0.1618
3-4-2	0.0984	0.0390	0.1982	5-1-2	0.0963	0.0379	0.1936
3-4-3	0.1118	0.0457	0.2307	5-1-3	0.1121	0.0459	0.2316
3-4-4	0.1258	0.0526	0.2695	5-1-4	0.1291	0.0542	0.2796
3-4-5	0.1405	0.0597	0.3184	5-1-5	0.1293	0.0304	0.3090
3-5-0	0.0913	0.0354	0.1825	5-2-0	0.0879	0.0337	0.1751
3-5-1	0.1055	0.0426	0.2150	5-2-1	0.1046	0.0421	0.2128
3-5-2	0.1204	0.0500	0.2538	5-2-2	0.1227	0.0511	0.2605
3-5-3	0.1362	0.0577	0.3029	5-2-3	0.1427	0.0608	0.3267
3-5-4	0.1529	0.0656	0.3715	5-2-4	0.1646	0.0710	0.4385
3-5-5	0.1707	0.0738	0.4795	5-2-5	0.1767	0.0503	0.5230
4-0-0	0.0381	0.0082	0.0809	5-3-0	0.1151	0.0474	0.2394
4-0-1	0.0461	0.0125	0.0942	5-3-1	0.1368	0.0580	0.3050
4-0-2	0.0563	0.0175	0.1126	5-3-2	0.1614	0.0695	0.4183
4-0-3	0.0668	0.0229	0.1323	5-3-3	0.1895	0.0821	0.5899
4-0-4	0.0777	0.0284	0.1537	5-3-4	0.2216	0.0957	0.7101
4-0-5	0.0890	0.0342	0.1773	5-3-5	0.2527	0.0814	0.7971
4-1-0	0.0484	0.0136	0.0983	5-4-0	0.1571	0.0676	0.3935
4-1-1	0.0592	0.0190	0.1181	5-4-1	0.1907	0.0826	0.5954
4-1-2	0.0705	0.0248	0.1395	5-4-2	0.2319	0.0999	0.7409
4-1-3	0.0822	0.0308	0.1631	5-4-3	0.2834	0.1196	0.8726
4-1-4	0.0945	0.0370	0.1894	5-4-4	0.3475	0.1417	1.0160
4-1-5	0.1072	0.0434	0.2193	5-4-5	0.4256	0.1437	1.1800
4-2-0	0.0626	0.0207	0.1244	5-5-0	0.2398	0.0762	0.7629
4-2-1	0.0748	0.0269	0.1479	5-5-1	0.3477	0.1172	1.0160
4-2-2	0.0875	0.0335	0.1742	5-5-2	0.5422	0.1791	1.4190
4-2-3	0.1009	0.0403	0.2041	5-5-3	0.9178	0.2672	2.2010
4-2-4	0.1150	0.0473	0.2392	5-5-4	1.6090	0.3837	4.1030
4-2-5	0.1299	0.0546	0.2820	5-5-5	>1.609000	0.3837	----

[a] Table was developed using the MPN calculator developed by Albert Klee (Reference 18.5)

13.2 Step 2: Convert to MPN / g (dry weight) and calculate MPN / 4 g total solids (dry weight):

For analysis and calculation of percent total solids, refer to Section 12.5.

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

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

Therefore, we may calculate MPN / 4 g total solids (dry weight) for liquid samples using the following equation:

$$\text{MPN / 4 g (dry weight)} = \frac{\text{[MPN / mL wet weight from Step 1]} \times 4}{\text{Percent total solids expressed as a decimal}}$$

Example calculations are provided in **Table 6**, below.

Table 6. Example Calculation of *Salmonella* Density

Example	Volume of homogenized sample used to inoculate TSB			Step 1: MPN / mL (wet weight)	Percent total solids (dry weight)	Step 2: MPN / 4 g (dry weight)
	20.0 mL	10.0 mL	1.0 mL			
A (liquid)	0/5	1/5	0/5	0.0067	1%	(0.0067) (4) / (0.01) = 2.68 MPN / 4 g
B (liquid)	3/5	1/5	1/5	0.0435	2%	(0.0435) (4) / (0.02) = 8.7 MPN / 4 g
C (liquid)	5/5	5/5	2/5	0.5422	3%	(0.5422) (4) / (0.03) = 72 MPN / 4 g
D (solid)	0/5	1/5	0/5	0.0067 / 0.1[1] = .067	96%	(0.067) (4) / (0.96) = 0.28 MPN / 4 g
E (solid)	4/5	4/5	4/5	0.1181 / 0.1[1] = 1.181	18%	(1.181) (4) / (0.18) = 26 MPN / 4 g
F (solid)	5/5	5/5	2/5	0.5422 / 0.1[1] = 5.422	43%	(5.422) (4) / (0.43) = 50 MPN / 4 g

[1]Dilution factor (1:10) for solid samples

14.0 Sample Spiking Procedure

14.1 Method 1682 QC requirements (Section 9) 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 as described below.

14.2 Preparation of Laboratory-Prepared Spiking Suspensions

14.2.1 Preparation

14.2.1.1 **Stock Culture**. Prepare a stock culture by inoculating a heart infusion agar (HIA) slant (or other non-selective media) with *Salmonella typhimurium* ATCC # 14028 and incubating at 36°C ± 1.5°C for 20 ± 4 hours. After incubation, the stock culture may be stored in the dark at room temperature for up to 30 days.

14.2.1.2 **1% Tryptic Soy Broth (TSB)**. Prepare a 1% solution of TSB by combining 99 mL of sterile phosphate buffered dilution water and 1 mL of sterile single-strength tryptic soy broth in a sterile screw cap bottle or re-sealable dilution water container. Shake to mix.

14.2.1.3 **Spiking Suspension (Undiluted)**. From the stock culture of *Salmonella typhimurium* ATCC # 14028, aseptically transfer a small loopful of growth to the 1% TSB solution and vigorously shake a minimum of 25 times. Incubate at 36°C ± 1.5°C for 20 ± 4 hours. The resulting spiking suspension contains approximately 1.0×10^7 to 1.0×10^8 *Salmonella typhimurium* colony forming units (CFU) per mL. This is referred to as the "undiluted spiking suspension".

14.3 Laboratory-Prepared Sample Spiking (Class A Biosolids)

Since the objective of spiking the biosolid sample is to establish percent recovery, it is necessary to determine the number of *Salmonella typhimurium* in the undiluted spiking suspension.

14.3.1 Sample spiking

14.3.1.1 Dilute spiking suspension

Mix the undiluted 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 phosphate buffered 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.

14.3.1.2 Use a sterile pipette to transfer 1.0 mL of spiking suspension dilution "A" to 99 mL of sterile phosphate buffered 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^{-4} mL of the original undiluted spiking suspension.

14.3.1.3 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "B" to 99 mL of sterile phosphate buffered 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^{-5} mL of the original undiluted spiking suspension.

14.3.1.4 Use a sterile pipette to transfer 11.0 mL of spiking suspension dilution "C" to 99 mL of sterile phosphate buffered 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^{-6} mL of the original undiluted spiking suspension.

14.3.2 Spike sample(s)

Since sample homogenization procedures in Method 1682 are specific to either liquid or solid samples, this spiking procedure is also liquid/solid-specific.

14.3.2.1 Liquid Samples: Homogenize an unspiked Class A biosolid sample (Section 11.1). To spike a liquid sample, add 0.5 mL of spiking suspension dilution "D" to 300 mL of pH adjusted unspiked homogenized sample, cover, and blend on high speed for 1 - 2 minutes. This is the "spiked" sample. The volume (mL) of undiluted spiking suspension added to each mL of the homogenized biosolid sample is 1.67 x 10^{-9} mL per mL [(0.5 mL x 10^{-6} mL) / 300 mL of biosolid], which is referred to as $V_{\text{spiked per unit biosolids}}$. Proceed to Section 11.2 (inoculation).

14.3.2.2 Solid Samples: Homogenize the Class A biosolid sample (Section 11.1). To spike a solid sample, add 0.5 mL of spiking suspension dilution "D" to 300 mL of pH adjusted unspiked homogenized sample (30 g of sample + 270 mL of sterile phosphate buffered dilution water), cover, and blend on high speed for 1 - 2 minutes. This is the "spiked" sample. The volume (mL) of undiluted spiking suspension added to each g (wet weight) of the homogenized biosolid sample is 1.67 × 10^{-8} mL per g [(0.5 mL × 10^{-6} mL) / 30 g of biosolid], which is referred to as $V_{\text{spiked per unit biosolids}}$. Proceed to Section 11.2 (inoculation).

14.4 Enumeration of undiluted spiking suspension

14.4.1 Prepare heart infusion agar (HIA) according to Section 7.12, 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.

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

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

14.4.3 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.

14.4.4 Allow inoculum to absorb into the medium completely.

14.4.5 Invert plates and incubate at 36°C ± 1.5°C for 24 ± 4 hours.

14.4.6 Count and record number of colonies per plate.

14.5 Calculation of Laboratory-Prepared Spike Percent Recovery

Spiked *Salmonella typhimurium* percent recovery will be calculated 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.

14.5.1 Step 1: Calculate Concentration of *Salmonella typhimurium* (CFU / mL) in Undiluted Spiking Suspension

14.5.1.1 The number of *Salmonella typhimurium* CFU / mL in the undiluted spiking suspension will be calculated using all HIA plates yielding counts within the ideal range of 30 to 300 CFU per plate.

14.5.1.2 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).

14.5.1.3 Calculate the concentration of *Salmonella typhimurium* (CFU / mL) in the undiluted spiking suspension according to the following equation. (Example calculations are provided in **Table 7**, below.)

$$Salmonella_{\text{undiluted spike}} = (CFU_1 + CFU_2 + ... + CFU_n) / (V_1 + V_2 + ... + V_n)$$

Where,

Salmonella $_{\text{undiluted spike}}$	=	*Salmonella typhimurium* 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 on 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

Table 7. Example Calculations of *Salmonella typhimurium* Spiking Suspension Concentration

Examples	CFU / plate (triplicate analyses) from HIA plates			*Salmonella* CFU / mL in undiluted spiking suspension (*Salmonella* $_{\text{undiluted spike}}$) [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] *Salmonella* $_{\text{undiluted spike}}$ is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

14.5.2 Step 2: Calculate the Concentration of Spiked *Salmonella* CFU / mL or CFU / g (wet weight)

14.5.2.1 The volume of undiluted spiking suspension per unit (mL or g) of spiked biosolid samples ($V_{\text{spiked per unit biosolids}}$) is provided in **Table 8**, below.

Table 8. Volume of Undiluted Spiking Suspension per Unit (mL or g) of Spiked Biosolid Samples ($V_{\text{spiked per unit biosolids}}$)

Description of spiked sample	$V_{\text{spiked per unit biosolids}}$
Class A liquid	1.67×10^{-9} mL per mL of biosolids
Class A solid	1.67×10^{-8} mL per g of biosolids (wet weight)

14.5.2.2 Calculate concentration of Spiked *Salmonella* $_{\text{wet weight}}$ (CFU / mL or CFU / g) according to the following equation. Example calculations are provided in **Table 9**, below.

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

Where,

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

Table 9. Example Calculations of Spiked *Salmonella* $_{\text{wet weight}}$

Salmonella $_{\text{undiluted spike}}$ **(Table 7 above)**	**V** $_{\text{spiked}}$ **(Table 8 above)**	**Spiked *Salmonella*** $_{\text{wet weight}}$ **[CFU / mL or CFU / g (wet weight)]**
Example 1: 2.6×10^{7} CFU / mL	Liquid: 1.67×10^{-9} mL per mL of biosolids	(2.6×10^{7} CFU / mL) x (1.67×10^{-9} mL / mL) = 0.043 CFU / mL
	Solid: 1.67×10^{-8} mL per g of biosolids (wet weight)	(2.6×10^{7} CFU / mL) x (1.67×10^{-8} mL / g) = 0.43 CFU / g (wet weight)
Example 2: 3.7×10^{8} CFU / mL	Liquid: 1.67×10^{-9} mL per mL of biosolids	(3.7 x 108 CFU / mL) x (1.67×10^{-9} mL / mL) = 0.62 CFU / mL
	Solid: 1.67×10^{-8} mL per g of biosolids (wet weight)	(3.7×10^{8} CFU / mL) x (1.67×10^{-8} mL / g) = 6.2 CFU / g (wet weight)

14.5.3 Step 3: Convert to "True" Spiked *Salmonella* CFU / 4 g Total Solids (dry weight)

14.5.3.1 Convert to "true" spiked CFU / 4 g total solids dry weight (T) using the spiked *Salmonella* mL or g (wet weight) as the numerator in the equation. Examples are provided in **Table 10**, below.

Table 10. Examples of Conversion to "True" Spiked *Salmonella* CFU / 4 g Total Solids (Dry Weight)

Example Total Solids (Method 1684)		**[(Spiked *Salmonella* $_{\text{wet weight}}$ from Table 9 above) / percent total solids] × 4 = True spiked *Salmonella* CFU / 4 g dry weight**
Example 1	Class A liquid: 5%	(0.043 / 0.05) × 4 = 3.5 CFU / 4 g dry weight
	Class A solid: 82%	(0.43 / 0.82) × 4 = 2.1 CFU / 4 g dry weight
Example 2	Class A liquid: 7%	(0.62 / 0.07) × 4 = 35 CFU / 4 g dry weight
	Class A solid: 88%	(6.2 / 0.88) × 4 = 28 CFU / 4 g dry weight

14.5.4 Step 4: Calculate Percent Recovery

14.5.4.1 Calculate percent recovery (R) using the following equation.

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

Where,

R	=	Percent recovery
N_s	=	*Salmonella* MPN / 4 g (dry weight) in the spiked sample
N_u	=	*Salmonella* MPN / 4 g (dry weight) in the unspiked sample
T	=	True spiked *Salmonella* CFU / 4 g (dry weight) in spiked sample

14.5.4.2 Example percent recovery calculations are provided in **Table 11**.

Table 11. Example Percent Recovery Calculations

N_s	N_u	T	Percent recovery (R)
2.6	0.268	Example 1: 3.5	100 x (2.6 - 0.268) / 3.5 = **67%**
2.4		Example 1: 2.1	100 x (2.4 - 0.268) / 2.1 = **101%**
46		Example 2: 35	100 x (46 - 0.268) / 35 = **130%**
16		Example 2: 28	100 x (16 - 0.268) / 28 = **56%**

14.6 BioBall™ Sample Spiking (Class A Biosolids) and Enumeration

14.6.1 Sample spiking

Since sample homogenization procedures in Method 1682 are specific to either liquid or solid samples, this spiking procedure is also liquid/solid-specific.

14.6.1.1 Liquid Samples: Homogenize an unspiked Class A biosolid sample. Open BioBall™ vial by removing the crimp and cap. To spike a liquid sample, aseptically add 1 BioBall™ to 300 mL of pH adjusted unspiked homogenized sample, cover, and blend on high speed for 1 - 2 minutes. This is the "spiked" sample. Proceed to Section 11.2 (inoculation).

14.6.1.2 Solid Samples: Homogenize the Class A biosolid sample (Section 11.1). Open BioBall™ vial by removing the crimp and cap. To spike a solid sample, aseptically add 1 BioBall™ to 300 mL of pH adjusted unspiked homogenized sample (30 g of sample + 270 mL of sterile phosphate buffered dilution water), cover, and blend on high speed for 1 - 2 minutes. This is the "spiked" sample. Proceed to Section 11.2 (inoculation).

14.7 Enumeration of BioBall™

14.7.1 Prepare heart infusion agar (HIA) according to Section 7.12, add 10 - 15 mL of HIA per 100 x 15 mm petri dish, and allow to solidify. For larger plates, adjust volume appropriately. 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.

14.7.2 Each of the following will be conducted in triplicate, resulting in the evaluation of three spread plates:

- Open BioBall™ vial by removing the crimp and cap. Aseptically place one BioBall™ onto the center of each HIA plate by tipping the vial over the medium.
- Immediately pipette 200 µL of sterile physiological saline solution (0.85%) directly onto the BioBall™.
- Allow the BioBall™ to dissolve.

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

14.7.4 Allow inoculum to absorb into the medium completely.

14.7.5 Invert plates and incubate at 36°C ± 1.5°C for 24 ± 4 hours.

14.7.6 Count and record number of colonies per plate.

14.8 Calculation of BioBall™ Spike Percent Recovery

Spiked BioBall™ percent recovery will be calculated in 4 steps as indicated. *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.

14.8.1 Step 1: Calculate Concentration of *Salmonella typhimurium* (CFU) per BioBall™

14.8.1.1 The number of *Salmonella typhimurium* (CFU) in the BioBalls will be calculated using all HIA plates. Count the number of CFUs on all three plates and calculate the mean. (An example is provided in **Table 12**.)

Table 12. Example calculation for mean *Salmonella* CFU per BioBall™

CFU / plate (triplicate analyses) from HIA plates			Mean *Salmonella* CFU per BioBall™
HIA plate count #1	HIA plate count #2	HIA plate count #3	
40	38	48	(40 + 38 + 48) / 3 = **42**

14.8.2 Step 2: Calculate the Concentration of Spiked *Salmonella* in the Homogenized Sample [CFU / mL or CFU / g (wet weight)]

14.8.2.1 Since sample homogenization procedures in Method 1682 are specific to either liquid or solid samples, the concentration of spiked *Salmonella* in the homogenized sample will be reported as CFU / mL for liquid samples or CFU / g for solid samples. Calculate the concentration of spiked *Salmonella* in the homogenized sample using the equation below. Examples are provided in **Table 13**, below.

$$\text{Spiked } Salmonella_{\text{wet weight}} = \frac{Salmonella_{\text{mean CFU}}}{V_{\text{homogenized sample}}}$$

Where,

Spiked *Salmonella* $_{\text{wet weight}}$	=	Number of spiked *Salmonella* CFU per mL or g of biosolid (wet weight)
Salmonella $_{\text{mean CFU}}$	=	Mean CFU of *Salmonella* in BioBalls
$V_{\text{homogenized sample}}$	=	mL or g of spiked homogenized biosolid sample

Table 13. Example Calculation of Spiked *Salmonella* in the Homogenized Sample (*Salmonella* $_{\text{wet weight}}$)

Salmonella $_{\text{mean CFU}}$ **(Table 12 above)**	$V_{\text{homogenized sample}}$	**Spiked *Salmonella*** $_{\text{wet weight}}$ **[CFU / mL or CFU / g (wet weight)]**
42 CFU	Liquid: 300 mL	42 CFU / 300 mL = 0.14 CFU / mL (wet weight)
	Solid: 30 g	42 CFU / 30 g = 1.4 CFU / g (wet weight)

14.8.3 Step 3: Convert to "True" Spiked *Salmonella* CFU / 4 g Total Solids (dry weight)

14.8.3.1 Convert to "true" spiked CFU / 4 g total solids dry weight (T) using the spiked *Salmonella* per mL or g (wet weight) as the numerator in the equation. Examples are provided in **Table 14**, below.

Table 14. Examples of Conversion to "True" Spiked *Salmonella* CFU / 4 g Total Solids (Dry Weight)

Example Total Solids	[(Spiked *Salmonella* $_{\text{wet weight}}$ from Table 13 above) / percent total solids] × 4 = True spiked *Salmonella* CFU / 4 g (dry weight)
Class A liquid: 5%	(0.14 / 0.05) × 4 = 11.2 CFU / 4 g dry weight
Class A solid: 82%	(1.4 / 0.82) × 4 = 6.8 CFU / 4 g dry weight

14.8.4 Step 4: Calculate Percent Recovery

14.8.4.1 Calculate percent recovery (R) using the following equation.

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

Where,

R = Percent recovery
N_s = *Salmonella* MPN / 4 g (dry weight) in the spiked sample
N_u = *Salmonella* MPN / 4 g (dry weight) in the unspiked sample
T = True spiked *Salmonella* CFU / 4 g (dry weight) in spiked sample

14.8.4.2 Example percent recovery calculations are provided in **Table 15**.

Table 15. Example Percent Recovery Calculations

N_s	N_u	T	Percent recovery (R)
12	0.268	11.2	100 x (12 - 0.268) / 11.2 = **105%**
4.8		6.8	100 x (4.8 - 0.268) / 6.8 = **67%**

15.0 Method Performance

15.1 Interlaboratory Validation of Method 1682

15.1.1 Twelve volunteer laboratories and a referee laboratory participated in the U.S. Environmental Protection Agency's (EPA's) interlaboratory validation study of EPA Method 1682. 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 study and results are provided in the validation study report (Reference 18.2). 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 March 2003 draft version of EPA Method 1682.

15.1.2 Recovery - Method 1682 mean recoveries of *Salmonella* from compost, thermophilically digested liquid, thermophilically digested solid, and alkaline-stabilized biosolids, spiked with BioBall™ spikes were 42%, 13%, 91%, and 19%, respectively. Mean recoveries of *Salmonella* from compost, thermophilically digested liquid, thermophilically digested solid, and alkaline-stabilized biosolids, spiked with laboratory-spiked spikes were 100%, 5.6%, 87%, and 55%, respectively. Mean *Salmonella* recoveries for Milorganite® (reference matrix) samples spiked with BioBalls and laboratory-prepared spikes were 81% and 120%, respectively.

15.1.3 Precision - Method 1682 overall relative standard deviations (RSDs) from biosolids, spiked with BioBalls ranged from 62% to 150%. For biosolid samples spiked with laboratory-prepared spiking suspensions, RSDs ranged from 61% to 180%.

16.0 Pollution Prevention

16.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

16.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

17.0 Waste Management

17.1 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).

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

17.3 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.

18.0 References

18.1 American Public Health Association, American Water Works Association, and Water Environment Federation. 1998. *Standard Methods for Water and Wastewater*. 20th Edition. Sections 9020, 9030, 9040, 9050, and 9221.

18.2 USEPA. *Results of the Interlaboratory Validation of EPA Method 1682 (MSRV) for Salmonella in Biosolids*. EPA-821-B-04-008. September 2004.

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

18.4 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.

18.5 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.

19.0 Figures

Figures 1 and 2. Liquid and Solid Samples

Method 1682 Class A Sample Homogenization and Inoculation

Figure 1. Liquid Sample (Sections 11.1.1 and 11.2.1)

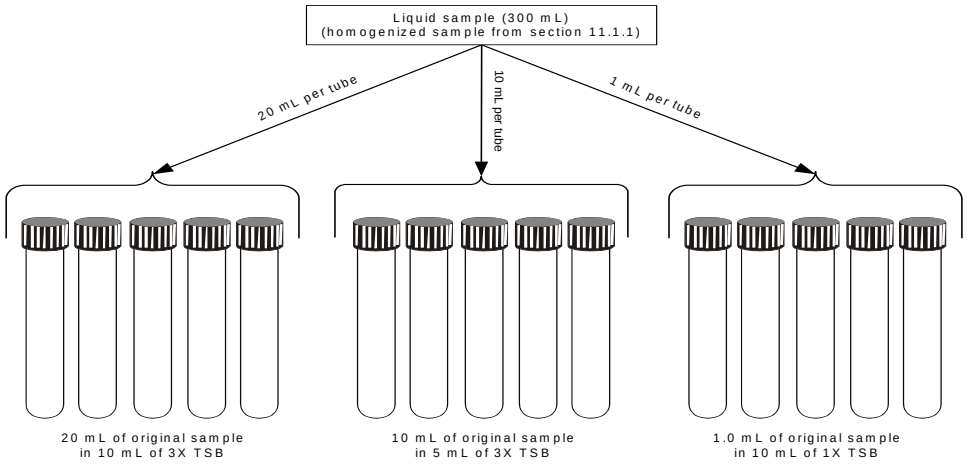


Figure 2. Solid Sample (Sections 11.1.2 and 11.2.2)

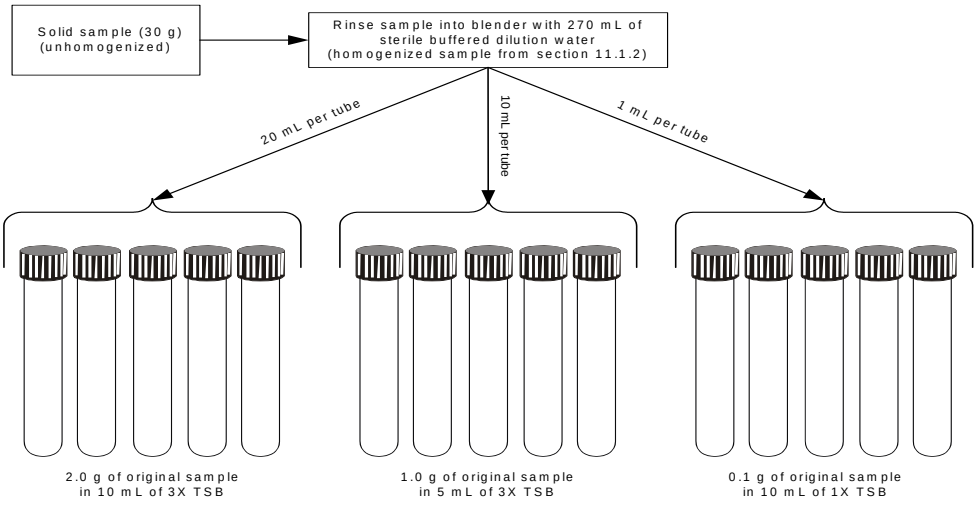
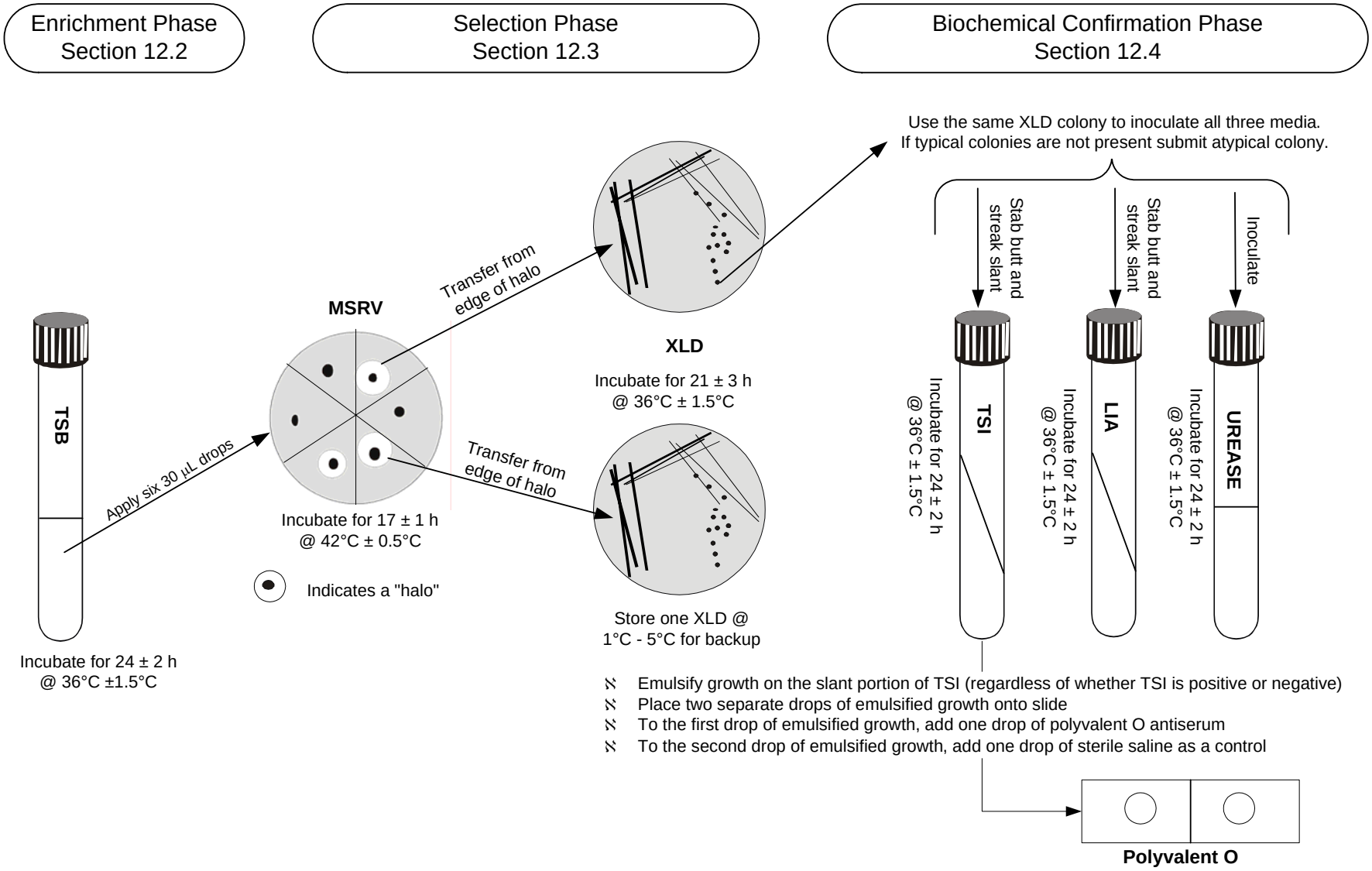


Figure 3. Method 1682 MSRV Procedure
(Repeat the following for each TSB tube)



20.0 Glossary of Definitions and Purposes

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

20.1 Units of weight and measure and their abbreviations

20.1.1 Symbols

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

20.1.2 Alphabetical characters

ASTM	American Society for Testing and Materials
ATCC	formerly known as American Type Culture Collection
CFR	Code of Federal Regulations
EPA	United States Environmental Protection Agency
g	gram
L	liter
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

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

Analyte—The microorganism tested for by this method. The analyte in this method is *Salmonella*.

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

Liquid samples—Generally, 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).