

Method 1643: Male-specific (F+) and Somatic Coliphage in Secondary (No Disinfection) Wastewater by the Single Agar Layer (SAL) Procedure

Acknowledgments

This method was prepared under the direction of Robin K. Oshiro and Lemuel Walker of the Engineering and Analysis Division within the U.S. Environmental Protection Agency's (EPA) Office of Water.

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Volunteer Analytical Laboratories:

- County Sanitation Districts of L.A. County – JWPCP: Kathy Walker, Michele Padilla, Jason Gregory, and Lavern Gullledge
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Section 11.4.13 Figure 1: Somatic Coliphage Plaques (CN-13) (Source: Jeremy Olstadt, Wisconsin State Laboratory of Hygiene) and Figure 2: Male-specific Coliphage Plaques (F_{amp}) (Source: Richard Danielson, IEH BioVir)

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

Lemuel Walker
Engineering and Analysis Division (4303T)
U.S. EPA Office of Water, Office of Science and Technology
1200 Pennsylvania Avenue, NW
Washington, DC 20460
walker.lemuel@epa.gov or OSTCWAMethods@epa.gov

Introduction

This EPA method was developed to address stakeholder needs for a validated coliphage (bacterial virus) method for monitoring secondary (no disinfection) wastewater matrices under the Clean Water Act. Method 1643 reflects the results of a multi-laboratory study of EPA Method 1602 for 100 mL secondary (no disinfection) wastewater samples for somatic and male-specific coliphage.

The results of the MLV study enabled performance characterization of Method 1643 in the reference matrix (phosphate buffered saline [PBS]) and secondary (no disinfection) wastewater samples. Results also enabled the development of initial precision and recovery/ongoing precision and recovery (IPR/OPR) and matrix spike (MS) quality control (QC) acceptance criteria.

The highly variable levels of coliphage (both male-specific and somatic) in secondary (no disinfection) wastewater effluents should be taken into consideration when implementing this method. During the multi-laboratory study, most of the secondary (no disinfection) wastewater samples had high levels of both somatic and male-specific coliphage. It is recommended that range-finding analyses be conducted for each new wastewater matrix to determine appropriate sample volumes.

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1.0 Scope and Application

- 1.1 Method 1643 assays for both male-specific (F⁺) and somatic coliphage using the single agar layer (SAL) procedure. The SAL procedure detects and allows for enumeration of male-specific and somatic coliphage (also referred to as phage in the method) in secondary (no disinfection) wastewater samples.
- 1.2 This method is based on EPA *Method 1602: Male-specific (F⁺) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure* (Reference 17.1), developed for the enumeration of coliphage in ground water.
- 1.3 Each laboratory and analyst that uses this method must demonstrate the ability to generate acceptable results using the procedures in Sections 8 and 11 prior to analyzing field samples.
- 1.4 The highly variable levels of coliphage (both male-specific and somatic) in secondary (no disinfection) wastewater samples should be taken into consideration when determining the volume of sample that may be needed for analyses. Range-finding analyses should be conducted to determine appropriate sample volumes for each source from which the laboratory has not previously analyzed samples.

2.0 Summary of Method

Method 1643 describes the SAL procedure. A 100 mL (undiluted or diluted [e.g., 1:10]) secondary (no disinfection) wastewater sample is assayed by adding magnesium chloride, (MgCl₂), appropriate antibiotic, log-phase host bacteria (*Escherichia coli* [*E. coli*] F_{amp} for male-specific (F⁺) coliphage and *E. coli* CN-13 for somatic coliphage), and 100 mL of molten double-strength tryptic soy broth (TSB) with 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 overnight incubation, plaques (circular lysis zones) 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 (QC) purposes, both a coliphage-positive phosphate buffered saline (PBS) sample (ongoing precision and recovery [OPR]) and an unspiked PBS (method blank) sample are analyzed for each type of coliphage with each sample batch.

3.0 Acronyms, Abbreviations and Definitions

3.1 Acronyms and Abbreviations

ASTM	American Society for Testing and Materials
°C	Degrees Celsius
DAL	Double agar layer
DNA	Deoxyribonucleic acid
EPA	U. S. Environmental Protection Agency
F ⁺	Male-specific coliphage
g	Gram
lb	Pound
IPR	Initial precision and recovery
K ₂ HPO ₄	Dipotassium phosphate
L	Liter
M	Molar
MgCl ₂	Magnesium chloride
MgCl ₂ •6H ₂ O	Magnesium chloride hexahydrate
mL	Milliliter
MLV	Multi-laboratory validation
mm	Millimeter
MS	Matrix spike
MS2	F ⁺ RNA group I coliphage
MSDS	Material Safety Data Sheet
Na ₂ HPO ₄	Disodium phosphate
NaH ₂ PO ₄	Monosodium phosphate
NIST	National Institute of Standards and Technology
nm	Nanometer
OD	Optical density
OPR	Ongoing precision and recovery
PBS	Phosphate buffered saline
PFU	Plaque forming unit
psi	Pounds per square inch
QA	Quality assurance
QC	Quality control
RNA	Ribonucleic acid
RSD	Relative standard deviation
rpm	Revolutions per minute
SAL	Single agar layer
strep/amp	Streptomycin/ampicillin
TNTC	Too numerous to count
TSB	Tryptic soy broth
µL	Microliter
µm	Micrometer
X	times

3.2 Definitions

- 3.2.1 Coliphages are a group of 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.
- 3.2.2 F-factor is the fertility factor in certain strains of *E. coli*. It is a plasmid that, when present, codes for the formation of a pilus termed the F-pilus. This F-pilus allows for transfer of nucleic acid from one bacterium to another.
- 3.2.3 Male-specific coliphages (F⁺) are ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) viruses that infect via the F-pilus of male strains of *E. coli*.
- 3.2.4 MS2 is a strain of F⁺ RNA (group I) coliphage.
- 3.2.5 Somatic coliphages are DNA viruses that infect host cells via the outer cell membrane.

4.0 Interferences

High background levels of microorganisms may prevent the host bacteria from producing a confluent lawn of growth. In addition, high background (ambient) phage levels could result in plates that are too numerous to count (TNTC).

5.0 Safety

- 5.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology and/or molecular biology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment. The laboratory must follow all regulations regarding proper disposal of contaminated materials.
- 5.2 This method does not address all of the safety issues associated with its use. It is the responsibility of the laboratory to establish appropriate safety and health practices prior to use of this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in Method 1643 analyses.
- 5.3 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

6.1 Equipment

- 6.1.1 Autoclave, capable of achieving and maintaining 121°C (15 lb pressure per square inch [psi]) for a minimum of 15 minutes
- 6.1.2 Balance, capable of accuracy to 0.01 g
- 6.1.3 Refrigerator, 4°C
- 6.1.4 Freezer, -20°C or -80°C
- 6.1.5 Incubator, capable of maintaining 36°C ± 1.0°C
- 6.1.6 Shaker incubator, capable of 36°C ± 1.0°C and 100 to 150 revolutions per minute (rpm), Thermo Scientific MaxQ™ 6000, or equivalent, or a shaker, Labnet Orbit™ 1900, or equivalent
- 6.1.7 Water bath, capable of maintaining 36°C ± 1.0°C and 45°C – 46°C
- 6.1.8 Visible wavelength spectrophotometer, capable of measuring at 595 nm
- 6.1.9 Stir plate, Fisher 14-493-120SQ, or equivalent
- 6.1.10 pH meter
- 6.1.11 Vortex mixer, Vortex Genie, or equivalent
- 6.1.12 Light box, VWR 89131-472, or equivalent or a Quebec Colony Counter, VWR 23610-157, or equivalent

6.2 Supplies

- 6.2.1 Test tubes, sterile, screw cap, borosilicate glass, 16 × 125 mm or 16 × 150 mm
- 6.2.2 Test tube rack
- 6.2.3 Pipets, sterile, T.D. bacteriological or Mohr, disposable glass or plastic, of appropriate volume
- 6.2.4 Inoculation loops – Nichrome or platinum wire, disposable, sterile plastic loops, at least 3 mm in diameter or 10 µL volume
- 6.2.5 Burner, Alcohol or Bunsen
- 6.2.6 Petri dishes, sterile, plastic or glass, 100 × 15 mm or 150 × 15 mm with loose fitting lids
- 6.2.7 Beakers, 800 mL, 2 L and 4 L, sterile, polypropylene, glass, or polycarbonate
- 6.2.8 Erlenmeyer flasks, sterile, 250 – 500 mL, 1 L, and 2 L
- 6.2.9 Graduated cylinders, sterile, 100 mL, 250 mL, and 1 L

- 6.2.10 Freezer vials, sterile, 5 mL screw cap
- 6.2.11 Stir bars, Fisher 14-513-51, or equivalent
- 6.2.12 Disposable powder-free gloves
- 6.2.13 Sterile, cotton-tipped applicators
- 6.2.14 Disposable powder-free gloves
- 6.2.15 Cuvettes
- 6.2.16 Shaker flasks, fluted Erlenmeyer, 125 mL with slip cap or sterile plug, Fisher 09-552-33, or equivalent
- 6.2.17 Thermometers, 0°C to 100°C
- 6.2.18 Flask weights, VWR 29700-004, or equivalent
- 6.2.19 Lint-free tissues, KimWipes, or equivalent
- 6.2.20 Weigh boats
- 6.2.21 0.22- μ m, sterile, membrane filtration units
- 6.2.22 Filter flasks

7.0 Reagents and Standards

7.1 General reagents

- 7.1.1 Reagent-grade water should conform to Specification D 1193, *Annual Book of ASTM Standards* (Reference 17.2).
- 7.1.2 Stock magnesium chloride (MgCl_2 [80X, 4M]) – Add 814 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to 300 mL reagent-grade water. Stir to dissolve. Bring to a final volume of 1 L, and mix thoroughly. Autoclave at 121°C (15 psi) for 15 minutes.
- 7.1.3 Glycerol – Sigma-Aldrich G6279, or equivalent. Autoclave at 121°C (15 psi) for 15 minutes. Remove promptly to avoid scorching. Store at room temperature.
- 7.1.4 Household bleach
- 7.1.5 Ethanol – 70% or greater

7.2 Antibiotic stocks

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

Stock nalidixic acid is added to all growth media for E. coli CN-13. Stock streptomycin/ampicillin is added to all growth media for E. coli F_{amp}.

7.2.1 Stock nalidixic acid (nalidixic [E. coli CN-13]) *Note:* Nalidixic acid is considered toxic. Wear suitable protective clothing, gloves, and eye/face protection and use in a chemical fume hood.

Dissolve 1 g of nalidixic acid sodium salt (Sigma-Aldrich N4382, or equivalent) in 100 mL reagent-grade water. Filter sterilize the solution using a 0.22 µm 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 solution thoroughly, prior to use.

7.2.2 Stock streptomycin/ampicillin (strep/amp [E. coli F_{amp}])

Dissolve 0.15 g of streptomycin sulfate (Sigma-Aldrich S6501, or equivalent) and 0.15 g of ampicillin sodium salt (Sigma-Aldrich A9518, or equivalent) in 100 mL of reagent-grade water, and mix thoroughly. Filter sterilize the solution using a 0.22 µm 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 solution thoroughly, prior to use.

7.3 Tryptic (or trypticase) Soy Broth (TSB) (BD™ 211825, or equivalent)

7.3.1 Composition:

Tryptone	17.0 g
Soytone	3.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium phosphate (K ₂ HPO ₄)	2.5 g
Reagent-grade water	1.0 L

7.3.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Autoclave at 121°C (15 psi) for 15 minutes. Final pH should be 7.3 ± 0.2. *Note:* 9 mL volumes of TSB without antibiotics are used in Section 13.4, as a diluent for the coliphage spiking suspensions. Cool to 48°C ± 1.0°C prior to adding nalidixic or strep/amp.

7.3.2.1 TSB with nalidixic (*E. coli* CN-13): Aseptically add 10 mL of stock nalidixic (Section 7.2.1) to 1 L of autoclaved, cooled ($48^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) TSB and mix. Aseptically dispense appropriate volumes (e.g., 25 mL) into screw cap bottles (Section 11.1.1.1).

7.3.2.2 TSB with strep/amp (*E. coli* F_{amp}): Aseptically add 10 mL of stock strep/amp (Section 7.2.2) to 1 L of autoclaved, cooled ($48^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) TSB and mix. Aseptically dispense appropriate volumes (e.g., 25 mL) into screw cap bottles (Section 11.1.1.3).

7.4 Phosphate Buffered Saline (PBS)

7.4.1 Composition:

Monosodium phosphate (NaH_2PO_4)	0.58 g
Disodium phosphate (Na_2HPO_4)	2.5 g
Sodium chloride	8.5 g
Reagent-grade water	1.0 L

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

7.5 Single Agar Layer (SAL) Media

7.5.1 Double-strength TSB with agar (2X TSB with agar): Double all components of TSB (Section 7.3) except reagent-grade water and add **18 g** of agar per liter. While stirring, heat to dissolve agar. Autoclave at 121°C (15 lb psi) for 15 minutes. Cool to $48^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ and mix molten medium thoroughly to ensure even distribution. Medium may become darker after autoclaving, but this should not impact performance.

7.5.1.1 2X TSB with agar and nalidixic (*E. coli* CN-13): Aseptically add 20 mL of stock nalidixic (Section 7.2.1) to 1 L of autoclaved, cooled ($48^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) 2X TSB with agar and mix to ensure even distribution. Keep molten at $45^{\circ}\text{C} - 46^{\circ}\text{C}$ in water bath until use. ***Agar must be used on the day of preparation.***

7.5.1.2 2X TSB with agar and strep/amp (*E. coli* F_{amp}): Aseptically add 20 mL of stock strep/amp (Section 7.2.2) to 1 L of autoclaved, cooled ($48^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$) 2X TSB with agar and mix to ensure even distribution. Keep molten at $45^{\circ}\text{C} - 46^{\circ}\text{C}$ in water bath until use. ***Agar must be used on the day of preparation.***

7.6 Double Agar Layer (DAL) Media

7.6.1 Tryptic soy broth (TSB) with 1.5% agar – To be used in streak plates (Section 7.8.2.1) and as bottom layer of agar (Section 13.4.2.2) for the double agar layer (DAL) procedure. Prepare TSB (Section 7.3) and add **15 g** of agar per liter. While stirring,

heat to dissolve agar. Autoclave at 121°C (15 lb psi) for 15 minutes. Place medium in a water bath and cool to 48°C ± 1.0°C.

7.6.1.1 TSB with 1.5% agar and nalidixic (*E. coli* CN-13): Aseptically add 10 mL of stock nalidixic (Section 7.2.1) to 1 L of autoclaved, cooled (48°C ± 1.0°C) TSB with 1.5% agar and mix to ensure even distribution. Once cooled, aseptically dispense 17 – 18 mL per 100-mm plate. Allow to solidify with lids off in a laminar flow hood for several minutes prior to use. Plates may be stored at 4°C ± 1°C for up to two weeks.

7.6.1.2 TSB with 1.5% agar and strep/amp (*E. coli* F_{amp}): Aseptically add 10 mL of stock strep/amp (Section 7.2.2) to 1 L of autoclaved, cooled (48°C ± 1.0°C) TSB with 1.5% agar and mix to ensure even distribution. Once cooled, aseptically dispense 17 – 18 mL per 100-mm plate. Allow to solidify with lids off in a laminar flow hood for several minutes prior to use. Plates may be stored at 4°C ± 1°C for up to two weeks.

7.6.2 TSB with 0.7% agar: “Soft” agar for use as the top layer of agar (Section 13.4.2.1) for the DAL procedure. Prepare TSB (Section 7.3) and add **7 g** of agar per liter. While stirring, heat to dissolve agar. Autoclave at 121°C (15 lb psi) for 15 minutes. Place medium in a water bath and cool to 48°C ± 1.0°C.

7.6.2.1 TSB with 0.7% agar and nalidixic (*E. coli* CN-13): Aseptically add 10 mL of stock nalidixic (Section 7.2.1) to 1 L of autoclaved, cooled (48°C ± 1.0°C) TSB with 0.7% agar and mix to ensure even distribution. Keep molten at 45°C – 46°C in water bath until use. Aseptically dispense 5 mL aliquots into sterile 16 × 125 mm tubes. ***Agar must be used on the day of preparation.***

7.6.2.2 TSB with 0.7% agar and strep/amp (*E. coli* F_{amp}): Aseptically add 10 mL of stock strep/amp (Section 7.2.2) to 1 L of autoclaved, cooled (48°C ± 1.0°C) TSB with 0.7% agar and mix to ensure even distribution. Keep molten at 45°C – 46°C in water bath until use. Aseptically dispense 5 mL aliquots into sterile 16 × 125 mm tubes. ***Agar must be used on the day of preparation.***

7.7 Coliphage Stocks

7.7.1 Somatic stock coliphage (phi-X174 [ATCC® #13706-B1™])

7.7.2 Male-specific stock coliphage (MS2 [ATCC® #15597-B1™])

7.7.3 Procedure for Preparing Coliphage Stocks

7.7.3.1 Using a loopful of growth from an isolated colony on 1.5% TSA (working stocks [*E. coli* F_{amp} ATCC® 700891™ and *E. coli* CN-13 ATCC® 700609™]), inoculate 2, 5 mL tubes of TSB (one per host) and incubate for 16 – 18 hours at 36°C ± 1°C.

- 7.7.3.2** After 16 – 18 hour incubation, transfer 1 mL of each culture to a tube/flask of (25 – 30 mL) of TSB with strep/amp or nalidixic, as appropriate. Incubate for 4 hours at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with gentle shaking. After the four hour incubation the host cultures should be in log phase.
- 7.7.3.3** Rehydrate stock cultures of MS2 (ATCC® 15597-B1™) and somatic coliphage (ATCC® 13706-B1™) by adding 1 mL of TSB to each culture.
- 7.7.3.4** Add 1 mL of each of the rehydrated phage stocks to the appropriate log phase host and incubate at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$, with gentle shaking overnight.
- 7.7.3.5** After overnight incubation (16 – 24 hours), centrifuge suspensions at $3500 \times g$ for 10 minutes to remove bacterial cell debris.
- 7.7.3.6** Filter supernatant through a $0.22 \mu\text{m}$ filter. The resulting phage suspensions contain approximately 1.0×10^8 plaque forming units (PFU) per mL.
- 7.7.3.7** Enumerate suspensions using the DAL procedure (Section 13.4).

7.8 Host Bacteria Stock Cultures

7.8.1 Reference Bacterial Cultures

- 7.8.1.1** *E. coli* CN-13 (somatic coliphage host [ATCC® #700609™]) – Nalidixic acid-resistant mutant of *E. coli* C
- 7.8.1.2** *E. coli* F_{amp} – *E. coli* HS (pF_{amp}) R (male-specific coliphage host [ATCC® #700891™])

7.8.2 Procedure for Preparing Host Bacteria Working Stock Cultures – The laboratory shall use reference bacterial cultures to establish pure frozen host stock cultures that are maintained by the laboratory. Working stocks are used as inoculum for overnight host bacteria stock cultures (Section 11.1.1).

- 7.8.2.1** Establish pure frozen stock cultures by streaking host bacteria onto 1.5% TSB with agar plates with appropriate antibiotic (Section 7.6.1) to attain isolated colonies.
- 7.8.2.2** Incubate inoculated plates overnight, pick an individual colony and inoculate aseptically into TSB with appropriate antibiotics (Section 7.3.2), and grow to log phase.
- 7.8.2.3** Harvest broth by mixing sterile glycerol and TSB with log-phase host bacteria in a ratio of 1:4 in a 5-mL freezer vial. (Example: 200 μL sterile glycerol plus 800 μL log-phase host).
- 7.8.2.4** Label with *E. coli* strain and date of harvest.

- 7.8.2.5** Freeze host bacteria stock cultures at -80°C , if possible. Cultures can be frozen at -20°C if the laboratory does not have the capability to freeze samples at -80°C . Cultures frozen at -20°C must be maintained in a freezer that does not have a defrost cycle.
- 7.8.2.6** Host bacteria stored at -80°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.
- 7.8.2.7** Prior to analyses, thaw vials of the stock host cultures and store at $<10^{\circ}\text{C}$ for up to one week. These vials will serve as working stock cultures. **Note:** Inoculum from the refrigerated working stock bacterial host culture will reach log-phase more rapidly than inoculum from frozen stock cultures.

8.0 Quality Control

- 8.1** Each laboratory that uses Method 1643 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Additional recommendations for QA and QC procedures for microbiological laboratories are provided in Reference 17.3.
- 8.2** The minimum analytical QC requirements for the analysis of samples using Method 1643 include an initial demonstration of laboratory capability through performance of initial precision and recovery (IPR) analyses (Section 8.3), ongoing demonstration of laboratory capability through performance of ongoing precision and recovery (OPR) analysis (Section 8.4) and matrix spike (MS) analysis (Section 8.5), routine analysis of method blanks (Section 8.6), and media sterility checks (Section 8.7). For the IPR, OPR, and MS analyses, it is necessary to spike samples with laboratory-prepared spiking suspensions as described in Section 13.4.3.

Note: Recovery and relative percent difference are based on each laboratory's enumeration of the referee-prepared spiking suspensions using the DAL procedure (Section 13) and enumeration of the recovery by SAL during the multi-laboratory validation study. Using the SAL procedure to enumerate coliphage spiking suspensions may affect recoveries of somatic (ϕ -X174) and male-specific (MS2) coliphage and is being investigated.

- 8.3 Initial precision and recovery (IPR)** – IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and should be performed by each laboratory before the method is used for monitoring field samples. EPA recommends, but does not require, that an IPR be performed by each analyst. IPR samples should be accompanied by an acceptable method blank (Section 8.6) and appropriate media sterility checks (Section 8.7). IPR analyses are performed as follows:

- 8.3.1** Prepare four, 100 mL samples of PBS per phage type. Spike four samples with laboratory-prepared ϕ -X174 suspension and four samples with laboratory-prepared

MS2 coliphage suspension according to Section 13.4.3. Process each IPR sample according to the procedures in Section 11 and calculate the number of somatic and male-specific coliphage per sample (PFU/100 mL) according to Section 12.

- 8.3.2** Calculate the percent recovery (R) for each IPR sample using the appropriate equations in Section 13.5.
- 8.3.3** Using the percent recoveries of the four analyses per phage type, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.
- 8.3.4** Compare the mean recovery and RSD with the corresponding IPR criteria in **Table 1**. If the mean and RSD for recovery of phi-X174 and MS2 coliphage meet acceptance criteria, system performance is acceptable and analysis of field samples may begin. If the mean recovery or the RSD fall outside of the required range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls; correct the problem and repeat IPR analyses.

Table 1. Calculated IPR^a and OPR^b Acceptance Criteria

Method	Phage	IPR ^a Mean Recovery (%)	IPR ^a RSD ^c (%)	OPR ^b Recovery (%)
Method 1643	Somatic (phi-X174)	139 – 278%	16%	134 – 283%
	Male-specific (MS2)	9 – 100%	17%	9 – 100%

^a Initial precision and recovery

^b Ongoing precision and recovery

^c Relative standard deviation

8.4 Ongoing precision and recovery (OPR) – To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze reference matrix spike samples. At a minimum, the laboratory should analyze one OPR sample per phage type each week that samples are analyzed. OPR analysis is performed as follows:

- 8.4.1** Prepare one, 100 mL reference matrix (PBS) sample per phage type as indicated in Section 8.3.1. Filter and process each OPR sample according to the procedures in Section 11 and calculate the number of somatic and male-specific coliphage per sample (PFU/100 mL) according to Section 12.
- 8.4.2** Calculate the percent recovery (R) for each OPR sample using the appropriate equations in Section 13.5.
- 8.4.3** Compare the OPR results (percent recovery) with the corresponding OPR recovery criteria in Table 1, above. If the OPR results meet the acceptance criteria for recovery,

method performance is acceptable and analysis of field samples may continue. If the OPR results fall outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, reagents, and controls; correct the problem and repeat OPR analyses.

8.4.4 As part of the laboratory QA program, results for OPR and IPR samples should be charted and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1643 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the accuracy as a recovery interval from $R - 2s_r$ to $R + 2s_r$.

8.5 Matrix spikes (MS) – MS analyses are performed to determine the effect of a particular matrix on coliphage recoveries. The laboratory should analyze one MS sample when secondary (no disinfection) wastewater samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given source should include a MS sample. MS samples must be accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site or of another unspiked aliquot of the same field sample, an acceptable method blank (Section 8.6), and appropriate media sterility checks (Section 8.7). MS analysis is performed as follows:

8.5.1 Prepare two, 100 mL field samples that were sequentially collected from the same site or different aliquots of the same field sample per phage type. Spike one of the samples with laboratory-prepared phi-X174 suspension and the other sample with laboratory-prepared MS2 coliphage suspension according to Section 13.4.3. The other two samples will remain unspiked. The unspiked samples will be analyzed to determine the background or ambient concentration of somatic and male-specific coliphage for calculating MS recoveries (Section 13.5). The spiked samples will serve as the MS samples.

8.5.2 Calculate the percent recovery (R) for the MS samples using the appropriate equations in Section 13.5.

8.5.3 Compare the MS results (percent recovery) with the appropriate method performance criteria in **Table 2**. If the MS recoveries meet the acceptance criteria, system performance is acceptable and analysis of field samples from this matrix may continue. If the MS recoveries are unacceptable and the OPR sample results associated with this batch of samples are acceptable, matrix interference may be causing the poor results. If the MS recoveries are unacceptable, all associated field data should be qualified.

8.5.4 Acceptance criteria for MS recovery (Table 2) are based on data from spiked secondary (no disinfection) wastewater matrices and are not appropriate for use with other matrices (e.g., fresh water).

Table 2. Matrix Spike Precision and Recovery Acceptance Criteria

Method	Matrix	Phage	MS/MSD ^a Recovery (%)	MS/MSD ^a RPD ^b
Method 1643	Wastewater Effluent (secondary, no disinfection)	Somatic (Phi-X174)	7 – 385%	51
		Male-specific (MS2)	Detect – 100%	79

^a Matrix spike/matrix spike duplicate

^b Relative percent difference

- 8.5.5** Laboratories should record and maintain a control chart comparing MS recoveries for all matrices to batch-specific and cumulative OPR sample results analyzed using Method 1643. These comparisons should help laboratories recognize matrix effects on method recovery and may also help to recognize inconsistent or sporadic matrix effects from a particular source.
- 8.6 Method blank** – The laboratory should analyze a method blank (100 mL unspiked sterile PBS) to demonstrate freedom from contamination according to Section 11. On an ongoing basis, the laboratory should analyze a method blank every day that samples are analyzed.
- 8.7 Media sterility check** – The laboratory should test media sterility by incubating one unit (tube or plate) of each batch of medium at 36°C ± 1.0°C for 24 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.
- 9.0 Calibration and Standardization**
- 9.1** Check temperatures in incubators and water baths twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.
- 9.2** Check temperatures in refrigerators and freezers daily to ensure operation within stated limits.
- 9.3** Check thermometers at least annually against a National Institute of Standards and Technology (NIST) certified thermometer or one that meets the requirements of NIST Monograph SP 250 - 23. Check columns for breaks.
- 9.4** The spectrophotometer should be calibrated each day of use using optical density (OD) calibration standards between 0.01 – 0.5. Follow manufacturer instructions for calibration.
- 9.5** Use sterile TSB without antibiotics as a blank.
- 9.6** Micropipettors should be calibrated at a minimum annually, ideally monthly, and tested for accuracy on a weekly basis. Follow manufacturer instructions for calibration.
- 9.7** 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.

9.8 Calibrate balances annually using ASTM-certified Class 2 reference weights.

10.0 Sample Collection, Preservation, and Storage

Sampling procedures are briefly described below. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Process and analyze samples as soon as possible after collection. Samples not collected according to these procedures should not be analyzed.

10.1 Sample Collection

Collect a 500 mL grab bulk wastewater sample for the analyses of the two coliphage types (somatic and male-specific). For range-finding analyses collect a 100 mL grab sample. *Note:* Each wastewater treatment facility may have different sampling locations and sampling procedures (e.g., sampling port, bucket sampling) in place.

10.2 Storage Temperature and Handling Conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. Use insulated containers to assure proper maintenance of storage temperature. Ensure that sample containers (e.g., bottles, carboys) are tightly closed and are not totally immersed in water during transit. Sample holding times described in the appropriate regulation should be followed.

11.0 Single Agar Layer (SAL) Procedure for Sample Analysis

(Procedures in Sections 11.2 and 11.4 are summarized in Section 18, SAL Flow Chart)

11.1 Host Cultures

11.1.1 Propagation of overnight host bacteria stock cultures

11.1.1.1 Dispense 25 mL of TSB with nalidixic acid (Section 7.3.2.1) into a sterile 125 mL shaker flask. Each culture flask of host bacteria should contain 25 – 30 mL of medium to ensure optimum growth conditions.

11.1.1.2 Aseptically inoculate the flask with a loopful of *E. coli* CN-13 from the working stock culture (Section 7.8.2).

11.1.1.3 Repeat Sections 11.1.1.1 and 11.1.1.2 using TSB with strep/amp as the medium (Section 7.3.2.2) and *E. coli* F_{amp} as the bacterial host.

11.1.1.4 Place a sterile slip cap or plug on the shaker flasks, label flasks, and secure in shaker.

11.1.1.5 Incubate at $36^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ and set shaker to 100 to 150 rpm. Shake for 16 – 18 hours.

11.1.1.6 Chill on wet ice or at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ until ready to inoculate into TSB for the 4 hour log-phase cultures.

11.1.2 Propagation of log-phase host bacteria stock cultures

11.1.2.1 To a 125 mL shaker flask containing 25 mL of TSB with nalidixic acid (Section 7.3.2.1) add 0.1 to 1.0 mL of overnight *E. coli* CN-13 host culture (Section 11.1.1). Each culture flask of host bacteria should contain 25 to 30 mL of medium to ensure optimum growth conditions. Each 100 mL sample analyzed using the SAL procedure will require a 10 mL inoculum of log-phase host bacteria. As a result, several flasks of host bacteria may have to be prepared based on the number of samples and controls being run each day.

11.1.2.2 Repeat Section 11.1.2.1 using TSB with strep/amp (Section 7.3.2.2) as the medium and *E. coli* F_{amp} as the host culture.

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

11.1.2.4 Incubate at $36^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$ and 100 to 150 rpm for approximately 4 hours or until cultures are visibly turbid (cloudy), indicating log-phase growth.

Note: It is not necessary to take OD readings of the log-phase (4 hour) host culture unless there is concern that there is not sufficient growth (e.g., none or very little indication of turbidity in the flask). In most cases the log-phase culture will be visually turbid.

11.1.2.5 If cultures are not visibly turbid or turbidity is weak, aseptically remove 1 mL of culture from the flask, dispense into a cuvette (Section 6.2.14), and read absorbance at 520 nm. An absorbance reading between 0.1 and 0.5 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.

11.1.2.6 Chill on wet ice or at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ to slow replication until ready to add to the samples (Section 11.4). It is recommended that the log-phase cultures be used immediately (within 6 hours).

11.2 Preparation of media for testing

Note: The use of commercially pre-prepared media is acceptable as long as testing demonstrates that method performance is equivalent.

11.2.1 Prepare 100 mL of 2X TSB with agar and nalidixic acid for *E. coli* CN-13 as described in Section 7.5.1.1 for each sample analyzed for somatic coliphage.

11.2.2 Add the 100 mL of 2X TSB with agar and nalidixic acid to a sterile 250 – 500 mL Erlenmeyer flask for each sample analyzed for somatic coliphage and place in a 45°C – 46°C water bath to equilibrate. The liquid in the water bath must come up to the level of the media. To ensure that the flask does not tip-over in the water bath, the use of a flask weight is recommended.

11.2.3 Prepare 100 mL of 2X TSB with agar and strep/amp for *E. coli* F_{amp} as described in Section 7.5.1.2 for each sample analyzed for male-specific coliphage.

11.2.4 Add the 100 mL of 2X TSB with agar and strep/amp to a sterile 250 – 500 mL Erlenmeyer flask for each sample analyzed for male-specific coliphage and place in a 45°C – 46°C water bath to equilibrate. The liquid in the water bath must come up to the level of the media. To ensure that the flask does not tip-over in the water bath, the use of a flask weight is recommended.

11.2.5 Keep the agar molten between 45°C – 46°C until use in the SAL assay.

11.3 Range-finding (Optional)

Range-finding analyses should be conducted when the laboratory receives wastewater samples from a treatment facility from which they have not previously analyzed samples. Range-finding results will help determine appropriate sample volumes (e.g., 100 mL, 10 mL).

11.3.1 Dispense two, 10 mL aliquots, from a 100 mL grab secondary (no disinfection) wastewater sample, into two sterile 125 – 250 mL bottles containing 90 mL of sterile PBS, these will serve as the unspiked 100 mL samples. **Note:** Depending on anticipated coliphage levels, smaller sample volumes (e.g., 1.0 mL, 0.1 mL) may need to be analyzed to obtain countable plates. When analyzing smaller volumes, the volume of PBS should be increased (e.g., 1.0 mL sample and 99 mL of PBS).

11.3.2 Analyze one unspiked sample for somatic coliphage and the other unspiked sample for male-specific coliphage according to Section 11.4.

11.4 Single Agar Layer (SAL)

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

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

Note: As a precaution against contamination, disinfect a work space near the water baths with a 1:10 dilution of household bleach or a 70% ethanol solution.

11.4.2 Dispense two, 100-mL aliquots of each sample into separate, sterile, 250 – 500 mL Erlenmeyer flasks.

11.4.3 Dispense 100 mL of reagent water into a 250 – 500 mL Erlenmeyer flask (this will be used to determine sample temperature changes in the following steps and will be referred to as the “temperature flask”).

11.4.4 Add 0.5 mL of sterile stock MgCl_2 (Section 7.1.2) to each sample flask except the temperature flask.

11.4.5 Place the flasks into the $36^\circ\text{C} \pm 1.0^\circ\text{C}$ water bath for 5 minutes or until sample just reaches the water bath temperature according to the temperature flask.

Note: All components should be warmed before the assay to avoid solidification prior to pouring plates. The temperature must be monitored closely to ensure that coliphage are not inactivated and the agar does not solidify prematurely.

11.4.6 Add 10 mL of log-phase *E. coli* CN-13 (Section 11.1.2.6) to one sample.

11.4.7 Add 10 mL of log-phase *E. coli* F_{amp} (Section 11.1.2.6) to the other sample.

11.4.8 Add an additional 10 mL of water to the temperature flask (110 mL total).

11.4.9 Immediately transfer temperature flask and flasks containing samples and log-phase bacteria to the $45^\circ\text{C} - 46^\circ\text{C}$ water bath. The approximate temperature of the samples should be determined by monitoring the temperature of the water in the temperature control flask. When water in the temperature control flask reaches $43^\circ\text{C} \pm 1.0^\circ\text{C}$, remove samples from the water bath and proceed to the next step immediately.

Note: During the SAL procedure, the sample and host bacteria should not remain in contact with each other for more than 10 minutes prior to plating. After plating, the agar must harden within 10 minutes. Increased contact time or agar-solidification 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 solidification of agar plates should not exceed 20 minutes.

11.4.10 Add the sample/*E. coli* CN-13 mixture (Section 11.4.9) to the 100 mL of 2X TSB with agar and nalidixic acid (Section 11.2.5) in the $45^\circ\text{C} - 46^\circ\text{C}$ water bath. Allow sample/*E. coli* CN-13 mixture to remain in contact with host for a minimum of three minutes before plating. 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.

11.4.11 Add the sample/*E. coli* F_{amp} mixture (Section 11.4.9) to the 100 mL of 2X TSB with agar and strep/amp (Section 11.2.5) in the $45^\circ\text{C} - 46^\circ\text{C}$ water bath. 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.

11.4.12 Allow the agar to solidify, cover, invert, and incubate for 16 – 24 hours at $36^\circ\text{C} \pm 1.0^\circ\text{C}$. *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.

- 11.4.13** 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. See **Figures 1 and 2** for somatic and male-specific plaques, respectively. Count all plaques per plate series, record results, and calculate the number of somatic and male-specific phage per sample (PFU/100 mL) according to Section 12. **Note:** The use of a light box (Section 6.1.12) to evaluate sample results is recommended.

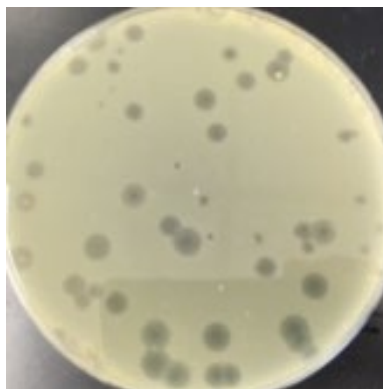


Figure 1: Somatic Coliphage Plaques (CN-13)

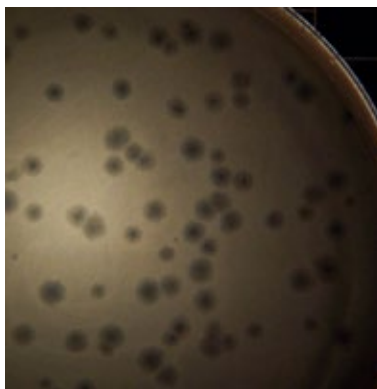


Figure 2: Male-specific Coliphage Plaques (F_{amp})

12.0 Data Analysis and Calculations

Use the following general rules to calculate the coliphage count per 100 mL of sample:

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

$$\text{Coliphage} / 100 \text{ mL} = \text{Plt}_1 + \text{Plt}_2 + \text{Plt}_3 + \text{Plt}_4 + \text{Plt}_5$$

- 12.2** Report results as PFU per 100 mL of sample.

13.0 Sample Spiking Procedure

- 13.1** Method 1643 QC requirements (Section 8) include the preparation and analysis of spiked reference (PBS) and matrix samples in order to monitor initial and ongoing method performance. For the IPR (Section 8.3), OPR (Section 8.4), and MS (Section 8.5) analyses it is necessary to spike samples with laboratory-prepared spiking suspensions.
- 13.2** The DAL procedure (Section 18 DAL Flow Chart) is used to enumerate phi-X174 and MS2 coliphage stock and spiking suspensions.
- 13.3** Preparation of laboratory-prepared spiking suspension and host cultures.

13.3.1 Use coliphage stocks prepared in Sections 7.7.1 (phi-X174) and 7.7.2 (MS2).

13.3.2 Prepare overnight host bacteria stock cultures (Section 11.1.1).

13.3.3 Prepare log-phase host bacteria stock cultures (Section 11.1.2) the day of enumeration.

13.4 Laboratory-Prepared Coliphage Spiking Suspension Enumeration and Sample Spiking

Since the objective of spiking the sample is to establish percent recovery, it is necessary to determine the concentration of phi-X174 and MS2 phage in the laboratory-prepared undiluted spiking suspensions. This section provides instructions for spiking suspension enumeration and sample spiking.

*Please be sure to vortex the spiking suspensions in the steps below to ensure accurate sample spiking and spiking suspension enumeration. **Note:** Additional dilutions may be necessary.*

13.4.1 Dilute coliphage spiking suspensions

Perform 13.4.1.1 – 13.4.1.3 using the laboratory-prepared undiluted spiking suspension (Section 13.3.1).

13.4.1.1 Mix the undiluted spiking suspension by vortexing for five seconds on a medium-high setting (if available) or until thoroughly mixed. Use a sterile pipet to transfer 1.0 mL of the undiluted spiking suspension to 9 mL of sterile TSB without antibiotics (Section 7.3); cap and mix by vortexing. This is 0.1 (10^{-1}) mL of the original undiluted spiking suspension.

13.4.1.2 Mix the 0.1 (10^{-1}) mL spiking suspension by vortexing for five seconds on a medium-high setting (if available) or until thoroughly mixed. Use a sterile pipet to transfer 1.0 mL of the 0.1 (10^{-1}) mL spiking suspension to 9 mL of sterile TSB without antibiotics (Section 7.3); cap and mix by vortexing. This is 0.01 (10^{-2}) mL of the original undiluted spiking suspension.

13.4.1.3 Mix the 0.01 (10^{-2}) mL spiking suspension by vortexing for five seconds on a medium-high setting (if available) or until thoroughly mixed. Use a sterile pipet to transfer 1.0 mL of the 0.01 (10^{-2}) mL spiking suspension to 9 mL of sterile TSB without antibiotics (Section 7.3); cap and mix by vortexing. This is 0.001 (10^{-3}) mL of the original undiluted spiking suspension.

13.4.2 Enumeration of Coliphage Spiking Suspensions

Eighteen tubes (9 per phage type) are necessary to enumerate the four dilutions in duplicate and 1 method blank (sterile TSB without antibiotics). Nine of the top agar tubes should contain nalidixic (Section 7.3.2.1) for growth of *E. coli* CN-13; the other nine should contain strep/amp (Section 7.3.2.2) for growth of *E. coli* F_{amp}. **Note:** Laboratories are not permitted to modify or omit any aspects of the coliphage spiking suspension enumeration procedure. As a result, magnesium chloride must not be added to the sample or media.

13.4.2.1 Prepare TSB with 0.7 % agar (Section 7.6.2) with appropriate antibiotics. Place the top agar tubes with antibiotics (Sections 7.6.2.1 and 7.6.2.2) in a 45°C –

46°C water bath. The top agar should remain molten in the water bath until ready for use.

13.4.2.2 Prepare TSB with 1.5% agar (Section 7.6.1) with appropriate antibiotics. Assemble 1.5% bottom agar plates with appropriate antibiotics and label so that the following information is identifiable: dilution of stock (e.g., 0.1, 0.01) or method blank; host (*E. coli* CN-13 or *E. coli* F_{amp}); date; and time.

13.4.2.3 Addition of host and spiking suspension to 0.7% tubes.

Note: To ensure viability of bacterial host, do not add bacterial host and coliphage spiking suspension until ready to plate.

13.4.2.3.1 With the top agar tubes in the water bath, aseptically inoculate two top agar tubes containing strep/amp or nalidixic with 100 µL (0.1 mL) of log-phase *E. coli* CN-13 or *E. coli* F_{amp}, as appropriate.

13.4.2.3.2 Immediately add 500 µL (0.5 mL) of undiluted coliphage stock to each of the two tubes.

13.4.2.3.3 Mix the inoculum by rolling the tubes briefly in palm of hand.

13.4.2.3.4 Pour contents into the two bottom agar plates marked “undiluted *E. coli* CN-13” or “undiluted *E. coli* F_{amp}”, as appropriate.”

13.4.2.3.5 Repeat Sections 13.4.2.3.1 – 13.4.2.3.4 for each dilution (0.1, 0.01, and 0.001) and the method blanks for each phage type.

13.4.2.4 Let agar solidify, cover, invert plates and incubate at 36°C ± 1.0°C for 16 – 24 hours. *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.

13.4.2.5 Circular zones of clearing (typically 1 to 10 mm in diameter) in the host lawn after 16 – 24 hours of incubation are plaques. Count the number of plaques on each plate. *Note:* The use of a light box (Section 6.1.12) to evaluate results is recommended.

13.4.3 Spike Samples

Use the enumerated (titered) spiking suspensions to spike samples. Spike the 100 mL samples (field and QC) with the appropriate dilution and volume of each titered stock (phi-X174 and MS2) to achieve a spike level of approximately 100 PFU of each phage type per 100 mL sample. *Note:* The volume and dilution will vary depending on the titer of the stock.

13.5 Calculation of Spiked Phage Percent Recovery

The spiked phage (phi-X174 and MS2) percent recovery will be calculated as indicated in Sections 13.5.1 – 13.5.4, below. *Note:* The calculated numbers in the tables provided below have been rounded at the end of each step. The percent recovery may be slightly different if your

laboratory recalculates the examples using a spreadsheet and rounds only after the final calculation (Section 13.5.5).

13.5.1 The titer of the undiluted coliphage spiking suspensions will be calculated using all DAL plates that yield plaque counts within the desired range of 1 – 100 PFU per plate for phi-X174 coliphage and 1 – 300 PFU per plate for MS2 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 TNTC.

13.5.2 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.

13.5.3 Sum the undiluted sample volumes used to inoculate all replicate plates at all dilutions having useable counts (as defined above).

13.5.4 Divide the sum of PFU by the sum of the undiluted sample volume to obtain PFU/mL in the spiking suspension. An example calculation is provided in **Table 3**.

13.5.5 The equation for Sections 13.5.1 – 13.5.4 is as follows:

$$\text{Phage}_{\text{Und spike}} = (\text{PFU}_1 + \text{PFU}_2 \dots \text{PFU}_n) / (V_1 + V_2 \dots V_n)$$

Where:

Phage_{Und spike} = Phage (PFU/mL) in undiluted spiking suspension

PFU = number of plaque forming units from plates yielding counts within the ideal range of 1 to 100 (phi-X174) or 1 to 300 (MS2)

V = volume of undiluted sample on each plate yielding counts within the ideal range of in all plates with countable plaques 1 to 100 (phi-X174) or 1 to 300 (MS2)

n = number of plates with counts within the ideal range

Table 3. Example Calculation of Phage Spiking Suspension Concentration

Undiluted	PFU/plate (duplicate analyses) ^a			Phage PFU/mL in undiluted spiking suspension (Phage _{und spike})
	0.1	0.01	0.001	
TNTC, TNTC	35, 37	0, 3	0, 0	$(35 + 37 + 3) / (0.05 + 0.05 + 0.005) = 75 / 0.105 = 714 \text{ PFU/mL}$

^a 0.5 mL per dilution was enumerated per plate (e.g., 0.5 mL of the 0.1 [10⁻¹] dilution is equal to 0.05 mL of the undiluted suspension)

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

13.5.6.1 Use a dilution of the coliphage spiking suspension that will result in a bulk spike volume between 0.1 and 3.0 mL for the spike concentration.

13.5.6.2 Use the following equation to determine the spiking volume:

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

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

Example, for IPR (Section 8.3):

T = 100 PFU is needed per 100 mL sample

B = Four, 100 mL samples

C = 714 PFU/mL

The equation would be solved as follows:

$$S = (100 \text{ PFU} \times 4) / (714 \text{ PFU/mL}) = 0.56$$

As a result, 0.56 mL of the undiluted spiking suspension would be required to spike all four samples. Each 100 mL sample would be spiked with 0.14 mL of the undiluted spiking suspension. Each 100 mL sample should contain approximately 100 PFU. Alternatively, 0.56 mL of the undiluted spiking suspension could be spiked into a 400 mL bulk sample. The 400 mL bulk sample would be mixed and four, 100 mL aliquots dispensed.

13.5.7 Calculate “True” Spiked Phage (PFU/100 mL)

Calculate the true concentration of spiked phage (PFU/100 mL) according to the following equation. Example calculation is provided in **Table 4**.

$$T_{\text{Spiked Phage}} = (\text{Phage}_{\text{undiluted spike}}) \times (V_{\text{spiked per 100 mL sample}})$$

Where,

- $T_{\text{Spiked Phage}}$ = Number of spiked Phage (PFU/100 mL)
- $\text{Phage}_{\text{und spike}}$ = Phage (PFU/mL) in undiluted spiking suspension
- $V_{\text{spiked per 100 mL sample}}$ = mL of undiluted spiking suspension per 100 mL sample

Table 4. Example Calculation of Spiked Phage

Phage undiluted spike	V spiked per 100 mL sample	T Spiked Phage
714 PFU/mL	1.4×10^{-1} mL/ 100 mL of sample	$(714 \text{ PFU/mL}) \times (1.4 \times 10^{-1} \text{ mL/ } 100 \text{ mL}) = 100 \text{ PFU/100 mL}$

13.5.8 Calculate Percent Recovery

Calculate percent recovery (R) using the following equation.

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

Where,

R = Percent recovery

N_s = Phage (PFU/100 mL) in the spiked sample

N_u = Phage (PFU/100 mL) in the unspiked sample

$T_{\text{Spiked Phage}}$ = True spiked Phage (PFU/100 mL) in spiked sample

Example percent recovery calculation is provided in **Table 5**.

Table 5. Example Percent Recovery Calculation

N_s (PFU/100 mL)	N_u (PFU/100 mL)	$T_{\text{Spiked Phage}}$ (PFU/100 mL)	Percent recovery (R)
40	3	50	$100 \times (40 - 3)/50 = 74\%$

14.0 Method Performance

Fourteen volunteer laboratories participated in the EPA MLV study of Method 1643 in secondary wastewater (no disinfection) and PBS. The purposes of the study were to characterize method performance across multiple laboratories and matrices and to develop QC acceptance criteria. A detailed description of the study and results are provided in the validation study report (Reference 17.4). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with Method 1643 and study-specific instructions. Method performance summary data are provided below.

14.1 Secondary Wastewater, no disinfection (Ten of the fourteen laboratories analyzed wastewater samples.)

14.1.1 Recovery

14.1.1.1 Somatic – Mean laboratory-specific recoveries, of phi-X174 from secondary wastewater (no disinfection) samples spiked with referee- or laboratory-

prepared phi-X174 phage suspensions ranged from 45% to 368%, with an overall mean recovery of 199%.

- 14.1.1.2** Male-specific – Mean laboratory-specific recoveries, of MS2 from secondary wastewater (no disinfection) samples spiked with referee- or laboratory-prepared MS2 phage suspensions ranged from 8% to 54%, with an overall mean recovery of 27%.

14.1.2 Precision

- 14.1.2.1** Somatic – Method 1643 was characterized by laboratory-specific relative standard deviations (RSDs) from secondary wastewater (no disinfection) samples spiked with referee- or laboratory-prepared phi-X174 phage suspensions ranging from 8% to 55%, with an overall pooled, within-laboratory RSD of 30%.

- 14.1.2.2** Male-specific – Method 1643 was characterized by laboratory-specific RSDs from secondary wastewater (no disinfection) samples spiked with referee- or laboratory-prepared MS2 phage suspensions ranging from 4% to 79%, with an overall pooled, within-laboratory RSD of 35%.

14.2 PBS (Fourteen laboratories analyzed PBS samples.)

14.2.1 Recovery

- 14.2.1.1** Somatic – Mean laboratory-specific recoveries of somatic phage from PBS samples spiked with referee-prepared phi-X174 phage suspensions ranged from 68% to 411%, with an overall mean recovery of 215%.

- 14.2.1.2** Male-specific – Mean laboratory-specific recoveries, of MS2 from PBS samples spiked with referee-prepared MS2 phage suspensions ranged from 13% to 114%, with an overall mean recovery of 32%.

14.2.2 Precision

- 14.2.2.1** Somatic – Method 1643 was characterized by laboratory-specific RSDs from PBS samples spiked with referee-prepared phi-X174 phage suspensions, ranged from 1% to 51%, with an overall pooled, within-laboratory RSD of 22%.

- 14.2.2.2** Male-specific – Method 1643 was characterized by laboratory-specific RSDs from PBS samples spiked with referee-prepared MS2 coliphage suspensions, ranged from 5% to 14%, with an overall pooled, within-laboratory RSD of 10%.

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

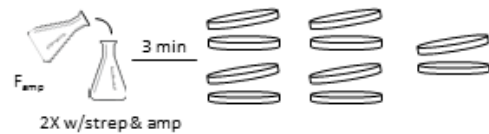
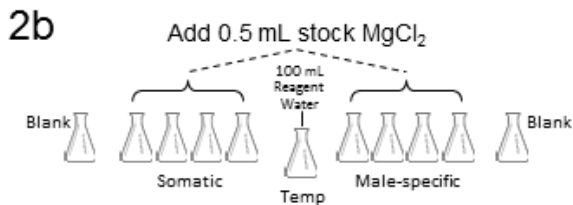
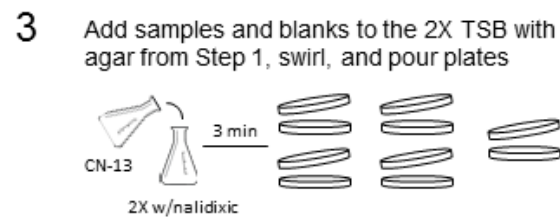
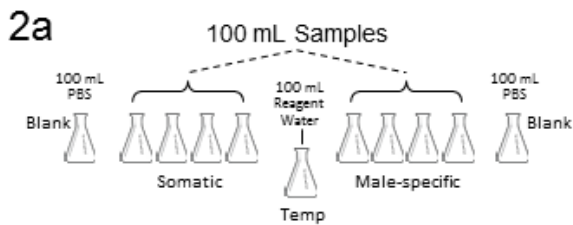
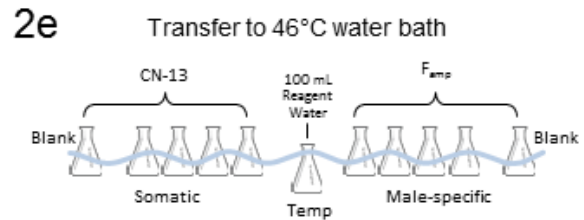
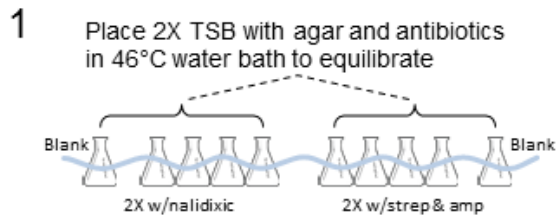
- 16.1 The laboratory is responsible for complying with all Federal, State, and local regulations governing waste management, particularly hazardous waste identification rules and land disposal restrictions, and for protecting the air, water, and land by minimizing and controlling all releases from fume 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 coliphage 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

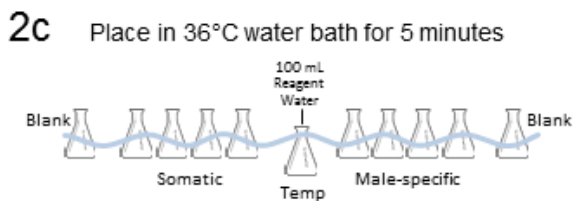
- 17.1 USEPA. 2001. Method 1602: Male-specific (F⁺) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure. EPA 821-R-01-029. Office of Water, Engineering and Analysis Division, Washington, DC 20460.
- 17.2 *Annual Book of ASTM Standards*. Vol. 11.01. American Society for Testing and Materials. Philadelphia, PA 19103.
- 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 USEPA. 2017. Results of the Multi-Laboratory Validation of Method 1602 for Coliphage in Fresh and Marine Recreational Waters and Wastewater Effluent (Advanced Treatment) Samples that have been concentrated using Ultrafiltration and 100-mL Secondary Wastewater (No Disinfection) Effluents. Draft report.

18.0 Flow Charts

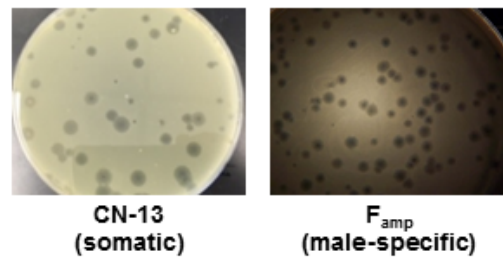
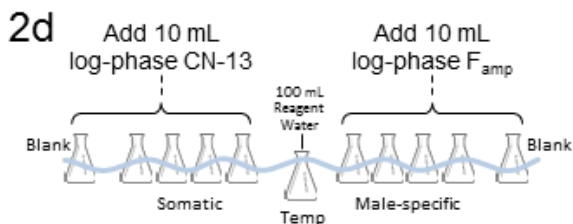
Single Agar Layer



Invert and incubate at 36°C for 16-24 hours

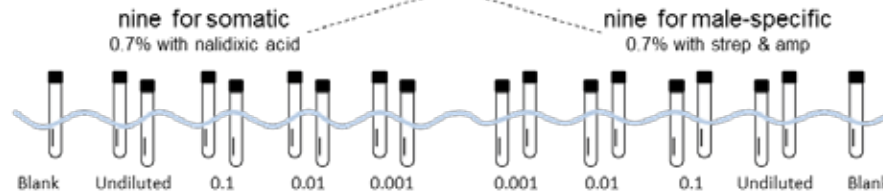


4 After incubation, count plaques, and record results

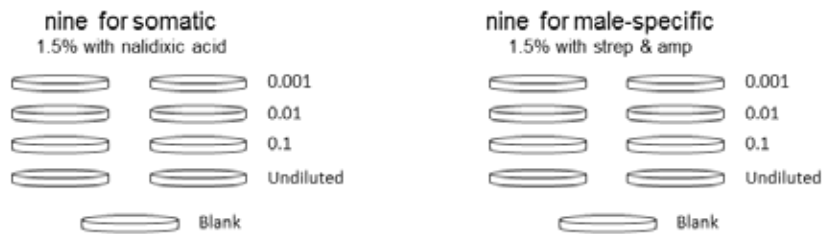


Double Agar Layer

- 1 Label and pour eighteen 0.7% top agar tubes with antibiotics in 46.5°C water bath

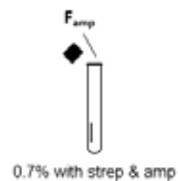
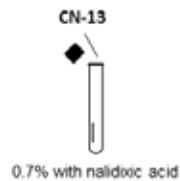


- 2 Label eighteen corresponding 1.5% bottom agar plates containing antibiotics

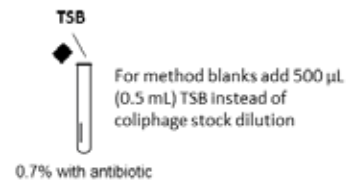


- 3 Prepare 0.7% top agar in 46°C water bath

Add 100 μ L (0.1 mL) log-phase host bacteria to tube with appropriate antibiotic



Immediately, add 500 μ L (0.5 mL) from appropriate coliphage stock dilution tube



Gently mix tube in palm and pour into appropriate bottom agar plate

Let agar solidify, then invert and incubate at 36°C for 16-24 hours

- 4 After incubation, count plaques, and record results