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Method 1642: Male-specific (F+) and Somatic Coliphage in Recreational Waters and Wastewater by Ultrafiltration (UF) and Single Agar Layer (SAL) Procedure

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- Anatek Labs, Inc.
- Bay County Laboratory
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- CH Diagnostics
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- Hallsdale-Powell Utility District
- James Madison University
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Section 11 Figures 1 and 2: Ultrafiltration and Elution Set up, respectively (Source: Jennipher Quach-Cu, San Jose Creek Water Quality Laboratory – County Sanitation Districts of L.A. County)

Section 11.5.13 Figure 4: Somatic Coliphage Plaques (CN-13) (Source: Jeremy Olstadt, Wisconsin State Laboratory of Hygiene) and Figure 5: Male-specific Coliphage Plaques (F_{amp}) (Source: Richard Danielson, IEH BioVir)

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Introduction

Currently, the U. S. Environmental Protection Agency (EPA) has a number of bacterial methods to quantify fecal indicators, but has received requests to develop a coliphage (bacterial virus) method to detect fecal contamination in fresh and marine recreational waters and wastewater effluents. Because EPA did not have a validated, approved method for these matrices, stakeholders could not use such a method in their National Pollutant Discharge Elimination System and other permits where an EPA approved method is required.

In response to stakeholders' needs for a validated method for coliphage for monitoring recreational waters and wastewater effluents, EPA conducted a single-laboratory validation (SLV) study of *Standard Method* 9224F (Reference 17.1) in 100 mL fresh and marine waters and advanced treatment wastewater effluents (e.g., secondary with disinfection, tertiary). Due to inconsistent results, issues with clogged filters, and an increased sample volume (1 L sample per phage type [somatic and male-specific]), EPA then evaluated the use of EPA *Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure* (Reference 17.2) for recreational water and wastewater analyses. Because EPA Method 1602 was developed to assay 100 mL sample volumes, an EPA dead-end ultrafiltration (UF) procedure was used to concentrate the larger sample volumes (2 L) required for recreational water monitoring. After UF the final sample volume is approximately 200 mL, which can then be assayed for both somatic and male-specific coliphage using the SAL procedure.

Based on the results of the Method 1642 (EPA UF procedure in conjunction with Method 1602) analyses, EPA conducted a multi-laboratory validation (MLV) study of Method 1642 for somatic and male-specific coliphage in fresh and marine recreational waters and advanced treatment (secondary with disinfection, tertiary) wastewater effluents.

Results of the MLV study enabled performance characterization of Method 1642 in the reference matrix (phosphate buffered saline [PBS]), marine and fresh water matrices, and advanced treatment wastewater effluents. 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 advanced treatment wastewater effluents should be taken into consideration when determining if it is necessary to analyze 1 L samples per phage type. In most cases it will be necessary to analyze 1 L samples; however, this may not be the case for all advanced treatment wastewater effluents. During the multi-laboratory study, some of the secondary disinfected effluent samples had very high levels of both male-specific and somatic phage. It is recommended that range-finding analyses be conducted for each new wastewater effluent and recreational waters that are suspected to have high levels of background coliphage to determine if 1 L samples are appropriate. If 1 L samples are not appropriate, 100 mL samples may be analyzed using the SAL procedure without ultrafiltration.

Table of Contents

Method 1642: Male-specific (F^+) and Somatic Coliphage in Recreational Waters and Wastewater by Ultrafiltration (UF) and Single Agar Layer (SAL) Procedure

April 2018

1.0 Scope and Application

1.1 Method 1642 employs dead-end ultrafiltration (UF) to concentrate larger sample volumes (2 L) required for recreational water monitoring. Following concentration, the final sample volume is assayed 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 fresh and marine waters and advanced treatment (e.g., secondary with disinfection, tertiary) wastewater effluents.

1.2 This method is based on an EPA dead-end UF procedure and EPA *Method 1602: Male-specific (F^+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure* (Reference 17.2), developed for the enumeration of coliphage in ground water.

1.3 Each laboratory and analyst that uses this method must first 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 (male-specific and somatic) in advanced treatment wastewater effluents and recreational waters 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. If smaller sample volumes (e.g., 100 mL) are more appropriate due to high background levels of coliphage refer to the procedures in EPA Method 1643.

2.0 Summary of Method

Method 1642 describes a dead-end UF concentration procedure with enumeration by the SAL method. A 2-L recreational water (fresh or marine) or advanced treatment wastewater effluent sample is concentrated using a hollow-fiber ultrafilter, the filter is then eluted resulting in a final sample volume of 200 mL which is split into two 100 mL aliquots. The 100 mL aliquots are then assayed by adding magnesium chloride ($MgCl_2$), 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 an 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)/ L. 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_2HPO_4	Dipotassium phosphate
L	Liter
M	Molar
$MgCl_2$	Magnesium chloride
$MgCl_2 \cdot 6H_2O$	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_2HPO_4	Disodium phosphate
NaH_2PO_4	Monosodium phosphate
$Na_2S_2O_3$	Sodium thiosulfate
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
SLV	Single-laboratory validation
strep/amp	Streptomycin/ampicillin
TNTC	Too numerous to count
TSB	Tryptic soy broth
UF	Ultrafiltration

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

Water samples with high turbidity or algae may clog the filter, preventing filtration. High background levels of microorganisms may prevent the host bacteria from producing a confluent lawn of growth.

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 1642 analyses.

5.3 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

6.1 General 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, Fisher14-493-120SQ, 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 Equipment and supplies for collection and ultrafiltration of samples

6.2.1 Sterile, wide-mouth, polypropylene, 2 L bottles or carboys with screw caps

6.2.2 Hollow-fiber ultrafilter, Dial Medical Supply Rexeed 15S, or equivalent

6.2.3 Peristaltic pump, Masterflex L/S, or equivalent

6.2.4 Vinyl tubing, (e.g., Cole Parmer EW-06405-18, or equivalent)

6.2.5 Silicone tubing, L/S 24 (Cole-Parmer SK-96410-24, or equivalent)

6.2.6 Jiffy jack

6.2.7 Luer locks (DIN adapter), Molded Products MPC-855, or equivalent

6.2.8 Tubing clamps

6.2.9 Ring stand and clamps

6.3 General Supplies

6.3.1 Test tubes, sterile, screw cap, borosilicate glass, 16 × 125 mm or 16 × 150 mm

6.3.2 Test tube rack

6.3.3 Pipets, sterile, T.D. bacteriological or Mohr, disposable glass or plastic, of appropriate volume

6.3.4 Inoculation loops − Nichrome or platinum wire, disposable, sterile plastic loops, at least 3 mm in diameter or 10 µL volume

6.3.5 Burner, Alcohol or Bunsen

6.3.6 Petri dishes, sterile, plastic or glass, 100 × 15 mm or 150 × 15 mm with loose fitting lids

6.3.7 Beakers, 800 mL, 2 L and 4 L, sterile, polypropylene, glass, or polycarbonate

6.3.8 Erlenmeyer flasks, sterile, 250 − 500 mL, 1 L and 2 L

6.3.9 Graduated cylinders, sterile, 100 mL, 250 mL, and 1 L

6.3.10 Freezer vials, sterile, 5 mL screw cap

6.3.11 Stir bars, Fisher 14-513-51, or equivalent

6.3.12 Disposable powder-free gloves

6.3.13 Cuvettes

6.3.14 Shaker flasks, fluted Erlenmeyer, 125 mL with slip cap or sterile plug, Fisher 09-552-33, or equivalent

6.3.15 Thermometers, 0°C to 100°C

6.3.16 Flask weights, VWR 29700-004, or equivalent

6.3.17 Lint-free tissues, KimWipes, or equivalent

6.3.18 Weigh boats

6.3.19 0.22-µm, sterile, membrane filtration units

6.3.20 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 17.3).

7.1.2 10% (w/v) Sodium thiosulfate − Add 10 g sodium thiosulfate ($Na_2S_2O_3$) per 90 mL reagent-grade water and mix until dissolved. Bring to a final volume of 100 mL and autoclave at 121°C (15 psi) for 15 minutes.

7.1.3 Stock magnesium chloride ($MgCl_2$ [80X, 4M]) − Add 814 g of $MgCl_2 \cdot 6H_2O$ 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.

7.1.4 Glycerol – Sigma-Aldrich G6279, or equivalent. Autoclave at 121°C (15 psi) for 15 minutes. Remove promptly to avoid scorching. Store at room temperature.

7.1.5 Household bleach

7.1.6 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_2HPO_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.

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°C ± 1.0°C) TSB and mix.

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°C ± 1.0°C) TSB and mix.

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 tryptic soy broth 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 psi) for 15 minutes. Cool to 48°C ± 1.0°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°C ± 1.0°C) 2X TSB with agar and mix. Keep molten at 45°C − 46°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°C ± 1.0°C) 2X TSB with agar and mix. Keep molten at 45°C − 46°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 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 gently stir to ensure even distribution of antibiotic. 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 gently stir to ensure even distribution of antibiotic. 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 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. 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. 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 (F^+) 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* $_{Famp}$ 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°C ± 1°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°C ± 1°C, with gentle shaking overnight.

7.7.3.5 After overnight incubation (16 – 24 hours), centrifuge suspensions at 3500 × g for 10 minutes to remove bacterial cell debris.

7.7.3.6 Filter supernatant through a 0.22 µm 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°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.

7.9 Ultrafiltration Reagents

7.9.1 **Tween® 80 solution –** Add 30 g of Tween® 80 to 100 mL of reagent-grade water, heat to dissolve, and filter sterilize using a 0.22 µm filter. Store in screw-cap tubes for up to 3 months.

7.9.2 **Elution solution –** Add 0.1 g sodium polyphosphate, 0.1 mL Tween® 80 solution, 0.01 mL Y-30 antifoam to 1 L reagent-grade water. Filter sterilize using a 0.22 µm filter. Store at room temperature. Use within 1 week.

8.0 Quality Control

8.1 Each laboratory that uses Method 1642 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 17.4.

8.2 The minimum analytical QC requirements for the analysis of samples using Method 1642 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 (phi-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 (Section 8.7) checks. IPR analyses are performed as follows:

8.3.1 Prepare four, 2 L samples of PBS and spike each sample with laboratory-prepared phi-X174 and MS2 coliphage suspensions according to Section 13.4.3. Filter and process each IPR sample according to the procedures in Section 11 and calculate the number of male-specific and somatic coliphage per sample (PFU/L) 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 MS2 and phi-X174 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 (%)	OPR [b] Recovery (%)
Method 1642 (2 L)	Somatic (Phi-X174)	68 – 397%	28%	59 – 406%
	Male-specific (MS2)	Detect – 100%	31%	Detect – 100%

[a] Initial precision and recovery
[b] Ongoing precision and recovery

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 each week that samples are analyzed. OPR analysis is performed as follows:

8.4.1 Prepare one, 2 L reference matrix sample as indicated in Section 8.3.1. Filter and process the OPR sample according to the procedures in Section 11 and calculate the number of somatic and male-specific coliphage per sample (PFU/L) according to Section 12.

8.4.2 Calculate the percent recovery (R) for the OPR sample using the appropriate equations in Section 13.5

8.4.3 Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 1, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, reagents, and controls; correct the problem and repeat OPR analysis.

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 1642 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 recreational water and wastewater effluent 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 (Section 8.7) checks. MS analysis is performed as follows:

8.5.1 Prepare two, 2 L field samples that were sequentially collected from the same site or different aliquots of the same field sample. Spike one of the samples with laboratory-prepared phi-X174 and MS2 coliphage suspensions according to Section 13.4.3. The other sample will remain unspiked. Filter the two, 2 L field samples (See Section 11.3). The unspiked 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 sample will serve as the MS sample

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

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

8.5.4 Results for MS samples should be charted to monitor matrix effects on method recovery to help recognize inconsistent or sporadic matrix effects from specific sources.

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

Table 2. Matrix Spike Precision and Recovery Acceptance Criteria

Method	Matrix	Phage	MS/MSD [a] Recovery (%)	MS/MSD [a] RPD [b]
Method 1642 (2 L)	Fresh Water	Somatic (Phi-X174)	Detect – 450%	59
		Male-specific (MS2)	Detect – 152%	130
	Marine Water	Somatic (Phi-X174)	66 – 303%	55
		Male-specific (MS2)	9 – 100%	53
	Advanced Treatment Wastewater Effluent	Somatic (Phi-X174)	Detect – 388%	84
		Male-specific (MS2)	10 – 100%	87

[a] Matrix spike/matrix spike duplicate
[b] Relative percent difference

8.5.6 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 1642. 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 (2 L 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

10.1 Sampling procedures are briefly described below. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these procedures should not be analyzed.

10.2 Sample Collection

Collect a minimum of 2 L per sample for concentration by UF for the analyses of the two coliphage types (male-specific and somatic).

10.3 Sampling Techniques

Fresh and Marine Waters

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge or bank adjacent to surface water. The sampling depth for surface water samples should be 6 - 12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to provide head space for proper mixing before analyses. Note sample location (GPS coordinates or closest street intersections), date and time that the sample was collected, sampling conditions, and anything unusual at sampling location on the data reporting form.

Wastewater (Advanced Treatment Wastewater Effluents)

Collect a 2 L grab bulk wastewater effluent sample. When samples such as chlorinated wastewaters are collected, the sample container must contain a dechlorinating agent (e.g., 2 mL of a 10% sodium thiosulfate solution per 2 L sample). ***Note***: Each wastewater treatment facility may have different sampling locations and sampling procedures (e.g., sampling port, bucket sampling) in place.

10.4 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.

10.5 Process and analyze samples as soon as possible after collection.

11.0 Single Agar Layer (SAL) Procedure for Sample Analysis

(Procedures in Section 11 are summarized in Figures 1-3, and 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 to 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°C ± 1.0°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°C ± 1°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 bacteria stock 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 bacterial host.

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°C ± 1.0°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 6.3.14), 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°C ± 1°C to slow replication until ready to add to the samples (Section 11.6). It is recommended that the log-phase cultures be used immediately (within 6 hours).

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

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 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 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 Ultrafiltration

Note: For IPR, OPR and MS samples spike the 2 L bulk sample prior to UF according Section 13.4.3.

11.3.1 One hollow-fiber ultrafilter (Section 6.2.2) will be used for each sample. Label each filter with the corresponding sample identifier.

11.3.2 Secure the first filter to a ring stand with clamp with the inlet at the top and the outlet at the bottom. **Figure 1** shows an ultrafiltration set up using a hollow-fiber ultrafilter (Section 6.2.2), where the orange capped end is inlet and the blue is outlet.

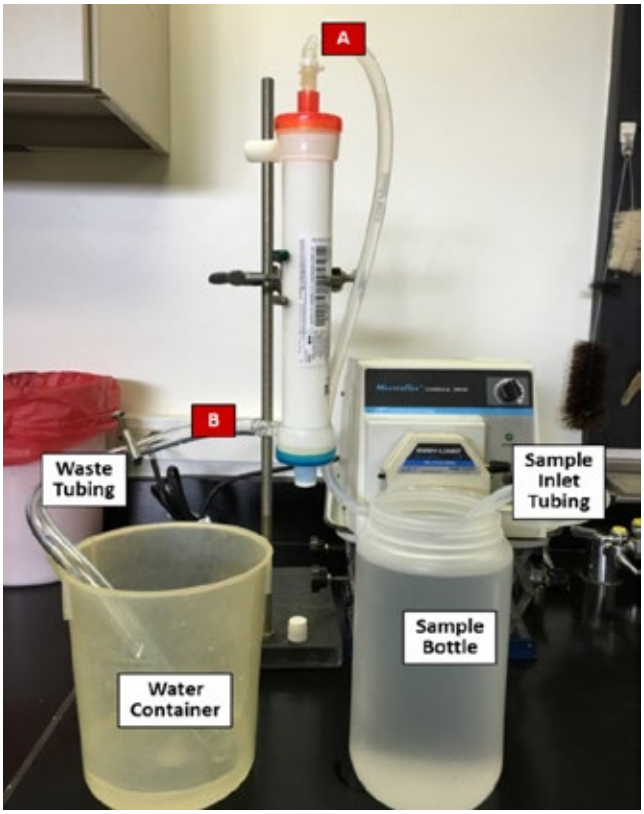


Figure 1: Ultrafiltration Set Up

11.3.3 Remove the side outlet port cap (B, Figure 1) at the lower end of the filter unit (***Note***: On the Rexeed 15S shown, this is not the blue bottom cap, but the one on the side). Attach 2 feet of vinyl tubing (e.g., EW-06405-18) to the side port and secure with a clamp, and run the other end of the tubing into the waste container. This corresponds to the waste tubing going into the waste container in Figure 1, which can be any clean beaker 2 L or larger.

11.3.4 Slide a plastic luer lock onto 3 feet of sterile L/S 24 tubing. Remove the cap at the top, inlet end of the ultrafilter (A) and connect the sterile L/S 24 tubing via the luer lock to (A), tightening only with your hand (screwing down too hard may cause the filter unit to break) and secure the connection with a clamp. This is the sample inlet tubing in Figure 1. ***Note***: The sample tubing should be attached to the shaft of the ultrafilter to prevent it from moving and potentially loosening the connection at (A).

11.3.5 Run the sample inlet tubing through the peristaltic pump and lock the tubing in place (on the Masterflex L/S shown in Figure 1, the pump lever should be turned clockwise from left to right).

Note: The peristaltic pump should be set up on the jiffy jack to prevent leaks.

11.3.6 Prepare a 10 mL pipet by breaking off the tip without removing the plastic cover. Remove the cotton. Then attach pipet to the sample inlet tubing, which will go into the sample bottle (Figure 1), which should be a sterile, wide-mouth, polypropylene 2-L bottle or carboy.

11.3.7 Ensure that the pump is set to pump the water from the sample bottle to the ultrafilter.

11.3.8 Turn the pump on and set to the appropriate rpm (e.g., 300 rpm) for a flow rate of approximately 0.5 – 0.8 L/minute. The flow rate setting may vary based on the pump. Ensure that the flow rate results in the sample moving through the tubing at a constant rate (e.g., not pulsating).

11.3.9 Begin filtering the sample; when 2 mL of sample is left, add 50 mL of sterile PBS and continue filtering. To prevent air from entering the filter, stop filtering before the entire 50 mL of PBS is gone.

11.4 Elution

11.4.1 With the pump stopped, slowly remove the L/S 36 tubing from the waste discharge (B) and replace the cover on the filter side port. See **Figure 2** and the Elution Flow Diagram (**Figure 3**) for set up.

11.4.2 Pour 200 mL of elution solution (Section 7.8.2) into an 800 mL sterile beaker (Elution Beaker, Figure 2).

11.4.3 Attach the luer lock to port B, and then attach 1 foot of sterile L/S 24 tubing to the outlet at the bottom end of the filter (C, Figure 2) for elution. Secure connection of tubing with a clamp. This is the elution tubing in Figure 2. Place the other end of the elution tubing into the elution beaker.

11.4.4 Take the sample inlet tubing out of the sample bottle and place it into the elution beaker.

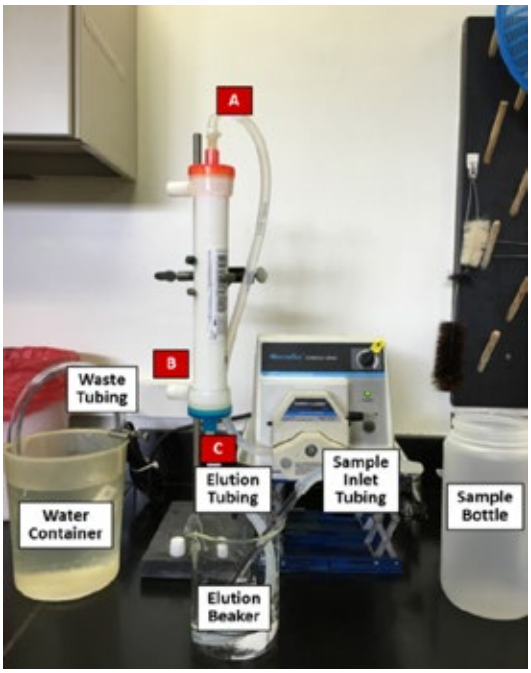


Figure 2. Elution Set Up

11.4.5 Reverse the pump; it should now be set to pump the elution solution clockwise through the filter assembly, i.e., from the elution beaker to (C) to (A). Ensure both pieces of tubing are in the elution buffer and then cover the beaker with aluminum foil to prevent any aerosolization of the solution.

11.4.6 Turn the pump on and set to the appropriate rpm (e.g., 300 rpm) for a flow rate of approximately 0.5 – 0.8 L/minute. The flow rate setting may vary based on the pump. Ensure that the flow rate results in the sample moving through the tubing at a constant rate (e.g., not pulsating). Recirculate the elution solution for one cycle of clockwise flow (one minute), then stop the pump, reverse the flow, and circulate the solution counterclockwise for one cycle (one minute).

11.4.7 Push as much solution through the tubing and into the beaker as possible.

11.4.8 Measure and record the final volumes.

11.4.9 Dispense two, 100 mL aliquots of the concentrated sample into separate, sterile Erlenmeyer flasks 250 mL − 500 mL in capacity; if there is less than 200 mL of sample, divide the volume into two equal aliquots and record the volumes. At this time, also dispense 100 mL of reagent-grade water into a third 250 to 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 control flask."

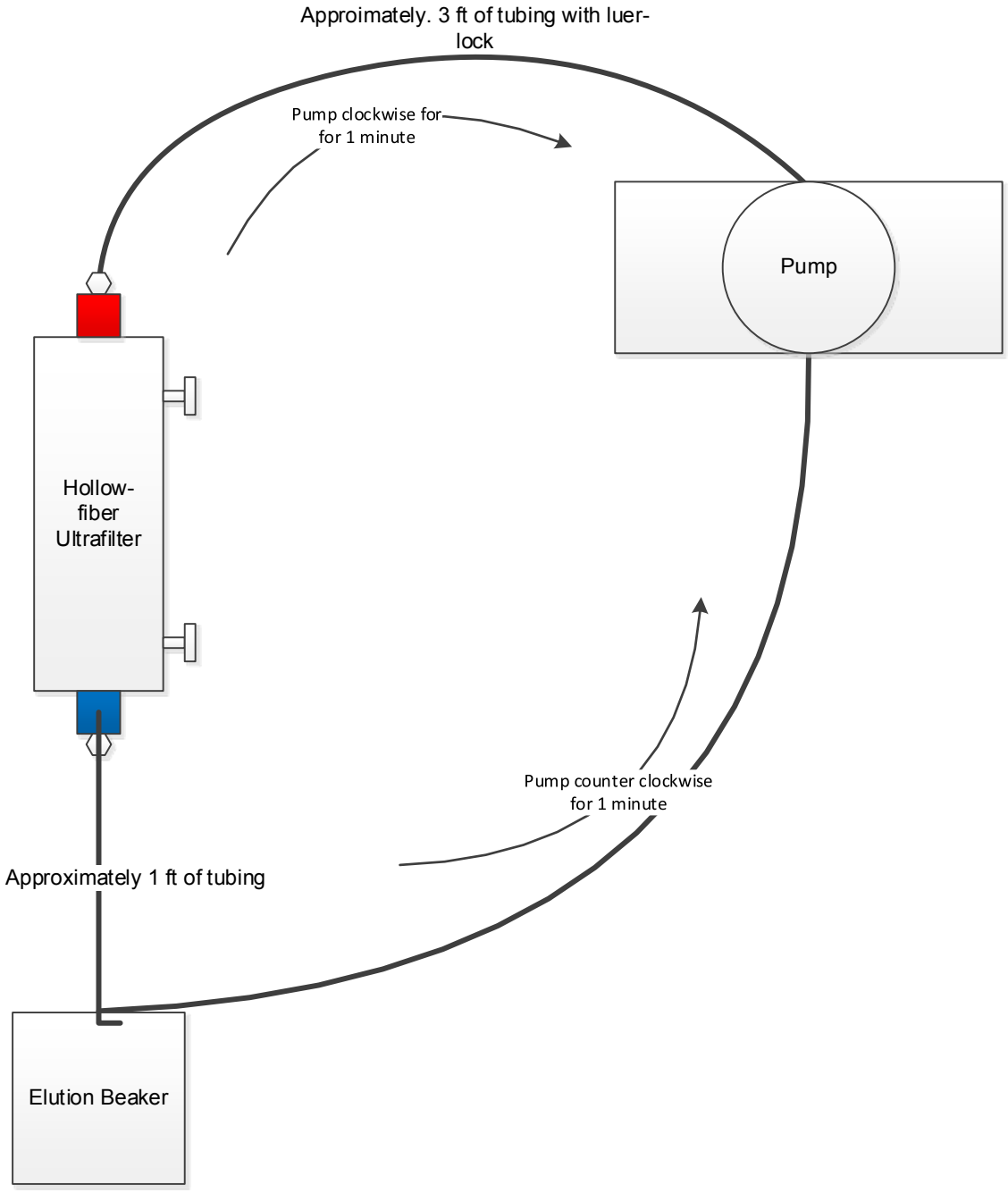


Figure 3. Elution Flow Diagram

11.5 Range-finding (Optional)

Range-finding analyses should be conducted when the laboratory receives matrix samples (e.g., fresh water, advanced treatment wastewater) from a source (e.g., lake, wastewater treatment plant) which they have not previously analyzed samples. Range-finding results will help determine if a 2 L sample (1 L per coliphage type) is appropriate.

11.5.1 Collect a 2 L sample and concentrate according to Section 11.3. Split the final volume into two, 100 mL aliquots

11.5.2 Analyze one unspiked 100 mL aliquot for somatic coliphage and the other unspiked 100 mL aliquot for male-specific coliphage according to Section 11.6. If plates are not countable, then a smaller volume (e.g., 100 mL) may need to be analyzed due to high ambient concentrations of coliphage. For analysis of volumes less than 2 L (e.g., 100 mL) refer to EPA Method 1643.

11.6 Single Agar Layer (SAL)

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

11.6.1 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.

11.6.2 Dispense two, 100-mL aliquots of sample into separate, sterile, 250 − 500 mL Erlenmeyer flasks.

11.6.3 Dispense 100 mL of reagent water into a third 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 control flask").

11.6.4 Add 0.5 mL of sterile stock $MgCl_2$ (Section 7.1.3) to each sample flask except the temperature control flask.

11.6.5 Place the flasks into the 36°C ± 1.0°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.

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

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

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

11.6.9 Immediately transfer temperature control flask and flasks containing samples and log-phase bacteria to the 45°C − 46°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°C ± 1.0°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.

11.6.10 Add the sample/*E. coli* CN-13 mixture (Section 11.6.9) to the 100 mL of 2X TSB with agar and nalidixic acid (Section 11.2.5) in the 45°C − 46°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.

11.6.11 Add the sample/*E. coli* F_{amp} mixture (Section 11.6.9) to the 100 mL of 2X TSB with agar and strep/amp (Section 11.2.5) in the 45°C − 46°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.

11.6.12 Allow the agar to solidify, cover, invert, and incubate for 16 − 24 hours at 36°C ± 1.0°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.

11.6.13 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 4** and **5** for somatic and male-specific coliphage plaques, respectively. Count and sum plaques for all plates from a single sample, record results, and proceed to Section 12 for calculation of PFU/L. ***Note:*** The use of a light box (Section 6.1.12) to evaluate sample results is recommended.

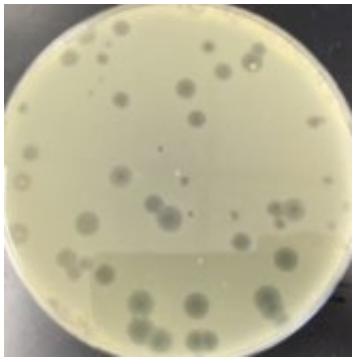


Figure 4: Somatic Coliphage Plaques (CN-13)

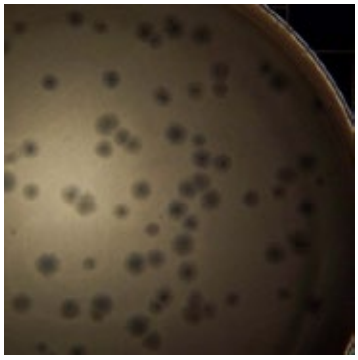


Figure 5: Male-specific Coliphage Plaques (F_{amp})

12.0 Data Analysis and Calculations

Use the following general rules to calculate the coliphage count per 1 L 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.

$$\text{Coliphage / 1 L} = Plt_1 + Plt_2 + Plt_3 + Plt_4 + Plt_5$$

12.2 Report results as PFU per 1 L of sample.

13.0 Sample Spiking Procedure

13.1 Method 1642 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}.

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 to 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 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* F_{amp} or *E. coli* CN-13, 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* F_{amp}" or "undiluted *E. coli* CN-13", 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 2 L 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 2 L sample prior to ultrafiltration. ***Note***: 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 "too numerous to count" (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:

$$\text{Phage}_{\text{Undiluted spike}} = (PFU_1 + PFU_2 \ldots PFU_n)/(V_1 + V_2 \ldots. V_n)$$

Where:

$\text{Phage}_{\text{Undiluted spike}}$ = Phage (PFU/mL) in undiluted spiking suspension

PFU = number of plaque forming units from plates yielding counts within the ideal range of 1 to100 (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 to100 (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

PFU/plate (duplicate analyses) [a]				Phage PFU/mL in undiluted spiking suspension (Phage $_{\text{undiluted spike}}$)
Undiluted	**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 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 2 L sample

B = Four, 2 L 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 spiked into the 8 L bulk sample. The 8 L bulk sample would be mixed and four, 2 L aliquots dispensed. Each 2 L sample should contain approximately 100 PFU. ***Note***: It may not be possible to spike an 8 L bulk sample

due to potential mixing and dispensing difficulties. In that case each 2 L sample may be spiked individually.

13.5.7 Calculate "True" Spiked Phage (PFU/2 L)

Calculate the true concentration of spiked phage (PFU/2 L) according to the following equation. Example calculation is provided in **Table 4**.

$$T_{\text{Spiked Phage}} = (\text{Phage}_{\text{undiluted spike}}) \times (V_{\text{spiked per 2 L sample}})$$

Where,

$T_{\text{Spiked Phage}}$	=	Number of spiked Phage (PFU/2 L)
$\text{Phage}_{\text{undiluted spike}}$	=	Phage (PFU/mL) in undiluted spiking suspension
$V_{\text{spiked per 100 mL sample}}$	=	mL of undiluted spiking suspension per 2 L sample

Table 4. Example Calculation of Spiked Phage

$\textit{Phage}_{\text{undiluted spike}}$	$V_{\text{spiked per mL sample}}$	$T_{\text{Spiked Phage}}$
714 PFU/mL	1.4×10^{-1} mL/ 2 L of sample	(714 PFU/mL) × (1.4×10^{-1}mL/ 2 L) = 100 PFU/ 2 L

Note: Because the final volume is split between both phage types the "true" spike value is 50 PFU/L based on the example provided in Table 4.

13.5.8 Calculate Percent Recovery

Calculate percent recovery (R) using the following equation.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R	=	Percent recovery
N_s	=	Phage (PFU/L) in the spiked sample
N_u	=	Phage (PFU/L) in the unspiked sample
$T_{\text{Spiked Phage}}$	=	True spiked Phage (PFU/L) in spiked sample

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

Table 5. Example Percent Recovery Calculation

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

14.0 Method Performance

Seventeen volunteer laboratories participated in the EPA MLV study of EPA Method 1642 in fresh and marine waters and advanced treatment wastewater effluents (e.g., secondary with disinfection, tertiary). The purposes of the study were to characterize method performance across multiple laboratories and multiple matrices and to develop QC acceptance criteria. A detailed description of the study and results are provided in the validation study report (Reference 17.5). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with Method 1642 and study-specific instructions. Method performance summary data are provided below.

14.1 Fresh Water (Nine of the seventeen laboratories analyzed fresh water matrices.)

14.1.1 Recovery

14.1.1.1 Somatic − Mean laboratory-specific recoveries of phi-X174 phage from fresh water samples spiked with referee- or laboratory- prepared somatic phage suspensions ranged from 50% to 323%, with an overall mean recovery of 195%.

14.1.1.2 Male-specific − Mean laboratory-specific recoveries, of MS2 from fresh water samples spiked with referee- or laboratory- prepared MS2 phage suspensions ranging from 15% to 106%, with an overall mean recovery of 51%.

14.1.2 Precision

14.1.2.1 Somatic − Method 1642 was characterized by laboratory-specific relative standard deviations (RSDs) from fresh water samples spiked with referee- or laboratory- prepared phi-X174 phage suspensions ranging from 7% to 30%, with an overall pooled, within-laboratory RSD of 19%.

14.1.2.2 Male-specific − Method 1642 was characterized by laboratory-specific relative standard deviations (RSDs) from fresh water samples spiked with referee- or laboratory- prepared MS2 phage suspensions ranging from 3% to 57%, with an overall pooled, within-laboratory RSD of 30%.

14.2 Marine Water (Ten of the seventeen laboratories analyzed marine water matrices.)

14.2.1 Recovery

14.2.1.1 Somatic − Mean laboratory-specific recoveries of phX174 phage from marine water samples spiked with referee-prepared somatic phage suspensions ranged from 105% to 401%, with an overall mean recovery of 215%.

14.2.1.2 Male-specific − Mean laboratory-specific recoveries, of MS2 from marine water samples spiked with referee-prepared MS2 phage suspensions ranging from 9% to 32%, with an overall mean recovery of 21%.

14.2.2 Precision

14.2.2.1 Somatic − Method 1642 was characterized by laboratory-specific RSDs from marine water samples spiked with referee-prepared phi-X174 phage suspensions ranging from 5% to 30%, with an overall pooled, within-laboratory RSD of 17%.

14.2.2.2 Male-specific − Method 1642 was characterized by laboratory-specific RSDs from marine water samples spiked with referee-prepared MS2 phage suspensions ranging from 10% to 28%, with an overall pooled, within-laboratory RSD of 18%.

14.3 Advanced Treatment Wastewater Effluent (Ten of the seventeen laboratories analyzed advanced treatment wastewater effluent matrices.)

14.3.1 Recovery

14.3.1.1 Somatic − Mean laboratory-specific recoveries of phi-X174 phage from advanced treatment wastewater effluent samples spiked with referee- or laboratory- prepared phi-X174 phage suspensions ranged from 116% to 331%, with an overall mean recovery of 197%.

14.3.1.2 Male-specific − Mean laboratory-specific recoveries, of MS2 from advanced treatment wastewater effluent samples spiked with referee- or laboratory-prepared MS2 phage suspensions ranging from 20% to 97%, with an overall mean recovery of 40%.

14.3.2 Precision

14.3.2.1 Somatic − Method 1642 was characterized by laboratory-specific RSDs from advanced treatment wastewater effluent samples spiked with referee- or laboratory- prepared phi-X174 phage suspensions ranging from 9% to 50%, with an overall pooled, within-laboratory RSD of 24%.

14.3.2.2 Male-specific − Method 1642 was characterized by laboratory-specific RSDs from advanced treatment wastewater effluent samples spiked with referee- or laboratory- prepared MS2 phage suspensions ranging from 6% to 39%, with an overall pooled, within-laboratory RSD of 25%.

14.4 PBS (Fourteen of the seventeen laboratories analyzed PBS samples.)

14.4.1 Recovery

14.4.1.1 Somatic − Mean laboratory-specific recoveries of somatic phage from PBS samples spiked with referee-prepared phi-X174 phage suspensions ranged from 43% to 3573%, with an overall mean recovery of 495%. The overall mean recovery was heavily influenced by the results from one laboratory (mean recovery 3573%). Mean recoveries for individual laboratories without including that laboratory ranged from 43% to 343%, with an overall mean of 215%.

14.4.1.2 Male-specific − Mean laboratory-specific recoveries, of MS2 from PBS samples spiked with referee-prepared MS2 phage suspensions ranged from 5% to 131%, with an overall mean recovery of 31%.

14.4.2 Precision

14.4.2.1 Somatic − Method 1642 was characterized by laboratory-specific RSDs from PBS samples spiked with referee-prepared phi-X174 phage suspensions, ranged from 5% to 141%, with an overall pooled, within-laboratory RSD of 44%. The precision and accuracy were heavily influenced by the results from one laboratory. The RSDs for the individual laboratories without including that laboratory ranged from 5% to 30%, with an overall pooled, within-laboratory RSD of 18%.

14.4.2.2 Male-specific − Method 1642 was characterized by laboratory-specific RSDs from PBS samples spiked with referee-prepared MS2 phage suspensions, ranged from 3% to 39%, with an overall pooled, within-laboratory RSD of 20%.

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 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 USEPA. 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 (April 2001).

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

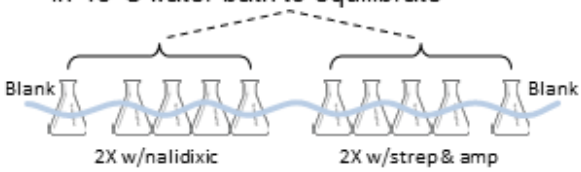
17.4 Bordner, R., J.A. Winter, and P.V. Scarpino (eds.). 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA-600/8-78-017. Office of Research and Development. USEPA.

17.5 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.

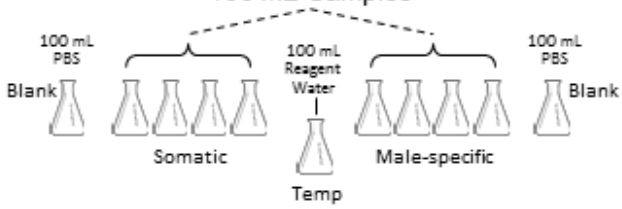
18.0 Flow Charts

Single Agar Layer

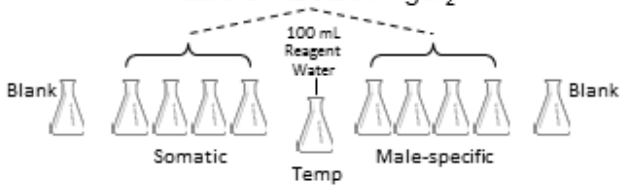
1 Place 2X TSB with agar and antibiotics in 46°C water bath to equilibrate



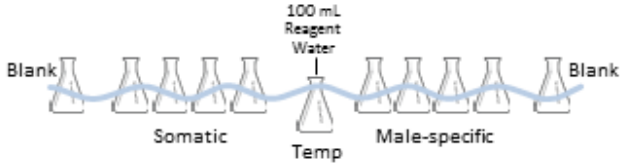
2a 100 mL Samples



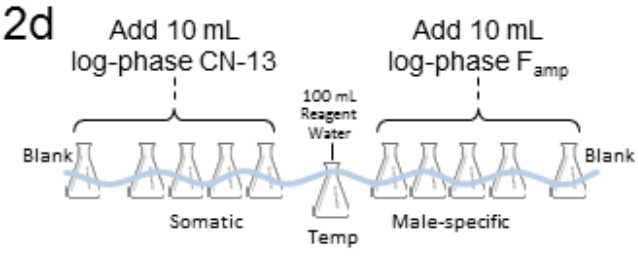
2b Add 0.5 mL stock $MgCl_2$



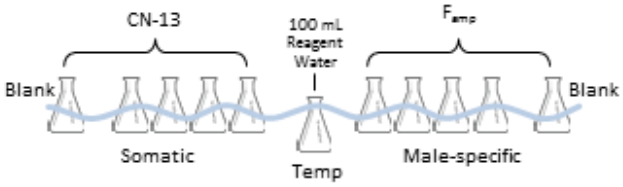
2c Place in 36°C water bath for 5 minutes



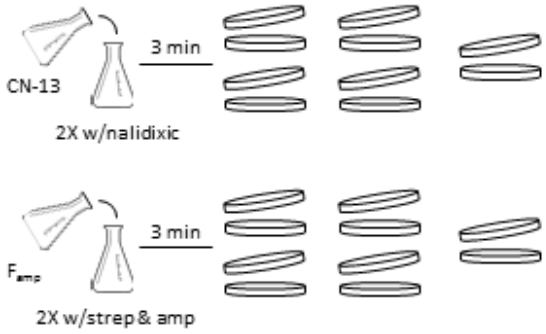
2d Add 10 mL log-phase CN-13 / Add 10 mL log-phase F_{amp}



2e Transfer to 46°C water bath

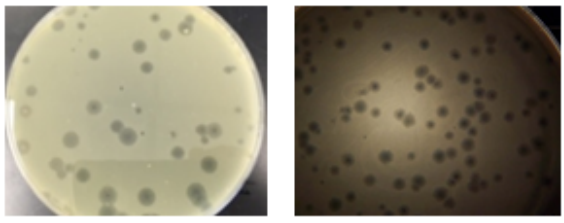


3 Add samples and blanks to the 2X TSB with agar from Step 1, swirl, and pour plates



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

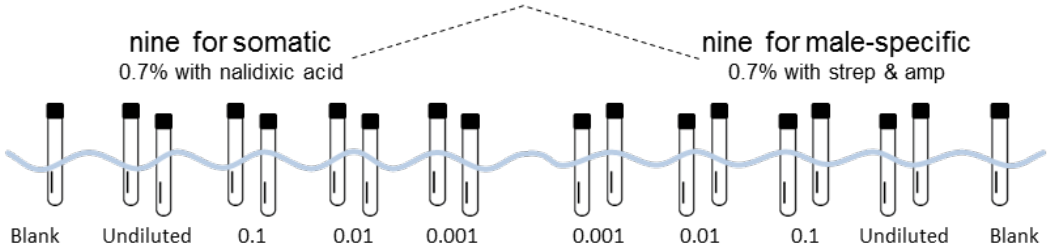
4 After incubation, count plaques, and record results



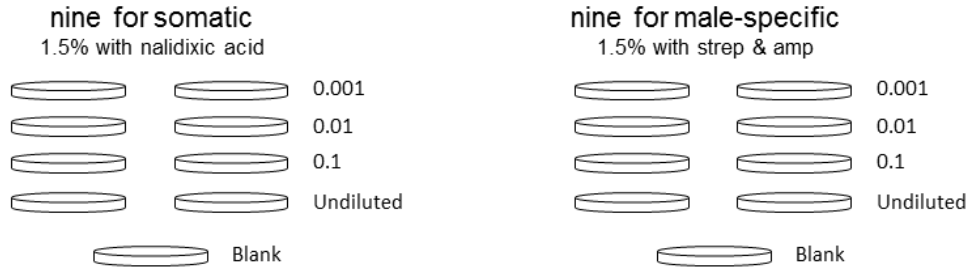
CN-13 (somatic) | **F_{amp} (male-specific)**

Double Agar Layer

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



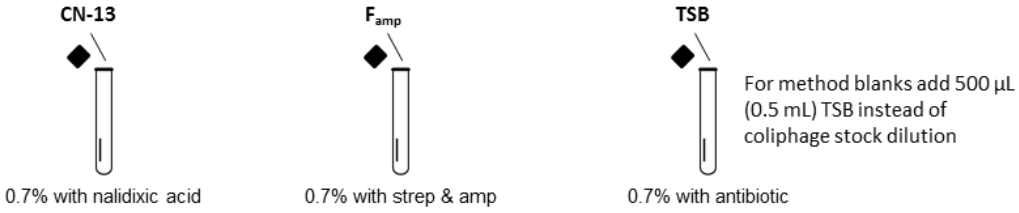
2 Label eighteen corresponding 1.5% bottom agar plates containing antibiotics



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

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

Immediately, add 500 µL (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