

STANDARD OPERATING PROCEDURE Procedure for the Detection and Enumeration of Male-Specific (F+) Coliphage Using Double Agar Layer Technique (DAL)

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STANDARD OPERATING PROCEDURE
Procedure for the Detection and Enumeration of Male-Specific (F+)
Coliphage Using Double Agar Layer Technique (DAL)

BACKGROUND

The Great Ships Initiative (GSI) is a regional effort devoted to ending the problem of ship-mediated invasive species in the Great Lakes-St. Lawrence Seaway System and globally. In support of that goal, the GSI has established superlative freshwater ballast treatment evaluation capabilities at three scales—bench, land-based, and on board ship. Each scale is dedicated to addressing specific evaluation objectives. These include:

GSI Bench-Scale Tests

- Range finding for effective doses under a range of ambient conditions;
- Chemical degradation over time under a range of ambient conditions;
- Detection of any residual toxicity under a range of ambient conditions; and
- Confirmation of treatment process.

GSI Land-Based Tests

- Detection of scale-up, mechanical operation issues;
- Effectiveness of a dose with respect to the full range of ambient organisms; and
- Detection of any whole water effluent toxicity.

GSI Shipboard Tests

- Confirmation of biological and operational performance as expected in the ship environment; and
- Confirmation of performance as expected under a broad range of ambient conditions.

The GSI awards its independent status-testing services to developers of ballast treatment systems and processes determined to be promising. GSI status-testing is performed at the scale appropriate to the state of development of the target treatment system, with the goal of facilitating the rapid progression of meritorious ballast treatment systems through the research and development and approval processes to a market-ready condition.

GSI has no involvement, intellectual or financial, in the mechanics, design or market success of the actual treatment systems it tests. To ensure that GSI tests are uncompromised by any real or perceived individual or team bias relative to test outcomes, GSI test activities are subject to rigorous quality assurance/quality control (QAQC) procedures and documentation. This attention to QAQC assures high quality and credible evaluation of GSI and its findings.

INTRODUCTION

This GSI Standard Operating Procedure (SOP) describes the procedure used to detect and enumerate male specific (F+) coliphage in a water sample. The objective of this method is to determine the effectiveness of a potential ballast water treatment on bacteriophages, using the bacteriophage MS2 as a model test organism. This method is a subset of USEPA method 1601 (U.S. Environmental Protection Agency, 2001), which has been adapted by GSI as a quantitative method for the testing of potential ballast water treatment systems at the bench-scale. It involves enumerating MS2 (F-specific) coliphage from MS2 spiked, control and treated test waters. Log-phase host *E. coli* F_{amp} (see figure 1) is added to soft agar with 500 µL of the sample and plated using the double agar layer (DAL) procedure. After incubation, zones of lysis, which indicate the presence of coliphage, are counted to determine system efficacy.

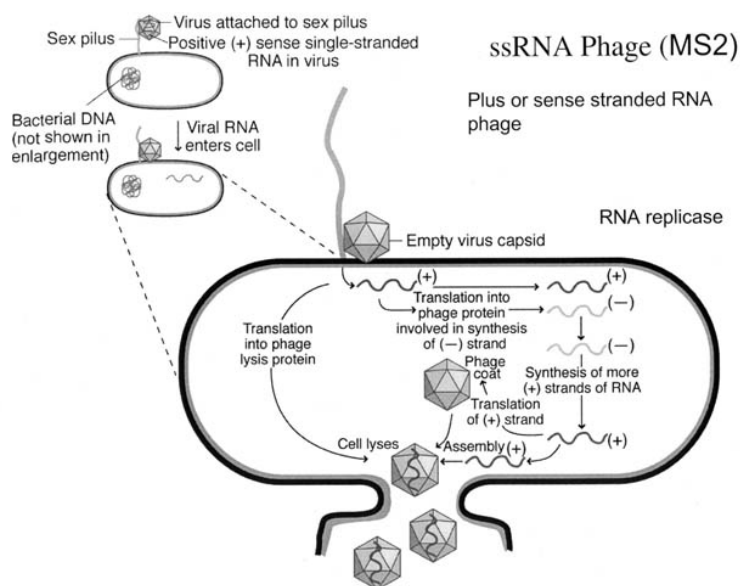


Figure 1. Replication diagram of bacteriophage MS2 infecting the host bacterium *E. coli* F_{amp}.
Accessed from: <http://faculty.washington.edu/jclara/301/images/ssRNA.jpg>, May 2010.

DEFINITIONS

Bacteriophage MS2: An icosahedral bacteriophage with a diameter of 27-34 nm and an isoelectric point (pI) of 3.9. MS2 phage can be propagated in *Escherichia coli*, commonly *E. coli* ATCC 15597.¹

Coliphage (Bacteriophage): A virus that infects *E. coli* and is an indicator of fecal contamination.

¹ Accessed from: http://en.wikipedia.org/wiki/Bacteriophage_MS2, May 2010.

F-Factor: The fertility factor in certain strains of *E. coli*. It is a plasmid that when present, codes for pilus formation. The pilus allows for transfer of nucleic acid from one bacterium to another.

Host Bacteria: Those bacteria that allow the bacteriophage to penetrate and replicate within them, ultimately lysing and resulting in the release of the progeny bacteriophage. Host bacteria are essential for virus replication. The host used in this method is *E. coli* F_{amp} (*E. coli* HS(pFamp)R).

Lysis Zone: Typically, a circular zone of clearing indicating a sample is positive for coliphages, also known as a plaque.

Male-Specific (F⁺) Coliphage: A RNA or DNA virus that infects via the F-pilus of male strains of *E. coli*.

Plaque: Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria on double-agar-layer plates after incubation.

Plaque-Forming Units (PFU): A measure of the number of particles capable of forming [plaques](#) per unit volume, such as [virus](#) particles. It is a functional measurement rather than a measurement of the absolute quantity of particles: viral particles that are defective or which fail to infect their target cell will not produce a plaque and thus will not be counted. For example, a solution of [Tick-borne encephalitis virus](#) with a concentration of 1,000 PFU/[μL](#) indicates that there are 1,000 infectious virus particles in one microliter of solution.²

EQUIPMENT LIST

- Sterile dilution tubes with caps (16 x 100mm) and test tube rack to accommodate.
- Vortex mixer.
- Sterile inoculation loops at least 3 mm in diameter or 10 μL volume.
- Sterile disposable Petri dishes.
- 125 mL sterile flasks with slip caps or plugs.
- Beakers and/or graduated cylinders for preparing media.
- Pipettors and associated sterile, aerosol resistant, pipette tips capable of delivering 0.1 mL to 10 mL volumes, or appropriate volumes, for dilutions.
- Incubator at 36 °C ± 1.0 °C.
- Balance.
- Spatula.
- Weighing dishes.
- Light box or equivalent.
- Water bath capable of maintaining 36 °C ± 1.0 °C and 45 °C to 48 °C and capable of shaking 100-150 rpm.

² Accessed from: http://en.wikipedia.org/wiki/Plaque_forming_units, May 2010.

- Spectrophotometer capable of holding 13 x 100 mm cuvettes or equivalent.
- Cuvettes-1 cm light path.
- KimWipes or lint-free tissues.
- Autoclave.
- Thermometer-Range of 0 °C to 100 °C.
- Bunsen burner.
- 1:100 household bleach solution or 70 % ethanol.
- Gloves.
- Goggles.
- Lab coat.
- Prepared labels or labeling tape and permanent marker.
- Prepared datasheets or laboratory notebook.

MEDIA AND REAGENT PREPARATION

Reagent Water

1. Obtain Milli-pore® water from Barstow-3 or equivalent.

10 % (w/v) Sodium Thiosulfate

1. Add 10 g $\text{Na}_2\text{S}_2\text{O}_3$ to 90 mL reagent water.
2. Mix well to dissolve.
3. Bring volume to 100 mL with reagent water.
4. Autoclave 15 min at 121 °C and 15 psi.

50 % Glycerol (e.g., Sigma G6279)

1. In a 100 mL graduated cylinder, measure 50 mL of glycerol.
2. Bring volume to 100 mL with reagent water.
3. Pour into an autoclavable 250mL bottle and mix well.
4. Autoclave 15 min at 121 °C and 15 psi.
5. Remove promptly to avoid scorching.
6. Store at room temperature. If there appears to be growth or any cloudiness, remake the solution. Note: If sterile technique is used, shelf life is more than a year.

Ampicillin/streptomycin antibiotic solution (100x, 1.5 mg/mL)

1. Dissolve 0.15 g ampicillin sodium salt (e.g., Sigma A9518) and 0.15 g streptomycin sulfate (e.g., Sigma S6501) in 100 mL reagent water.
2. Filter through 0.22 μm pore size membrane filtration assembly to sterilize.
3. Dispense 5 mL aliquots into 5 mL freezer vials.
4. Label and date.
5. Store at ≤ -70 °C for up to one year.

6. Thaw at room temperature or in $36\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ water bath and mix well prior to use.
Note: Any leftover solution may be stored at $2\text{ }^{\circ}\text{C}$ to $8\text{ }^{\circ}\text{C}$ for up to 24 hours.

Tryptic Soy Broth (TSB)

1. Prepare as specified on the bottle of media

Please note for the following media: Antibiotics must always be added after media has been autoclaved and cooled.

TSB With Ampicillin/Streptomycin Sulfate

1. Aseptically add 10 mL of ampicillin/streptomycin sulfate to 1 L of autoclaved, cooled ($<48\text{ }^{\circ}\text{C}$) TSB.
2. Mix well.
3. Store at $2\text{ }^{\circ}\text{C}$ to $8\text{ }^{\circ}\text{C}$ for up to 2 weeks.

1.5 % Tryptic Soy Agar (TSA) Plus Ampicillin/Streptomycin

Note: Used for streak plates and bottom layer of agar for double agar layer technique.

1. Prepare TSB as above and add 15 g agar/L, OR prepare Trypticase Soy Agar (e.g., Fisher BD 211043) as specified on bottle of media.
2. Stir while heating to dissolve agar.
3. Autoclave 15 min at $121\text{ }^{\circ}\text{C}$ and 15 psi.
4. Cool to $48\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$.
5. Pour plates as described in "Plate Preparation" section of *GSI-SOP-BS-RA-MP-1 General Microbiology Preparation Procedures*.
6. Plates may be dried with lids cracked open in incubator or laminar flow hood and used immediately. If not used immediately, replace lids and store plates inverted in a sealed plastic container at $4\text{ }^{\circ}\text{C}$ for up to two weeks.

0.7 % TSA Top Agar Tubes Plus Ampicillin/Streptomycin

Note: Used for top agar layer in double agar layer technique.

1. Prepare TSB as above and add 7 g agar/L.
2. Autoclave 15 min at $121\text{ }^{\circ}\text{C}$ and 15 psi.
3. Cool to $48\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$.
4. Aseptically add 10 mL ampicillin/streptomycin solution (100x, 1.5 mg/mL) per liter.
5. Swirl to mix well and dispense 5 mL of media per 10 mL sterile tube and keep at $45\text{ }^{\circ}\text{C}$ to $48\text{ }^{\circ}\text{C}$ until use.
6. Tubes must be used the day they are prepared.

Spot Plates

Notes: May be prepared for qualitative presence/absence procedure. Prepare only enough plates that will be used within 4 days. **Log phase *E. coli* F_{amp} must be prepared in advance.** See “Preparing a Log Phase Culture” section of *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures*.

1. Dissolve 30g TSB and 7.5 g agar/L reagent water.
2. Stir while heating to dissolve agar.
3. Autoclave 15 min at 121 °C and 15 psi.
4. Cool to 45 °C to 48 °C.
5. Aseptically add 20 mL of log phase *E. coli* F_{amp} and 10 mL ampicillin/streptomycin solution (100x, 1.5 mg/mL) per liter.
6. Swirl flask to mix well and dispense 20 mL medium per sterile plate with grids.
7. Plates may be used immediately or may be stored at 4 °C ± 1.0 °C for up to four days before use. *Note: If spot plates have condensation, incubate plates for approximately 10 minutes to reduce condensation prior to inoculation.*

PREPARATION

Note: Follow aseptic technique throughout procedure. Wash counter with 1:100 bleach solution and work around a Bunsen burner flame. Refer to *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures* for more on aseptic technique.

Two or More Days Before Sample Preparation and Analysis

1. Titer the MS2 coliphage (ATCC #15597-B1) stock as described in *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures*. Note: should be done monthly
2. Prepare bottom agar layer plates (1.5 % Tryptic soy agar (TSA) plus ampicillin/streptomycin).

One Day Before Sample Preparation and Analysis

1. Prepare the host Bacteria (a or b)
 - a. Prepare an overnight culture from a stock culture of *E. coli* (F_{amp})-(ATCC #700891) using TSB with ampicillin/streptomycin sulfate as the growth medium. Refer to the “**Preparing an Overnight Culture**” section of *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures*.
 - b. Prepare a streak plate from a stock culture of *E. coli* (pFamp)-(ATCC #700891) using 1.5% TSA with ampicillin/streptomycin sulfate as the growth medium. Refer to the “**Preparing a Streak Plate**” section of *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures*.

On the Day of Assay

1. Prepare 0.7 % TSA top agar tubes plus ampicillin/streptomycin as directed above. Should remain molten in 44-48 °C water bath until use.
2. Prepare a log phase culture:
 - a. Using TSB with ampicillin/streptomycin sulfate and an overnight culture or streak plate of the host bacteria, prepare a log phase culture of *E. coli* (Famp)-(ATCC #700891). Refer to “**Preparing a Log Phase Culture**” section of *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures*. Several flasks may be needed to prepare enough volume to add 100µL per sample/plate.
 - b. Once culture has reached an absorbance of 0.1-0.5, refrigerate flasks or put them on ice until ready to use (up to 48 hours, but best if used within 6 hours).
3. Sample Collection:
 - a. Follow *GSI/SOP/BS/RA/MA/2 - Procedure for Assessing Antimicrobial Activity Using Time-Kill Method* for test initiation and sample collection.
 - b. Inactivate or neutralize any active substance at the time of sample collection or when a defined exposure period has been reached (i.e., sodium thiosulfate to neutralize chlorine).
 - c. Store samples in the refrigerator or in a cooler on ice at 2 °C – 8 °C until time of analysis.
 - d. Analyze samples within 4-6 hours of collection. In exceptional circumstances, i.e., if there is a delay, store samples under the above conditions for a maximum of 24 hours before beginning enumeration analysis.
4. Enumerating coliphage using double agar layer procedure (DAL)

Note: The following number of bottom-layer plates and top agar tubes are required for this part of the assay: one for each sample per dilution, one method blank per day, one positive control per day, and one additional plate for every ten samples (minimum of 10 % of the samples are analyzed duplicate).

 - a. Clean work surface with 1:100 bleach solution or 70 % ethanol.
 - b. Label 1.5 % TSA bottom agar plates with the following information:
 - i. Sample ID, dilution, and volume used
 - ii. Bacterial host
 - iii. Coliphage type
 - iv. Date
 - c. With top agar still in water bath, aseptically inoculate a top agar tube with 100 µL of log-phase (abs=0.1-0.5) *E. coli* F_{amp}.
 - d. Immediately add 500 µL of sample to the tube. Mix the inoculum by rolling each tube briefly in the palm of hand. Try to not make bubbles.
 - e. Pour contents of tube into bottom agar plate labeled with appropriate sample ID.
 - f. Repeat steps c - e for each sample/dilution. *Note: Dilutions of samples may be done using TSB if samples are expected to have a high coliphage concentration (i.e., controls and less effective treatment concentrations). The goal is to count*

between zero and 300 PFU per plate. See section on “**Preparing a Dilution Series**” in *GSI/SOP/BS/RA/MP/1 - General Microbiology Preparation Procedures*.

- g. Prepare at least one positive control per analysis period. Typically this step can be omitted because samples will be from MS2 spiked tests and the three control replicates act as the positive control.
 - i. Add *E. coli* as in “c” above, and add 500 µl of diluted coliphage stock to yield approximately 60 pfu/mL.
 - ii. Mix and pour into bottom agar plate labeled “+Control”.
- h. Prepare at least one method blank per analysis period and media batch.
 - i. Add *E. coli* as in “c” above, but **DO NOT** add coliphage. Instead, add 500 µL of TSB.
 - ii. Mix and pour into bottom agar plate labeled “Blank”.
- i. Allow overlay to harden on all plates.
- j. Invert all plates and incubate for 16-24 hours at 36 °±1 °C.
- k. Record times in and out of incubator on prepared datasheets or in laboratory notebook.
- l. After incubation, count the number of plaques (circular zones of clearing, 1 to 10 mm in diameter) present on each plate. Use of a light box is recommended but counting plaques on dark bench top and holding plate up to light to double check for missed plaques works.
- m. Record the number of plaques on datasheets or in laboratory notebook. If there are more plaques than are in the ideal range (0 to 300 PFU per plate), or plaques are not discrete, record results as too numerous to count (TNTC).

CALCULATIONS

1. Calculate the number of plaque forming units (PFU) per mL sample
 - a. $PFU / mL = (PFU_1 + PFU_2 + \dots + PFU_n) / (V_1 + V_2 + \dots + V_n)$
 - PFU = number of plaque forming units for all countable sample dilutions (≥1PFU/per plate). Exclude zeros or TNTC plates.
 - V = volume of undiluted sample
 - N = number of usable counts

EXAMPLE CALCULATION OF CONTROL SAMPLE CONCENTRATION

Dilution	PFU/plate (for each duplicate plate)	Volume of undiluted sample (mL)
UNDILUTED	TNT, TNTC	0.5
1:10	178, 184	0.05
1:100	21, 16	0.005
1:1000	0, 3	0.0005

$$(178+184+21+16+3+0)/(0.05+0.05+0.005+0.005+0.0005+.0005) = 402/0.111 = 3621 \text{ PFU/mL}$$

Disposal

1. After all counts have been recorded, place plates in autoclave bags and autoclave for 45 minutes at 121 °C. Dispose of plates in accordance with UW-S Environmental Health and Safety procedures.
 - a. Place biohazard bag of autoclaved plates in a box lined with a garbage bag.
 - b. Label box “Sterilized Microbiology Waste-Nonpathogenic”.
 - c. Seal box with packing tape and dispose of box in dumpster.

Optional steps to Use As Needed

1. Removing interfering bacteria: If plate contains confluent bacterial growth distinct from the host lawn, and it is difficult to determine if lysis of host has occurred. Interfering bacteria may be removed, prior to analysis, by either of the following methods.
 - a. Push the sample through a sterile syringe filter (0.22 µm pore size, low protein binding) and collect the filtrate in a sterile tube.
 - b. Centrifuge an aliquot of the sample at 5,000-10,000G for 10 minutes and recover the supernatant.
2. Coliphage confirmation: Not required, but may be used to confirm questionable lysis zone.
 - a. Prepare spot plates as directed in section.
 - b. Pick lysis zone with sterile Pasteur pipette, stick, or loop and transfer to tube with 0.5 mL TSB.
 - c. Allow to stand 5 minutes at room temperature.
 - d. Vortex 5 seconds or until well mixed.
 - e. Spot 10 µL of the inoculated TSB to a spot plate. Record the position of spot.
 - f. Spot 10 µL of TSB(method blank) and 10 µL of MS2 stock (positive control) Record the position of spots.
 - g. Allow the “spots” to absorb into medium for 30 - 60minutes. Inocula must not be allowed to run across the plate
 - h. Invert plate and incubate for 16-24 hours at 36 °C.
 - i. Positive confirmation of coliphage may appear as one circular zone or as one or more small plaques within the spot. If spot lawn is indistinguishable from the background lawn, this indicates a negative result. A positive result may also appear as a zone of lysis containing small discrete colonies of phage resistant bacteria.

QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

1. Initial Demonstration of Capability (IDC)- the laboratory should demonstrate the ability to perform this procedure before analyzing samples.
 - a. Spike a one liter volume of test water with 13 PFU of male specific coliphage to achieve a target spike concentration of 1.3 PFU per sample. (See Determining Spiking Volume of Coliphage).

Example for an Initial Demonstration of Capability (IDC) test:
Target is to have 1.3 PFU per 100mL sample. Ten 100mL samples will be spiked in bulk.
A 1:1000000 or 10^{-6} dilution of the spiking suspension yields ~8.82PFU/mL.

$$S = ((1.3)(10))/(8.82\text{PFU/mL})$$

$$S = 1.47 \text{ mL}$$

So 1.47 mL of the 10^{-6} dilution of spiking suspension containing ~ 8.82 PFU/mL is spiked into the 1 L bulk sample to achieve the 1.3 PFU per 100 mL sample goal.

- b. Swirl the container to mix well and dispense 10 aliquots into individual containers.
 - c. Analyze the spiked samples as directed in the DAL procedure.
 - d. A minimum of five out of the ten samples must be positive for a successful test.
2. Conduct all QAQC procedures according to *GSI/QAQC/QAPP/BS/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Bench-Scale Tests (2010)* or *GSI/QAQC/QAPP/LB/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Land-Based Tests*.
 3. Follow all procedures outlined in this SOP. Any deviations known ahead of time must be approved by the GSI Principal Investigator and communicated to a GSI QAQC Officer
 4. At least 10 % of samples collected should be analyzed in duplicate.

DATA STORAGE AND ARCHIVING

1. Store and archive data according to *GSI/QAQC/QAPP/BS/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Bench-Scale Tests (2010)*.
2. Archive all original, raw data and records generated for a period of five years after the final report is approved.

REFERENCES AND RELATED DOCUMENTS

GSI/QAQC/QMP/1 – Great Ships Initiative Quality Management Plan (2010).

GSI/QAQC/QAPP/BS/1 - Quality Assurance Project Plan for Great Ships Initiative (GSI) Bench-Scale Tests (2010).

GSI/SOP/BS/RA/MA/2 - Procedure for Assessing Antimicrobial Activity Using Time-Kill Method.

GSI/SOP/G/RA/SC/4 – Procedure for Labeling GSI Bench Scale Samples.

GSI/SOP/BS/RA/MP/1- General Microbiology Preparation Procedures.

Great Ships Initiative website: www.greatshipsinitiative.org.

Great Ships Initiative Standard Operating Protocols: <http://www.nemw.org/GSI/protocols.htm>.

USEPA method 1601: Male –specific (F⁺) and Somatic Coliphage in Water by Two-step Enrichment Procedure, April 2001