Method 1200: Analytical Protocol for Non-Typhoidal *Salmonella* in Drinking Water and Surface Water

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Acknowledgments

This Analytical Protocol is based on procedures single-laboratory verified by the County Sanitation Districts of Los Angeles County, Joint Water Pollution Control Plant (JWPCP) Water Quality Laboratory, under direction of Sanjiv R. Shah at the National Homeland Security Research Center (NHSRC) within the U.S. Environmental Protection Agency's (EPA's) Office of Research and Development for analysis of non-typhoidal *Salmonella* in drinking water and surface water samples. This protocol was multi-laboratory validated by 10 volunteer laboratories. Technical support and data evaluation were provided by Computer Sciences Corporation under EPA Contract No. EP-C-05-045.

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Multi-Laboratory Validation Study Volunteer Participant Laboratories:

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- County Sanitation Districts of Los Angeles County, Joint Water Pollution Control Plant (JWPCP)
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The procedures described in this document are intended for use in laboratories when analyzing water samples in support of response and remediation efforts following a homeland security incident. The culture-based procedures provide viability determination, identification, and either qualitative or quantitative results. The sample preparation procedures are deemed the most appropriate for the wide variety of water matrices to be examined. To the extent possible, these procedures were developed to be consistent with other federal agency procedures. These procedures do not include the rapid screening, field techniques, or molecular techniques that may accompany laboratory analysis.

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Appendix A: Part II (General Operations), Section A (Sample Collection, Preservation, and Storage) (Taken from *Microbiological Methods for Monitoring the Environment: Water and Wastes* [Reference 17.14]).

Analytical Protocol for Non-Typhoidal *Salmonella* in Drinking Water and Surface Water

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1.0 SCOPE AND APPLICATION

1.1 This Analytical Protocol is for the identification, confirmation, and quantitation of non-typhoidal *Salmonella* (referred to as "*Salmonella*" in this document) in drinking water and surface water samples, using selective and non-selective media followed by biochemical and serological confirmation.

1.2 This protocol has been adapted from U.S. Environmental Protection Agency's (EPA's) Method 1682: *Salmonella* in Sewage Sludge (Biosolids) by Modified Semisolid Rappaport-Vassiliadis (MSRV) Medium (Reference 17.1) and is for use by laboratories when analyzing samples in support of EPA homeland security efforts.

1.3 *Salmonella* are the causative agents of salmonellosis. Due to the infectious nature of these bacteria and the potential for transmission to humans, all procedures should be performed in laboratories that use, at a minimum, biological safety level (BSL)-2. Use of a biological safety cabinet is recommended for any aerosol-generating procedures (Reference 17.2).

1.4 All sample handling, analysis, and reporting of results must be performed in accordance with established guidelines. Laboratories must have requisite resources in place prior to use of these procedures.

1.5 This method is not intended for analyses of microorganisms other than *Salmonella* spp. and the matrices described.

2.0 SUMMARY OF METHOD

2.1 *Salmonella* can be identified in a variety of water samples using selective media, morphological, biochemical, and serological analyses. Bacterial densities can be estimated by the Most Probable Number (MPN) technique.

2.2 For qualitative results, samples are diluted 1:1 in double-strength tryptic soy broth (TSB) and incubated at 36.0°C ± 1.5°C for 24 ± 2 hours.

2.3 For quantitative results, samples are analyzed as received. All samples are analyzed using a 15-tube MPN. Inoculated TSB tubes are incubated at 36.0°C ± 1.5°C for 24 ± 2 hours.

2.4 TSB tubes (MPN and qualitative analysis) exhibiting growth (turbidity) are spotted onto MSRV plates and incubated at 42.0°C ± 0.5°C for 16 – 18 hours.

2.5 MSRV plates are examined for a whitish halo around the colony, which is evidence of motility. Presumptive colonies are sub-cultured onto xylose lysine desoxycholate (XLD) agar and incubated at 36.0°C ± 1.5°C for 18 – 24 hours. Presumptive positive *Salmonella* colonies are confirmed using lysine iron agar (LIA), triple sugar iron (TSI) agar, and urea broth, followed by positive serological typing using *Salmonella* O antiserum, polyvalent A-I and Vi.

2.6 TSB tubes (MPN and qualitative analysis) exhibiting growth (turbidity) or growth from MSRV plates may be confirmed by real-time polymerase chain reaction (PCR) in place of biochemical and serological confirmation.

2.7 Quantitation of *Salmonella* is determined using the MPN technique (Flowchart 18.1). Tubes that are confirmed positive for *Salmonella* are used to determine MPN.

3.0 ACRONYMS AND ABBREVIATIONS

ATCC®	American Type Culture Collection
BSL	Biological safety level
cm	Centimeter
°C	Degrees Celsius
EPA	U.S. Environmental Protection Agency
g	Gram
IPR	Initial precision and recovery
JWPCP	Joint Water Pollution Control Plant
LACSD	Los Angeles County Sanitation District
L	Liter
LIA	Lysine iron agar
µL	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimeter
MPN	Most probable number
MS	Matrix spike
MSRV	Modified semisolid Rappaport-Vassiliadis (agar)
N	Normal – one equivalent weight per liter
NCTC	National Collection of Type Cultures
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OPR	Ongoing precision and recovery

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPE	Personal protective equipment
psi	Pounds per square inch
QA	Quality assurance
QC	Quality control
RSD	Relative standard deviation
SD	Standard deviation of the percent recoveries
TSB	Tryptic soy broth
TSI	Triple sugar iron
w/v	Weight to volume ratio
XLD	Xylose lysine desoxycholate

4.0 INTERFERENCES AND CONTAMINATION

4.1 During the multi-laboratory validation study (Study Report, Reference 17.3), differences in mean MPN/100 mL were observed in surface water matrices. This may be due to varying degrees of laboratory proficiency with the method, as at least one laboratory retrospectively suggested that, they should have submitted more "questionable colonies" from MSRV to confirmation. In addition, as indicated in Section 6 of the Study Report, another laboratory indicated that background bacteria from the TSB enrichment made it very difficult to identify presumptively positive colonies on MSRV. As a result, it is critical that laboratories become proficient with this protocol prior to analyzing samples from complex surface water matrices, as inappropriately low results may be reported.

4.2 *Salmonella* recoveries may be impacted by the presence of high numbers of competing or inhibitory organisms (e.g., other Enterobacteriaceae), or toxic substances (e.g., metals or organic compounds).

4.3 A viable but non-culturable state of *Salmonella* may also account for low recoveries (Reference 17.4).

5.0 SAFETY

5.1 Laboratory Hazards

In order to prevent transmission, disposable gloves should be worn when working with this organism. Hands should be washed immediately following removal of gloves. Direct and indirect contact of intact and broken skin with cultures and contaminated laboratory surfaces, accidental parenteral inoculation, and rarely, exposure to infectious aerosols are the primary hazards to laboratory personnel. Staff should apply safety procedures used for pathogens when handling all samples.

5.2 Recommended Precautions

5.2.1 *Salmonella* are BSL-2 pathogens and all procedures should be performed in laboratories that use, at a minimum, BSL-2 practices (Reference 17.2). This includes prohibiting eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food and drink in the laboratory.

5.2.2 A Class II biological safety cabinet is recommended for sample manipulations where the risk of aerosol production is high. Production of aerosols should be avoided.

5.2.3 Disposable materials are recommended for sample manipulation.

5.2.4 Mouth-pipetting is prohibited.

5.2.5 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.

5.2.6 Personal Protective Equipment (PPE)

5.2.6.1 Disposable nitrile gloves should be worn at all times to prevent contact with infectious materials. Gloves should be changed whenever they are visibly soiled. Aseptic technique should be used when removing gloves and other protective clothing.

5.2.6.2 Protective goggles and/or non-breakable, chemical-resistant glasses should be worn, as appropriate.

5.2.6.3 Laboratory coats, covering arms and clothing and closed in the front, should be worn at all times. Laboratory coats that become soiled should be changed.

5.2.7 This protocol does not address all safety issues associated with its use. Please refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition (Reference 17.2) for additional safety information. A reference file of Material Safety Data Sheets should be available to all personnel involved in analyses.

6.0 EQUIPMENT AND SUPPLIES

6.1 Autoclave or steam sterilizer, capable of achieving 121°C (15 pounds per square inch [psi]) for 15 minutes

6.2 Autoclave bags, aluminum foil, or kraft paper

6.3 Balance, top loading, with ASTM International Class S reference weights, capable of weighing 100 g ± 0.1 g

6.4 Beakers, glass or plastic (assorted sizes)

6.5 Biological safety cabinet, Class II (optional but recommended)

6.6 Borosilicate glass or plastic screw-cap, wide-mouth bottles, sterile (e.g., 250 mL)

6.7 Borosilicate glass culture tubes, with autoclavable screw or snap caps (25 × 150 mm)

6.8 Borosilicate glass culture tubes, with autoclavable screw-caps (16 × 100 mm)

6.9 Erlenmeyer flasks (500 mL, 1 L, 2 L)

6.10 Filters and filter syringes, sterile, for reagent sterilization (0.22 µm pore size)

6.11 Graduated cylinders (assorted sizes)

6.12 Gloves, sterile, nitrile, or equivalent

6.13 Incubators, microbiological type, maintained at 36.0°C ± 1.0°C and 42.0°C ± 1.0°C

6.14 Inoculation loops, sterile, disposable

6.15 Micropipettor

6.16 Parafilm® or equivalent

6.17 Petri dishes, sterile, plastic (15 × 100 mm)

6.18 pH meter

6.19 Pipettes, standard tip, sterile, plastic, disposable (assorted sizes)

6.20 Pipetting device (automatic or equivalent)

6.21 Pipette tips (e.g., 2 µL – 100 µL), sterile

6.22 Stirring hotplates and stir bars

6.23 Test tube racks

6.24 Thermometer, National Institute of Standards and Technology (NIST)-traceable

6.25 Tissues, lint-free (Kimwipes® or equivalent)

6.26 Waterbath maintained at 45°C – 50°C for tempering agar

6.27 Weigh paper and boats

7.0 REAGENTS AND STANDARDS

7.1 Reagent-grade chemicals must be used in all analyses. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 17.5). For suggestions regarding the testing of reagents not listed by the American Chemical Society, see *AnalaR Standards for Laboratory Chemicals* (Reference 17.6) and *United States Pharmacopeia and National Formulary 24* (Reference 17.7).

7.2 Whenever possible, use commercially available culture media. The agar used in the preparation of culture media must be of microbiological grade. Storage temperatures and times for prepared media and reagents are provided in **Table 2** (Section 7.15).

7.3 Reagent-grade water must conform to specifications in *Standard Methods for the Examination of Water and Wastewater*, 21st Edition (Reference 17.8).

7.4 Phosphate Buffered Saline (PBS)

Prepare reagent according to the following and store at <10°C and above freezing for a maximum of two weeks in tubes with loose caps or three months in screw-cap tubes.

7.4.1 Composition:

Monosodium phosphate (NaH_2PO_4)	0.58 g
Disodium phosphate (Na_2HPO_4)	2.50 g
Sodium chloride	8.50 g
Reagent-grade water	1.0 L

7.4.2 Dissolve reagents in 1 L reagent-grade water and dispense appropriate volumes in screw-cap bottles or tubes and autoclave at 121°C (15 psi) for 15 minutes. Final pH should be 7.4 ± 0.2.

7.5 Tryptic Soy Broth (TSB)

Commercially prepared medium is recommended. Dehydrated medium (Bacto™ 211824 or equivalent) may be used. If commercially prepared medium is not available, prepare according to Sections 7.5.1 and 7.5.2.

7.5.1 Composition:

	1X	2X	3X
Pancreatic digest of casein	17.0 g	34.0 g	51.0 g
Enzymatic digest of soybean meal	3.0 g	6.0 g	9.0 g
Sodium chloride	5.0 g	10.0 g	15.0 g
Dipotassium phosphate (K_2HPO_4)	2.5 g	5.0 g	7.5 g
Dextrose	2.5 g	5.0 g	7.5 g
Reagent-grade water	1.0 L	1.0 L	1.0 L

7.5.2 Add reagents to 950 mL reagent-grade water and mix thoroughly using a stir bar and heat to dissolve completely. Adjust pH to 7.3 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. For 1X TSB, aseptically dispense 10 mL into 25 × 150 mm tubes. For 3X TSB, aseptically dispense 5 and 10 mL volumes into 25 × 150 mm tubes. For 2X TSB, dispense in appropriate volumes (e.g., 100 mL). Autoclave at 121°C (15 psi) for 15 minutes.

7.6 Modified Semisolid Rappaport-Vassiliadis (MSRV) Agar with Novobiocin

Commercially prepared medium is recommended. Dehydrated medium (Difco™ 218681 or equivalent), with the addition of novobiocin supplement (Difco™ 231971 or equivalent), may be used. If commercially prepared medium is not available, prepare according to Sections 7.6.1 through 7.6.4.

7.6.1 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
Agar	2.70 g
Reagent-grade water	1.0 L

7.6.2 Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for one minute with rapid stir bar agitation 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 and bring to 1 L. Cool to 45°C – 50°C in a waterbath.

7.6.3 Prepare 2% novobiocin stock solution by dissolving 500 mg of sodium novobiocin in 25 mL of reagent-grade water and filter sterilizing, using a 0.22-µm pore-size filter. Dispense 1.1 mL of the stock solution into 2.0 mL cryogenic vials and freeze at -20°C.

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

7.6.4 Add 1.0 mL of the 2% solution of novobiocin per liter of medium. Mix well by swirling the medium. Immediately pour approximately 25 mL into each 15 × 100 mm sterile Petri plate. ***Do not invert plates to store***. **MSRV plates must be used within 48 hours of preparation**.

Note: *If using a commercially prepared novobiocin antibiotic solution that is not 2%, add sufficient volume of the novobiocin to achieve a final concentration of 0.002%.*

7.7 Xylose Lysine Desoxycholate (XLD) Agar

Commercially prepared medium is recommended. Dehydrated medium (Difco™ 278850 or equivalent) may be used. If commercially prepared medium is not available, prepare according to Sections 7.7.1 and 7.7.2.

7.7.1 Composition:

Xylose	3.75 g
L-lysine	5.0 g
Lactose	7.5 g
Saccharose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	0.08 g

Sodium desoxycholate	2.5 g
Sodium thiosulfate	6.8 g
Ferric ammonium citrate	0.8 g
Agar	15.0 g
Reagent-grade water	1.0 L

7.7.2 Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Heat with agitation just until the medium boils. Do not overheat, as overheating causes precipitation. ***Do not autoclave***. Adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. Cool to 45°C – 50°C in a waterbath. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri plate.

Note: *Heating medium to boiling sterilizes the medium; overheating or autoclaving may cause precipitation.*

7.8 Triple Sugar Iron (TSI) Agar

Commercially prepared medium is recommended. Dehydrated medium (Difco™ 226540 or equivalent) may be used. If commercially prepared medium is not available, prepare according to Sections 7.8.1 and 7.8.2.

7.8.1 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
Phenol red	0.024 g
Agar	12.0 g
Reagent-grade water	1.0 L

7.8.2 Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for one minute with rapid stir bar agitation to dissolve completely. Adjust pH to 7.4 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. Aseptically dispense 5 – 7 mL aliquots into 16 × 100 mm screw-cap tubes, cap, and autoclave at 121°C (15 psi) for 15 minutes. Cool in a slanted position so that the surface area is equally divided between the slant and butt. Let medium warm to room temperature prior to inoculation.

7.9 Lysine Iron Agar (LIA)

Commercially prepared medium is recommended. Dehydrated medium (Difco™ 284920 or equivalent) may be used. If commercially prepared medium is not available, prepare according to Sections 7.9.1 and 7.9.2.

7.9.1 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

7.9.2 Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for one minute with rapid stir bar agitation to dissolve completely. Adjust pH to 6.7 ± 0.2 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. Aseptically dispense 5 – 7 mL aliquots into 16 × 100 mm screw-cap tubes, cap, and autoclave at 121°C (15 psi) for 12 minutes. Cool in a slanted position so that the surface area is equally divided between the slant and butt. Let medium warm to room temperature prior to inoculation.

7.10 Urea Broth

Commercially prepared medium is recommended. Dehydrated medium (Difco™ 227210 or equivalent) may be used. If commercially prepared medium is not available, prepare according to Sections 7.10.1 and 7.10.2.

7.10.1 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

7.10.2 Add reagents to 950 mL of reagent-grade water and mix thoroughly using a stir bar. ***Do not boil or autoclave***. Adjust pH to 6.8 ± 0.1 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide and bring to 1 L. Filter sterilize. Aseptically dispense 3.0 mL aliquots into sterile 16 × 100 mm screw-cap tubes. Let medium warm to room temperature prior to inoculation.

7.11 Saline, physiological (0.85% w/v): Dissolve 0.85 g NaCl in 100 mL of reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes. Cool to room temperature.

7.12 *Salmonella* O antiserum Polyvalent Groups A-I and Vi (BD™ 222641 or equivalent).

7.13 BioBall® (30 CFU) *Salmonella typhimurium* (ATCC® 14028™/NCTC 12023) BTF, Pty (a bioMérieux Company) in North Ryde, Australia.

7.14 Positive and negative control cultures that are to be used with this protocol are listed in **Table 1**. Use of these controls is discussed in Section 9.

Table 1. Positive and Negative Control Cultures

Media/Reagents	Positive Control	Negative Control
MSRV	*Salmonella typhimurium* (ATCC® 14028™)	*Escherichia coli* (ATCC® 25922™)
XLD	*Salmonella typhimurium* (ATCC® 14028™)	*Escherichia coli* (ATCC® 25922™)
TSI	*Salmonella typhimurium* (ATCC® 14028™)	*Escherichia coli* (ATCC® 25922™)
LIA	*Salmonella typhimurium* (ATCC® 14028™)	*Escherichia coli* (ATCC® 25922™)
Urea broth	*Proteus hauseri* (formerly *vulgaris*) (ATCC® 13315™)	*Salmonella typhimurium* (ATCC® 14028™)
Salmonella O antiserum polyvalent A-I and Vi	*Salmonella typhimurium* (ATCC® 14028™)	*Escherichia coli* (ATCC® 25922™)

7.15 Storage temperatures and times for prepared media and reagents are provided in Table 2. Follow manufacturers' guidelines for storage and expiration of all commercially prepared reagents.

Table 2. Storage Temperatures and Times for Prepared Media and Reagents [1]

Media/Reagents	Storage Temperature	Storage Time
PBS, saline in screw-cap containers	Room temperature	3 months
Tubes: TSB, TSI, and LIA slants, urea broth	<10°C and above freezing	2 weeks in loose cap tubes 3 months in screw-cap tubes
Plates: XLD	<10°C and above freezing	2 weeks
Plates: MSRV	<10°C and above freezing	48 hours

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

8.0 SAMPLE COLLECTION, STORAGE, AND HOLDING TIME REQUIREMENTS

8.1 Drinking Water Sample Collection (200 mL)

8.1.1 Select a cold water line faucet and remove aerator, if present**.**

8.1.2 Clean the faucet exterior with disinfection solution (e.g., 10% household bleach).

8.1.3 Open the tap to obtain a smooth-flowing stream at moderate pressure without splashing.

8.1.4 Allow water to run at least 2 – 3 minutes.

8.1.5 Remove the cap from a sterile bottle containing 1 mL of a 10% sodium thiosulfate solution (dechlorinating agent).

8.1.6 Avoid contaminating the sample bottle lip or inside the cap.

8.1.7 Reduce the water flow to fill the bottle without splashing and fill to within 2.5 cm – 5 cm (1" – 2") of the top for proper mixing before analyses.

8.1.8 Do not rinse dechlorinating agent out of the bottle.

8.1.9 Tightly cap the container and transport to the laboratory on ice (do not freeze).

8.2 Surface Water Sample Collection (200 mL)

8.2.1 Collect samples by hand or with a sampling pole if the sampling site has difficult access such as a dock, bridge, or bank adjacent to surface water.

8.2.2 The sampling depth should be 6" – 12" below the water surface.

8.2.3 Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point.

8.2.4 After removal of the container from the water, a small portion of the sample should be discarded to leave a headspace of 2.5 cm – 5 cm (1" – 2") for proper mixing before analyses.

8.2.5 Transport to the laboratory on ice (do not freeze).

8.3 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.

8.4 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 31 hours of sample collection. **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 (QC)

9.1 General

Each laboratory that uses this method is required to operate a formal quality assurance program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Specific quality control (QC) procedures for use with this method are discussed below.

The minimum analytical QC requirements for the analysis of samples using this protocol include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.2), ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery (OPR) analysis (Section 9.3), matrix spike (MS) analysis (Section 9.4), and the routine analysis of negative and positive controls (Sections 9.5 and 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 BioBalls as described in Section 13.1.

9.2 Initial precision and recovery (IPR)

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. If field samples will be analyzed on the day of sample collection (ideally within 6 hours), all IPR sample analyses should begin within 30 minutes of spiking and results compared to the 0-Hour IPR Criteria provided in **Table 3**, below. If field samples are shipped overnight for analyses (e.g., samples analyzed within 30 ± 1 hours of sample collection); IPRs should be spiked and refrigerated at <10°C and above freezing for 30 ± 1 hours prior to analyses, and results compared to the 30-Hour IPR Criteria provided in Table 3.

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:

9.2.1 Prepare four, 200-mL PBS samples and spike each sample with a single *Salmonella typhimurium* (ATCC® 14028™/NCTC 12023) BioBall® according to the spiking procedure in Section 13 and analyze according to Section 11.

9.2.2 Calculate the percent recovery (R) for each IPR sample using the equation in Section 13.

9.2.3 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.

9.2.4 Compare the mean percent recovery and RSD with the corresponding IPR criteria in Table 3. If the mean percent recovery and RSD meet acceptance criteria, system performance is acceptable and analysis of field samples may begin. If the mean percent recovery or the RSD fall outside of the IPR 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 IPR analyses.

Table 3. Calculated IPR and OPR Criteria Based on 95% Prediction Interval

Holding Time	IPR/OPR	Performance Test	Analytical Method Acceptance Criteria for 200-mL PBS Samples
0-Hour	IPR (4 PBS samples)	Mean percent recovery	61% - 151%
		Precision [a]	67%
	OPR (1 PBS sample)	Percent recovery	20% - 191 %
30-Hour	IPR (4 PBS samples)	Mean percent recovery	31% - 171%
		Precision [a]	75%
	OPR (1 PBS sample)	Percent recovery	detect - 204%

[a] Precision as maximum RSD

9.3 Ongoing precision and recovery (OPR)

To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked PBS samples. The laboratory should analyze one OPR sample after every 20 field and MS samples or one per week that samples are analyzed, whichever occurs more frequently. If field samples will be analyzed on the day of sample collection (ideally within 6 hours), OPR samples should be analyzed within 30 minutes of spiking and results compared to the 0-Hour OPR Criteria provided in Table 3, above. If field samples are shipped overnight for analyses (e.g., samples analyzed within 30 ± 1 hours of sample collection); OPRs should be spiked and refrigerated at <10°C and above freezing for 30 ± 1 hours prior to analyses, and results compared to the 30-Hour OPR Criteria provided in Table 3.

EPA recommends but does not require that an OPR be performed by each analyst. OPR samples must be accompanied by an acceptable method blank (Section 9.7) and appropriate media sterility checks (Section 9.8). OPR analyses are performed as follows:

9.3.1 Prepare one, 200-mL PBS sample and spike sample with a single *Salmonella typhimurium* (ATCC® 14028™/NCTC 12023) BioBall® according to the spiking procedure in Section 13.

9.3.2 Calculate the percent recovery (R) for the OPR sample using the equation in Section 13.

9.3.3 Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 3, 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 and all associated field data should be flagged or considered invalid. 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.

9.3.4 As part of the laboratory quality assurance (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 this protocol by calculating the average mean percent recovery (R) and the standard deviation of the percent recoveries (sr). Express the accuracy as a recovery interval from R - 2sr to R + 2sr.

9.4 Matrix Spikes (MS)

MS analyses are performed to determine the effect of a particular matrix on Salmonella recoveries. The laboratory should analyze one MS sample when drinking water (DW) or surface water (SW) 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 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 9.3). The MS analysis is performed as follows:

9.4.1 Prepare two, 200-mL drinking water or surface water 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 a single *Salmonella typhimurium* (ATCC® 14028™/NCTC 12023) BioBall® according to the spiking procedure in Section 13. If field samples will be analyzed on the day of sample collection (ideally within 6 hours), process and analyze both the unspiked and spiked matrix samples immediately according to the procedures in Section 11 and calculate the *Salmonella* MPN / 100 mL according to Section 12. If field samples will be shipped overnight (analyzed within 30 ± 1 hours of sample collection), refrigerate both the unspiked matrix and the MS samples for 30 ± 1 hours prior to analyses.

9.4.2 For the MS sample, calculate the *Salmonella* MPN / 100 mL according, including adjusting the density based on the ambient concentration of *Salmonella* observed in the unspiked matrix sample, as described in Section 13.

9.4.3 Calculate the percent recovery (R) for the MS sample according to Section 13.

9.4.4 Compare the MS result (percent recovery) with the appropriate method performance criteria in **Table 4**. If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data should be flagged.

Table 4. Matrix Spike (MS) Criteria Based on 95% Prediction Interval

Matrix	Holding Time	Performance Test	Analytical Method Acceptance Criteria 200-mL Samples
Drinking Water	0-Hour	Percent recovery	11% - 206%
	30-Hour	Percent recovery	Detect – 320%
Surface Water	0-Hour	Percent recovery	Detect – 150%
	30-Hour	Percent recovery	Detect – 150%

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

9.5 Negative Controls

9.5.1 The laboratory should analyze negative controls to ensure that all media and reagents are performing properly. Negative controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should analyze a negative control every day that samples are analyzed. Recommended negative control organisms are provided in Table 1 (Section 7.14), and descriptions of negative results are provided in **Table 5** (Section 11.6).

9.5.2 Analysis of negative controls is conducted by inoculating media and conducting biochemical and serological analyses with known negative control organisms as described in Section 11. The negative control is treated as a sample and submitted to the same analytical procedures.

9.5.3 If a negative control fails to exhibit the appropriate response, check and/or replace the associated media, reagents, and/or negative control organism, and re-analyze the appropriate negative control and corresponding sample(s).

9.5.4 Viability of the negative controls should be demonstrated on a monthly basis, at a minimum, using a non-selective medium (e.g., trypticase soy agar).

9.6 Positive Controls

9.6.1 The laboratory should analyze positive controls to ensure that all media and reagents are performing properly. Positive controls should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should analyze a positive control every day that samples are analyzed. Recommended positive control organisms are provided in Table 1 (Section 7.14), and descriptions of positive results are provided in Table 5 (Section 11.6).

9.6.2 Analysis of positive controls is conducted by inoculating media and conducting biochemical and serological tests with known positive organisms as described in Section 11. The positive control is treated as a sample and submitted to the same analytical procedures.

9.6.3 If a positive control fails to exhibit the appropriate response, check and/or replace the associated media, reagents, and/or positive control organism, and re-analyze the appropriate positive control and corresponding sample(s).

9.7 Method Blank

On an ongoing basis, the laboratory should analyze a method blank every day that samples are analyzed using sterile PBS (Section 7.4) to verify the sterility of equipment, materials, and supplies. The method blank is treated as a sample, and subjected to the same analytical procedures. Absence of growth indicates freedom from contamination by the target organisms.

9.8 Media Sterility Check

Test sterility of PBS and media (TSB, MSRV, XLD, TSI, LIA, urea broth) by incubating one unit (tube or plate) from each batch at 36.0°C ± 1.5°C for 24 hours and observe for growth. Absence of growth indicates the media are sterile. On an ongoing basis, media sterility checks should be done every day that samples are analyzed.

10.0 CALIBRATION AND STANDARDIZATION

10.1 Check temperature in incubators twice daily, a minimum of four hours apart, to ensure operation is within stated limits of the method. Record daily measurements in an incubator log book.

10.2 Check temperature in refrigerators/freezers at least once daily to ensure operation is within stated limits of the method. Record daily measurements in a refrigerator/freezer log book.

10.3 Calibrate thermometers and incubators annually against a NIST-certified thermometer or against a thermometer that meets the requirements of NIST Monograph SP 250-23 (Reference 17.9). Check mercury columns for breaks.

10.4 Calibrate pH meter prior to each use with two of three standards (e.g., pH 4.0, 7.0, or 10.0) closest to the range being tested.

10.5 Calibrate analytical and top-loading balances with ASTM International Class S reference weights once per month, at a minimum. Check each day prior to use with Class S weights.

10.6 Calibrate micropipettors once per year. Spot-check micropipettor accuracy once per month by weighing a measured amount of reagent-grade water (1 µL = 1 mg).

10.7 Re-certify biological safety cabinets once per year. Re-certification must be performed by a qualified technician.

11.0 PROCEDURES

Salmonella is a pathogen and all samples should be handled with caution, using BSL-2 procedures and PPE. A Class II biological safety cabinet is recommended for sample manipulation where the risk of aerosol production is high.

11.1 Qualitative Sample Analyses

Add a sample volume (e.g., 100 mL) to an equal volume of double-strength TSB (Section 7.5) and incubate at 36°C ± 1.5° C for 24 ± 2 hours. After incubation, proceed to Section 11.3 for isolation of *Salmonella*.

11.2 Quantitative Sample Analyses

A multiple-tube assay incorporating differential sample volumes is used to estimate *Salmonella* densities in undiluted or diluted samples. If low levels of *Salmonella* are suspected, larger sample volumes (20 mL of original sample) should be used to inoculate the first row of tubes in the series. If high levels of *Salmonella* are suspected, additional serial dilutions should be used. See Flowchart 18.1 for an overview of the sample dilution and inoculation scheme. A minimum sample volume of 155 mL is required if 20 mL volumes are used to inoculate the first row of tubes.

11.2.1 Sample inoculation

Arrange TSB tubes in three rows (10 mL of 3X, 5 mL of 3X, and 10 mL of 1X) of five tubes each. Inoculate the first row of tubes (10 mL of 3X TSB) with 20 mL of the undiluted sample. Inoculate 10 mL of the undiluted sample into each of the tubes in the second row (5 mL of 3X TSB). Inoculate 1 mL from the undiluted sample into each of the tubes in the third row (10 mL of 1X TSB). See Flowchart 18.1 for an overview of the sample inoculation scheme.

11.2.2 Sample dilutions

Samples may require serial dilution prior to inoculation due to high levels of *Salmonella*. If analyzing serially diluted samples, 1.0 mL of each dilution will be used to inoculate each tube of 1X TSB, as appropriate.

11.2.3 Growth

Incubate tubes at 36.0°C ± 1.5°C for 24 ± 2 hours. After incubation, proceed to Section 11.3 for isolation of *Salmonella*.

11.3 Isolation on MRSV Plates

See Flowchart 18.2 for an overview of the colony identification procedures.

11.3.1 Select all TSB (qualitative and quantitative) tubes exhibiting growth within 24 ± 2 hours. Apply six discrete, 30 µL drops from each TSB tube onto separate MSRV plates. Space the drops evenly over the entire plate. ***Do not invert the plates, as the semisolid***

medium will not remain intact. Allow the drops to absorb into the agar for approximately one hour at room temperature before incubating the plates at 42.0°C ± 0.5°C for 16 – 18 hours.

11.3.2 On MSRV plates, presumptive *Salmonella* colonies produce a whitish halo around the colony, an indication of motility.

11.4 Isolation on XLD Plates

11.4.1 Examine plates for the appearance of motility surrounding the inoculations, as evidenced by a whitish halo.

11.4.2 Using a sterile inoculating loop, stab into a halo at the outer edge of a target colony on the MSRV plate and streak for isolation onto an XLD plate. Since *Salmonella* are predominately located within the MSRV medium, the loop should penetrate the MSRV at least half-way. Incubate the plates at 36.0°C ± 1.5°C for 18 – 24 hours.

11.4.3 Seal the MSRV plates with Parafilm® and store at <10°C and above freezing as backup, for a maximum of one week.

11.4.4 Use isolated colonies from the XLD plates for biochemical and serological analyses.

11.5 Biochemical and Serological Analyses

Salmonella produce black and/or pink to red colonies with black centers on XLD plates. Use a single well-isolated colony exhibiting *Salmonella* morphology from the XLD plate to inoculate all three media (TSI, LIA, and urea broth) used for biochemical confirmation.

11.5.1 TSI Agar

Using a sterile inoculating needle, stab the butt of a slant of TSI agar with a portion of the isolated colony and streak the slant. Loosen the cap to prevent anaerobic conditions and incubate at 36.0°C ± 1.5°C for 24 ± 2 hours. *Salmonella* have an acid (yellow) butt and alkaline (red) slant, with or without (rare) H_2S gas production. When H_2S gas production is present, the butt may appear black.

11.5.2 LIA

Using a sterile inoculating needle, stab the butt of a slant of LIA agar with a portion of the isolated colony and streak the slant. Loosen the cap to prevent anaerobic conditions and incubate at 36.0°C ± 1.5°C for 24 ± 2 hours. *Salmonella* have an alkaline (purple) butt and alkaline (purple) slant, with or without (rare) H_2S gas production. When H_2S gas production is present, the butt may appear black.

11.5.3 Urea Broth

Using a sterile loop, inoculate a urea broth tube with a portion of the isolated colony. Loosen the cap to prevent anaerobic conditions and incubate at 36.0°C ± 1.5°C for 24 ± 2 hours. If the bacterium is urease-positive, it will hydrolyze the urea in the broth, producing ammonia, and making the broth alkaline. This will turn the broth from orange

to pink or deep purplish-red. A negative urease test will exhibit no color change. *Salmonella* are urease-negative.

11.5.4 Serological Analyses

Emulsify growth from the TSI slant using sterile physiological saline (Section 7.11). Place two discrete drops of emulsified growth onto a slide. To the first drop of emulsified growth, add one drop of *Salmonella* O antiserum, polyvalent A-I and Vi (Section 7.12). 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. *Salmonella* are agglutination-positive for O, polyvalent A-I and Vi. Results should be compared with those for positive and negative controls (Table 1) analyzed at the same time.

Note: *If using antiserum equivalent to BD™ 222641(Section 7.12), the laboratory should verify each lot of the antiserum with control organisms.*

11.6 Description of Quality Control and *Salmonella* Results

Typical results are provided in Table 5.

Table 5. Positive and Negative Result Descriptions and *Salmonella* Results

Medium/Test	*Salmonella* Results	Positive Control Result and Description	Negative Control Result and Description
MSRV	Positive	Growth with whitish halo around the colony	No growth or growth without halos
XLD	Positive	Black and/or pink to red colonies with black centers	Yellow colonies
TSI (slant/butt)	Alkaline slant (red) with acid butt (yellow), with or without H_2S production	Alkaline slant (red) with acid butt (yellow), with or without H_2S production	Acid slant (yellow) with acid butt (yellow), without H_2S production
LIA (slant/butt)	Alkaline slant (purple) with alkaline butt (purple), with or without H_2S production	Alkaline slant (purple) with alkaline butt (purple), with or without H_2S production	Alkaline slant (red) with acid butt (yellow), without H_2S production
Urea broth	Negative	Pink to purplish red color change	No color change
Salmonella O antiserum polyvalent A-I and Vi	Positive	Agglutination	No agglutination

12.0 DATA ANALYSIS AND BACTERIAL DENSITY CALCULATION

12.1 Most Probable Number (MPN) Technique

Estimation of bacterial densities may be determined based on the number of tubes positive for *Salmonella* either by morphological, biochemical, and serological results or PCR.

12.2 Calculation of MPN

If only three rows of tubes were analyzed, identify appropriate MPN value using either **Table 7** or **8**, depending on volumes assayed. If more than three rows of tubes were analyzed, the appropriate rows must be selected and MPN value calculated as described in Sections 12.2.1 and 12.2.2. Table 7 should only be used for volumes of 20.0 mL, 10.0 mL, and 1.0 mL. If volumes of 10.0 mL or less were analyzed, refer to Table 8 for the MPN values (Sections 12.2.1 and 12.2.2).

12.2.1 Selection of Tubes

If more than three rows of tubes are inoculated with sample (e.g., volumes/dilutions), select the most appropriate rows of tubes according to the criteria provided below. Examples of row selections and MPN/100 mL values are provided in **Table 6**.

12.2.1.1 Choose the smallest volume or the highest dilution giving positive results in all five tubes inoculated, plus the two succeeding lower concentrations. In Example A from Table 6, 10 mL is a smaller volume than 20 mL, and is the lowest volume giving positive results in all five tubes.

12.2.1.2 If the largest volume tested has less than five tubes with positive results, select it and the next two volumes (Table 6, Examples B and C).

12.2.1.3 When a positive result occurs in a smaller volume than the three rows selected according to the rules above, change the selection to the largest volume that has less than five positive results, and the next two smaller volumes (Table 6, Example D).

12.2.1.4 When the selection rules above have left unselected any smaller volumes with positive results, add those positive tubes to the row of tubes for the smallest volume selected (Table 6, Example E).

12.2.1.5 If there were not enough lower volumes analyzed to select three dilutions using the rules above, then select the three smallest volumes (Table 6, Example F).

Table 6. Examples of Appropriate Tube Selection and MPN/100 mL [1]

Example	20 mL	10 mL	1.0 mL	0.1 mL	Significant Dilutions	Table	MPN Index	MPN/100 mL
A	5/5	5/5	3/5	0/5	5-3-0	8	0.792	79.2
B	4/5	5/5	1/5	0/5	4-5-1	7	0.1524	15.24
C	0/5	1/5	0/5	0/5	0-1-0	7	0.0067	0.67
D	5/5	3/5	1/5	1/5	3-1-1	8	0.137	13.7
E	4/5	4/5	0/5	1/5	4-4-1	7	0.1181	11.81
F	5/5	5/5	5/5	2/5	5-5-2	8	5.422	542.2

[1] Appropriate volumes are underlined and the largest sample volumes analyzed are highlighted.

12.2.2 For calculation of MPN/100 mL when additional dilutions are analyzed (e.g., 10^{-2}, 10^{-3}), obtain the MPN index value from Table 8 using the number of positive tubes in the three selected dilutions. Calculate MPN/100 mL using the equation below:

$$\text{MPN/100 mL} = \frac{\text{MPN Index from Table 8}}{\text{Middle volume analyzed in the series used for MPN determination}} \times 100$$

For example, a dilution series of 10^{-3}, 10^{-4}, 10^{-5}, with the following positive tubes 5, 1, 0, respectively, would be:

$$\text{MPN/100 mL} = \frac{0.329}{10^{-4}} \times 100 = 3.29 \times 10^{5}$$

Table 7. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Volume and Inoculation Volumes are 20.0, 10.0, and 1.0 mL [1]

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

Table 7. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Volume and Inoculation Volumes are 20.0, 10.0, and 1.0 mL (cont) [1]

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	>1.6090	0.3837	----

[1] Table was developed using the MPN calculator developed by Albert Klee (Reference 17.10).

Table 8. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Volume and Inoculation Volumes are 10.0, 1.0, and 0.1 mL [1]

Combination of Positives	MPN Index	95% Confidence Limits		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.018	---	0.063	1-3-0	0.083	0.012	0.196
0-0-1	0.018	0.003	0.063	1-3-1	0.104	0.020	0.243
0-0-2	0.036	0.003	0.101	1-3-2	0.125	0.029	0.296
0-0-3	0.054	0.003	0.137	1-3-3	0.147	0.038	0.364
0-0-4	0.072	0.008	0.174	1-3-4	0.169	0.048	0.460
0-0-5	0.091	0.015	0.212	1-3-5	0.191	0.057	0.566
0-1-0	0.018	0.003	0.063	1-4-0	0.105	0.021	0.245
0-1-1	0.036	0.003	0.101	1-4-1	0.127	0.030	0.300
0-1-2	0.055	0.003	0.138	1-4-2	0.148	0.039	0.370
0-1-3	0.073	0.008	0.175	1-4-3	0.170	0.048	0.468
0-1-4	0.091	0.015	0.214	1-4-4	0.193	0.058	0.575
0-1-5	0.110	0.023	0.256	1-4-5	0.215	0.067	0.657
0-2-0	0.037	0.003	0.102	1-5-0	0.128	0.030	0.303
0-2-1	0.055	0.003	0.139	1-5-1	0.150	0.040	0.375
0-2-2	0.074	0.008	0.176	1-5-2	0.172	0.049	0.477
0-2-3	0.092	0.015	0.215	1-5-3	0.195	0.058	0.583
0-2-4	0.111	0.023	0.258	1-5-4	0.217	0.068	0.664
0-2-5	0.129	0.031	0.307	1-5-5	0.240	0.077	0.731
0-3-0	0.056	0.003	0.140	2-0-0	0.045	0.003	0.119
0-3-1	0.074	0.009	0.177	2-0-1	0.068	0.006	0.164
0-3-2	0.093	0.016	0.217	2-0-2	0.091	0.015	0.213
0-3-3	0.112	0.023	0.260	2-0-3	0.115	0.025	0.269
0-3-4	0.130	0.031	0.310	2-0-4	0.139	0.035	0.338
0-3-5	0.149	0.039	0.372	2-0-5	0.164	0.046	0.437
0-4-0	0.075	0.009	0.179	2-1-0	0.068	0.006	0.166
0-4-1	0.094	0.016	0.219	2-1-1	0.092	0.015	0.216
0-4-2	0.112	0.024	0.263	2-1-2	0.116	0.025	0.272
0-4-3	0.131	0.032	0.313	2-1-3	0.141	0.036	0.343
0-4-4	0.150	0.040	0.377	2-1-4	0.166	0.046	0.447
0-4-5	0.169	0.048	0.462	2-1-5	0.192	0.057	0.571
0-5-0	0.094	0.016	0.221	2-2-0	0.093	0.016	0.218
0-5-1	0.113	0.024	0.265	2-2-1	0.118	0.026	0.276
0-5-2	0.133	0.032	0.317	2-2-2	0.143	0.036	0.349
0-5-3	0.152	0.040	0.382	2-2-3	0.168	0.047	0.456
0-5-4	0.171	0.048	0.470	2-2-4	0.194	0.058	0.581
0-5-5	0.190	0.056	0.563	2-2-5	0.221	0.069	0.675
1-0-0	0.020	0.003	0.068	2-3-0	0.119	0.026	0.279
1-0-1	0.040	0.003	0.108	2-3-1	0.144	0.037	0.355
1-0-2	0.060	0.003	0.149	2-3-2	0.170	0.048	0.467
1-0-3	0.081	0.011	0.191	2-3-3	0.197	0.059	0.591
1-0-4	0.101	0.019	0.236	2-3-4	0.223	0.070	0.683
1-0-5	0.122	0.028	0.287	2-3-5	0.251	0.082	0.759
1-1-0	0.040	0.003	0.109	2-4-0	0.146	0.038	0.361
1-1-1	0.061	0.003	0.150	2-4-1	0.172	0.049	0.477
1-1-2	0.081	0.011	0.192	2-4-2	0.199	0.060	0.600
1-1-3	0.102	0.019	0.238	2-4-3	0.226	0.072	0.692
1-1-4	0.123	0.028	0.290	2-4-4	0.254	0.083	0.768
1-1-5	0.144	0.037	0.354	2-4-5	0.282	0.094	0.836
1-2-0	0.061	0.003	0.151	2-5-0	0.174	0.050	0.488
1-2-1	0.082	0.012	0.194	2-5-1	0.201	0.061	0.610
1-2-2	0.103	0.020	0.240	2-5-2	0.229	0.073	0.700
1-2-3	0.124	0.029	0.293	2-5-3	0.257	0.084	0.776
1-2-4	0.146	0.038	0.359	2-5-4	0.286	0.095	0.845
1-2-5	0.167	0.047	0.451	2-5-5	0.315	0.107	0.910

Table 8. MPN Index and 95% Confidence Limits for Various Combinations of Positive Results When Five Tubes are Used per Volume and Inoculation Volumes are 10.0, 1.0, and 0.1 mL (cont.) [1]

Combination of Positives	MPN Index	95% Confidence Levels		Combination of Positives	MPN Index	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-0-0	0.079	0.010	0.188	4-3-0	0.271	0.090	0.809
3-0-1	0.106	0.021	0.246	4-3-1	0.326	0.111	0.934
3-0-2	0.135	0.033	0.323	4-3-2	0.386	0.132	1.060
3-0-3	0.165	0.046	0.440	4-3-3	0.451	0.154	1.192
3-0-4	0.196	0.059	0.589	4-3-4	0.521	0.176	1.331
3-0-5	0.229	0.073	0.699	4-3-5	0.593	0.196	1.477
3-1-0	0.107	0.022	0.250	4-4-0	0.335	0.114	0.953
3-1-1	0.137	0.034	0.329	4-4-1	0.398	0.137	1.084
3-1-2	0.167	0.047	0.452	4-4-2	0.466	0.159	1.223
3-1-3	0.199	0.060	0.601	4-4-3	0.539	0.181	1.368
3-1-4	0.232	0.074	0.710	4-4-4	0.615	0.202	1.521
3-1-5	0.267	0.088	0.800	4-4-5	0.693	0.223	1.681
3-2-0	0.138	0.035	0.335	4-5-0	0.411	0.141	1.111
3-2-1	0.170	0.048	0.464	4-5-1	0.483	0.164	1.256
3-2-2	0.202	0.062	0.613	4-5-2	0.559	0.187	1.409
3-2-3	0.236	0.076	0.720	4-5-3	0.639	0.209	1.570
3-2-4	0.271	0.090	0.810	4-5-4	0.722	0.230	1.739
3-2-5	0.308	0.104	0.894	4-5-5	0.806	0.250	1.916
3-3-0	0.172	0.049	0.477	5-0-0	0.240	0.076	0.763
3-3-1	0.205	0.063	0.624	5-0-1	0.314	0.106	0.908
3-3-2	0.240	0.077	0.731	5-0-2	0.427	0.146	1.142
3-3-3	0.276	0.092	0.821	5-0-3	0.578	0.192	1.446
3-3-4	0.313	0.106	0.906	5-0-4	0.759	0.239	1.816
3-3-5	0.352	0.120	0.989	5-0-5	0.953	0.165	2.234
3-4-0	0.209	0.064	0.636	5-1-0	0.329	0.112	0.940
3-4-1	0.244	0.079	0.742	5-1-1	0.456	0.156	1.202
3-4-2	0.281	0.093	0.833	5-1-2	0.631	0.207	1.553
3-4-3	0.319	0.108	0.918	5-1-3	0.839	0.257	1.985
3-4-4	0.358	0.123	1.002	5-1-4	1.062	0.304	2.485
3-4-5	0.399	0.137	1.086	5-1-5	1.293	0.304	3.090
3-5-0	0.248	0.080	0.753	5-2-0	0.493	0.167	1.276
3-5-1	0.286	0.095	0.844	5-2-1	0.700	0.224	1.694
3-5-2	0.325	0.110	0.931	5-2-2	0.944	0.280	2.213
3-5-3	0.365	0.125	1.017	5-2-3	1.205	0.331	2.843
3-5-4	0.407	0.140	1.103	5-2-4	1.479	0.381	3.714
3-5-5	0.450	0.154	1.189	5-2-5	1.767	0.503	5.230
4-0-0	0.130	0.031	0.311	5-3-0	0.792	0.247	1.886
4-0-1	0.166	0.046	0.445	5-3-1	1.086	0.308	2.544
4-0-2	0.207	0.064	0.631	5-3-2	1.406	0.368	3.445
4-0-3	0.253	0.082	0.764	5-3-3	1.750	0.434	5.131
4-0-4	0.302	0.102	0.881	5-3-4	2.122	0.529	6.798
4-0-5	0.355	0.121	0.996	5-3-5	2.527	0.814	7.971
4-1-0	0.169	0.048	0.460	5-4-0	1.299	0.348	3.108
4-1-1	0.212	0.066	0.646	5-4-1	1.724	0.429	4.975
4-1-2	0.258	0.085	0.779	5-4-2	2.212	0.563	7.087
4-1-3	0.310	0.105	0.898	5-4-3	2.781	0.882	8.600
4-1-4	0.365	0.125	1.016	5-4-4	3.454	1.159	10.110
4-1-5	0.425	0.145	1.138	5-4-5	4.256	1.437	11.800
4-2-0	0.216	0.067	0.661	5-5-0	2.398	0.762	7.629
4-2-1	0.264	0.087	0.794	5-5-1	3.477	1.172	10.160
4-2-2	0.317	0.108	0.915	5-5-2	5.422	1.791	14.190
4-2-3	0.375	0.129	1.037	5-5-3	9.178	2.672	22.010
4-2-4	0.438	0.150	1.164	5-5-4	16.090	3.837	41.030
4-2-5	0.504	0.171	1.297	5-5-5	>16.090	3.837	-----

[1] Table was developed using the MPN calculator developed by Albert Klee (Reference 17.10).

13.0 SAMPLE SPIKING AND PERCENT RECOVERY CALCULATION

QC requirements (Section 9) include the preparation and analysis of spiked reference (PBS) samples in order to assess initial and ongoing method performance and matrix effects. For IPR (Section 9.2), OPR (Section 9.3), and MS (Section 9.4) analyses, it is necessary to spike samples with *Salmonella typhimurium* (ATCC® 14028™/NCTC 12023) BioBall® as described below.

13.1 Sample Spiking

13.1.1 Open vial and aseptically add 1 BioBall® to 200 ml of sample. Mix by vigorously shaking the bottles a minimum of 25 times.

13.1.2 Analyze samples according to Section 11.

13.2 Calculation of BioBall® Spike Percent Recovery

Since one BioBall® is spiked per 200 mL sample, use the *S. typhimurium* lot mean value provided by the manufacturer as the "true" spiked *S. typhimurium*. That is:

$$T_{\text{Spiked } S.\ typhimurium}\ (\text{CFU/200 mL}) = \text{BioBall® lot mean value}$$

Calculate percent recovery (R) using the following equation:

$$R = \frac{100 \times (N_s - N_u)}{T_{\text{Spiked } S.\ typhimurium}}$$

Where,

R = Percent recovery
N_s = *S. typhimurium* (CFU/200 mL) in the spiked sample)
N_u = *S. typhimurium* (CFU/200 mL) in the unspiked sample

Note: *For recovery calculations, when no background is observed in the unspiked sample, the detection limit should be used for N_u in the calculations, instead of zero. See first example in **Table 9**, below.*

Percent recovery example calculations are provided in Table 9.

Table 9. Percent Recovery Example Calculations

N_s (CFU/200 mL)	N_u (CFU/200 mL)	$T_{\text{Spiked } S.\ typhimurium}$ (CFU/200 mL)	Percent recovery (R)
25	<1	27	100 × (25 - 1) / 27 = **89%**
39	10	27	100 × (39 - 10) / 27 = **107%**

14.0 PROTOCOL PERFORMANCE

A summary of protocol performance based on the single-laboratory verification and multi-laboratory validation studies are provided in Sections 14.1 and 14.2, respectively.

14.1 Single-Laboratory Verification Study

Culture-based procedures were evaluated for *S. typhimurium* in a reference matrix (PBS) and two matrices of interest (drinking water, surface water) during a single-laboratory verification study. Results are based upon recovery of a single strain laboratory strain of *Salmonella* (*S. typhimurium* ATCC® 14028™); results may vary when assaying for environmental strains. Details regarding procedure performance are provided in the study report (Reference 17.11). Single laboratory verification results are provided in **Table 10**.

Table 10. *S. typhimurium* Results for PBS, Drinking Water, and Surface Water Samples

Sample ID	Spike Level (CFU/100 mL) [a]	MPN Combo	*S. typhimurium* (MPN/100 mL)	Percent Recovery	Mean Recovery (%)	SD [b] (%)	RSD [c] (%)
PBS Samples							
Unspiked	NA	0-0-0	< 0.6473	NA			
Spiked	15.25	5-3-1	13.68	85.46	100.95	14.65	14.51
		5-4-1	19.07	120.80			
		5-4-0	15.71	98.77			
		5-4-0	15.71	98.77			
Drinking Water Samples							
Unspiked	NA	0-0-0	< 0.6473	NA			
		0-0-0	< 0.6473	NA			
Spiked	15.25	4-3-1	9.37	57.20	78.66	17.06	21.69
		5-4-0	15.71	98.77			
		4-3-3	12.45	77.39			
		4-5-0	13.04	81.26			
Surface Water Samples							
Unspiked	NA	0-0-0	< 0.6473	NA			
		0-0-0	< 0.6473	NA			
Spiked	15.25	5-5-0	23.98	153.00	129.05	47.90	37.12
		5-5-0	23.98	153.00			
		4-3-1	9.37	57.20			
		5-5-0	23.98	153.00			

[a] Colony forming unit per 100 milliliter
[b] Standard deviation
[c] Relative standard deviation

14.2 Multi-Laboratory Validation Study

Ten volunteer laboratories participated in EPA's multi-laboratory validation study of evaluating this protocol. A detailed description of the study and results are provided in the validation study report (Reference 17.3). The Study included the analysis of PBS, drinking water, and surface water samples spiked with *S. typhimurium* BioBall® spikes. The results of this Study were used to assess method performance (i.e., recovery and precision) across multiple laboratories and matrices, compare effect of holding time (0 − 8 hours after sample spiking, compared to 30 ± 1 hours after sample spiking), assess reproducibility of results from analyses of "blind" samples collected from each of two sites per matrix (drinking water and surface water), and develop QC acceptance criteria. 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 April 2010 draft version of the protocol**.**

14.2.1 Validation and Holding Time Analyses – Recovery and Precision

For this assessment, Laboratory 7's data were excluded even though the 0-Hour data were valid, because including 0-Hour results when there were no 30-hour results (30-Hour analyses were not conducted) would bias the analysis.

14.2.1.1 PBS

For PBS, the mean recovery measured at Time-0 did not differ significantly from the mean recovery measured at the 30-Hour holding time (p-value=0.0797). The overall mean recovery for Time-0 in PBS, excluding Laboratory 7, was 137.41% with a pooled within-laboratory relative standard deviation (RSD) of 57.62%; laboratory-specific mean recoveries ranged from 95.05% to 270.56%. The overall mean recovery for 30-hour PBS was 101.06% with a pooled within-laboratory RSD of 37.13%; and laboratory specific mean recoveries ranged from 60.87% to 162.39%.

14.2.1.2 Drinking Water

For drinking water, the mean recovery measured at Time-0 did not differ significantly from the mean recovery measured at the 30-Hour holding time (p-value=0.7915). The overall mean recovery for Time-0 drinking water, excluding Laboratory 7, was 117.65% with a pooled within-laboratory RSD of 47.71%; laboratory-specific mean recoveries ranged from 69.62% to 162.77%. For the 30-hour holding time, drinking water had an overall mean recovery of 122.71% and a pooled within-laboratory RSD of 61.79%; laboratory-specific mean recoveries ranged from 41.12% to 182.92%.

14.2.1.3 Surface Water

For surface water, the mean recovery measured at Time-0 differed significantly from the mean recovery measured at the 30-Hour holding time (p-value=0.0423). The overall mean recovery for Time-0 surface water, excluding Laboratory 7, was 79.60% with a pooled within-laboratory RSD of 79.25%; laboratory-specific mean recoveries ranged from 1.14% to 199.82%. For the 30-Hour holding time, there was an overall recovery of 58.10% and a pooled within-laboratory RSD of 66.01%; laboratory-specific mean recoveries ranged from 0.00% to 95.17%. It should be noted that the significant

difference in holding time was strongly influenced by the Laboratory 10, Time-0 recoveries which were 71.23%, 223.76%, 351.3%, and 153.00% resulting in a mean laboratory-specific recovery of 199.82% – more than twice as high as the other laboratories. When data from Laboratory 10 are removed, there is not a significant difference in recoveries between Time-0 and 30-hour results (p-value=0.2221).

For PBS and drinking water matrices, recoveries at Time-0 did not differ significantly compared to the 30-Hour holding time, indicating that these samples can be held at <10°C, but above freezing for up to 30 hours without detrimental effect (i.e., samples can be shipped overnight). While a significant difference was observed between Time-0 and 30-Hour results for surface water, this was largely driven by unusual results from a single laboratory. In most cases, holding surface water samples for 30 hours did not appear to have a detrimental effect on recovery.

14.2.2 Assessment of Reproducibility

Reproducibility data were analyzed for each matrix (drinking water sample 1, drinking water sample 2, surface water sample 1, and surface water sample 2). The main goal of this analysis was to assess whether any laboratory yielded significantly different mean MPN/100 mL results from any other laboratory, thereby indicating that results were not fully reproducible at different laboratories when the same "blind" sample was being analyzed across all laboratories.

14.2.2.1 Drinking Water

Laboratory-specific mean MPN/100 mL for drinking water sample 1 ranged from 11.39 to 34.52 with laboratory-specific RSDs ranging from 13.15% to 110.96%. For drinking water sample 2, laboratory specific mean MPN/100 mL ranged from 11.59 to 24.05 with laboratory-specific RSDs ranging from 1.29% to 86.41%.

There was not a significant difference between laboratories for either drinking water matrix (F-test p-values of 0.465 and 0.516 for drinking water samples 1 and 2, respectively).

14.2.2.2 Surface Water

Laboratory-specific mean MPN/100 mL for surface water sample 1 one ranged from 0.69 to 21.09 with laboratory-specific RSDs ranging from 5.16% to 82.53%. For surface water sample 2, laboratory-specific mean MPN/100 mL ranged from 1.09 to 16.23 with laboratory-specific RSDs ranging from 16.42% to 107.79.

Significant differences in mean MPN/100 mL were observed between at least two laboratories for surface water sample 1 and also for surface water sample 2 (F-test p-values of 0.013 and 0.017 for surface water samples 1 and 2, respectively).

Because there were no significant differences in mean MPN/100 mL values between laboratories for drinking water matrices, it appears that laboratory results are reproducible. The differences in mean MPN/100 mL values observed in the surface water matrices may be due to varying degrees of laboratory proficiency with the method,

as at least one laboratory retrospectively suggested that, they should have submitted more "questionable colonies" from MSRV to confirmation. In addition, another laboratory indicated that background bacteria from the TSB enrichment made it very difficult to identify presumptively positive colonies on MSRV. As a result, it is critical that laboratories become proficient with this protocol prior to analyzing samples from complex surface water matrices, as inappropriately low results may be reported.

15.0 POLLUTION PREVENTION

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

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

16.0 WASTE MANAGEMENT

16.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect 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.

16.2 Samples, reference materials, and equipment known or suspected to be contaminated with viable *Salmonella* must be sterilized prior to disposal.

16.3 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 17.12) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 17.13), both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

17.0 REFERENCES

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17.11 U.S. Environmental Protection Agency. *Single-Laboratory Verification of Culture-Based Analytical Procedure for Non-Typhoidal Salmonella in Drinking Water and Surface Water.* Publication forthcoming; date and number to be determined.

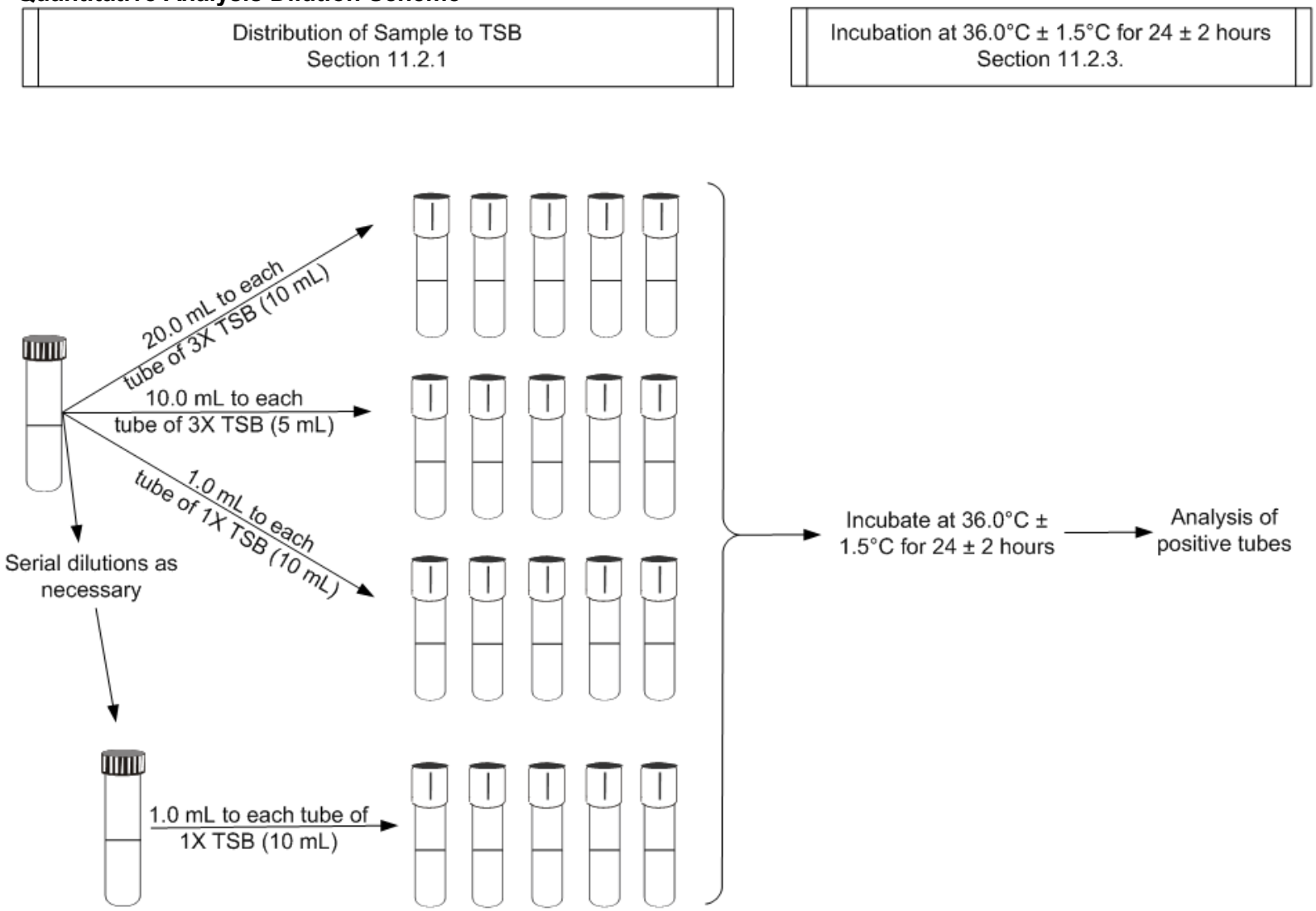
17.12 American Chemical Society (ACS). 1990. *The Waste Management Manual for Laboratory Personnel.* Washington, DC: American Chemical Society Department of Government Relations and Science Policy.

17.13 American Chemical Society (ACS). 1985. *Less Is Better: Laboratory Chemical Management for Waste Reduction.* Washington, DC: American Chemical Society Department of Government Relations and Science Policy.

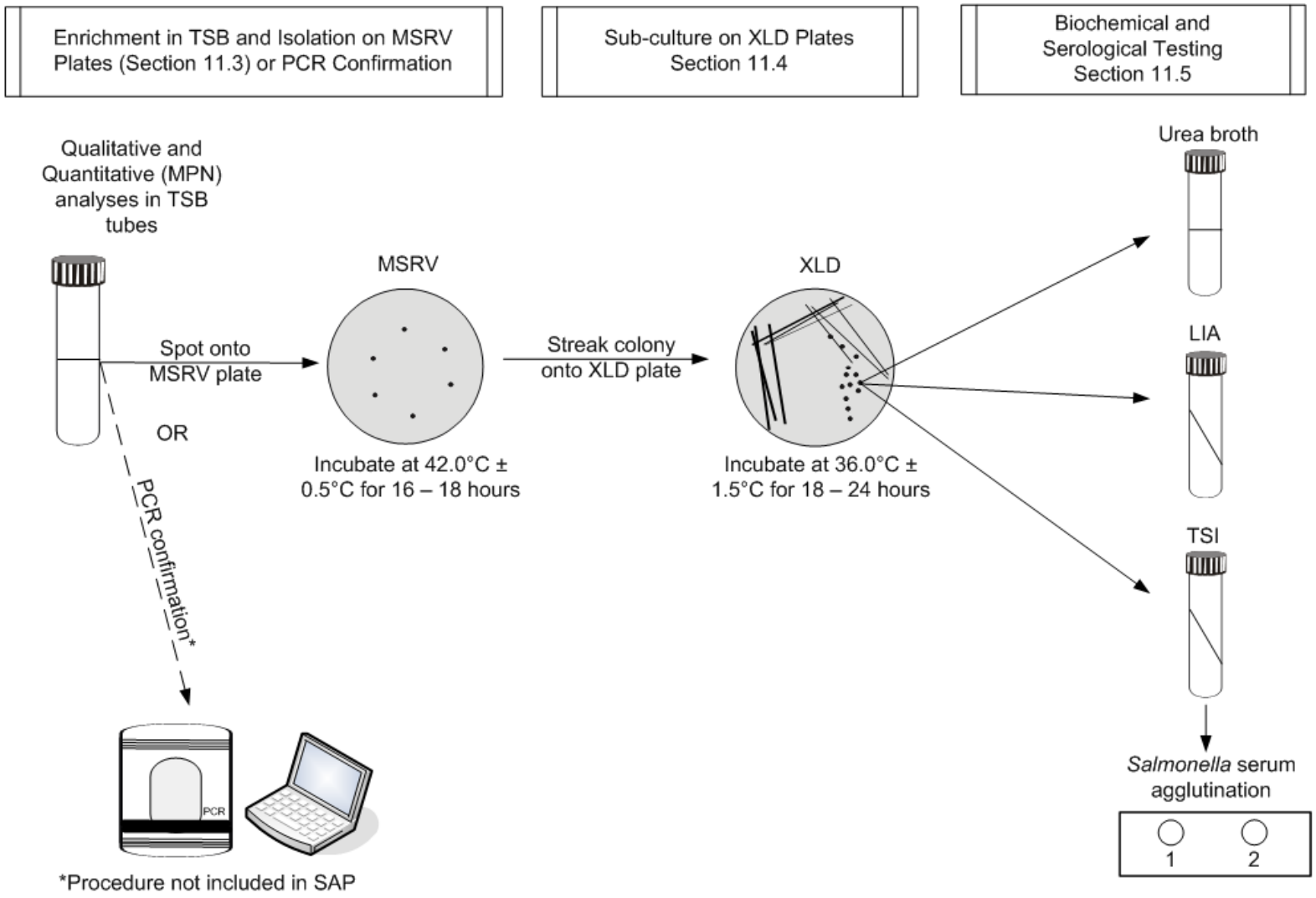
17.14 Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). Microbiological Methods for Monitoring the Environment: Water and Wastes, EPA-600/8-78-017. Cincinnati, OH: U.S. Environmental Protection Agency, 1978.

18.0 FLOWCHARTS

18.1 Quantitative Analysis Dilution Scheme



18.2 Identification Flowchart



Appendix A:

Part II (General Operations), Section A (Sample Collection, Preservation, and Storage)

The following is an excerpt from Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). Microbiological Methods for Monitoring the Environment: Water and Wastes, EPA-600/8-78-017. Cincinnati, OH: U.S. Environmental Protection Agency, 1978 [Reference 17.14].

Sample Collection[1]

1.0 Sample Containers

1.1 Sample Bottles: Bottles must be resistant to sterilizing conditions and the solvent action of water. Wide-mouth borosilicate glass bottles with screw-cap or ground-glass stopper or heat-resistant plastic bottles may be used if they can be sterilized without producing toxic materials (see examples A and C in Figure 1). Screw-caps must not produce bacteriostatic or nutritive compounds upon sterilization.

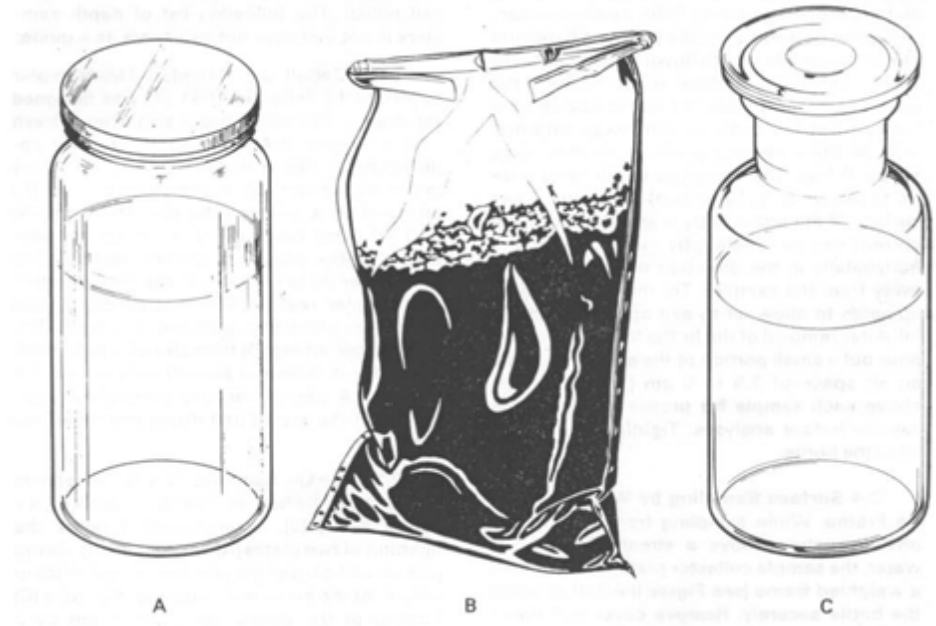


Figure 1. Suggested sample containers.

1.2 Selection and Cleaning of Bottles: Samples bottles should be at least 125 mL volume for adequate sampling and for good mixing. Bottles of 250 mL, 500 mL, and 1000 mL volume are often used for multiple analyses. Discard bottles which have chips, cracks, and etched surfaces. Bottle closures must be water-tight. Before use, thoroughly cleanse bottles and closures with detergent and hot water, followed by a hot water rinse to remove all trace of detergent. Then rinse them three times with laboratory-pure water.

1.3 Dechlorinating Agent: The agent must be placed in the bottle when water and wastewater samples containing residual chlorine are anticipated. Add sodium thiosulfate to the bottle before sterilization at a concentration of 0.1 mL of a 10% solution for each 125 mL sample volume. This concentration will neutralize approximately 15 mg/L of residue chlorine.

1.4 Chelating Agent: A chelating agent should be added to sample bottles used to collect samples suspected of containing >0.01 mg/L concentrations of heavy metals such as copper, nickel or zinc, etc.

Add 0.3 mL of a 15% solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt, for each 125 mL sample volume prior to sterilization.

1.5 Wrapping Bottles: Protect the tops and necks of glass stoppered bottles from contamination by covering them before sterilization with aluminum foil or kraft paper.

1.6 Sterilization of Bottles: Autoclave glass or heat-resistant plastic bottles at 121°C for 15 minutes. Alternatively, dry glassware may be sterilized in a hot oven at 170°C for not less than two hours. Ethylene oxide gas sterilization is acceptable for plastic containers that are not heat-resistant. Sample bottles sterilized by gas should be stored overnight before being used to allow the last traces of gas to dissipate.

1.7 Plastic Bags: The commercially available bags (Whirl-pak) (see example B in Figure 1) are a practical substitute for plastic or glass samples bottles in sampling soil, sediment, or biosolids. The bags are sealed in manufacture and opened only at time of sampling. The manufacturer states that such bags are sterilized.

2.0 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a bridge or bank adjacent to a surface water.

2.1 Chlorinated Samples: When samples such as treated waters, chlorinated wastewaters or recreational waters are collected, the sample bottle must contain a dechlorinating agent (see section 1.3 above).

2.2 Composite Sampling: In no case should a composite sample be collected for bacteriologic examination. Data from individual samples show a range of values. A composite sample will not display this range. Individual results will give information about industrial process variations in flow and composition. Also, one or more portions that make up a composite sample may contain toxic or nutritive materials and cause erroneous results.

2.3 Surface Sampling by Hand: A grab sample is obtained using a sample bottle prepared as described in (1) above. Identify the sampling site on the bottle label and on a field log sheet. Remove the bottle covering and closure and protect from contamination. Grasp the bottle at the base with one hand and plunge the bottle mouth down into the water to avoid introducing surface scum (Figure 2). Position the mouth of the bottle into the current away from the hand of the collector and, if applicable, away from the side of the sampling platform. The sampling depth should be 15-30 cm (6-12 inches) below the water surface. If the water body is static, an artificial current can be created, by moving the bottle horizontally in the direction it is pointed and away from the sampler. Tip the bottle slightly upwards to allow air to exit and the bottle to fill. After removal of the bottle from the stream, pour out a small portion of the sample to allow an air space of 2.5-5 cm (1-2 inches) above each sample for proper mixing of the sample before analyses. Tightly stopper the bottle and place on ice (do not freeze) for transport to the laboratory.

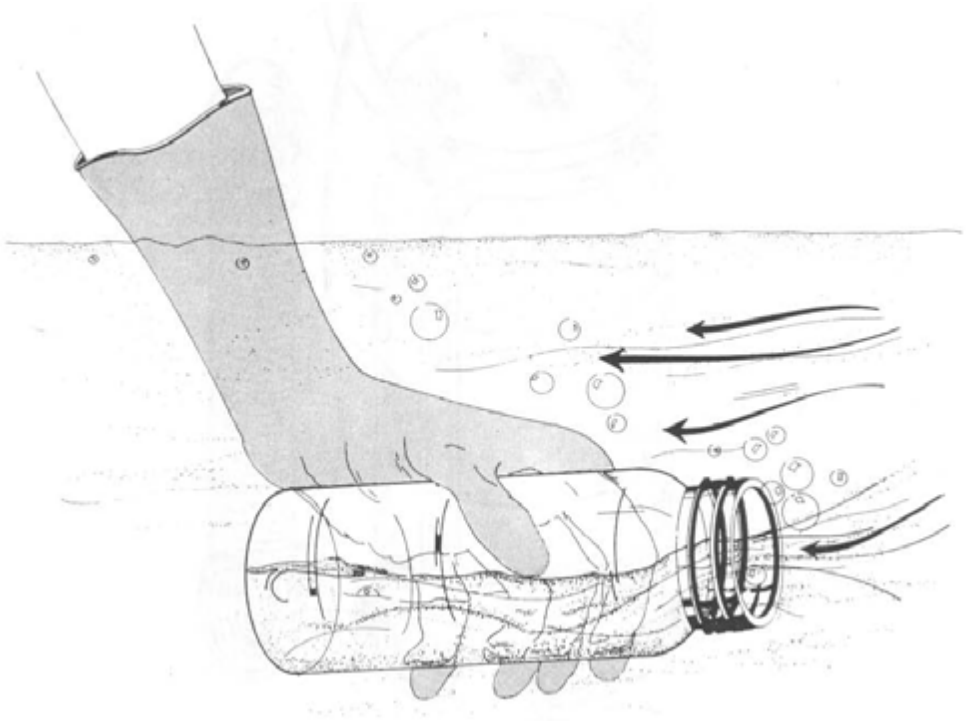


Figure 2. Grab sampling technique for surface waters.

3.0 Selection of Sampling Sites and Frequency

These will be described for streams, rivers, estuarine, marine, and recreational waters as well as domestic and industrial wastewaters.

3.1 Stream Sampling: The objectives of the initial survey dictate the location, frequency and number of samples to be collected.

3.1.1 Selection of Sampling Sites: A typical stream sampling program includes sampling locations upstream of the area of concern, upstream and downstream of waste discharges, upstream and downstream from tributary entrances to the river and upstream of the mouth of the tributary. For more complex situations, where several waste discharges are involved, sampling includes sites upstream and downstream from the combined discharge area and samples taken directly from each industrial or municipal waste discharge. Using available bacteriological, chemical and discharge rate data, the contribution of each pollution source can be determined.

3.1.2 Small Streams: Small streams should be sampled at background stations upstream of the pollution sources and at stations downstream from pollution sources. Additional sampling sites should be located downstream to delineate the zones of pollution. Avoid sampling areas where stagnation may occur (e.g., backwater of a tributary) and areas located near the inside bank of a curve in the stream which may not be representative of the main channel.

3.1.3 Large Streams and Rivers: Large streams are usually not well mixed laterally for long distances downstream from the pollution sources. Sampling sites below point source pollution should be established to provide desired downstream travel time and dispersal as determined by flow rate measurements. Particular care must be taken to establish the proper sampling points. Occasionally, depth samples are necessary to determine vertical mixing patterns.

3.2 Estuarine and Marine Sampling: Sampling estuarine and marine waters requires the consideration of other factors in addition to those usually recognized in fresh water sampling. They include tidal cycles, current patterns, bottom currents and counter-currents, stratification, seasonal fluctuations, dispersion of discharges and multi-depth samplings. The frequency of sampling varies with the objectives. When a sampling program is started, it may be necessary to sample every hour around the clock to establish pollution loads and dispersion patterns. The sewage discharges may occur continuously or intermittently.

When the sampling strategy for a survey is planned, data may be available from previous hydrological studies done by the Coast Guard, Corps of Engineers, National Oceanic and Atmospheric Administration (NOAA), U.S. Geological Survey, or university and private research investigations. In a survey, float studies and dye studies are often carried out to determine surface and undercurrents. Initially depth samples are taken on the bottom and at five feet increments between surface and bottom. A random grid pattern for selecting sampling sites is established statistically.

3.2.1 Estuarine Sampling: When a survey is made on an estuary, samples are often taken from a boat, usually making an end to end traverse of the estuary. Another method involves taking samples throughout a tidal cycle, every hour or two hours from a bridge or from an anchored boat at a number of fixed points. In a large bay or estuary where many square miles of area are involved, a grid or series of stations may be necessary. Two sets of samples are usually taken from an area on a given day, one at ebb or flood slack water, and the other three hours earlier, or later, at the half tidal interval. Sampling is scheduled so that the mid-sampling time of each run coincides with the calculated occurrence of the tidal condition. In location sampling sites, one must consider points at which tributary waters enter the main stream or estuary, location of shellfish beds and bathing beaches. The sampling stations can be adjusted as data accumulate. For example, if a series of stations half mile apart consistently show similar values, some of these stations may be dropped and other stations added in areas where data shows more variability. Considerable stratification can occur between the salt water from the sea and the fresh water supplied by a river. It is essential when starting a survey of an unknown estuary to find out whether there is any marked stratification. This can be done by chloride determinations at different locations and depths. It is possible for stratification to occur in one part of an estuary and not in another.

On a flood tide, the more dense salt water pushing up into the less dense fresh river water will cause an overlapping with the fresh water flowing on top. A phenomenon called a salt water wedge can form. As a result, stratification occurs. If the discharge of pollution is in the salt water layer, the contamination will be concentrated near the bottom at the flood tide. The flow or velocity of the fresh water will influence the degree of stratification which occurs. If one is sampling only at the surface, it is possible that the data will not show the polluted underflowing water which was contaminated at the point below the fresh water river. Therefore, where stratification is suspected, samples at different depths will be needed to measure vertical distribution.

3.3 Recreational Waters (Bathing Beaches): Sampling sites at bathing beaches or other recreational areas should include upstream or peripheral areas and locations adjacent to natural drains that would discharge stormwater, or run-off areas draining septic wastes from restaurants, boat marinas, or garbage collection areas. Samples of bathing beach water should be collected at locations and times of heaviest use. Daily sampling, preferably in the afternoon, is the optimum frequency during the season. Weekends and holidays which are periods of highest use must be included in the sampling program. Samples of estuarine bathing waters should be obtained at high tide, ebb tide and low tide in order to determine the cyclic water quality and deterioration that must be monitored during the swimming season.