



Method 1602: Male-specific (F⁺) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure

April 2001

Acknowledgments

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Introduction

Coliphage presence in ground water is an indication of fecal contamination. Method 1602 is a performance-based method for enumerating male-specific (F⁺) and somatic coliphage in ground water and other waters. Laboratories are permitted to modify or omit any steps or procedure, with the exception of the coliphage stock enumeration procedure (Section 11.3), provided that all performance requirements set forth in the validated method are met. The laboratory may not omit any quality control analyses.

This single agar layer procedure requires the addition of host bacteria, magnesium chloride, and double-strength molten agar medium to the sample, followed by pouring the total volume of the mixture into plates. All plates from a single sample are examined for plaque formation (zones of bacterial host lawn clearing). The quantity of coliphage in a sample is expressed as plaque forming units (PFU) / 100 mL.

This method is for use in the Environmental Protection Agency's (EPA's) data gathering and monitoring programs under the Safe Drinking Water Act and the Clean Water Act.

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1.0 Scope and Application

- 1.1 The single agar layer (SAL) procedure detects and enumerates male-specific (F⁺) and somatic coliphages in ground water and other waters. This method is intended to help determine if ground water is affected by fecal contamination.
- 1.2 Although this method may be used for water matrices other than ground water, it has only been validated for use in ground water.
- 1.3 This method is based on procedures developed for the determination of coliphage in water in the Supplement to the 20th Edition of *Standard Methods for the Examination of Water and Wastewater* (Reference 17.1).
- 1.4 This method is not intended for use in biosolids samples or as a test for microorganisms other than coliphage. This method may be used in ground water and other water matrices where coliphage is suspected to be present.
- 1.5 Each laboratory and analyst that uses this method must first demonstrate the ability to generate acceptable results using the procedures in Section 9.0.
- 1.6 Any modification of the method beyond those expressly permitted is subject to the application and approval of alternate test procedures under 40 CFR parts 136.4 and 136.5, and/or 141.27.

2.0 Summary of Method

- 2.1 Method 1602 describes the single agar layer (SAL) procedure. A 100-mL ground water sample is assayed by adding MgCl₂ (magnesium chloride), log-phase host bacteria (*E. coli* F_{amp} for F⁺ coliphage and *E. coli* CN-13 for somatic coliphage), and 100 mL of double-strength molten tryptic soy agar to the sample. The sample is thoroughly mixed and the total volume is poured into 5 to 10 plates (dependent on plate size). After an overnight incubation, circular lysis zones (plaques) are counted and summed for all plates from a single sample. The quantity of coliphage in a sample is expressed as plaque forming units (PFU) / 100 mL. For quality control purposes, both a coliphage-positive reagent water sample (OPR) and a negative reagent water sample (method blank) are analyzed for each type of coliphage with each sample batch.

3.0 Definitions

- 3.1 Coliphages are viruses (bacteriophages) that infect *E. coli* and are indicators of fecal contamination. This method is capable of detecting two types of coliphages: male-specific (F⁺) and somatic.
- 3.2 F-factor is the fertility factor in certain strains of *E. coli*. It is a plasmid that, when present, codes for pilus formation. The pilus allows for transfer of nucleic acid from one bacterium to another.

- 3.3 Male-specific coliphages (F⁺) are RNA or DNA viruses that infect via the F-pilus of male strains of *E. coli*.
- 3.4 Somatic coliphages are DNA viruses that infect host cells via the outer cell membrane.
- 3.5 Definitions for other terms used in this method are given in the glossary in Section 19.3.

4.0 Interferences

- 4.1 During the single agar layer procedure the sample and host bacteria should not remain in contact with each other for more than 10 minutes prior to plating and after plating the agar must harden within 10 minutes. Increased contact time or agar hardening time may result in replication of phages such that the initial phage concentration is overestimated. The entire plating procedure from combining sample with host to hardening of single-agar layer plates should not exceed 20 minutes.

5.0 Safety

- 5.1 The biohazards and the risk of infection by pathogens associated with handling raw sewage are high in this method. Use good laboratory practices when working with potentially harmful samples.
- 5.2 Method 1602 does not purport to address all of the safety problems associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. The analyst/technician must know and observe the safety procedures required in a laboratory that handles biohazardous material while preparing, using, and disposing of cultures, reagents, and materials. The analyst/technician must use proper safety procedures while operating sterilization equipment. Equipment and supplies that have come into contact with biohazardous material or are suspected of containing biohazardous material must be sterilized prior to disposal or re-use. 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 handling pathogens to all samples.
- 5.3 The laboratory is responsible for maintaining a current awareness file of Occupational Safety and Health Administration (OSHA) regulations regarding the safe handling of the chemicals specified in this method. A reference file of material safety data sheets should be made available to all personnel involved in these analyses. Additional information on laboratory safety can be found in Section 16.0 Waste Management.
- 5.4 Samples may contain high concentrations of biohazardous agents and must be handled with gloves. Any positive reference materials also must be handled with gloves in an appropriate laboratory hood. The analyst/technician must never place gloves near the face after exposure to media known or suspected to contain pathogenic microorganisms. Laboratory personnel must change gloves after handling raw sewage or any other items which may carry pathogenic microorganisms.
- 5.5 Mouth pipetting is prohibited.

6.0 Equipment and Supplies

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

- 6.1 Equipment for collection and transport of samples
 - 6.1.1 Bottles for collection of water—Sterile, wide-mouth, polypropylene, 4-L (or smaller) bottles or carboys with screw caps
 - 6.1.2 Ice chest—Igloo, Coleman, styrofoam box or equivalent
 - 6.1.3 Ice
 - 6.1.3.1 Wet ice—purchased locally, or
 - 6.1.3.2 Ice packs—Blue Ice, UTek cat. no. 429, or equivalent, frozen for use
 - 6.1.4 Bubble wrap
- 6.2 Equipment and supplies for growth of microorganisms
 - 6.2.1 Sterile dilution tubes with screw caps—Reusable or disposable, 16 × 150 mm, or 16 × 100 mm
 - 6.2.2 Test tube rack—Size to accommodate tubes specified in Section 6.2.1
 - 6.2.3 Glass or plastic, plugged, sterile serological pipettes—To deliver, of appropriate volume(s) (Falcon, Kimble, or equivalent)
 - 6.2.4 Pipet bulbs, automatic pipetter—Pipet-Aid or equivalent
 - 6.2.5 Inoculation loops—Nichrome or platinum wire, disposable, sterile plastic loops, or wooden applicator, at least 3 mm in diameter or 10 µL volume (VWR, Fisher, DIFCO, or equivalent)
 - 6.2.6 Micropipettors, adjustable—10- to 200-µL, and 100- to 1000-µL, with appropriate aerosol resistant tips, Gilson, Eppendorf, or equivalent. *Please note: To avoid cross-contamination, micropipettors should be wiped down with a 1 : 100 solution of household bleach followed by a 10% solution of sodium thiosulfate. Alternatively, disposable pipets (Serological, Pasteur, or equivalent) may be used.*
 - 6.2.7 Burner—Alcohol, Bunsen, Fisher, or equivalent
 - 6.2.8 Sterile disposable petri dishes—100-mm -diameter dishes (Falcon # 1029) or 150-mm-dishes (Falcon #1058) or equivalent
 - 6.2.9 Incubator capable of maintaining 36°C ± 1.0 °C for growth of microorganisms
 - 6.2.10 Beakers—2- and 4-L, sterile, polypropylene, glass, or polycarbonate
 - 6.2.11 Polypropylene, glass, or polycarbonate bottles—Wide-mouth, 100-mL or 1-L, square or round, autoclavable with screw cap
 - 6.2.12 Erlenmeyer flasks—1-L and 2-L, sterile, Corning, Nalgene, Kimble or equivalent
 - 6.2.13 Stir bar—Fisher cat. no. 14-511-93, or equivalent
 - 6.2.14 Stir plate—Fisher cat. no. 14-493-120S, or equivalent
 - 6.2.15 Water bath capable of maintaining 36°C ± 1.0°C and 45°C to 48°C —Precision, VWR Scientific, or equivalent

- 6.2.16 Sterilization filtration equipment—Millex type for syringe or larger Millipore type, sterile, 0.22- μ m pore size
- 6.2.17 Sterile, cotton-tipped applicators
- 6.2.18 Latex gloves for handling samples, supplies, and equipment—Microflex, San Francisco, CA, stock no. UL-315-L, or equivalent
- 6.2.19 pH meter—Beckman, Corning, or equivalent
- 6.2.20 Vortex mixer—Vortex Genie, or equivalent
- 6.2.21 Spectrophotometer or colorimeter (with wavelengths in visible range)—Spectronic 20, Spectrum Instruments, Inc., or equivalent, with cell holder for 1/2" diameter cuvettes (Model # 4015) or 13 mm \times 100 mm cuvettes
- 6.2.22 Cuvettes—1-cm light path, Beckman, Bausch and Lomb, or equivalent
- 6.2.23 Shaker flasks—Fluted Erlenmeyer, 125-mL with slip cap or sterile plug, Fisher (09-552-33, 10-140-6, 10-041-5A) or equivalent or equivalent
- 6.2.24 Shaker incubator—Capable of 36°C \pm 1.0 °C and 100 to 150 rpm, New Brunswick, PsychoTherm, Innova, or equivalent or an ordinary shaker in an incubator
- 6.2.25 Flask weights—VWR 29700-004 or equivalent
- 6.3 Supplies for collection and filtration of raw sewage (Section 7.4.3)
 - 6.3.1 Disposable filter disks—25-mm-diameter, 0.45- μ m pore size, sterile, low protein binding (Gelman Acrodisc HT Tuffryn, No. 4184, cellulose acetate Corning No. 21053-25, or equivalent)
 - 6.3.2 Syringes—Sterile, disposable, 5-, 10-, or 20-mL
 - 6.3.3 Polypropylene dilution tubes—Sterile, 10- to 20-mL, Falcon or equivalent
 - 6.3.4 Sterile glass or polypropylene 250-mL bottles for collection of raw sewage
- 6.4 Miscellaneous lab ware and supplies
 - 6.4.1 Lint-free tissues—KimWipes or equivalent
 - 6.4.2 Weigh boats
 - 6.4.3 Graduated cylinders—Sterile, polypropylene or glass, 100-mL, 250-mL, and 1-L
 - 6.4.4 Autoclave
 - 6.4.5 Thermometers—Range of 0°C to 100°C
 - 6.4.6 Balance—Capable of weighing to 0.1 mg for samples having a mass up to 200 g
 - 6.4.7 Freezer vials—Sterile, 5-mL screw cap, Nunc or equivalent
 - 6.4.8 Light box—VWR 21475-460 or equivalent

7.0 Reagents and Standards

7.1 General reagents

- 7.1.1 Reagent water—Should conform to Specification D 1193, Annual Book of ASTM Standards (Reference 17.5).
- 7.1.2 10% (w/v) Sodium thiosulfate—Add 10 g sodium thiosulfate (Na₂S₂O₃) per 90 mL reagent water. Mix until dissolved. Bring to a final volume of 100 mL and autoclave for 15 minutes at 121°C and 15 psi.

- 7.1.3** Stock magnesium chloride (80X, 4M)—To 814 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, add 300 mL reagent grade water. Stir to dissolve. Bring to a final volume of 1 L, and mix thoroughly. Autoclave for 15 minutes at 121°C and 15 psi.
- 7.1.4** Glycerol—Sigma #G6279 or equivalent. Autoclave for 15 minutes at 121°C and 15 psi. Remove promptly to avoid scorching. Store at room temperature.
- 7.1.5** Household bleach
- 7.1.6** Ethanol—70% or greater
- 7.2 Antibiotic stocks**—Antibiotics must always be added to medium *after* the medium has been autoclaved.
- 7.2.1** Stock nalidixic acid (Sigma N4382, or equivalent)—**Please note:** *Nalidixic acid is considered toxic. Wear suitable protective clothing, gloves, and eye/face protection and use in a chemical fume hood.*
- 7.2.1.1** For growth of *E. coli* CN-13, the host bacteria for somatic coliphage.
- 7.2.1.2** Dissolve 1 g of nalidixic acid sodium salt in 100 mL reagent water. Filter through a sterile, 0.22- μm -pore-size membrane filter assembly. Dispense 5 mL per 5-mL freezer vial, date vial, and store frozen at -20°C for up to one year. Thaw at room temperature or rapidly in a 36°C \pm 1.0°C water bath. Mix well prior to use.
- 7.2.2** Stock ampicillin/streptomycin
- 7.2.2.1** For growth of *E. coli* F_{amp}, the host bacteria for male-specific coliphage.
- 7.2.2.2** Dissolve 0.15 g of ampicillin sodium salt (Sigma A9518) and 0.15 g streptomycin sulfate (Sigma S6501) in 100 mL of reagent water. Filter through a sterile 0.22- μm -pore-size membrane filter assembly. Dispense 5 mL per 5-mL freezer vial, date vial, and store frozen at -20°C for up to one year. Thaw prior to use at room temperature or rapidly in a 36°C \pm 1.0°C water bath. Mix well prior to use.
- 7.3 Media**
- 7.3.1 Tryptic (or trypticase) soy broth (TSB)**—(DIFCO 0370-15-5, or equivalent)
- 7.3.1.1** TSB—Follow procedure as specified on bottle of media. If dehydrated medium is not available, prepare the media by adding 17.0 g of tryptone, 3.0 g of soytone, 2.5 g of dextrose, 5.0 g of sodium chloride, and 2.5 g of dipotassium phosphate to 1L of reagent water and heat to dissolve. Adjust pH to 7.3 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary. Autoclave at 121°C and 15 psi for 15 minutes. Check pH again after autoclaving by aseptically removing an aliquot of medium. Adjust pH as necessary. Discard aliquot after checking pH, to ensure that the medium is not contaminated.
- 7.3.1.2** TSB with nalidixic acid (for growth of *E. coli* CN-13)—Aseptically add 10 mL of stock nalidixic acid (Section 7.2.1) to 1 L of autoclaved, cooled (48°C \pm 1.0°C) TSB (Section 7.3.1.1) and mix. **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.*
- 7.3.1.3** TSB with streptomycin/ampicillin (for growth of *E. coli* F_{amp})—Aseptically add 10 mL of stock streptomycin/ampicillin sulfate (Section 7.2.2) to 1 L of autoclaved, cooled (48°C \pm 1.0°C) TSB (Section 7.3.1.1) and mix. **Please note:**

Antibiotics must always be added to medium after the medium has been autoclaved and cooled.

- 7.3.2 1.5% tryptic soy agar (TSA)**—To be used in streak plates (Section 7.5.2.1) and as bottom layer of agar (Section 11.3.1.3) during the double agar layer (DAL) coliphage stock QC sample spiking suspension enumeration procedure. Prior to autoclaving the TSB without antibiotics, prepared as described in Section 7.3.1.1, add 15 g of agar per liter of TSB. While stirring, heat to dissolve agar. Autoclave for 15 minutes at 121°C and 15 psi. Cool to 48°C ± 1.0°C and mix molten medium well to ensure even distribution.
- 7.3.2.1** For growth of somatic coliphages using *E. coli* CN-13 as host bacteria, aseptically add 10 mL of stock nalidixic acid (Section 7.2.1) per liter of autoclaved 1.5% TSA (Section 7.3.2). **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Swirl flask until well mixed and aseptically dispense 17 - 18 mL per 100-mm plate. Allow to solidify with lids off in a biohazard hood for several minutes prior to use. If not used immediately, replace lids and store inverted at 4°C ± 1°C for up to 2 weeks.
- 7.3.2.2** For growth of male-specific (F⁺) coliphages using *E. coli* F_{amp} as host bacteria, aseptically add 10 mL stock ampicillin/streptomycin sulfate (Section 7.2.2) per liter of autoclaved 1.5% TSA (Section 7.3.2). **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Swirl flask until well mixed and aseptically dispense 17 - 18 mL per 100-mm plate. Allow to solidify with lids off in a biohazard hood for several minutes prior to use. If not used immediately, replace lids and store inverted at 4°C ± 1°C for up to 2 weeks.
- 7.3.3 0.7% tryptic soy agar (TSA)**—“Soft” agar for use as the top layer of agar (Section 11.3.1.1) during the double agar layer (DAL) coliphage stock QC sample spiking suspension enumeration procedure. Prior to autoclaving the TSB without antibiotics, prepared as described in Section 7.3.1.1, add 7 g of agar per liter of TSB. While stirring, heat to dissolve agar. Autoclave for 15 minutes at 121°C and 15 psi. Cool to 48°C ± 1.0°C.
- 7.3.3.1 0.7% TSA top agar tubes with nalidixic acid** (for growth of *E. coli* CN-13)—To 1 L of autoclaved 0.7% TSA (soft agar) (Section 7.3.3), aseptically add 10 mL of stock nalidixic acid (Section 7.2.1). **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Dispense 5 mL per sterile 10-mL tube, label, and keep at 45°C to 48°C until use. Tubes must be used the day they are prepared.
- 7.3.3.2 0.7% TSA top agar tubes with ampicillin/streptomycin** (for growth of *E. coli* F_{amp})—To 1 L of autoclaved 0.7% TSA (soft agar) (Section 7.3.3), aseptically add 10 mL of stock ampicillin/streptomycin (Section 7.2.2). **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Dispense 5 mL per sterile 10-mL tube, label, and keep at 45°C to 48°C until use. Tubes must be used the day they are prepared.
- 7.3.4 Single agar layer**
- 7.3.4.1 Double-strength tryptic soy agar (2X TSA)**—Double all components of TSB without antibiotics from Section 7.3.1.1 (except reagent water) and add 18 g of agar per liter. Heat to dissolve while stirring. Autoclave for 15 minutes at 121°C and 15 psi. Cool to 48°C ± 1.0 °C. After autoclaving, swirl to mix.

Medium may become darker after autoclaving but this should not impact media performance.

- 7.3.4.2** 2X TSA with nalidixic acid (for growth of *E. coli* CN-13)—To 1 L of autoclaved 2X TSA, aseptically add 20 mL of stock nalidixic acid (Section 7.2.1) and mix well. **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Keep molten at 45°C to 48°C in water bath until use. Agar must be used on the day of preparation.
- 7.3.4.3** 2X TSA with ampicillin/streptomycin (for growth of *E. coli* F_{amp})—To 1 L of autoclaved 2X TSA, aseptically add 20 mL of stock ampicillin/streptomycin (Section 7.2.2) and mix well. **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Keep molten at 45°C to 48°C in water bath until use. Agar must be used on the day of preparation.
- 7.3.5 Spot plates**—To be used during the plaque confirmation procedure (Section 12.6). **Please note:** *Condensation may accumulate at the edges of stored spot plates and may drip over agar surface if tilted, ruining the spot pattern. If the stored spot plates have condensation, incubate plates for approximately 10 minutes to eliminate condensation prior to inoculation.*
- 7.3.5.1** Log-phase host bacteria must be prepared in advance (Section 7.5.4). Dissolve 3 g TSB (Section 7.3.1.1) and 0.75 g bacteriological grade agar per 100 mL of reagent grade water. Heat and mix to dissolve. Autoclave for 15 minutes at 121°C and 15 psi. Cool to 45°C to 48°C in a water bath.
- 7.3.5.2** Add 2 mL of log-phase host bacterium prepared as directed in Section 7.5.4 and 1 mL stock antibiotic (Section 7.2). **Please note:** *Antibiotics must always be added to medium after the medium has been autoclaved and cooled. Nalidixic acid is used with *E. coli* CN-13, and ampicillin/streptomycin is used with *E. coli* F_{amp}.* Swirl to mix, and pour 20 mL per 100-mm diameter, sterile petri plate. Allow to solidify. Label plates with name of host bacterium. Plates may be used that day or stored at 4°C ± 1°C for up to four days before use. Divide the bottom of the plate into a grid of 1-cm squares using a permanent marking pen. Number each square for ease of reference. Other alternatives include: 1) gridded petri dishes, 2) adhesive grids, or 3) creating the 1-cm grid on a circular plastic dish and attaching to the bottom exterior of the plate with cellophane tape.

7.4 Coliphage stock

- 7.4.1** MS2 stock coliphage (ATCC#15597-B1)—Male-specific (F⁺) coliphage. Refer to <http://www.atcc.org/SearchCatalogs/faqBacteriology.cfm#q10> for initial preparation of coliphage stock. May be stored at 2°C to 8°C for up to 5 years.
- 7.4.2** phi-X 174 stock coliphage (ATCC#13706-B1)—Somatic coliphage. Refer to <http://www.atcc.org/SearchCatalogs/faqBacteriology.cfm#q10> for initial preparation of coliphage stock. May be stored at 2°C to 8°C for up to 5 years.
- 7.4.3** Coliphage stock from sewage filtrate—This filtrate will be used as a spiking suspension for QC samples.
- 7.4.3.1** Collect approximately 100 mL of raw sewage in a 250-mL collection bottle.

- 7.4.3.2 Transport to the laboratory on ice. Analysis of raw sewage filtrate should begin within 24 hours of collection.
- 7.4.3.3 Allow the raw sewage to settle at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 1 to 3 hours. This will make the filtration process easier.
- 7.4.3.4 Remove a sterile, 20-mL syringe from its package, aseptically remove plunger from barrel, and attach a filter disk to the syringe barrel.
- 7.4.3.5 Pipet 10 to 15 mL of supernatant from settled sewage into the syringe barrel.
- 7.4.3.6 Hold the assembly over a sterile 15-mL polypropylene tube with screw-cap or snap-cap, insert the plunger into the syringe barrel, and push the sewage through the filter into the sterile tube. If filter clogs, change it as necessary and continue to filter sewage until at least 10 mL of filtered sewage is obtained in the 15-mL polypropylene tube (filtration may require use of numerous filters).
- 7.4.3.7 Cap the tube, label with source, date, and initials, and store the filtrate at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ until ready to assay. If filtrate is stored more than 24 hours, it must be re-titered before use.

7.5 Host bacteria stock cultures

7.5.1 Pure host bacteria cultures

- 7.5.1.1 *E. coli* CN-13 (somatic coliphage host)—Nalidixic acid-resistant mutant of *E. coli* C; originated by Pierre Payment, Institute Armand Frappier, University of Quebec, Montreal, Canada, frozen stock. ATCC#700609.
- 7.5.1.2 *E. coli* F_{amp}—*E. coli* HS(pFamp)R (male-specific coliphage host)—originated by Victor Cabelli, formerly of the Department of Microbiology, University of Rhode Island, Kingston, RI, USA, frozen stock. ATCC#700891.

7.5.2 Frozen host bacteria stock cultures—The laboratory shall obtain reference host bacterial cultures (Sections 7.5.1.1 and 7.5.1.2) and use these to establish pure frozen host stock cultures that are maintained by the laboratory. Frozen stocks are used as inoculum for overnight host bacteria stock cultures (Section 7.5.3).

- 7.5.2.1 Establish pure frozen stock cultures by streaking host bacteria onto 1.5% TSA plates with appropriate antibiotic (Section 7.3.2) to attain isolated colonies.
- 7.5.2.2 Incubate inoculated plates overnight, pick an individual colony and inoculate into tryptic soy broth with appropriate antibiotics (Sections 7.3.1.2 and 7.3.1.3), and grow to log phase (Section 7.5.4).
- 7.5.2.3 Harvest broth by mixing sterile glycerol and broth with log-phase host bacteria in a ratio of 1:4 in a 5-mL freezer vial. Prepare log-phase host bacteria as described in Section 7.5.4, below. (Example: 200 μL sterile glycerol plus 800 μL log-phase *E. coli*).
- 7.5.2.4 Label with *E. coli* strain and date of harvest.
- 7.5.2.5 Freeze host bacteria stock cultures at -70°C , if possible. Cultures can be frozen at -20°C if the laboratory does not have the capability to freeze samples at -70°C .
- 7.5.2.6 Host bacteria stored at -70°C may be retained for up to one year. If stored at -20°C , the host bacteria may be retained for up to two months.

7.5.3 Overnight host bacteria stock cultures—Inoculum from an overnight bacterial host culture will reach log-phase more rapidly than inoculum from frozen stock.

- 7.5.3.1 Dispense 25 mL of tryptic soy broth (TSB) with nalidixic acid (Section 7.3.1.2) into a sterile 125-mL shaker flask. For proper growth conditions, each flask should always contain 25 to 30 mL of medium.
- 7.5.3.2 Inoculate the flask with a loopful of *E. coli* CN-13 from the frozen stock culture (Section 7.5.2).
- 7.5.3.3 Repeat Sections 7.5.3.1 and 7.5.3.2 using TSB with streptomycin/ampicillin as the medium (Section 7.3.1.3) and *E. coli* F_{amp} as the bacterial host.
- 7.5.3.4 Place a sterile slip cap or plug on the shaker flasks, label flasks, and secure in shaker.
- 7.5.3.5 Incubate at 36°C ± 1.0°C and set shaker to 100 to 150 rpm overnight (18 to 20 hours).
- 7.5.3.6 Chill on wet ice or at 4°C ± 1°C until ready for use.
- 7.5.4 **Log-phase host bacteria stock cultures** (Section 18, Flow chart 1)
 - 7.5.4.1 To a 125-mL shaker flask containing 25 mL of TSB with nalidixic acid (Section 7.3.1.2) add 0.1 to 1.0 mL of overnight *E. coli* CN-13 host bacteria stock culture (Section 7.5.3 or 7.5.4.7). For proper growth conditions, each culture flask of host bacteria should contain 25 to 30 mL of medium. As a result, several flasks of host bacteria may have to be prepared (this depends on the number of samples and controls being run each day). Each 100-mL sample analyzed using the single agar layer procedure (Section 12) will require a 10-mL inoculum of log-phase host bacteria.
 - 7.5.4.2 Repeat Section 7.5.4.1 using TSB with streptomycin/ampicillin (Section 7.3.1.3) as the medium and *E. coli* F_{amp} as the bacterial host.
 - 7.5.4.3 After inoculation, place a sterile slip-cap or plug on the shaker flasks and secure in shaker incubator.
 - 7.5.4.4 Incubate at 36°C ± 1.0°C and 100 to 150 rpm for approximately 4 hours or until cultures are visibly turbid (cloudy), indicating log-phase growth.
 - 7.5.4.5 Aseptically remove 1 mL of culture from flask, dispense into a cuvette (Section 6.2.22), and read absorbance at 520 nm. An absorbance reading between 0.1 and 0.5 optical density (OD) units is an indication of log-phase growth. If proper OD has not been reached, place cultures back into shaker incubator and take readings every 30 minutes until an OD of between 0.1 and 0.5 is reached.
 - 7.5.4.6 Chill on wet ice or at 4°C ± 1°C to slow replication until ready for use. The suspension may be stored up to 48 hours. However, the best results occur when cultures are used immediately (within 6 hours).
 - 7.5.4.7 Store remaining bacterial host culture at 4°C ± 1°C overnight to inoculate flasks for the preparation of new log-phase bacterial hosts.

8.0 Sample Collection, Preservation, and Storage

Please note: Unless the sample is known or suspected to contain infectious agents (e.g., during an outbreak), samples should be shipped as noninfectious and should not be marked as infectious. U.S. Department of Transportation (DOT) regulations (49 CFR 172) prohibit interstate shipment of more than 4 L of solution known to contain infectious materials. State regulations may contain similar regulations for intrastate commerce. If an outbreak is suspected, ship less than 4 L at a time.

- 8.1 Samples are collected in plastic bottles or carboys and shipped to the laboratory for analysis. Samples must be shipped at 2°C to 8°C using wet ice, Blue Ice®, or similar products to maintain temperature. Samples must be stored at 4°C ± 1°C. Do not freeze.
- 8.2 Sample collection: Collect 250 mL of sample for each of the two coliphage types to allow for sample re-analysis, if necessary.
- 8.3 The sampling team must maintain a log book with the following information for each sample:
 - 8.3.1 Facility name and location
 - 8.3.2 Date and time of collection
 - 8.3.3 Name of analytical facility, contact, and phone number
 - 8.3.4 Sample number
 - 8.3.5 Sample location
- 8.4 The sample container must indicate the following:
 - 8.4.1 Sample number
 - 8.4.2 Date and time of collection
 - 8.4.3 Sample collection location
- 8.5 Holding times. The following are maximum holding times beyond which the sample cannot be retained for testing.
 - 8.5.1 Single agar layer procedure—Between collection of sample and beginning of analysis: 48 hours
 - 8.5.2 Raw sewage sample—Between collection of sewage sample and analysis: 24 hours, unless re-titered and titer has not decreased by more than 50%. If titer has not decreased by more than 50%, the sample can be stored for up to 72 hours.
- 8.6 Dechlorination procedure—Although this method was validated for use with unchlorinated ground water, it potentially can be used with chlorinated ground waters. If the sample has been chlorinated, add 0.5-mL 10% sodium thiosulfate per 1-L of sample at time of sample.

9.0 Quality Control

9.1 Each laboratory that uses Method 1602 is required to operate a formal quality assurance (QA) program. The minimum QA requirements consist of an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) test (Section 9.3), analysis of spiked samples to evaluate and document data quality, and analysis of standards and blanks as tests of continued acceptable performance. Laboratory performance is compared to established performance criteria to determine if the results of analyses meet the performance criteria of the method. Specific quality control (QC) requirements for Method 1602 are provided below. General recommendations on QA and QC for facilities, personnel, laboratory equipment, instrumentation, and supplies used in microbiological analyses are provided in the USEPA Microbiology Methods Manual, Part IV, C (Reference 17.3).

9.2 **General QC requirements**—All spiked QC samples must be spiked with enumerated sewage filtrate or equivalent (Section 11).

9.2.1 **Initial precision and recovery (IPR).** The laboratory shall demonstrate the ability to generate acceptable performance with this method by performing an IPR test before

analyzing any field samples. The procedure for performing the IPR is described in Section 9.3.

- 9.2.2 Method blanks.** The laboratory shall analyze method blanks (reagent water sample containing no coliphage) to demonstrate freedom from contamination. The procedures and criteria for analysis of a method blank are described in Section 9.4. The laboratory shall analyze one method blank with each analytical batch. An analytical batch is defined as all samples analyzed during a single day, up to a maximum of 20 samples (field samples and matrix spike samples) per coliphage type.
- 9.2.3 Matrix spikes (MS).** The laboratory shall spike a separate sample aliquot from the same ground water source to monitor method performance. This MS test is described in Section 9.5. The laboratory shall analyze one MS sample (Section 9.5) when samples are first received from a ground water source for which the laboratory has never before analyzed samples. The MS analysis is performed on an additional (second) sample aliquot collected from the ground water source at the same time as the routine field sample. If the laboratory routinely analyzes samples from one or more ground water sources, one MS analysis must be performed per 20 field samples. For example, when a laboratory receives the first sample from a source, the laboratory must obtain a second aliquot of this sample to be used for the MS. When the laboratory receives the 20th sample from this site, a separate aliquot of this 20th sample must be collected and spiked.
- 9.2.4 Ongoing precision and recovery (OPR).** The laboratory shall, on an ongoing basis, demonstrate through analysis of OPR samples (Section 9.6) that the analytical system is in control. The laboratory shall analyze one OPR sample for each analytical batch. An analytical batch is defined as all samples analyzed during a single day, up to a maximum of 20 samples (field samples and matrix spike samples) per coliphage type. *Please note: the OPR serves as the positive control for Method 1602.*
- 9.2.5 Method modification validation/equivalency demonstration requirements.** Method 1602 is a performance-based method and the laboratory is permitted to modify certain method procedures to improve performance or lower the costs of measurements, provided that all quality control (QC) tests cited in Section 9.2.5 are performed and all QC acceptance criteria are met. The laboratory is not permitted to modify the double agar layer QC spiking suspension enumeration procedure (Section 11.3).
- 9.2.5.1 Method modifications at a single laboratory.** Each time a modification is made to this method for use in a single laboratory, the laboratory is required to validate the modification according to Tier 1 of EPA's performance-based measurement system (PBMS) (Table 3 and Reference 17.6) to demonstrate that the modification produces results equivalent or superior to results produced by this method as written. Briefly, each time a modification is made to this method, the laboratory is required to demonstrate acceptable modified method performance through the IPR test (Section 9.3). IPR results must meet the QC acceptance criteria in Table 2 in Section 14, and should be comparable to previous results using the unmodified procedure. Although not required, the laboratory also should perform a matrix spike/matrix spike duplicate (MS/MSD) test (Section 9.7) to demonstrate the performance of the modified method in at least one real-world matrix before analyzing field samples using the modified method.
- 9.2.5.2 Method modifications for nationwide approval.** If the laboratory or a manufacturer seeks EPA approval of a method modification for nationwide use, the laboratory or manufacturer must validate the modification according to Tier 2 of EPA's PBMS (Table 3 and Reference 17.6). Briefly, at least three

laboratories must perform IPR tests (Section 9.3) and MS/MSD tests (Section 9.7) using the modified method, and all tests must meet the QC acceptance criteria specified in Tables 2 in Section 14. Upon nationwide approval, laboratories electing to use the modified method still must demonstrate acceptable initial and ongoing performance in their laboratory according to the requirements in Section 9.2.

- 9.2.6 Media sterility check.** The laboratory shall test media sterility by incubating one unit (tube or plate) of each batch of medium at $36^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ for 48 to 72 hours and observing for growth. Also, if media is stored in the refrigerator after sterilization, the media must be stored overnight at room temperature and all media with growth discarded.
- 9.2.7 Record maintenance.** The laboratory shall maintain records to define the quality of data that are generated. Development of accuracy statements is described in Sections 9.5 and 9.6. The laboratory shall maintain a record of the date and results of all QC samples described in Section 9.2. A log book containing reagent and material lot numbers should be maintained along with samples analyzed using each of the lots.
- 9.2.8 Performance studies.** The laboratory should periodically analyze an external QC sample, such as a performance evaluation (PE) samples when available. The laboratory also should participate in available interlaboratory performance studies conducted by local, state, and federal agencies or commercial organizations. The laboratory should review results, correct unsatisfactory performance, and record corrective actions.
- 9.2.9** The specifications contained in this method can be met if the analytical system is maintained under control.

9.3 Initial precision and recovery (IPR)—The IPR test is performed to demonstrate acceptable performance with the method as written prior to analysis of field samples or to evaluate acceptable performance of a method modification. IPR tests must be accompanied by analysis of a method blank (Section 9.4). All spiked QC samples must be spiked with enumerated sewage filtrate or equivalent (Section 11).

- 9.3.1** A total of four reagent water samples for each coliphage type are required for the IPR test. The target spike concentration is 80 PFU per sample.
- 9.3.2** IPR samples must be spiked in bulk. For each coliphage type (somatic and F⁺), use an appropriate volume of the enumerated sewage filtrate stock spiking suspension (or equivalent) prepared in Section 7.4.3 and enumerated in Section 11, to spike a 400-mL “bulk” reagent water sample. Spike approximately 320 male-specific or somatic coliphages per 400-mL bulk reagent water sample to achieve the target spike concentration of approximately 80 PFU / sample and swirl to mix. Aliquot each bulk sample into four, 100-mL samples (see Section 11 for enumeration of coliphage stock and Section 13.2 for spiking volume calculations).
- 9.3.3** Analyze the four spiked 100-mL samples per coliphage type using the SAL procedure (Section 12).
- 9.3.4** Compute the coliphage percent recovery in each sample using the following equation:

$$R = 100 \times \frac{N}{T}$$

where

R = percent recovery

N = number of coliphage detected (PFU / sample)

T = number of coliphage spiked (PFU / sample)

9.3.5 Using all four sample results for each coliphage type from an IPR test, compute the average percent recovery (\bar{x}) and the relative standard deviation of the recovery (RSD_r). (See glossary for definition of RSD_r .) Compare RSD_r and \bar{x} with the corresponding limits for IPR (Section 14). If RSD_r and \bar{x} meet the acceptance criteria, the system performance is acceptable and analysis of samples may begin. If RSD_r or \bar{x} falls outside the range, system performance is unacceptable. In this event, identify and correct the problem and repeat the test.

9.4 Method blank—Performed at the frequency specified in Section 9.2.2.

9.4.1 For each coliphage type, prepare and analyze a reagent water sample containing no coliphage using the same procedure as used for analysis of the field or QC samples.

9.4.2 If coliphage, or any potentially interfering organisms are found in the blank, analysis of additional samples must be halted until the source of contamination is eliminated, and a repeat of the method blank analysis shows no evidence of contamination. Any sample in a batch associated with a contaminated blank should be recollected (if holding times have been violated) and reanalyzed. Samples from a batch that proves to have no coliphage in its blank may be reported.

9.5 Matrix spike (MS)—The laboratory shall spike and analyze a field sample aliquot to determine the effect of the matrix on the method's coliphage recovery. The MS shall be analyzed according to the frequency in Section 9.2.3. All spiked QC samples must be spiked with enumerated sewage filtrate or equivalent (Section 11).

9.5.1 For each coliphage type, analyze an unspiked, 100-mL field sample according to the SAL quantitative procedure (Section 12). For each coliphage type, add approximately 80 PFU to a second, 100-mL aliquot of the same field sample (see Section 11 for enumeration of coliphage stock and Section 13.2 for spike volume calculations).

9.5.2 Compute the percent recovery (R) of coliphage using the following equation:

$$R = 100 \times (N_{sp} - N_{usp}) / T$$

where

R is the percent recovery

N_{sp} is the number of coliphage detected in the spiked sample

N_{usp} is the number of coliphage detected in the unspiked sample

T is the number of coliphage spiked

9.5.3 Compare the coliphage recovery with the corresponding limits in Section 14. If the recovery for coliphage falls outside its limit, method performance is unacceptable for that sample. If the results for the OPR sample associated with this batch of samples are within their respective control limits, a matrix interference may be causing poor recovery. If the results for the OPR are not within their control limits, the laboratory is not in control. The problem must be identified and corrected. The matrix spike and associated field sample(s) should be reanalyzed. The recovery should be maintained on a control chart and updated on a regular basis.

9.6 Ongoing precision and recovery (OPR)—Performed at the frequency specified in Section 9.2.4. All spiked QC samples must be spiked with enumerated sewage filtrate or equivalent (Section 11). *Please note: the OPR serves as the positive control for Method 1602.*

- 9.6.1 For each coliphage type, spike one, 100-mL reagent water sample with approximately 80 PFU of coliphage (see Section 11 for enumeration of coliphage stock and Section 13.2 for spike volume calculations).
- 9.6.2 Analyze the spiked samples using the SAL procedure (Section 12).
- 9.6.3 Compute the percent recovery of coliphage in each OPR sample using the following equation:

$$R = 100 \times \frac{N}{T}$$

where

R = percent recovery

N = the number of coliphage detected

T = the number of coliphage spiked

- 9.6.4 Compare R with the corresponding limits for ongoing precision and recovery in Section 14. If R meets the acceptance criteria, system performance is acceptable and analysis of samples may continue. If R falls outside the range for recovery, system performance is unacceptable. Identify and correct the problem and perform another OPR test before continuing with the analysis of field samples.
 - 9.6.5 As part of the QA program for the laboratory, method precision for samples should be assessed and records retained. After the analysis of five OPR samples for which the reagent water spike recovery of coliphage is determined, the laboratory should compute the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the precision assessment as a percent recovery interval from $R - 2 s_r$ to $R + 2 s_r$ for each coliphage type. For example, if $R = 80\%$ and $s_r = 30\%$, the accuracy interval is expressed as 20% to 140%. The assessment should be maintained on a control chart and updated on a regular basis.
- 9.7 **Matrix spike/matrix spike duplicate (MS/MSD)**—If IPR and MS/MSD test performance is equal to or better than the criteria set forth in Table 2, then the modified version of the method is acceptable.

- 9.7.1 For each coliphage type, analyze an unspiked, 100-mL field sample according to the SAL quantitative procedure (Section 12). For each coliphage type, the laboratory shall spike and analyze two identical field sample aliquots (MS and MSD samples), as described in Section 9.5, using the modified version of the method.
- 9.7.2 For each coliphage type, calculate the percent recovery (R) using the equation in Section 9.5.2. Calculate the mean of the MS and MSD recoveries ($(R_{MS} + R_{MSD})/2$).
- 9.7.3 Calculate the relative percent difference (RPD) using the following equation:

$$RPD = 100 \frac{|N_{MS} - N_{MSD}|}{N_{mean}}$$

where

RPD is the relative percent difference

N_{MS} is the number of coliphage detected in the MS

N_{MSD} is the number of coliphage detected in the MSD

N_{mean} is the mean number of coliphage detected in the MS and MSD

- 9.7.4** Compare the mean MS/MSD recovery and RPD with the corresponding limits in Table 2 (Section 14) for each coliphage type.

10.0 Calibration and Standardization

- 10.1** At a minimum, check temperatures in water baths, refrigerators, -20°C freezers, and -70°C freezers daily to ensure operation within stated limits of method and record daily measurements in a log book.
- 10.2** At a minimum, check temperatures in incubators twice daily, at least 4 hours apart, to ensure operation within stated limits of method and record measurements in log book.
- 10.3** Check thermometers at least annually against an NIST-certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. The mercury column should not be separated.
- 10.4** Calibrate pH meter prior to use, using standards of pH 4.0, 7.0, and 10.0. To calibrate, use the two standards that are nearest to the desired pH.
- 10.5** Calibrate balances annually using ASTM-certified Class 2 reference weights.
- 10.6** Calibrate spectrophotometer prior to each use, following method described in owner's manual. Use sterile TSB without antibiotics as the blank.
- 10.7** Laboratories must adhere to all applicable quality control requirements set forth in Reference 17.4.

11.0 Enumeration of Coliphage QC Spiking Suspensions

- 11.1** The double agar layer (DAL) procedure is used to enumerate stock suspensions of somatic and male-specific coliphage for use in spiking quality control samples.
- 11.2** Dilution of coliphage stock (Section 7.4.1 or 7.4.2) or sewage filtrate (Section 7.4.3)—A minimum of four different volumes/dilutions are necessary for the double agar layer (DAL) enumeration of the coliphage stock or sewage filtrate (Section 18, Flow chart 2):
- Undiluted
 - 0.1
 - 0.01
 - 0.001
- Additional dilutions may be necessary. TSB without antibiotics (Section 7.3.1.1) is used as the diluent and as the method blank.
- 11.2.1** Aseptically add 9.0 mL of TSB without antibiotics (Section 7.3.1.1) into each of four (or more) sterile dilution tubes (Section 6.2.1). Label them as "0.1," "0.01," "0.001," "method blank," etc.
- 11.2.2** Add 1.0 mL of the coliphage stock or sewage filtrate to the tube of TSB labeled "0.1." Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
- 11.2.3** Add 1.0 mL of the well-mixed 0.1 dilution to a tube with 9 mL of TSB labeled "0.01". Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

- 11.2.4** Add 1.0 mL of the well-mixed 0.01 dilution to a tube with 9 mL of TSB labeled “0.001.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
- 11.2.5** Add 1.0 mL of TSB without antibiotics (Section 7.3.1.1) to the tube labeled “method blank.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
- 11.3** Coliphage spiking stock suspension enumeration by double agar layer (DAL) procedure (Section 18, Flow chart 3)—In this procedure, a tube of molten 0.7% TSA “top agar” with added host bacteria is inoculated with coliphage stock and will be poured into a 1.5% TSA “bottom agar” plate. Four dilutions of coliphage stock or sewage filtrate will be analyzed in duplicate for each coliphage type. As a result, nine double-agar layer plates will be required for each coliphage type: two plates per dilution (undiluted, 0.1, 0.01, and 0.001) and one method blank plate. **Please note:** *Laboratories are not permitted to modify or omit any aspects of the coliphage stock enumeration procedure (Section 11.3). As a result, magnesium chloride or calcium chloride must not be added the sample or media.*
- 11.3.1** Agar preparation
- 11.3.1.1** Place 0.7% TSA top agar tubes with antibiotics (Section 7.3.3.1 and 7.3.3.2) in a 45°C to 48°C water bath. The top agar should remain molten in the water bath until ready for use. 18 tubes are necessary to enumerate four dilution volumes in duplicate for each phage. The 18 tubes also includes an additional method blank tube for each phage type. Nine of the top agar tubes should contain nalidixic acid (Section 7.3.3.1) for growth of *E. coli* CN-13; the other nine should contain ampicillin/streptomycin (Section 7.3.3.2) for growth of *E. coli* F_{amp}.
- 11.3.1.2** As a precaution against contamination, disinfect a workspace near the water bath with a 1 : 100 dilution of household bleach and allow to dry. If workspace can be corroded by bleach use an ethanol solution of 70% or greater.
- 11.3.1.3** Assemble 1.5% TSA bottom agar plates (Section 7.3.2) and label so that the following information is identifiable:
- Dilution of stock filtrate or method blank
 - Bacterial host (*E. coli* CN-13 or *E. coli* F_{amp})
 - Coliphage type (somatic for the *E. coli* CN-13 bacterial host or male-specific for the *E. coli* F_{amp} bacterial host)
 - Date
 - Time

Please note: *The following steps are critical. To ensure viability of bacterial host and coliphage, do not add bacterial host and coliphage spiking suspension filtrate until ready to plate.*

- 11.3.2** Preparation of plates for enumeration of somatic coliphage
- 11.3.2.1** With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing nalidixic acid with 100 µL of log-phase *E. coli* CN-13.
- 11.3.2.2** Immediately add 500 µL (0.5 mL) of undiluted coliphage stock or sewage filtrate.
- 11.3.2.3** Mix the inoculum by rolling the tube briefly in palm of hand.

- 11.3.2.4** Pour contents into one of the two bottom agar plates marked “undiluted, *E. coli* CN-13, somatic.”
- 11.3.2.5** Duplicate analysis—Repeat Sections 11.3.2.1 through 11.3.2.4 for the duplicate.
- 11.3.2.6** Repeat Sections 11.3.2.1 through 11.3.2.5 for each dilution volume.
- 11.3.3** Preparation of plates for enumeration of male-specific (F⁺) coliphage—Repeat Section 11.3.2 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}
- 11.3.4** Preparation of somatic coliphage method blank
 - 11.3.4.1** With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing nalidixic acid with 100 µL of log-phase *E. coli* CN-13.
 - 11.3.4.2** Immediately add 500 µL (0.5 mL) of TSB from the “method blank” dilution tube.
 - 11.3.4.3** Mix the inoculum by rolling the tube briefly in palm of hand.
 - 11.3.4.4** Pour contents into a bottom agar plate marked “method blank, *E. coli* CN-13, somatic.”
- 11.3.5** Preparation of the male-specific (F⁺) coliphage method blank—Repeat Section 11.3.4 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}.
- 11.3.6** Store undiluted coliphage stock or sewage filtrate at 4°C ± 1.0°C for use in preparing new dilutions for positive controls, IDC, ODC, and MS samples.
- 11.3.7** After the top agar hardens, cover, invert the plates and incubate for 16 to 24 hours at 36°C ± 1.0°C.
- 11.3.8** Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 to 24 hours of incubation are plaques. Count the number of plaques on each plate. **Please note:** *The use of a light box (Section 6.4.8) to evaluate results is recommended.*
- 11.3.9** Proceed to Section 13.1 and calculate the PFU / mL for each coliphage.
- 11.3.10** Use the enumerated somatic and male-specific stocks to spike the single agar layer IPR, OPR, MS, and positive control samples as described in Section 9.

12.0 Single Agar Layer (SAL) Procedure for Sample Analysis (Procedures in Section 12 Also Are Summarized in Flow Chart 4)

- 12.1 Refer to Section 7.5.4 for preparation of log-phase host bacterial cultures.
- 12.2 Preparation of media for testing
 - 12.2.1 Prepare 100 mL of 2X TSA with nalidixic acid for *E. coli* CN-13 as described in Section 7.3.4.2.
 - 12.2.2 Add the 100 mL of 2X TSA with nalidixic acid to a 250-mL to 500-mL size Erlenmeyer flask and place in a 45°C to 48°C water bath to equilibrate. The liquid in the waterbath must come up to the level of the media. To ensure that the flask does not tip-over in the waterbath, the use of a flask weight (Section 6.2.25) is recommended.
 - 12.2.3 Prepare 100 mL of 2X TSA with ampicillin/streptomycin for *E. coli* F_{amp} as described in Section 7.3.4.3.
 - 12.2.4 Add the 100 mL of 2X TSA with ampicillin/streptomycin to a 250-mL to 500-mL size Erlenmeyer flask and place in a 45°C to 48°C water bath to equilibrate.
 - 12.2.5 Keep the agar molten between 45°C and 48°C until use in the SAL assay.
 - 12.2.6 Prepare method blanks as specified in Section 9.4.
- 12.3 As a precaution against contamination, disinfect a work space near the water baths with a 1 : 100 dilution of household bleach and allow to dry. If workspace can be corroded by bleach use an ethanol solution of 70% or greater.
- 12.4 Assemble and label plates with bacterial host (*E. coli* CN-13 or *E. coli* F_{amp}), date, and time.
- 12.5 Addition of sample and host bacteria to SAL media
 - 12.5.1 Dispense two, 100-mL aliquots of sample into separate, sterile, 250-mL to 500-mL size Erlenmeyer flasks. Also dispense 100 mL of reagent water into a third 250-mL to 500-mL size Erlenmeyer flask (this will be used to determine sample temperature changes in the following steps and will be referred to as the “temperature flask”).
 - 12.5.2 Add 0.5 mL of sterile stock magnesium chloride (Section 7.1.3) to each sample flask (not the temperature flask).
 - 12.5.3 Place the flasks into the 36 °C ± 1.0 °C water bath for 5 minutes or until sample water just reaches the water bath temperature according to the temperature flask.

Please note: All components should be warmed before assay to avoid solidification prior to pouring plates. Also, The following steps are critical. Temperature must be monitored closely to ensure that coliphages are not inactivated and also to ensure that the agar does not harden prematurely.

- 12.5.4 Add 10 mL of log-phase *E. coli* CN-13 (Section 7.5.4) to one flask of sample water.
- 12.5.5 Add 10 mL of log-phase *E. coli* F_{amp} (Section 7.5.4) to the other flask of sample water.
- 12.5.6 Add an additional 10 mL of water to the temperature flask (110 mL total).
- 12.5.7 Immediately transfer temperature flask and flasks containing sample and log-phase bacteria to the 45°C to 48°C water bath. The approximate temperature of the samples should be determined by monitoring the temperature of the water in the temperature flask. When water in the temperature flask reaches 43°C ± 1.0°C, remove samples from the water bath and proceed to the next step immediately.

Please note: During the single agar layer procedure, the sample and host bacteria should not remain in contact with each other for more than 10 minutes prior to plating. After plating, the agar must harden within 10 minutes. Increased contact time or agar-hardening time may result in replication of phages such that the initial phage concentration is overestimated. The entire plating procedure from combining sample with host to hardening of single-agar layer plates should not exceed 20 minutes. Samples also should remain in contact with host for a minimum of three minutes before plates are poured.

- 12.5.8** Add the sample/*E. coli* CN-13 mixture to the 100 mL of 2X TSA containing nalidixic acid. Allow sample/*E. coli* CN-13 mixture to remain in contact with host for a minimum of three minutes before plating.
 - 12.5.8.1** Pour the contents into a series of petri dishes at 20 mL per 100-mm-diameter dish or 40 mL per 150-mm-diameter dish. (This procedure requires either five, 150-mm plates or ten, 100-mm plates per 100-mL sample.)
- 12.5.9** Combine the sample/*E. coli* F_{amp} mixture with the 100 mL of 2X TSA containing ampicillin/streptomycin. Allow sample/*E. coli* F_{amp} mixture to remain in contact with host for a minimum of three minutes before plating.
 - 12.5.9.1** Pour the contents into a series of petri dishes at 20 mL per 100-mm diameter dish or 40 mL per 150-mm dish. **Please note:** Plates should be dry before they are inverted, as condensation drops on the agar surface may appear to be plaques. When reading plates examine plaques closely.
- 12.5.10** Allow the agar to harden, cover, invert, and incubate for 16 - 24 hours at 36°C ± 1.0°C.
- 12.5.11** Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria in SAL plates after 16 - 24 hours are considered to be plaques. Count all plaques per plate series, record results, and go to Section 13.3 for calculation of PFU / 100 mL. **Please note:** The use of a light box (Section 6.4.8) to evaluate sample results is recommended.
- 12.6** Spot plate plaque confirmation procedure—Although not required, laboratories may use the spot plate procedure for confirmation of plaques if one or more such plaques on a single agar layer plate are questionable.
 - 12.6.1** Pick plaque(s) with a sterile Pasteur (or other) pipette and transfer it to a tube with 0.5 mL TSB (Section 7.3.1.1).
 - 12.6.2** Allow the inoculated broth to stand 5 minutes at room temperature.
 - 12.6.3** Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
 - 12.6.4** Prepare spot plates according to Section 7.3.4. Once prepared, spot plates may be used on the same day or held at 4°C ± 1°C for up to 4 days prior to use.
 - 12.6.5** Spot 10 microliters of inoculated broth to a spot plate with appropriate *E. coli* host, using the same *E. coli* host on which the phage was initially isolated. Record spot time.
 - 12.6.6** Spot the method blank and positive control samples as specified in Section 9.4 and 9.5 of the April 2001 version of Method 1601 (Reference 17.7).
 - 12.6.7** Allow inocula to absorb into medium. This will take approximately 30 to 60 minutes. The inocula must not be allowed to run across the plate.
 - 12.6.8** After inocula absorption, cover, invert, and incubate the plate at 36°C ± 1.0°C for 16 to 24 hours.
 - 12.6.9** Lysis zone formation (typically a circular zone of clearing) indicates confirmation for

coliphages. If the spot contains an intact lawn of bacteria indistinguishable from the background lawn of bacteria, this indicates a negative result. However, other outcomes of the spot assay are possible. A positive confirmation also may appear as one or more small plaques or areas of clearing of the host bacteria lawn within the spot, despite the presence of some portion of the host bacteria lawn within the spot. A positive result may also appear as a zone of lysis containing small, discrete colonies of bacteria within the spot. These bacterial colonies are from phage-resistant mutants. **Please note:** *The use of a light box (Section 6.4.8) to evaluate sample results is recommended.*

13.0 Data Analysis and Calculations

13.1 Calculation of QC sample spiking suspension concentrations from the double agar layer (DAL) enumeration procedure (Section 11)

13.1.1 The number of plaque forming units (PFU) per mL in the coliphage spiking suspension will be calculated using DAL plates that yield plaque counts within the desired range of zero to 300 PFU per plate for male-specific (F⁺) coliphage and zero to 100 PFU per plate for somatic coliphage. There may be occasions when the total number of plaques on a plate will be above the ideal range. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as “too numerous to count” (TNTC).

13.1.2 For each coliphage type, sum the number of PFU from all dilutions with plaques (on either of the duplicate plates), excluding dilutions with all TNTC or all zeros. (See equation in Section 13.1.5)

13.1.3 Sum the undiluted sample volumes used to inoculate all replicate plates at all dilutions having useable counts (as defined above). (See equation in Section 13.1.5)

13.1.4 Divide the sum of the PFU by the sum of the undiluted sample volume to obtain PFU/ mL in the spiking suspension. (See equation in Section 13.1.5)

13.1.5 The equation for Sections 13.1.1 through 13.1.4 is as follows:

$$\text{Undiluted spiking suspension PFU / mL} = (\text{PFU}_1 + \text{PFU}_2 \dots \text{PFU}_n) / (\text{V}_1 + \text{V}_2 \dots \text{V}_n)$$

Where:

- PFU = number of plaque forming units from plates of all countable sample dilutions (dilutions with 1 or more PFU per plate, excluding dilutions with all TNTC or all zeros (0))
- V = volume of undiluted sample in all plates with countable plaques
- n = number of useable counts

Table 1. Example DAL data

Dilution	PFU / plate (for each duplicate plate)	Volume of <i>undiluted</i> spiking suspension (mL)
Undiluted	TNTC, TNTC	0.5 mL
1 : 10	35, 37	0.05
1 : 100	0, 3	0.005
1 : 1,000	0, 0	0.0005

Example: $(35 + 37 + 0 + 3)/(0.05 + 0.05 + 0.005 + 0.005) = 75/0.11 = 682$ PFU / mL

In this example, the undiluted spiking suspension contains approximately 682 PFU per mL, the 1 : 10 dilution contains approximately 68.2 PFU per mL, the 1 : 100 dilution contains approximately 6.82 PFU per mL, and the 1 : 1000 dilution contains approximately 0.682 PFU per mL.

13.2 Calculation for preparing IPR, OPR, MS, and positive control spikes

13.2.1 Use a dilution of the QC sample spiking suspension that will result in a bulk spike volume between 0.1 and 3.0 mL for the spike concentration specified in Section 9.

13.2.2 Use the following equation to determine the spiking volume:

$$S = \frac{(T)(B)}{(C)}$$

where,

S = Spike volume (mL)

T = Target number of coliphage per sample (PFU)

B = Number of samples that will be spiked (only necessary when multiple QC samples are spiked in bulk)

C = Concentration (PFU/mL) in the dilution to be used for spiking

13.2.3 For example, for the IPR test (Section 9.3):

T) A spike dose of 80 PFU is needed per 100-mL sample

B) A total of four, 100-mL samples will be spiked at the same time

C) The undiluted spiking solution contains 682 PFU / mL

The equation would be solved as follows:

$$S = \frac{(80 \text{ PFU})(4)}{(682 \text{ PFU / mL})} = 0.47 \text{ mL}$$

As a result, 0.47 mL of the undiluted spiking suspension would be spiked into the 400-mL bulk sample. The 400-mL bulk sample would be mixed and four, 100-mL aliquots dispensed. Each 100-mL sample should contain approximately 80 PFU.

13.3 Calculation for the SAL procedure (Section 12)

13.3.1 The SAL method (SAL) has been validated for use with 100 mL sample volumes. 100% of each sample should be plated.

13.3.2 For each sample, count the total number of plaques from all plates. If the plaques are not discrete, results should be recorded as “too numerous to count” (TNTC). The remaining sample should be diluted, as appropriate, and re-analyzed.

13.3.3 Total number of plaques per 100 mL sample = PFU / 100 mL.

14.0 Method Performance

14.1 The QC acceptance criteria listed in Table 2 are based on data generated through the interlaboratory validation of Method 1602 involving 10 laboratories and 10 raw ground water matrices.

Table 2. Quality control acceptance criteria

Performance test	Male-specific acceptance criteria	Somatic acceptance criteria
Initial precision and recovery (IPR) (Section 9.3)		
• Mean percent recovery	9% - 130%	86% - 177%
• Precision (as maximum relative standard deviation)	46%	23%
Ongoing precision and recovery (OPR) as percent recovery (Section 9.6)	4% - 135%	79% - 183%
Matrix spike (MS) (Section 9.5)		
• MS percent recovery	Detect - 120%	48% - 291%
Matrix spike, matrix spike duplicate (MS/MSD) (Section 9.7)		
• Mean percent recovery for MS/MSD	Detect - 120%	48% - 291%
• Precision (as maximum relative percent difference of MS/MSD)	57%	28%

14.2 Method 1602 is a performance-based method and the laboratory is permitted to modify certain method procedures to improve performance or lower the costs of measurements, provided that all quality control (QC) tests cited in Section 9.2.5 are performed and all QC acceptance criteria are met. The laboratory is not permitted to modify the double agar layer QC spiking suspension enumeration procedure (Section 11.3).

14.2.1 Method modifications at a single laboratory. Each time a modification is made to this method for use in a single laboratory, the laboratory is required to validate the modification according to Tier 1 of EPA’s performance-based measurement system (PBMS) (Table 3 and Reference 17.6).

14.2.2 Method modifications for nationwide approval. If the laboratory or a manufacturer seeks EPA approval of a method modification for nationwide use, the laboratory or manufacturer must validate the modification according to Tier 2 of EPA's PBMS (Table 2 and Reference 17.6). **Please note:** After a method modification is validated for nationwide use, each individual laboratory electing to use the modification still must demonstrate acceptable initial and on-going performance with the modified method through the analysis of method blanks, media sterility checks, positive controls, ODC samples, and MS samples.

Table 3. Tier 1 and Tier 2 Validation/Equivalency Demonstration Requirements

Test	Description	Tier 1 modification ⁽¹⁾	Tier 2 modification ⁽²⁾
IPR (Section 9.3)	4 replicates of spiked reagent water	Required. Must be accompanied by a method blank.	Required per laboratory
Method blank (Section 9.4)	Unspiked reagent water	Required	Required per laboratory
MS/MSD (Section 9.7)	2 replicates of spiked matrix water	Recommended, but not required. Must be accompanied by an unspiked field sample collected at the same time as the MS sample	Required per laboratory. Each laboratory must analyze a different water.

Please note:

- (1) If a modification will be used only in one laboratory, these tests must be performed and the results must meet all of the QC acceptance criteria in the method (these tests also are required the first time a laboratory uses the validated version of the method). After the initial demonstration that the modification is equivalent to the procedure specified in this method, the laboratory must continue to demonstrate acceptable ongoing performance with the modified method through the analysis of media sterility checks, method blanks, positive controls, ODC samples, and MS samples.
- (2) If nationwide approval of a modification is sought for one type of water matrix (such as ground water), a minimum of 3 laboratories must perform the tests and the results from each lab individually must meet all QC acceptance criteria in the method. If more than 3 laboratories are used in a study, a minimum of 75% of the laboratories must meet all QC acceptance criteria.

15.0 Pollution Prevention

- 15.1 The solutions and reagents used in this method 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 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).
- 16.2 Samples, reference materials, and equipment known or suspected to have bacteriophage attached or contained must be sterilized prior to disposal.

- 16.3** 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 N.W., Washington D.C. 20036.

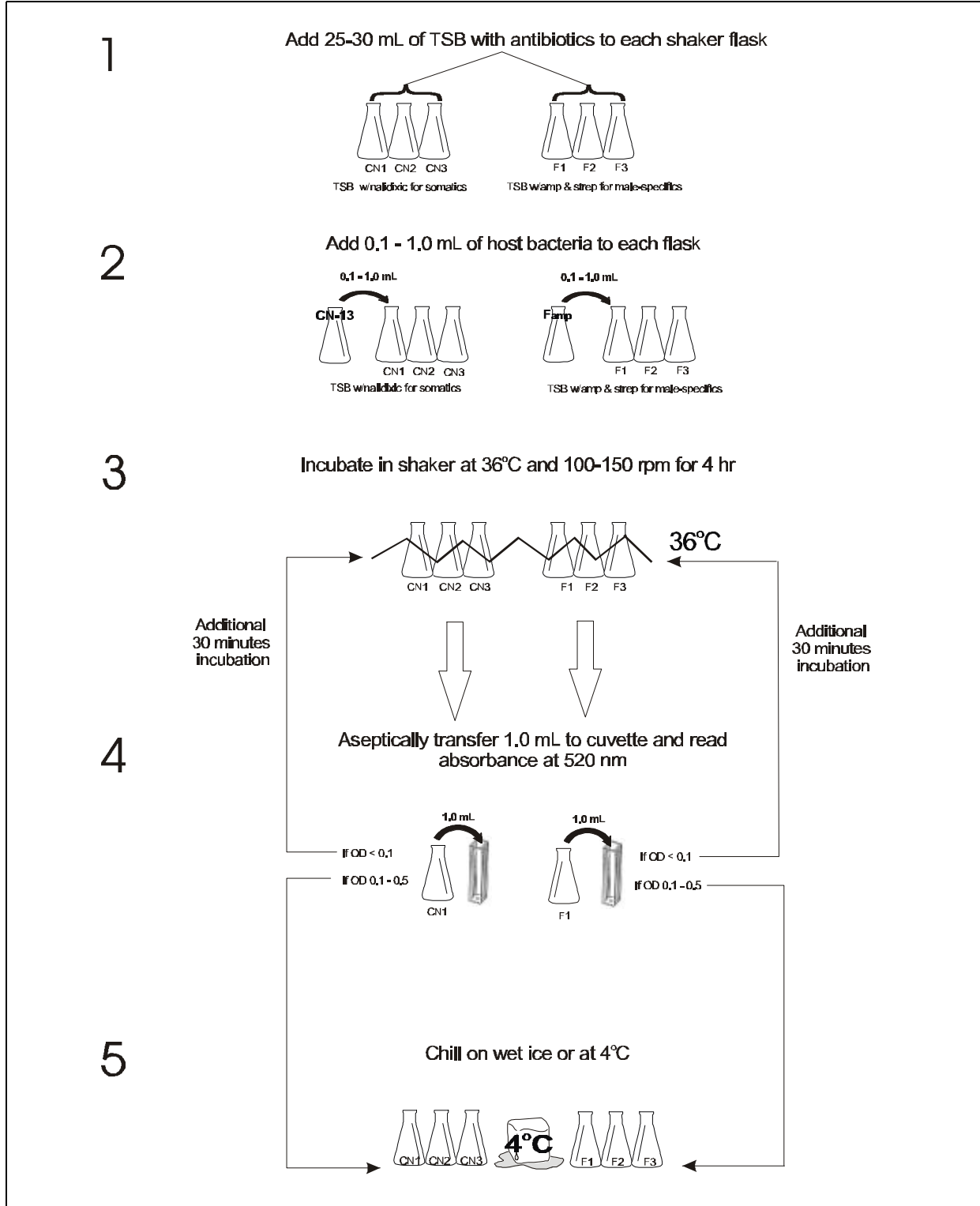
17.0 References

- 17.1** American Public Health Association, American Water Works Association, Water Environment Federation. Washington, D.C. Joint Task Group for Section 9224, 1997. Detection of Coliphages. For Standard Methods for the Examination of Water and Waste Water 20th Edition Supplement. (draft version - December 1997)
- 17.2** American Public Health Association, American Water Works Association, and Water Environment Federation. 1995. *Standard Methods for Water and Wastewater*. 20th Edition. Sections 9020, 9030, 9040, 9050, and 9221.
- 17.3** Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA-600/8-78-017. Office of Research and Development. USEPA.
- 17.4** *Manual for the Certification of Laboratories Analyzing Drinking Water*, EPA 815-B-97-001, Office of Ground Water and Drinking Water, Technical Support Center, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268.
- 17.5** *Annual Book of ASTM Standards*. Vol. 11.01. American Society for Testing and Materials. Philadelphia, PA 19103.
- 17.6** USEPA. *EPA Guide to Method Flexibility and Approval of EPA Water Methods*, EPA 821-D-96-004. Office of Water, Engineering and Analysis Division, Washington, DC 20460 (1996).
- 17.7** USEPA. Method 1601:Male-specific (F⁺) and Somatic Coliphage in Water by Two-Step Enrichment Procedure. EPA 821-R-01-030. Office of Water, Engineering and Analysis Division, Washington, DC 20460 (April 2001).

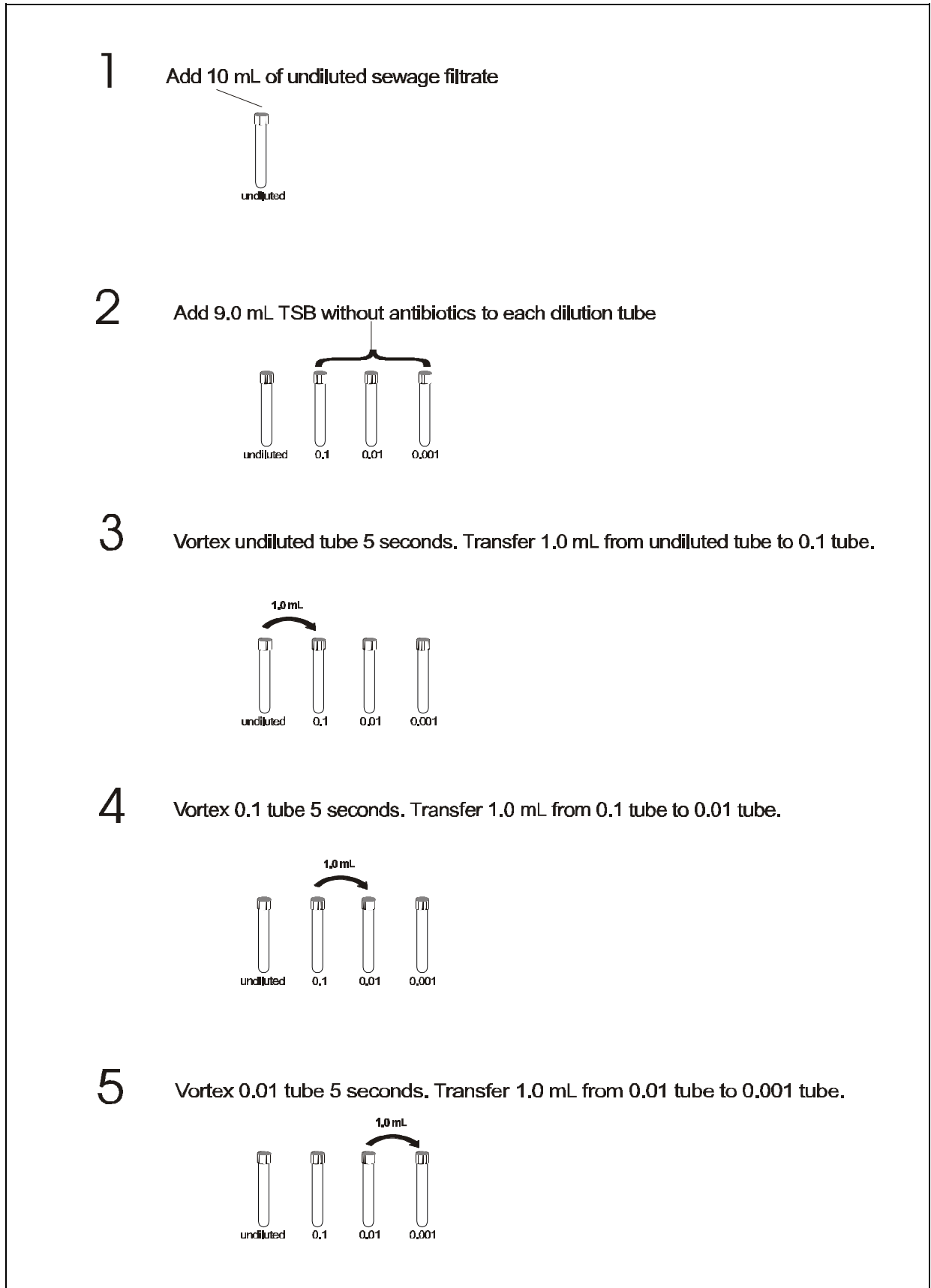
18.0 Flowcharts

We greatly appreciate Fred Williams (USEPA, Cincinnati, OH) for providing the original flow charts on which all of the following flowcharts are based.

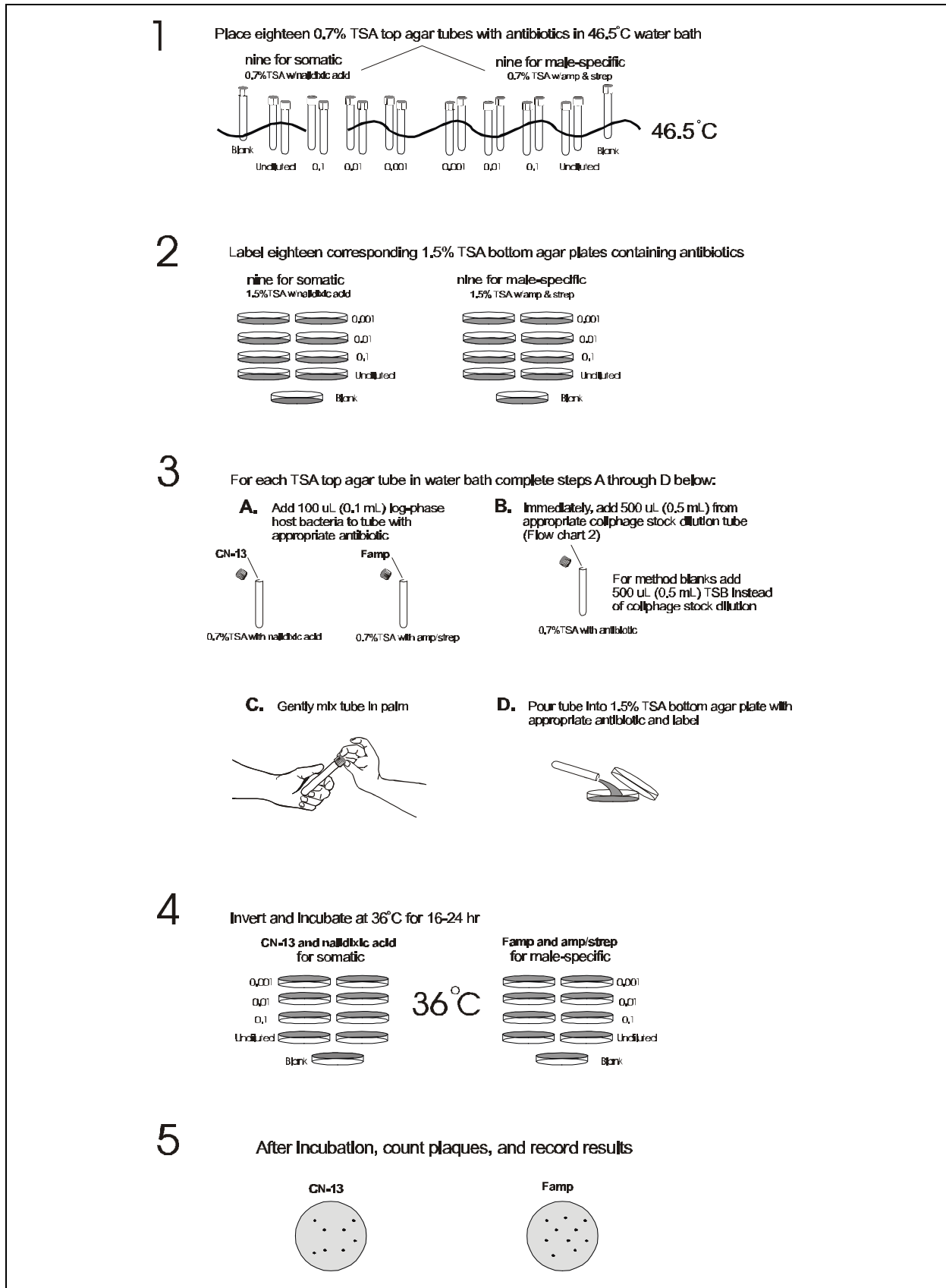
Flow chart 1. Preparation of log-phase host bacteria stock cultures (Section 7.5.4)



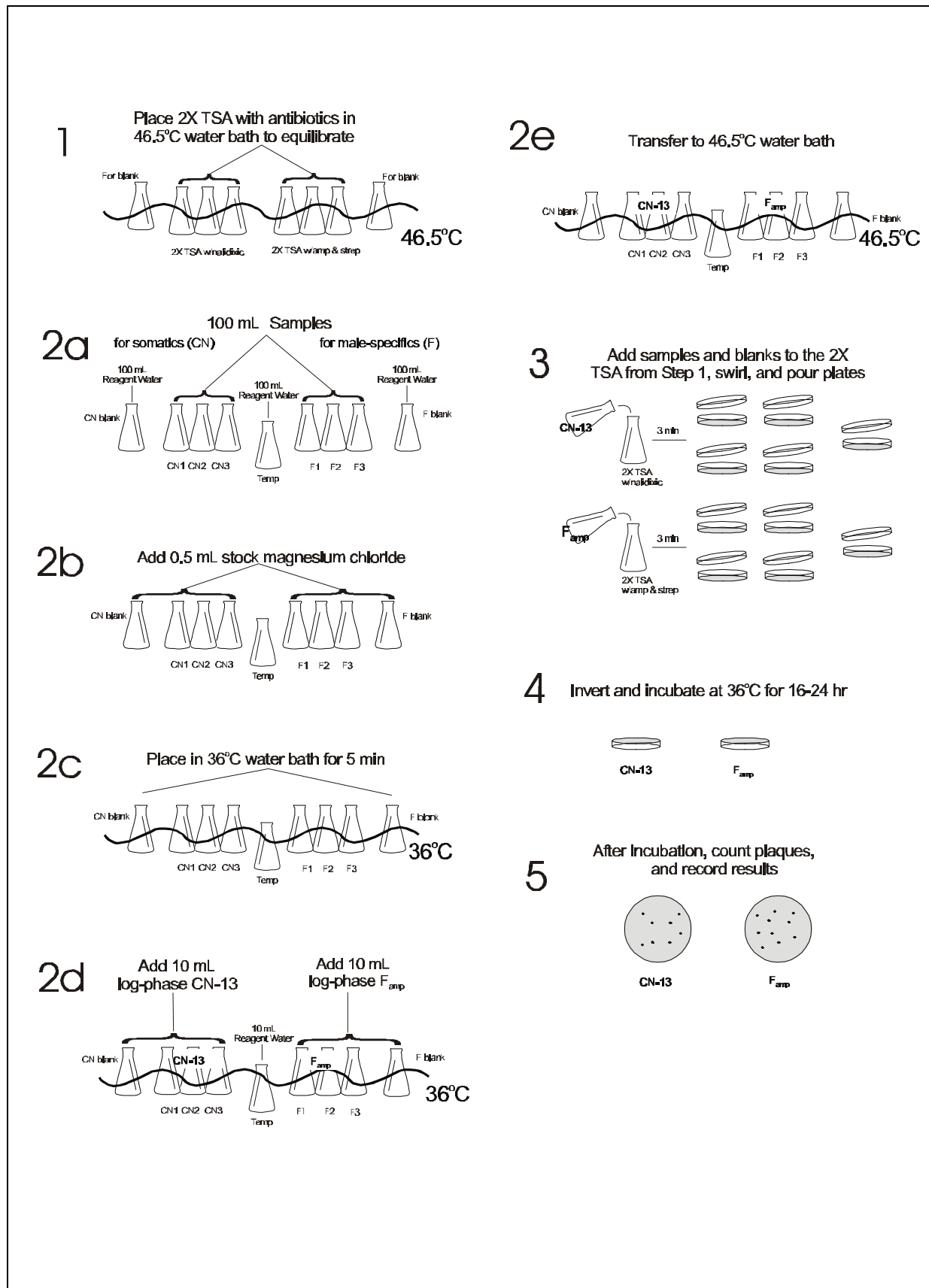
Flow chart 2. Dilution of coliphage QC spiking suspensions (Section 11.2)



Flow chart 3. Coliphage spiking suspension enumeration by double agar layer (DAL) procedure (Section 11.3)



Flow chart 4. Single agar layer (SAL) enumeration procedure for 100-mL samples (Section 12)



19.0 Glossary

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

19.1 Symbols

°C	degrees Celsius
μ	micro
#	number
%	percent

19.2 Alphabetical characters and acronyms

ASTM	American Society for Testing and Materials
CFR	Code of Federal Regulations
DAL	double agar layer method
DOT	Department of Transportation
g	gram
HCl	hydrochloric acid
IPR	initial precision and recovery
KH ₂ HPO ₄	potassium phosphate
L	liter
M	molar
mg	milligram
MgCl ₂ •6H ₂ O	magnesium chloride hexahydrate
mL	milliliter
mm	millimeter
MPN	most probable number
MS	matrix spike
NaOH	sodium hydroxide
Na ₂ S ₂ O ₃	sodium thiosulfate
NIST	National Institute of Standards and Technology
nm	nanometer
OD	optical density
OPR	ongoing precision and recovery
OSHA	Occupational Safety and Health Administration
PFU	plaque forming unit
psi	pounds per square inch
QA	quality assurance
QC	quality control
rpm	revolutions per minute
SAL	single agar layer method
TNTC	too numerous to count
TSA	tryptic soy agar
TSB	tryptic soy broth
USEPA	United States Environmental Protection Agency
X	“times”

19.3 Additional definitions

Accuracy—A measure of the degree of conformity of a single test result generated by a specific procedure to the assumed or accepted true value and includes both precision and bias.

Analyte—The organism tested for by this method. The analyte in this method is coliphage.

Bias—the persistent positive or negative deviation of the average value of a test method from the assumed or accepted true value.

Coliphage—Viruses that infect *E. coli*.

Host bacteria—Are those bacteria that allow the bacteriophage to penetrate and replicate within them, ultimately lysing, resulting in the release of the progeny bacteriophage. Host bacteria are essential for virus replication. The hosts used in this method are: *E. coli* CN-13, and *E. coli* F_{amp} (*E. coli* HS(pFamp)R).

Initial precision and recovery (IPR)—The IPR test is performed to establish the ability to demonstrate control over the analytical system and to generate acceptable precision and recovery.

Male-specific coliphage—Viruses (bacteriophages) that infect coliform bacteria only via the F-pilus.

Method blank—An aliquot of reagent water that is treated exactly as a sample and carried through all portions of the procedure until determined to be negative or positive. The method blank is used to determine if the sample has become contaminated by the introduction of a foreign microorganism through poor technique.

Ongoing precision and recovery—A reagent water sample method blank spiked with known quantities of analytes. The OPR is analyzed exactly like a sample. Its purpose is to assure that the results produced by the laboratory remain within the limits specified within this method for precision and recovery.

Plaque—Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria in SAL and DAL plates after incubation.

Precision—The degree of agreement of repeated measurements of the same property, expressed in terms of dispersion of test results about the arithmetical mean. Results are obtained by repetitive testing of a homogeneous sample under specified conditions. The precision of a test method is expressed quantitatively as the standard deviation computed from the results of a series of controlled determinations.

Relative Standard Deviation (RSD)—The standard deviation times 100 divided by the mean.

Reagent water—Water conforming to Specification D 1193, Annual Book of ASTM Standards (Reference 17.5) or specifications in Standard Methods 9020 B.4.d (Reference 17.2).

Somatic coliphage—Those coliphage that infect host cells via the outer cell membrane but do not infect host cells via the F-pilus.