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EPA

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

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Disclaimer

This method is in final draft form. The method has been validated through a 10-laboratory round-robin. The Agency welcomes comments on its technical merit. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Introduction

Coliphage presence in groundwater is an indication of fecal contamination. Method 1602 is a performance-based method for enumerating male-specific (F+) and somatic coliphage in groundwater and other waters. The 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 mixture into plates using the total volume. 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.

Note: *Although Method 1602 has been validated through a 10-laboratory round-robin validation study, the QC acceptance criteria and frequency of QC samples to be required in the method have not been finalized. Final QC criteria and frequency will be specified in the next draft of the method.*

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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Note: *Prior to finalization of this method, each step of this method must be performed as written. After the method is finalized, this method will be performance-based. At that point, the laboratory is permitted to modify or omit any steps or procedure, provided that all performance requirements set forth in the validated method are met. The laboratory may not omit any quality control analyses. The terms "shall," "must," and "may not" indicate steps and procedures required for producing reliable results. The terms "should" and "may" indicate optional steps that may be modified or omitted if the laboratory can demonstrate that the modified method produces results equivalent or superior to results produced by this method.*

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

April 2000 Draft

1.0 Scope and Application

1.1 The single agar layer (SAL) procedure detects and enumerates male-specific (F^+) and somatic coliphages in groundwater and other waters. This method is intended to help determine if groundwater is affected by fecal contamination.

Note: *Although this method may be used for water matrices other than groundwater, it has only been validated for use in groundwater.*

1.2 This method is designed to meet the monitoring requirements of the U.S. Environmental Protection Agency (EPA). It 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 18.1).

1.3 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 groundwater and other water matrices where coliphage is suspected to be present.

1.4 Each laboratory and analyst that uses this method must first demonstrate the ability to generate acceptable results using the procedures in Section 9.0.

1.5 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 groundwater sample is assayed by adding $MgCl_2$ (magnesium chloride), 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 coliform bacteria 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 bacteriophages 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 20.3.

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

Caution: *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.*

5.1 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.

5.2 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 17.0.

5.3 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.

5.4 Mouth pipetting is prohibited.

6.0 Equipment and Supplies

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 here, 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 (TD), 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

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-µm 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 ½" diameter cuvettes (Model # 4015) or 13 mm × 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 ± 1.0 °C and 100 to 150 rpm, New Brunswick, PsychoTherm, Innova, or equivalent or an ordinary shaker in an incubator

6.3 Supplies for collection and filtration of raw sewage for spiking (if not using stock coliphage, Sections 7.1.10 and 7.1.11)

6.3.1 Disposable filter disks—25-mm-diameter, 0.45-µm pore size, sterile, low protein binding (Gelman Acrodisc HT Tuffryn, No. 4184, cellulose acetate Corning No. 21053-25, or equivalent)

6.3.2 Syringe—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

7.0 Reagents and Standards

7.1 General reagents

7.1.1 Reagent water—Conforming to Specification D 1193, Annual Book of ASTM Standards (Reference 18.7).

7.1.2 *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.

7.1.3 *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.

7.1.4 Preparation of frozen stock cultures—The laboratory shall obtain reference host bacterial cultures (Sections 7.1.2 and 7.1.3) and use these to establish pure frozen stock cultures that are maintained for the laboratory. Stocks are used as inoculum for log-phase host bacterial cultures.

7.1.4.1 Establish pure frozen stock cultures by streaking host bacteria onto tryptic soy agar (Section 7.2.1) with appropriate antibiotic to attain isolated colonies.

7.1.4.2 Incubate overnight, pick an individual colony, and grow to log phase (Section 12) in tryptic soy broth with appropriate antibiotic (Sections 7.1.7.2 and 7.1.7.3).

7.1.4.3 Harvest broth by mixing sterile glycerol and broth with log-phase host bacteria in a ratio of 1:4 in a freezer vial (Example: 200 µL sterile glycerol plus 800 µL log-phase *E. coli*).

7.1.4.4 Label with *E. coli* strain and date of harvest.

7.1.4.5 Freeze stock cultures at -70°C, if possible. Cultures can be frozen -20°C if the laboratory does not have the capability to freeze samples at -70°C).

7.1.5 100X nalidixic acid (Sigma N4382)—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 freezer vial and store frozen at -20°C for up to one year. Thaw at room temperature or rapidly in a 36°C ± 1.0 °C water bath. Mix well prior to use.

7.1.6 100X ampicillin/streptomycin—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 freezer 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 ± 1.0 °C water bath.

Note: *100X nalidixic acid and 100X ampicillin/streptomycin are always added to medium after the medium has been autoclaved.*

7.1.7 Tryptic (or trypticase) soy broth (DIFCO 0370-15-5, or equivalent) (TSB)

7.1.7.1 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.

Note: *To determine pH after autoclaving, aseptically remove an aliquot of medium and measure pH. Discard aliquot after checking pH, to ensure that the medium is not contaminated.*

7.1.7.2 TSB with nalidixic acid (for growth of *E. coli* CN-13)—Aseptically add 10 mL of 100X nalidixic acid (Section 7.1.5) to 1 L of autoclaved, cooled TSB (Section 7.1.7.1) and mix.

7.1.7.3 TSB with streptomycin/ampicillin (for growth of *E. coli* F_{amp})—Aseptically add 10 mL of 100X streptomycin/ampicillin sulfate (Section 7.1.6) to 1 L of autoclaved, cooled TSB (Section 7.1.7.1) and mix.

7.1.7.4 10X Tryptic soy broth—Dissolve 300 g TSB per liter of reagent water. Autoclave for 15 minutes at 121°C and 15 psi. Be careful to remove broth as soon as possible from the autoclave to prevent scorching. Store at 4°C ± 1°C until use.

7.1.8 10% (w/v) Sodium thiosulfate—Add 10 g sodium thiosulfate ($Na_2S_2O_3$) per 90 mL reagent water. Mix until dissolved. Bring to 100 mL and autoclave for 15 minutes at 121°C and 15 psi.

7.1.9 Magnesium chloride 80X (4M)—To 814 g of $MgCl_2 \cdot 6H_2O$, 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.

7.1.10 MS2 stock coliphage (ATCC#15597-B1)

7.1.11 ϕX174 stock coliphage (ATCC#13706-B1)

7.1.12 Glycerol—Sigma#G6279 or equivalent. Autoclave for 15 minutes at 121°C and 15 psi. Remove promptly to avoid scorching. Store at room temperature.

7.2 Double agar layer reagents

7.2.1 Tryptic soy agar or bottom agar (1.5%)—Prior to autoclaving the TSB in Section 7.1.7.1, 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 for even distribution.

7.2.1.1 For growth of somatic coliphage using *E. coli* CN-13 as host bacteria, add 10 mL of 100X nalidixic acid (Section 7.1.5) per liter of autoclaved TSA. Swirl flask to mix well 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. Replace lids and store inverted at 4°C ± 1°C for up to 2 weeks.

7.2.1.2 For growth of male-specific (F^+) coliphage using *E. coli* F_{amp} as host bacteria, add 10 mL 100X ampicillin/streptomycin sulfate (Section 7.1.6) per liter of autoclaved TSA. Swirl flask to mix well 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. Replace lids and store inverted at 4°C ± 1°C for up to 2 weeks.

7.2.2 Top (soft) agar tubes (0.7%)—Prior to autoclaving the TSB in Section 7.1.7.1, 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.

7.2.2.1 Top agar with nalidixic acid (for growth of *E. coli* CN-13)—To 1 L of autoclaved TSA (soft agar) (Section 7.2.2), add 10 mL of 100X nalidixic acid. 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.

7.2.2.2 Top agar tubes with ampicillin/streptomycin (for growth of *E. coli* F_{amp})—To 1 L of autoclaved TSA (soft agar) (Section 7.2.2), add 10 mL of 100X ampicillin/streptomycin. 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.

Note: *When using top agar, add bacteria and sample to agar tubes immediately before plating to ensure viability of both bacterial host and coliphage.*

7.3 Single agar layer

7.3.1 2X tryptic soy agar for single agar layer (SAL) method (2X TSA)—To make double-strength (2X) TSA, double all components of TSB from Section 7.1.7.1 (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. Medium may become darker after autoclaving but this should not impact media performance.

7.3.1.1 2X TSA with nalidixic acid (for growth of *E. coli* CN-13)—To 1 L of autoclaved 2X TSA, add 20 mL of 100X nalidixic acid and mix well. Keep molten at 45°C to 48°C in water bath until use. Agar must be used on the day of preparation.

7.3.1.2 2X TSA with ampicillin/streptomycin (for growth of *E. coli* F_{amp})—To 1 L of autoclaved 2X TSA, add 20 mL of 100X ampicillin/streptomycin and mix well. Keep molten at 45°C to 48°C in water bath until use. Agar must be used on the day of preparation.

8.0 Sample Collection, Preservation, and Storage

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. 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.

Note: *Wet ice, Blue Ice® or similar products must be used to maintain a temperature of 2°C to 8°C during shipment. The samples must be protected from freezing.*

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.*

8.3 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.

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 (if necessary)—For 1-L of water, add 0.5-mL 10% sodium thiosulfate.

9.0 Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality assurance (QA) program. The minimum QA requirements for this program 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 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 18.4).

9.2 General QC requirements

Note: *Although Method 1602 has been validated through a 10-laboratory round-robin validation study, the QC acceptance criteria and frequency of QC samples to be required in the method have not been finalized. Final QC criteria and frequency will be specified in the next revision of the method.*

9.2.1 The laboratory shall demonstrate the ability to generate acceptable performance with this method by performing an IPR test. The procedure for performing the IPR is described in Section 9.3.

9.2.2 The laboratory shall analyze method blanks 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 for each analytical batch *[analytical batch to be defined]*.

9.2.3 The laboratory shall spike a separate sample aliquot from the same groundwater source to monitor method performance. This matrix spike (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 groundwater source for which the laboratory has never before analyzed samples. The MS analysis is performed on an additional (second) sample aliquot collected from the groundwater source at the same time as the routine field sample. If the laboratory routinely analyzes samples from one or more groundwater sources, one MS analysis must be performed per *[number to be determined]* 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 *[number to be determined]* sample from this site, a separate aliquot of this *[number to be determined]* sample must be collected and spiked.

9.2.4 The laboratory shall, on an ongoing basis, demonstrate through analysis of the ongoing precision and recovery (OPR) samples that the analytical system is in control (Section 9.6). The laboratory shall analyze one OPR sample for each analytical batch *[analytical batch to be defined]*.

9.2.5 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

9.2.6 The laboratory shall test media sterility by subjecting a representative portion of each media batch to incubation at 36°C ± 1.0 °C for 48 to 72 hours and observe for indications of growth. With respect to media, a batch is defined as 1 tube out of 50 in each lot or one tube, if the lot contains less than 50 tubes.

9.2.7 The laboratory should participate in available interlaboratory performance studies conducted by local, state, and federal agencies or commercial organizations. Review results to correct unsatisfactory performance and record corrective actions.

9.2.8 A log book containing reagent and material lot numbers should be maintained along with samples analyzed using each of the lots.

9.2.9 The laboratory shall maintain a record of the date and results of all QC samples described in Section 9.2. A record of sterility check, OPR, and MS sample results must be maintained.

9.3 Initial precision and recovery (IPR)—The IPR test is performed to demonstrate acceptable performance with the method.

Note: *IPR tests must be accompanied by analysis of a method blank (Section 9.4).*

9.3.1 A total of *[number to be determined]* spiked reagent water samples (*[number to be determined]* samples spiked with each coliphage type) are required for the IPR test, as is a method blank (Section 9.4) for each coliphage type.

9.3.2 For each coliphage type (somatic and F^+), use an appropriate volume of the enumerated stock suspension prepared in Section 11, to spike a *[number to be determined]*-mL "bulk" reagent water sample with approximately 320 coliphage (resulting in 80 PFU / sample). Aliquot each sample into *[number to be determined]*, 100-mL samples (see Section 11 for enumeration of coliphage stock and Section 14.2 for spiking volume calculations).

9.3.3 Analyze the *[number to be determined]* spiked samples and two method blanks (one for each coliphage type) using the SAL procedure (Section 13).

9.3.4 Compute the percent recovery of coliphage 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 sample results 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 15). If RSD_r and $\bar{x}$ meet the acceptance criteria, system performance is acceptable and analysis of blanks and samples may begin. If RSD_r or $\bar{x}$ falls outside the range for recovery, 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 Prepare and analyze a reagent water sample containing no coliphage using the same procedure as used for analysis of the field 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 blank shows no evidence of contamination. Any sample in a batch associated with a contaminated blank must be recollected if holding time limits have been violated. Samples from a batch that proves to have no coliphage in its blank may be reported along with that batch's sample data.

9.5 Matrix spike—The laboratory shall spike and analyze *[number to be determined]* separate 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.

9.5.1 Analyze an unspiked field sample according to the SAL quantitative procedure (Section 13). To a second aliquot of the same field sample, add a volume of stock coliphage equal to 80 PFU / 100 mL (see Section 11 for enumeration of coliphage stock and Section 14.2 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 15. 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 should be reanalyzed.

9.6 Ongoing precision and recovery ([OPR]; laboratory control sample) (performed at the frequency specified in Section 9.2.4)

9.6.1 Spike *[number to be determined]* 100-mL reagent water samples with enumerated coliphage stock suspension (Section 11), *[number to be determined]* sample for each coliphage type (somatic and F^+) will be spiked.

9.6.2 Spike 80 coliphage into each sample (see Section 11 for enumeration of coliphage stock and Section 14.2 for spiking volume calculations).

9.6.3 Analyze the *[number to be determined]* spiked samples using the SAL procedure (Section 13).

9.6.4 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

9.6.5 Compare R with the corresponding limits for ongoing precision and recovery in Section 15. If R meets the acceptance criteria, system performance is acceptable and analysis of samples may begin. If R falls outside the range for recovery, system performance is unacceptable. In this event, correct the problem and repeat the test.

9.6.6 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 updated on a regular basis (e.g., after each 5 to 10 new accuracy measurements).

9.7 The laboratory should periodically analyze an external QC sample, such as a performance evaluation when available. The laboratory also should periodically participate in interlaboratory comparison studies using the method.

9.8 The specifications contained in this method can be met if the analytical system is maintained under control. The standards used for initial (Section 9.3) and ongoing (Section 9.6) precision and recovery should be identical, so that the most precise results will be obtained.

10.0 Calibration and Standardization

10.1 Check temperatures in water baths, refrigerators, and -20°C freezers daily to ensure operation within stated limits of method and record daily measurements in a log book.

10.2 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. Check mercury columns for breaks.

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 18.6.

11.0 Enumeration of Coliphage Stock Suspension

11.1 The double agar layer (DAL) procedure is used to enumerate stock suspensions of somatic and F^+ coliphage for use in spiking quality control samples.

11.2 Prepare log-phase bacterial hosts from stock cultures incubated overnight. Inoculum from an overnight bacterial host culture will reach log-phase more rapidly than inoculum from frozen stock.

11.2.1 Preparation of overnight stock cultures

11.2.1.1 Dispense 25 mL of tryptic soy broth (TSB) with nalidixic acid (Section 7.1.7.2) into a sterile 125-mL shaker flask. Label the flask. For proper growth conditions, each flask should always contain 25 to 30 mL of medium.

11.2.1.2 Inoculate the flask with a loopful of *E. coli* CN-13 from the frozen stock culture (Section 7.1.2). Label the flask.

11.2.1.3 Repeat Sections 11.2.1.1 and 11.2.1.2 using TSB with streptomycin and ampicillin as the medium (Section 7.1.7.3) and *E. coli* F_{amp} (Section 7.1.3) as the bacterial host.

11.2.1.4 Place a sterile slip cap or plug on the shaker flasks and secure in shaker.

11.2.1.5 Incubate at 36°C ± 1.0 °C and set shaker to 100 to 150 rpm overnight (18 to 20 hours).

11.2.1.6 Chill on wet ice or at 4°C ± 1°C until ready for use.

11.2.2 Preparation of log-phase bacterial host cultures—See Section 12

11.3 Preparation of filtrate from raw sewage. This filtrate will be used as a QC spiking suspension. If coliphage stock is available, skip this section and proceed to Section 11.4.

11.3.1 Collect approximately 100 mL of raw sewage in a 250-mL collection bottle.

11.3.2 Transport to the laboratory on ice.

11.3.3 Allow the raw sewage to settle at 4°C ± 1°C for 1 to 3 hours. This will make the filtration process easier.

11.3.4 Remove a sterile, 20-mL syringe from its package, aseptically remove plunger from barrel, and attach a filter disk to the syringe barrel.

11.3.5 Pipet 10 to 15 mL of supernatant from settled sewage into the syringe barrel.

11.3.6 Hold the assembly over a 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).

11.3.7 Cap the tube, label with source, date, and initials, and store the filtrate at 4°C ± 1°C until ready to assay. The filtrate should not be stored more than 24 hours, unless re-titered.

Note: *Coliphage titer may decrease over time, therefore sewage filtrate should be assayed immediately for total coliphage and used within 24 hours of preparation. Re-titer this sewage filtrate the day it is used for IPR, OPR, and MS samples.*

11.4 Dilution of coliphage stock or sewage filtrate (Section 11.3)—A minimum of four different volumes/dilutions are used for DAL enumeration of the stock/filtrate:

- Undiluted
- 0.1
- 0.01
- 0.001

Additional dilutions may be necessary. TSB without antibiotics (Section 7.1.7.1) is used as the diluent and as the method blank.

11.4.1 Aseptically add 9.0 mL of TSB without antibiotics (Section 7.1.7.1) into each of three (or more) sterile dilution tubes (Section 6.2.1). Label them as "0.1," "0.01," "0.001," "method blank," etc.

11.4.2 Add 1.0 mL of the stock/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.

11.4.3 Add 1.0 mL of the well-mixed 0.1 dilution to the tube of TSB labeled "0.01". Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.4.4 Add 1.0 mL of the well-mixed 0.01 dilution to the tube of TSB labeled "0.001." Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.4.5 Continue as necessary for subsequent dilutions.

11.4.6 Add 1.0 mL of TSB without antibiotics to a tube labeled "method blank". Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

11.5 Coliphage stock suspension enumeration procedure—In this procedure, a tube of molten top agar with added host bacteria is inoculated with coliphage stock and will be poured into a bottom agar plate. Four dilutions of stock/filtrate will be analyzed in duplicate for each coliphage type. As a result, nine double-agar 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.

11.5.1 Agar preparation

11.5.1.1 Place top agar tubes in a 45°C to 48°C water bath. The top agar should remain molten in the water bath until ready for use. Four tubes are needed for each volume of stock/filtrate (two for each bacterial host). Be sure to include additional two top agar tubes for the method blanks (a method blank is required for each bacterial host). Half of the top agar tubes should contain nalidixic acid (Section 7.2.2.1) for growth of *E. coli* CN-13; the other half should contain ampicillin/streptomycin (Section 7.2.2.2) for growth of *E. coli* F_{amp}.

11.5.1.2 Disinfect a workspace near the water bath.

11.5.1.3 Assemble bottom agar plates and label or code 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 F^+ for the *E. coli* F_{amp} bacterial host)
- Date
- Time

Note: The following plate-preparation steps are critical. Do not add bacterial host and stock/filtrate until ready to plate.

11.5.2 Preparation of plates for enumeration of somatic coliphage (see Table 1 for inoculation scheme)

11.5.2.1 With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing nalidixic acid with 30 to 100 µL of log-phase *E. coli* CN-13.

11.5.2.2 Immediately add 500 µL of undiluted stock/filtrate.

11.5.2.3 Mix the inoculum by rolling the tube briefly in palm of hand.

11.5.2.4 Pour contents into one of the two bottom agar plates marked "undiluted, *E. coli* CN-13, somatic."

11.5.2.5 Duplicate analysis—Repeat Sections 11.5.2.1 through 11.5.2.4 for the duplicate.

11.5.2.6 Repeat Sections 11.5.2.1 through 11.5.2.5 for each dilution volume.

11.5.3 Preparation of plates for enumeration of F^+ coliphage—Repeat Section 11.5.2 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}. (see Table 1 for inoculation scheme)

11.5.4 Preparation of somatic coliphage method blank (see Table 1 for inoculation scheme)

11.5.4.1 With the top agar tube still in the water bath, aseptically inoculate a top agar tube containing nalidixic acid with 30 to 100 µL of log-phase *E. coli* CN-13.

11.5.4.2 Immediately add 500 µL of TSB.

11.5.4.3 Mix the inoculum by rolling the tube briefly in palm of hand.

11.5.4.4 Pour contents into a bottom agar plate marked "blank, *E. coli* CN-13, somatic."

11.5.5 Preparation of the F^+ coliphage method blank—Repeat Section 11.5.4 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp} (see Table 1 for inoculation scheme)

11.5.6 Retain undiluted stock/filtrate for use in preparing new dilutions for OPR and MS samples.

11.5.7 After the top agar hardens, cover, invert the plates and incubate for 16 to 24 hours at 36°C ± 1.0 °C.

11.5.8 Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 to 24 hours are plaques. Count the number of plaques on each plate.

11.5.9 Proceed to Section 14.1 and calculate the PFU / mL for each coliphage.

Top Agar Tubes Contain		Coliphage Source (stock or sewage filtrate)								
		Undiluted		0.1 Dilution in TSB		0.01 Dilution in TSB		0.001 Dilution in TSB		Method Blank
		500 µL ↓	500 µL ↓	500 µL ↓	500 µL ↓	500 µL ↓	500 µL ↓	500 µL ↓	500 µL ↓	500 µL ↓
TSA w/Nalidixic Acid	Add 30 - 100 µL of *E. coli* CN-13 per tube →	∪	∪	∪	∪	∪	∪	∪	∪	∪
TSA w/Ampicillin and Streptomycin sulfate	Add 30 - 100 µL of *E. coli* F_{amp} per tube →	∪	∪	∪	∪	∪	∪	∪	∪	∪

*Host bacteria are added first, then coliphage or TSB (for the blanks). Additional dilutions may be necessary.

12.0 Preparation of Log-Phase Host Bacterial Cultures

12.1 Inoculate a shaker flask containing 25 mL of TSB containing nalidixic acid (Section 7.1.7.2) with 0.1 to 1.0 mL of *E. coli* CN-13. 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 will require a 0.5-mL inoculum of log-phase host bacteria.

12.2 Repeat Section 12.1 using TSB with streptomycin and ampicillin (Section 7.1.7.3) as the medium and *E. coli* F_{amp} as the bacterial host.

12.3 After inoculation, place a sterile slip-cap or plug on the shaker flasks and secure in shaker incubator.

12.4 Incubate at 36°C ± 1.0 °C and 100 to 150 rpm for approximately 4 hours or until cultures are visibly turbid, indicating log-phase growth.

12.5 Aseptically remove 1 mL of culture from flask, dispense into a cuvette, and read absorbance at 520 nm (Section 6.2.22). 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.

12.5 Aseptically remove 1 mL of culture from flask, dispense into a cuvette, and read absorbance at 520 nm (Section 6.2.22). 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.

12.6 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).

12.7 Store remaining bacterial host culture at 4°C ± 1°C overnight to inoculate flasks for the preparation of new log-phase bacterial hosts.

13.0 Single Agar Layer (SAL) Procedure

13.1 Refer to Section 12 for preparation of log-phase host bacterial cultures.

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 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.*

13.2 Preparation of SAL media

13.2.1 Prepare 100 mL of 2X TSA with nalidixic acid for *E. coli* CN-13 as described in Section 7.3.1.1.

13.2.2 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 and care must be taken to ensure that the flask does not tip-over in the waterbath.

13.2.3 Prepare 100 mL of 2X TSA with ampicillin/streptomycin for *E. coli* F_{amp} as described in Section 7.3.1.2.

13.2.4 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.

13.2.5 Keep the agar molten between 45°C and 48°C until use in the SAL assay.

13.2.6 Prepare method blanks as specified in Section 9.4.

13.3 Disinfect a work space near the water baths.

13.4 Assemble and label plates with bacterial host (*E. coli* CN-13 or *E. coli* F_{amp}), date, and time.

13.5 Addition of sample and host bacteria to SAL media

13.5.1 Dispense two, 100-mL aliquots of sample into separate 250-mL to 500-mL size Erlenmeyer flasks. Also dispense 100 mL of reagent water into a 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").

13.5.2 Add 0.5 mL of sterile 80X $MgCl_2 \cdot 6H_2O$ (Section 7.1.9) to each flask.

13.5.3 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.

Note: *All components should be warmed before assay to avoid solidification prior to pouring plates.*

Note: *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.*

13.5.4 Add 10 mL of log-phase *E. coli* CN-13 (Section 12) to one flask of sample water.

13.5.5 Add 10 mL of log-phase *E. coli* F_{amp} (Section 12) to the other flask of sample water.

13.5.6 Add 10 mL of water to the temperature flask.

13.5.7 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.

Note: *Samples should remain in contact with host for a minimum of three minutes before plates are poured.*

13.5.8 Add the sample/*E. coli* CN-13 mixture to the 100 mL of 2X TSA containing nalidixic acid.

13.5.8.1 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.

Note: *This procedure requires either five, 150-mm plates or ten, 100-mm plates per 100-mL sample.*

13.5.9 Combine the sample/*E. coli* F_{amp} mixture with the 100 mL of 2X TSA containing ampicillin/streptomycin.

13.5.9.1 Pour the contents into a series of petri dishes at 20 mL per 100-mm diameter dish or 40 mL per 150-mm dish.

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.

13.5.10 Allow the agar to harden, cover, invert, and incubate for 16 - 24 hours at 36°C ± 1.0°C.

13.5.11 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 and go to Section 14.2 for calculation of PFU / 100 mL.

14.0 Data Analysis and Calculations

14.1 Calculation for the DAL procedure (Section 11)

14.1.1 Compute PFU / mL of filtered sewage using DAL plates that yield plaque counts within the desired range of 20 to 300 PFU per plate for F^+ coliphage and 20 to 100 PFU per plate for somatic coliphage. There may be occasions when the total number of plaques on a plate will be above or below the ideal range. If the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC).

14.1.2 The inoculation volume, as described in Section 11, is 500 µL (0.5 mL). Refer to Table 2 for dilution factors.

Table 2. Dilution factors for concentration of filtered sewage.

Sewage Concentration	Dilution Factor
Undiluted	1
0.1 Dilution	0.1
0.01 Dilution	0.01
0.001 Dilution	0.001

14.1.3 Use the following equation to calculate PFU / mL of coliphage stock or filtered sewage for each DAL plate:

$$\text{PFU / mL} = \frac{\text{number of plaques counted}}{(\text{inoculation volume in mL})(\text{dilution factor})}$$

14.1.4 If multiple plates are within the desired range, then PFU / mL of filtered sewage should be calculated for each of those plates and the average taken.

14.2 Calculation for the SAL procedure (Section 13)

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

14.2.2 For each sample, count total number of plaques from all plates.

Note: 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.

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

14.3 Calculation for preparing IPR, MS, and OPR spikes

14.3.1 The stock/filtrate enumerated in Section 11 will be used as the spiking suspension. Use a dilution of stock/filtrate that will result in a spike volume between 0.1 and 3.0 mL.

14.3.2 Use the following equation to determine the spiking volume:

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

where,
S = Spike volume (mL)
T = Target number of coliphage per sample (PFU)
V = Sample volume (mL)
B = Bulk sample volume (mL) (only necessary when multiple samples are spiked in bulk for QC purposes)
C = Concentration of coliphage in the dilution to be used for spiking (PFU / mL)

14.3.3 For example, if:
T) A spike dose of 2 PFU per sample is needed
V) 100-mL samples will be spiked
B) A total of four samples will be spiked at the same time
(bulk sample volume = 400 mL)
C) The 10^{-2} dilution contains 6 PFU / mL

The equation would be solved as follows:

$$1.3 \text{ mL} = \frac{(2 \text{ PFU} / 100 \text{ mL})(400 \text{ mL})}{(6 \text{ PFU} / \text{mL})}$$

As a result, 1.3 mL of the 10^{-2} dilution 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 2 PFU.

15.0 Method Performance

Note: Although Method 1602 has been validated through a 10-laboratory round-robin validation study, the QC acceptance criteria and frequency of QC samples to be required in the method have not been finalized. Final QC criteria and frequency will be specified in the next revision of the method.

15.1 Precision and accuracy statements will be placed within this document when they have been established.

16.0 Pollution Prevention

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

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

17.0 Waste Management

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

17.2 Samples, reference materials, and equipment known or suspected to have bacteriophage attached or contained must be sterilized prior to disposal.

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

18.0 References

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18.3 U.S. Environmental Protection Agency, 1998. Method 1691: Municipal Biosolids Sampling Guidance. Draft, September 1998. Office of Water, Washington, DC.

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

18.5 *ICR Microbial Laboratory Manual*, EPA/600/R-95/178, National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, 26 Martin Luther King Drive, Cincinnati, OH 45268.

18.6 *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.

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

19.0 Tables, Diagrams, Flowcharts, and Validation Data

[To be added]

20.0 Glossary

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

20.1 Symbols

°C	degrees Celsius
µ	micro
#	number
%	percent

20.2 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_2HPO_4	potassium phosphate
L	liter
M	molar
mg	milligram
$MgCl_2 \cdot 6H_2O$	magnesium chloride hexahydrate
mL	milliliter
mm	millimeter
MPN	most probable number
MS	matrix spike
NaOH	sodium hydroxide
$Na_2S_2O_3$	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"

20.3 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.

Analytical batch—*[analytical batch to be defined]*.

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 fecal coliforms, particularly *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 and are indicators of fecal contamination.

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

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

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

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.

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 18.7).

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

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