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Method 1601: Male-specific (F^+) and Somatic Coliphage in Water by Two-step Enrichment Procedure

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The contributions of the following persons and organizations to the development of this method are gratefully acknowledged:

Sobsey, Mark, Ming Jing Wu, and Greg Lovelace, University of North Carolina, Department of Environmental Sciences and Engineering, CB#7400, McGavran-Greenberg Building, Chapel Hill, NC 27599

Hsu, Fu-Chih, and Jim Larkin, Environmental Health Laboratories, 110 South Hill Street, South Bend, IN 46617

Chambers, Yildiz, City of San Diego Marine Microbiology Laboratory, 5530 Kiowa Drive, La Mesa, CA 91942

Cliver, Dean, Tadesse Mariam, and Mulugeta Tamene, University of California Davis, Department of Health and Reproduction, School of Veterinary Medicine, Davis, CA 95616-8743

Danielson, Richard, BioVir Laboratory, 685 Stone Road Unit # 6, Benicia, CA 94510

Fujioka, Roger and Geeta Rijal, University of Hawaii, Water Resources Center, Holmes Hall 283, 2540 Dole Street, Honolulu, HI 96822

Karim, Mohammad and Dale Young, American Water Works System Research Laboratory, 1115 South Illinois Street, Belleville, IL 62220-3731

Margolin, Aaron and Nicola Ballester, University of New Hampshire, Department of Microbiology, Biological Sciences Building, Rudman Hall Room 285, Durham, NH 03824

Pillai, Suresh and Elisa Camacho, Texas A & M University, Department of Poultry Science, Kleberg Center Room 418D, College Station, TX 77843

Pope, Misty, Kevin Connell, Ken Miller, Jason Kempton, and Jessica Pulz, DynCorp Information and Enterprise Technologies, 6101 Stevenson Avenue, Alexandria, VA 22304

Williams, Fred and Ron Stetler U.S. Environmental Protection Agency, 26 West Martin Luther King Drive, Cincinnati, OH, 45268

Yates, Marylynn, Omid Bakhtar, and Andre Salazar, University of California Riverside, Department of Environmental Sciences, 2217 Geology, Riverside, CA 92521-0424

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Introduction

Coliphage presence in ground water is an indication of fecal contamination. Method 1601 (two-step enrichment procedure) is a performance-based method for detecting the presence of 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.

The two-step enrichment procedure requires enrichment of coliphage in a nutrient broth with host bacteria followed by spotting onto a lawn of host bacteria and assessing lysis zone formation in the lawn. This two-step enrichment method was validated as a qualitative, presence-absence method, and Method 1601 was written with this use in mind. Although the method potentially may be used as a quantitative assay of coliphage concentrations in an MPN format, the two-step enrichment method has not been validated this way.

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.

Questions concerning this method or its application should be addressed to:

William A. Telliard
U.S. EPA Office of Water
Analytical Methods Staff
1200 Pennsylvania NW
Mail Code 4303
Washington, DC 20460
Email: telliard.william@epa.gov

Requests for additional copies of this publication should be directed to:

Water Resource Center
Mail Code RC-4100
401 M Street, SW
Washington, D.C. 20460
(202) 260-7786 or (202) 260-2814

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1.0 Scope and Application

1.1 The two-step enrichment procedure determines the presence or absence of male-specific (F^+) and somatic coliphages in ground water and other waters. The two-step enrichment method was validated as a qualitative, presence-absence method, and Method 1601 was written with this use in mind. The two-step enrichment method potentially may be used as a quantitative assay of coliphage concentrations in an MPN format, however, the method has not been validated this way. 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 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.

1.4 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 1601 describes a qualitative (presence/absence) two-step enrichment procedure for coliphage. A 100-mL or 1-L ground water sample is supplemented with $MgCl_2$ (magnesium chloride), log-phase host bacteria (*E. coli* F_{amp} for male-specific coliphage and *E. coli* CN-13 for somatic coliphage), and tryptic soy broth (TSB) as an enrichment step for coliphage. After an overnight incubation, samples are "spotted" onto a lawn of host bacteria specific for each type of coliphage, incubated, and examined for circular lysis zones, which indicate the presence of coliphages.

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 enrichment phase of the two-step enrichment procedure, other bacteria in the water sample can grow and interfere with the spot-test confirmation step. Bacteria may grow over the lysis zone and obscure visualization, resulting in a false negative. Generally, when bacteria have overgrown lysis zones, they appear as raised colonies or a confluent growth of raised colonies. As a result, they are distinguishable from the surrounding lawn of host bacteria. If this problem is noted, follow the procedure described in Section 12.2.11.

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 1601 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 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 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 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, 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, Gibson, 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 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, 125-mL, 1-L, or 1.5-L 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

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 (Section 7.4.3)

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 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 ($Na_2S_2O_3$) 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 $MgCl_2 \cdot 6H_2O$, 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.

7.1.4 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 ± 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 ± 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 ± 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 ± 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.*

7.3.1.4 10X Tryptic soy broth (TSB)—Add 300 g TSB per liter of reagent water and heat to dissolve. 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.

7.3.2 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 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 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 **Spot plates**—To be used during the two step enrichment procedure (Section 12). ***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 reduce condensation prior to inoculation.*

7.3.4.1 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.

7.3.4.2 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 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 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°C ± 1°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°C ± 1°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 two-step enrichment procedure (Section 12) will require a 0.5-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

8.2.1 **Two-step enrichment procedure using 100-mL samples:** Collect 250 mL of sample for each of the two coliphage types to allow for sample re-analysis, if necessary.

8.2.2 **Two-step enrichment procedure using 1-L samples:** Collect 2.5 L 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 Two-step enrichment 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 waters. If the sample has been chlorinated, add 0.5-mL of 10% sodium thiosulfate per 1-L of sample at time of sample collection.

9.0 Quality Control

9.1 Each laboratory that uses Method 1601 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 demonstration of capability (IDC) test (Section 9.3), ongoing analysis of spiked reagent water and field samples to evaluate and document data quality, and analysis of positive controls and method blanks as tests of continued acceptable performance. Spiked sample results are compared to acceptance criteria based on data generated during the interlaboratory validation of Method 1601 involving 10 laboratories and 10 raw ground water matrices. Specific quality control (QC) requirements for Method 1601 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—Positive control samples may be spiked with enumerated sewage filtrate or pure cultures of MS2 (male-specific) or phi-X 174 (somatic) coliphage. All other spiked QC samples must be spiked with enumerated sewage filtrate or equivalent.

9.2.1 Initial demonstration of capability (IDC). The laboratory shall demonstrate the ability to generate acceptable performance with this method by performing an IDC test before analyzing any field samples. The procedure for performing the IDC is described in Section 9.3. IDC tests must be accompanied by a method blank (Section 9.2.2) for each coliphage type.

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. At a minimum, the laboratory shall analyze one method blank per spot plate (Section 13). In an effort to determine if cross-contamination is an issue, the method blank should be spotted onto the lawn of host bacteria immediately following the positive control spot.

9.2.3 Positive controls. The laboratory shall analyze positive controls to ensure that stock coliphage suspensions, host bacterial cultures, and growth media are performing properly (Section 9.5). The laboratory shall inoculate one positive control spot per spot plate (Section 13). If multiple spot plates are inoculated with samples on the same day, a single enriched positive control sample may be used to inoculate multiple spot plates on that day.

9.2.4 Matrix spikes (MS). The laboratory shall analyze one set of MS samples for each coliphage type when samples are first received from a ground water source for which the laboratory has never before analyzed samples (Section 9.6). In addition, the laboratory shall analyze one set of MS samples on an ongoing basis after every 20th field sample for each ground water source. For example, when a laboratory receives the first sample from a source, the laboratory must obtain additional aliquots of the field samples to be used for the MS test. When the laboratory receives the 20th field sample from this site, additional aliquots of this sample must be collected and spiked. MS samples should be collected at the same time as routine field samples.

9.2.5 Ongoing demonstration of capability (ODC). The laboratory shall demonstrate that the analytical system is in control on an ongoing basis through analysis of ODC samples (Section 9.7). The laboratory shall analyze one set of ODC samples after every 20 field and MS samples or one per week, whichever occurs more frequently.

9.2.6 Method modification validation/equivalency demonstration requirements. Method 1601 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 9.2.6 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).

9.2.6.1 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 4 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 IDC test (Section 9.3). IDC results must meet the QC acceptance criteria in Table 1 in Section 9.3, and should be comparable to previous results using the unmodified procedure. Although not required, the laboratory also should perform an Expanded MS test (Section 9.8) to demonstrate the performance of the modified method in at least one real-world matrix before analyzing field samples using the modified method.

9.2.6.2 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 4 and Reference 17.6). Briefly, at least three laboratories must perform IDC tests (Section 9.3) and Expanded MS tests (Section 9.8) using the modified method, and all tests must meet the QC acceptance criteria specified in Tables 1 and 3 in Sections 9.3 and 9.8. 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.

9.2.7 Media sterility checks. The laboratory shall test media sterility by incubating one unit (tube or plate) of each batch of medium at 36°C ± 1.0°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.

9.2.8 Record maintenance. The laboratory shall maintain records to define the quality of data that are generated. 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, IDC, ODC, and MS sample results must be maintained. In addition, a log book containing reagent and material lot numbers should be maintained along with samples analyzed using each of the lots.

9.2.9 Performance studies. The laboratory should periodically analyze external QC samples, such as performance evaluation (PE) samples, when available. The laboratory should also 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.

9.2.10 The specifications contained in this method can be met if the analytical system is maintained under control.

9.3 Initial demonstration of capability (IDC)—The IDC 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. IDC test samples must be spiked with enumerated sewage filtrate or equivalent. IDC tests must be accompanied by analysis of a method blank (Section 9.4).

9.3.1 A total of 10 spiked reagent water samples per coliphage type (male-specific and somatic) and

sample volume are required for the IDC test. 100-mL reagent water samples should be used if the laboratory will analyze 100-mL field samples by this procedure, while 1-L reagent water samples should be used if the laboratory will analyze 1-L field samples.

9.3.2 IDC samples must be spiked in "bulk" for each coliphage type at concentrations as specified in Table 1. For example, for 1-L somatic IDC samples, spike 10-L of reagent water with 14 PFU to achieve the target spike concentration of 1.4 PFU per sample. Please refer to Section 11 for the coliphage spiking suspension enumeration procedure and Section 13.2 for spike volume calculations.

Table 1. Initial demonstration of laboratory capability (IDC)

Coliphage type	Sample size	Target spike concentration (PFU per sample)	"Bulk" volume to be spiked	Bulk spike concentration (PFU per bulk volume)	Minimum number of positive samples out of 10
F^+	100-mL	1.3	1000 mL	13	5
F^+	1-L	1.2	10 L	12	3
Somatic	100-mL	1.5	1000 mL	15	5
Somatic	1-L	1.4	10 L	14	5

9.3.3 Mix the spiked reagent water by swirling the container. For each coliphage type and volume, dispense 10 aliquots into individual containers.

9.3.4 Analyze the spiked IDC samples according to the two-step enrichment procedure (Section 12). See Table 1 for the minimum number of samples that must be positive for each set of 10 IDC samples. If system performance is unacceptable, identify and correct the problem and repeat the IDC 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 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 Positive controls—performed at the frequency specified in Section 9.2.3.

9.5.1 Positive controls for 100-mL samples—Add 100 mL of reagent water to each of two 250 to 500-mL sterile Erlenmeyer flasks. If spiking with sewage filtrate, spike each flask with one coliphage type (somatic or male-specific) at a concentration of approximately 20 PFU / 100 mL. If spiking with MS2 (male-specific) or phi-X 174 (somatic), spike each flask with one coliphage type at a concentration of approximately 60 PFU / 100 mL. See Section 11 for enumeration of stock/filtrate and Section 13.2 for spike volume calculations. Label each flask with the coliphage type and "+" or "positive." Analyze positive control sample by the two-step enrichment procedure as described in Section 12.

9.5.2 Positive controls for 1-L samples—Add 1 L of sterile reagent water to each of two sterile, 1-L bottles. If spiking with sewage filtrate, spike each flask with one coliphage type (somatic or male-specific) at a concentration of approximately 20 PFU / L. If spiking with MS2 (male-specific) or phi-X 174 (somatic), spike each flask with one coliphage type at a concentration of approximately 60 PFU / L. See Section 11 for enumeration of stock/filtrate and Section 13.2 for spike volume calculations. Label each flask with the coliphage type and "+" or "positive." Analyze positive control sample by the two-step enrichment procedure, as described in Section 12.

9.6 Matrix spike (MS) analyses for ongoing assessment of method performance—The laboratory shall analyze MS samples according to the frequency in Section 9.2.4. The laboratory shall spike and analyze field samples from each ground water source to assess method performance in each matrix. For each coliphage type and sample volume, at a minimum, one out of three MS samples must be positive. 100-mL MS samples must be analyzed if 100-mL field samples are being analyzed by this procedure, while 1-L MS samples must be analyzed if 1-L field samples are being analyzed by this procedure. MS test samples must be spiked with enumerated sewage filtrate or equivalent.

9.6.1 The ongoing MS sample must be collected and analyzed at the same time as the unspiked field sample from the same source.

9.6.2 MS samples must be spiked in "bulk" for each coliphage type and sample volume. Spike the bulk samples at concentrations according to Table 2. For example, for 1-L somatic MS samples, spike 3-L of ground water with 4.2 PFU of somatic coliphage to achieve a target spike concentration of 1.4 PFU per sample, based on stock coliphage enumeration. See Section 11 for enumeration of coliphage stock and Section 13.2 for spiking volume calculations.

9.6.3 Analyze MS samples according to the two-step enrichment procedure (Section 12).

9.6.4 One or more MS samples out of three must be positive for method performance to be considered acceptable for that ground water source. If the matrix spike results are unacceptable and the ODC sample and positive control sample results associated with this batch of samples are acceptable, a matrix interference may be causing the poor results.

9.7 Ongoing demonstration of capability (ODC)—Performed at the frequency specified in Section 9.2.5. To demonstrate that the laboratory is in control for each coliphage type, at a minimum, one out of three reagent water spiked samples must be positive. 100-mL reagent water samples should be used if 100-mL field samples are being analyzed by this procedure, while 1-L reagent water samples should be used if 1-L field samples are being analyzed by this procedure. ODC test samples must be spiked with enumerated sewage filtrate or equivalent.

9.7.1 ODC samples must be spiked in "bulk" for each coliphage type. Spike the bulk samples at concentrations according to Table 2. For example, for 1-L somatic ODC samples, spike 3-L of reagent water with 4.2 PFU of somatic coliphage to achieve a target spike concentration of 1.4 PFU per sample, based on stock coliphage enumeration. See Section 11 for enumeration of coliphage stock and Section 13.2 for spiking volume calculations.

9.7.2 Mix the spiked reagent water by swirling the container and dispense three aliquots into individual containers.

9.7.3 Analyze the spiked ODC samples according to Section 12. One or more out of three samples must be positive for each coliphage type and sample volume being evaluated. If not, method performance is unacceptable. Identify and correct the problem and perform another ODC test before continuing with the analysis of field samples.

Table 2. MS and ODC sample spiking requirements for ongoing evaluation of method performance

Coliphage type	Sample size	Target spike concentration (PFU per sample)	Number of samples that must be spiked (≥1 must be positive)	"Bulk" volume to be spiked	Bulk spike concentration (PFU per bulk volume)
F^+	100-mL	1.3	3	300-mL	3.9
F^+	1-L	1.2	3	3-L	3.6
Somatic	100-mL	1.5	3	300-mL	4.5
Somatic	1-L	1.4	3	3-L	4.2

9.8 **Expanded MS test**— If IDC and Expanded MS test performance is equal to or better than the criteria set forth in Tables 1 and 3, then the modified version of the method is acceptable. Expanded MS analyses for evaluation of modified method performance are as follows. ***Please note:*** *the expanded MS test must be accompanied by an unspiked matrix sample. If the unspiked matrix sample tests positive, the Expanded MS test must be re-run.*

9.8.1 A total of 10 spiked field samples per coliphage type (male-specific and somatic) and volume are required. 100-mL matrix spike samples should be used if 100-mL field samples are being analyzed by this procedure, while 1-L matrix spike samples should be used if 1-L field samples are being analyzed by this procedure.

9.8.2 Samples must be spiked in "bulk" for each coliphage type. Spike the bulk samples at concentrations according to Table 3. For example, for 1-L somatic IDC samples, spike 10-L of ground water with a target spike dose of 14 PFU of somatic coliphage. See Section 11 for enumeration of coliphage stock and Section 13.2 for spiking volume calculations.

Table 3. Expanded MS test requirements for evaluation of method modification performance

Coliphage type	Sample size	Target spike concentration (PFU per sample)	"Bulk" volume to be spiked	Target bulk Spike (PFU per bulk volume)	Minimum number of positive samples out of 10
F^+	100-mL	1.3	1000-mL	13	4
F^+	1-L	1.2	10-L	12	2
Somatic	100-mL	1.5	1000-mL	15	5
Somatic	1-L	1.4	10-L	14	5

9.8.3 Mix the spiked ground water by swirling the container. For each coliphage type and volume dispense 10 aliquots into individual containers.

9.8.4 Analyze the spiked ground water samples according to the two-step enrichment procedure (Section 12). Table 3 specifies the minimum number of samples that must be positive for each set of 10 Expanded MS samples.

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 (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 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 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 two-step enrichment IDC, ODC, MS, and positive control samples as described in Section 9.

12.0 Two-step Enrichment Procedure for Sample Analysis

12.1 Enrichment procedure—*Instead of adding antibiotics to each individual sample as described in Sections 12.1.1.8, 12.1.1.9, 12.1.2.8, and 12.1.2.9 laboratories may add antibiotics directly to the 10X TSB, AFTER the 10X TSB has been autoclaved and cooled to 48°C ± 1.0°C. Regardless of which technique is used, the laboratory's QC samples must be prepared and analyzed the same way that field samples are analyzed.*

12.1.1 100-mL samples (Section 18, Flow chart 4)

12.1.1.1 Dispense a 100-mL sample aliquot into each of two sterile, 100-mL or 125-mL bottles. If 100-mL bottles are used, be sure that there is ample head space for the addition of reagents.

12.1.1.2 Label bottle with the following information

- Sample number
- Bacterial host (*E. coli* CN-13 or *E. coli* F_{amp})
- Date
- Start time
- Sample volume

12.1.1.3 Record this information on a report form.

12.1.1.4 Add 1.25 mL of stock magnesium chloride (Section 7.1.3) to each ground water sample aliquot.

12.1.1.5 Add 0.5 mL of log-phase *E. coli* CN-13 (Section 7.5.4) to one sample aliquot for each sample.

12.1.1.6 Add 0.5 mL of log-phase *E. coli* F_{amp} (Section 7.5.4) to the other sample aliquot for each sample.

12.1.1.7 Add 5 mL of 10X TSB (Section 7.3.1.4) to all aliquots. This gives a final concentration of 0.5X TSB.

12.1.1.8 To those bottles with *E. coli* CN-13 as host, add 1 mL of stock nalidixic acid (Section 7.2.1).

12.1.1.9 To those bottles with *E. coli* F_{amp} as host, add 1 mL of stock ampicillin/streptomycin solution (Section 7.2.2).

12.1.1.10 Cap and invert each bottle five times to mix.

12.1.1.11 Prepare method blanks and positive controls as specified in Sections 9.4 and 9.5.

12.1.1.12 Incubate the bottles for 16 to 24 hours at 36°C ± 1.0°C with no further mixing. Proceed to Section 12.2.

12.1.2 1-L samples (Section 18, Flow chart 5)

12.1.2.1 Dispense a 1-L sample aliquot into each of two sterile, 1-L or 1.5-L bottles. If 1-L bottles are used, be sure that there is ample head space for the addition of reagents.

12.1.2.2 Label bottle with the following information

- Sample number
- Bacterial host (*E. coli* CN-13 or *E. coli* F_{amp})
- Date
- Time
- Sample volume

12.1.2.3 Record this information on a report form.

12.1.2.4 Add 12.5 mL of stock magnesium chloride (Section 7.1.3) to each ground water sample aliquot.

12.1.2.5 Add 5 mL of log-phase *E. coli* CN-13 (Section 7.5.4) to one sample aliquot for each sample.

12.1.2.6 Add 5 mL of log-phase *E. coli* F_{amp} (Section 7.5.4) to the other sample aliquot for each sample.

12.1.2.7 Add 50 mL of 10X TSB (Section 7.3.1.4) to all aliquots. This gives a final concentration of 0.5X TSB.

12.1.2.8 To those bottles with *E. coli* CN-13 as host, add 10 mL of stock nalidixic acid (Section 7.2.1).

12.1.2.9 To those bottles with *E. coli* F_{amp} as host, add 10 mL of stock ampicillin/streptomycin solution (Section 7.2.2).

12.1.2.10 Cap and invert each bottle 5 times to mix.

12.1.2.11 Prepare method blanks and positive controls as specified in Sections 9.4 and 9.5.

12.1.2.12 Incubate the bottles for 16 to 24 hours at 36°C ± 1.0°C with no further mixing. Proceed to Section 12.2.

12.2 Spot-plate procedure (Section 18, Flow charts 4 and 5)

12.2.1 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.

12.2.2 After the overnight enrichment of 16 to 24 hours, mix the sample by inverting the bottle 25 times or more.

Please note: *If the positive control or method blank exhibits an inappropriate response or if there is a problem with overlapping spots, it will be necessary to re-spot all associated samples. As a result, the enriched samples may be stored at 4°C ± 1°C for up to 48 hours.*

12.2.3 Spot 10 µL of enriched sample from the bottle containing *E. coli* CN-13 host bacteria onto the gridded spot plate that was prepared using *E. coli* CN-13. Record spot time on the report form.

12.2.4 Record the grid number and corresponding sample number into a log book.

12.2.5 Spot 10 µL of enriched sample from the bottle containing *E. coli* F_{amp} host bacteria onto the gridded spot plate that was prepared using *E. coli* F_{amp}. If the analyst is extremely careful, one 100-mm plate can be inoculated with up to 15 spots.

12.2.6 Record the grid number and corresponding sample number on the report form.

12.2.7 Spot the method blank and positive control samples as specified in Section 9.4 and 9.5.

12.2.8 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.

12.2.9 After inocula absorption, cover, invert, and incubate the plate at 36°C ± 1.0°C for 16 to 24 hours.

12.2.10 Lysis zone formation (typically a circular zone of clearing) indicates a sample is positive 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 result 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.*

12.2.11 It is possible that the circular spot area contains confluent bacterial growth or a very large number of bacterial colonies that are distinct from the background lawn of host bacteria. This result could make it difficult or impossible to determine if lysis of host bacteria has occurred. In this case, the bacteria must be removed from the enrichment culture material before re-spotting onto a new pre-poured lawn of host bacteria in a spot plate. The interfering bacteria are removed from the enrichment material by filtration or centrifugation.

Please note: *A preliminary examination of spot plates for lysis zones may be completed after 6 hours of incubation to determine if there will be bacterial interferences. Alternatively, the filtration or centrifugation step (Section 12.2.11.1 or Section 12.2.11.2) may be performed before the initial spot.*

12.2.11.1 To remove interfering bacteria by filtration, push a 0.5 to 1.0 mL volume of the enrichment sample through a 25-mm-diameter, 0.45-µm pore-size, sterile, low-protein binding filter (Section 6.3.1) using a 1 to 3 mL syringe, and collect the filtrate in a sterile microcentrifuge tube.

12.2.11.2 To remove interfering bacteria by centrifugation, pipet a 0.5 to 1-mL volume of the enrichment sample into a 1.8-mL capacity, sterile microcentrifuge tube. Centrifuge at 5,000 to 10,000 X G for 10 minutes. Recover most of the supernatant by aspirating with a micropipet. Place the recovered supernatant in a 1.5-mL capacity, sterile microcentrifuge tube.

12.2.11.3 Spot 10-µL volumes of filtered or centrifuged enrichment onto pre-poured lawns of host bacteria as described in Section 12.2.

12.3 Spot plate coliphage confirmation procedure—Although not required, laboratories may use the spot plate procedure for confirmation of lysis zones if one or more such zones on a spot plate are questionable.

12.3.1 Pick lysis zone with a sterile Pasteur (or other) pipette and transfer it to a tube with 0.5 mL TSB (Section 7.3.1.1).

12.3.2 Allow the inoculated broth to stand 5 minutes at room temperature.

12.3.3 Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.

12.3.4 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.

12.3.5 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.

12.3.6 Spot the method blank and positive control samples as specified in Section 9.4 and 9.5.

12.3.7 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.

12.3.8 After inocula absorption, cover, invert, and incubate the plate at 36°C ± 1.0°C for 16 to 24 hours.

12.3.9 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:

$$\textbf{Undiluted spiking suspension PFU / mL} = (PFU_1 + PFU_2 \ldots PFU_n)/(V_1 + V_2 \ldots V_n)$$

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

Example DAL data

Dilution	PFU / plate (for each duplicate plate)	Volume of *undiluted* 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 IDC, ODC, 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 1-L, somatic IDC samples (Section 9.3.2):
T) A spike dose of 1.4 PFU is needed per 1-L sample
B) A total of ten, 1-L samples will be spiked at the same time
C) The 1 : 100 dilution contains 6.82 PFU / mL

The equation would be solved as follows:

$$S = \frac{(1.4\ \text{PFU})(10)}{(6.82\ \text{PFU / mL})} = 2.05\ \text{mL}$$

As a result, approximately 2.1 mL of the 1 : 100 dilution would be spiked into the 10-L bulk sample. The 10-L mL bulk sample would be mixed and ten, 1-L aliquots dispensed. Each 1-L sample should contain approximately 1.4 PFU.

14.0 Method Performance

14.1 The QC acceptance criteria listed in Tables 1, 2 and 3, are based on data generated through the interlaboratory validation of Method 1601 involving 10 laboratories and 10 raw ground water matrices. Detailed Method QC procedures applicable to these criteria are discussed in Section 9.

Table 1. Initial demonstration of laboratory capability (IDC)

Coliphage type	Sample size	Target spike concentration (PFU per sample)	"Bulk" volume to be spiked	Bulk spike concentration (PFU per bulk volume)	Minimum number of positive samples out of 10
F^+	100-mL	1.3	1000 mL	13	5
F^+	1-L	1.2	10 L	12	3
Somatic	100-mL	1.5	1000 mL	15	5
Somatic	1-L	1.4	10 L	14	5

Table 2. MS and ODC sample spiking requirements for ongoing evaluation of method performance

Coliphage type	Sample size	Target spike concentration (PFU per sample)	Number of samples that must be spiked (≥1 must be positive)	"Bulk" volume to be spiked	Bulk spike concentration (PFU per bulk volume)
F^+	100-mL	1.3	3	300-mL	3.9
F^+	1-L	1.2	3	3-L	3.6
Somatic	100-mL	1.5	3	300-mL	4.5
Somatic	1-L	1.4	3	3-L	4.2

Table 3. Expanded MS test requirements for evaluation of method modification performance

Coliphage type	Sample size	Target spike concentration (PFU per sample)	"Bulk" volume to be spiked	Target bulk Spike (PFU per bulk volume)	Minimum number of positive samples out of 10
F^+	100-mL	1.3	1000-mL	13	4
F^+	1-L	1.2	10-L	12	2
Somatic	100-mL	1.5	1000-mL	15	5
Somatic	1-L	1.4	10-L	14	5

14.2 Method 1601 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 9.2.6 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 4 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 4 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 4. Tier 1 and Tier 2 Validation/Equivalency Demonstration Requirements

Test	Description	Tier 1 modification(1)	Tier 2 modification(2)
IDC (Section 9.3)	10 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
Expanded MS (Section 9.8)	10 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.

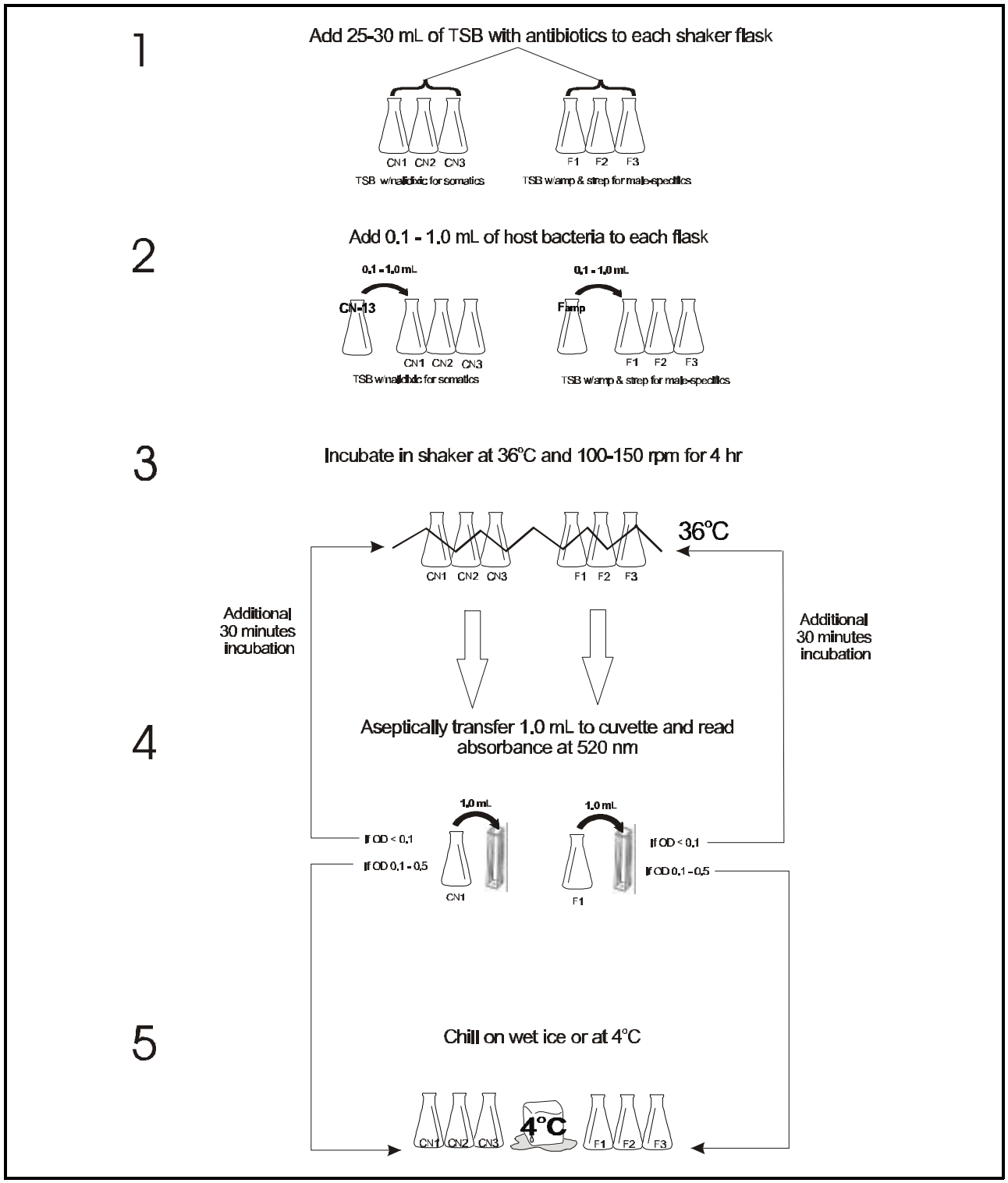
17.5 *Annual Book of ASTM Standards.* Vol. 11.01. American Society for Testing and Materials. Philadelphia, PA 19103.

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).

18.0 Flowcharts

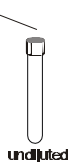
We greatly appreciate Fred Williams (USEPA, Cincinnati, OH) for providing the original flow charts on which all of the following flowcharts are based.

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

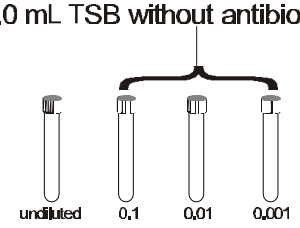


Flow chart 2. Dilution of coliphage QC spiking suspensions (Section 11.2)

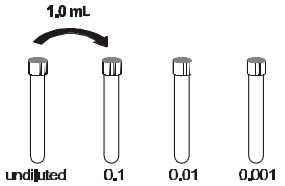
1 Add 10 mL of undiluted sewage filtrate



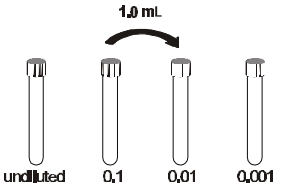
2 Add 9.0 mL TSB without antibiotics to each dilution tube



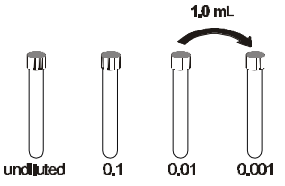
3 Vortex undiluted tube 5 seconds. Transfer 1.0 mL from undiluted tube to 0.1 tube.



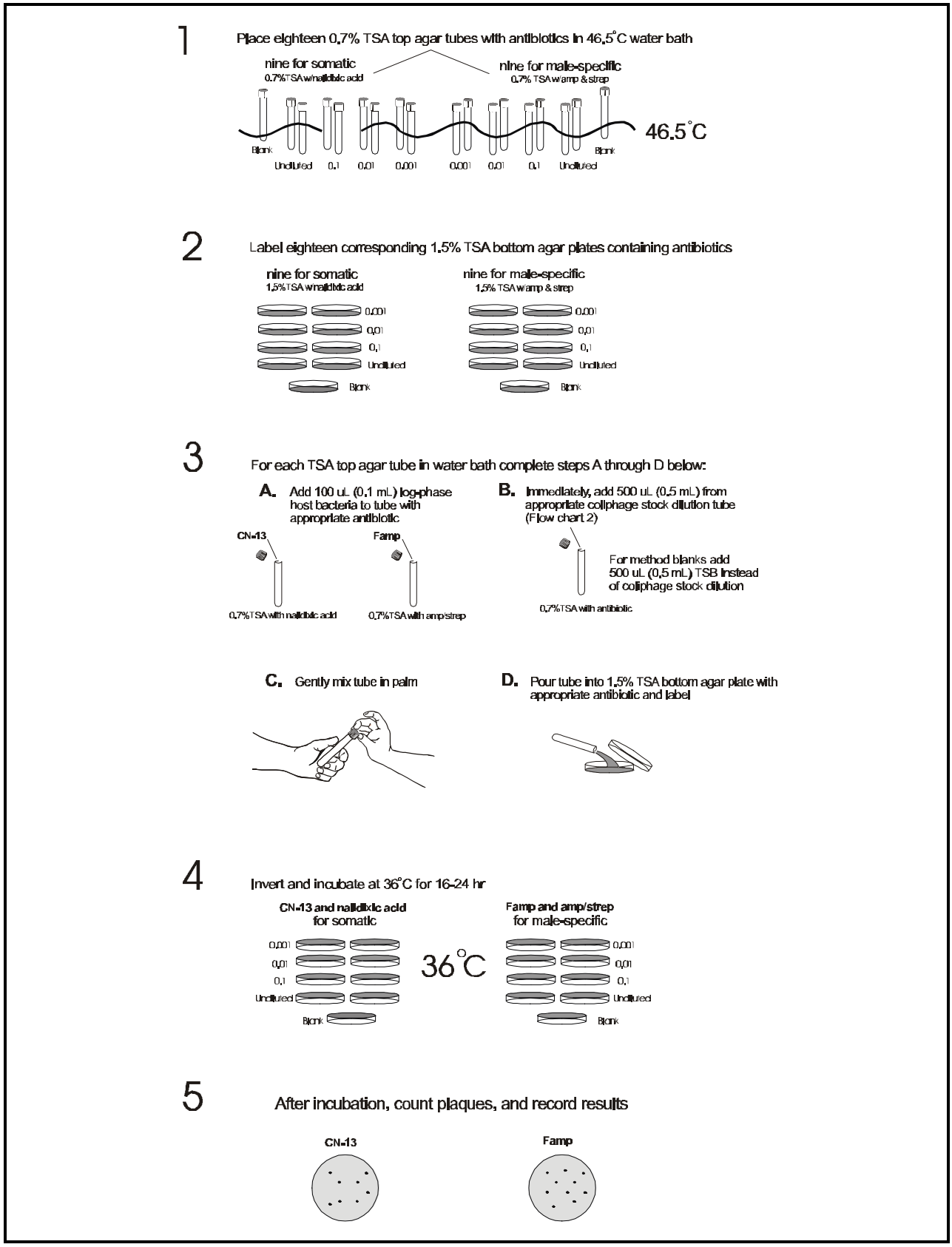
4 Vortex 0.1 tube 5 seconds. Transfer 1.0 mL from 0.1 tube to 0.01 tube.



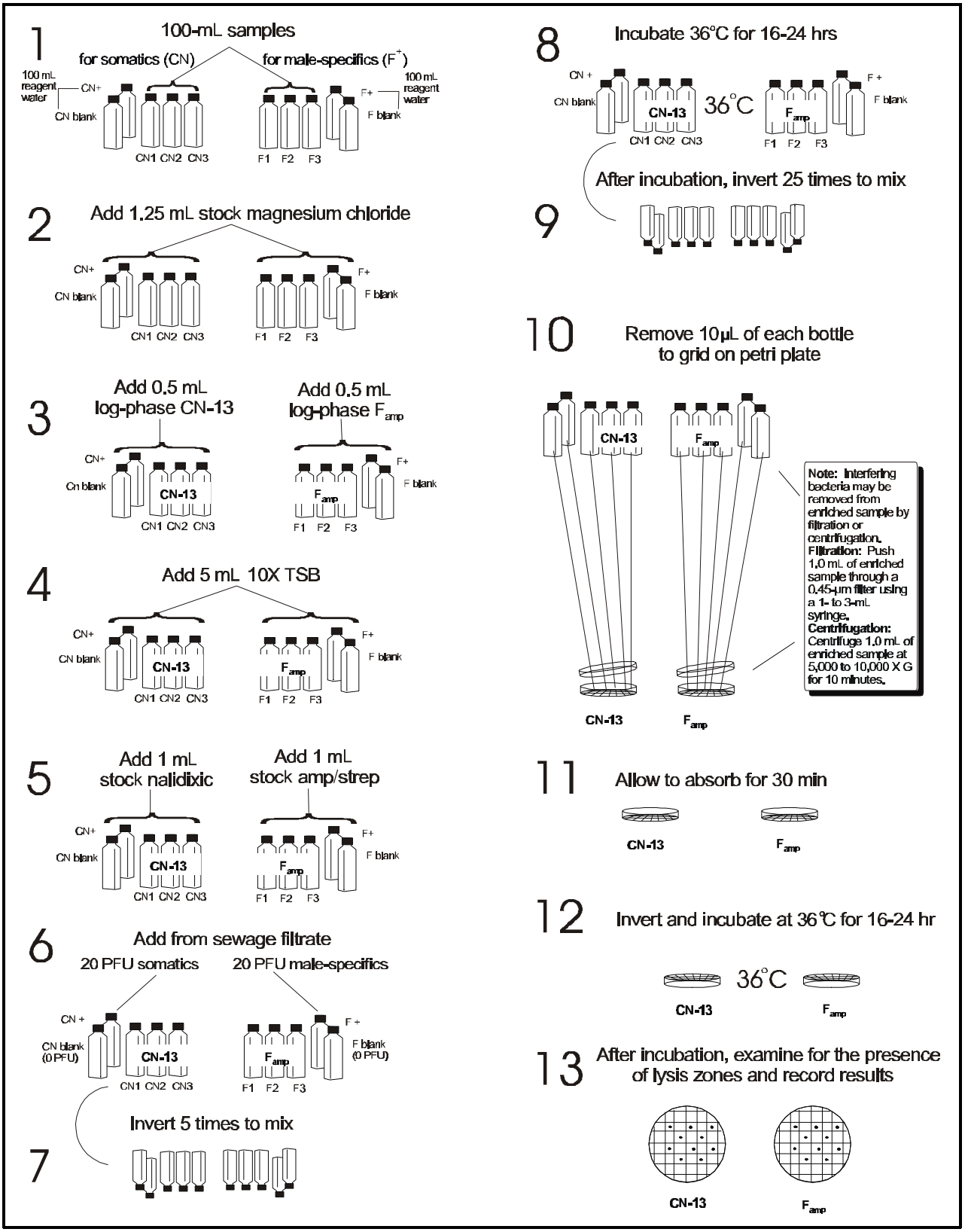
5 Vortex 0.01 tube 5 seconds. Transfer 1.0 mL from 0.01 tube to 0.001 tube.



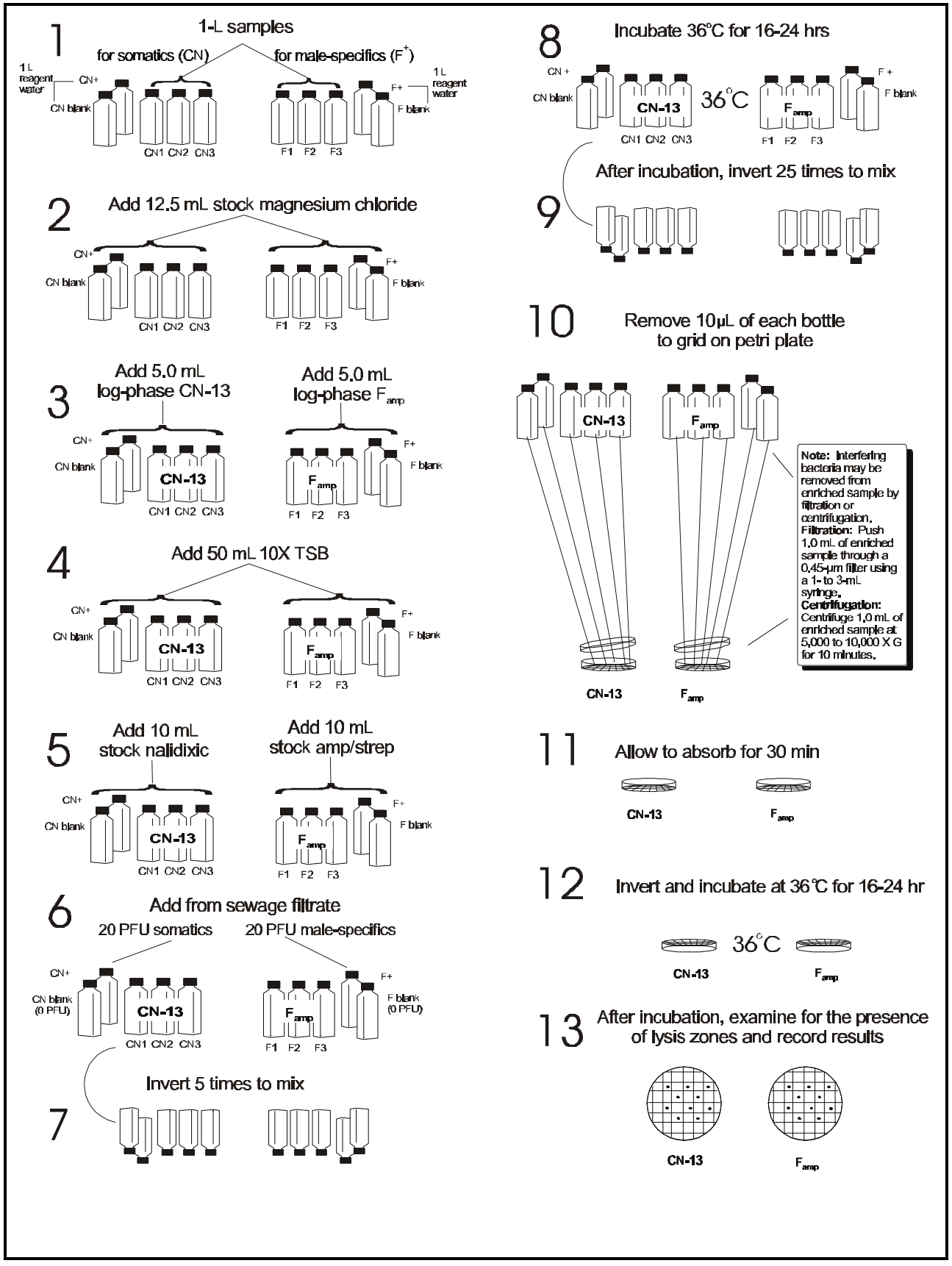
Flow chart 3. Coliphage spiking suspension enumeration by double agar layer (DAL) procedure (Section 11.3)



Flow chart 4. Two-step enrichment procedure for 100-mL samples (Sections 12.1.1 and 12.2)



Flow chart 5. Two-step enrichment procedure for 1-L samples (Sections 12.1.2 and 12.2)



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
IDC	initial demonstration of capability
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
ODC	ongoing demonstration of capability
OSHA	Occupational Safety and Health Administration
psi	pounds per square inch
QA	quality assurance
QC	quality control
rpm	revolutions per minute
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*.

Enrichment—In this method, enrichment is meant as the increase in number of bacteriophage through the addition to the growth medium of host bacteria allowing coliphage replication.

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 demonstration of capability (IDC)—The IDC test is performed to establish the ability to demonstrate control over the analytical system and to demonstrate acceptable performance.

Lysis zone—In this method, typically a circular zone of clearing indicating a sample is positive for coliphages.

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 demonstration of capability (ODC)—Reagent water samples spiked with known quantities of analytes and analyzed exactly like a field sample. The purpose of this test is to assure that the results produced by the laboratory remain within limits specified in this method.

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

Presence-absence—A qualitative method for detection of a microorganism where the result indicates whether or not the microorganism is present in the sample. The presence-absence method will not give the numbers of virus present.

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.