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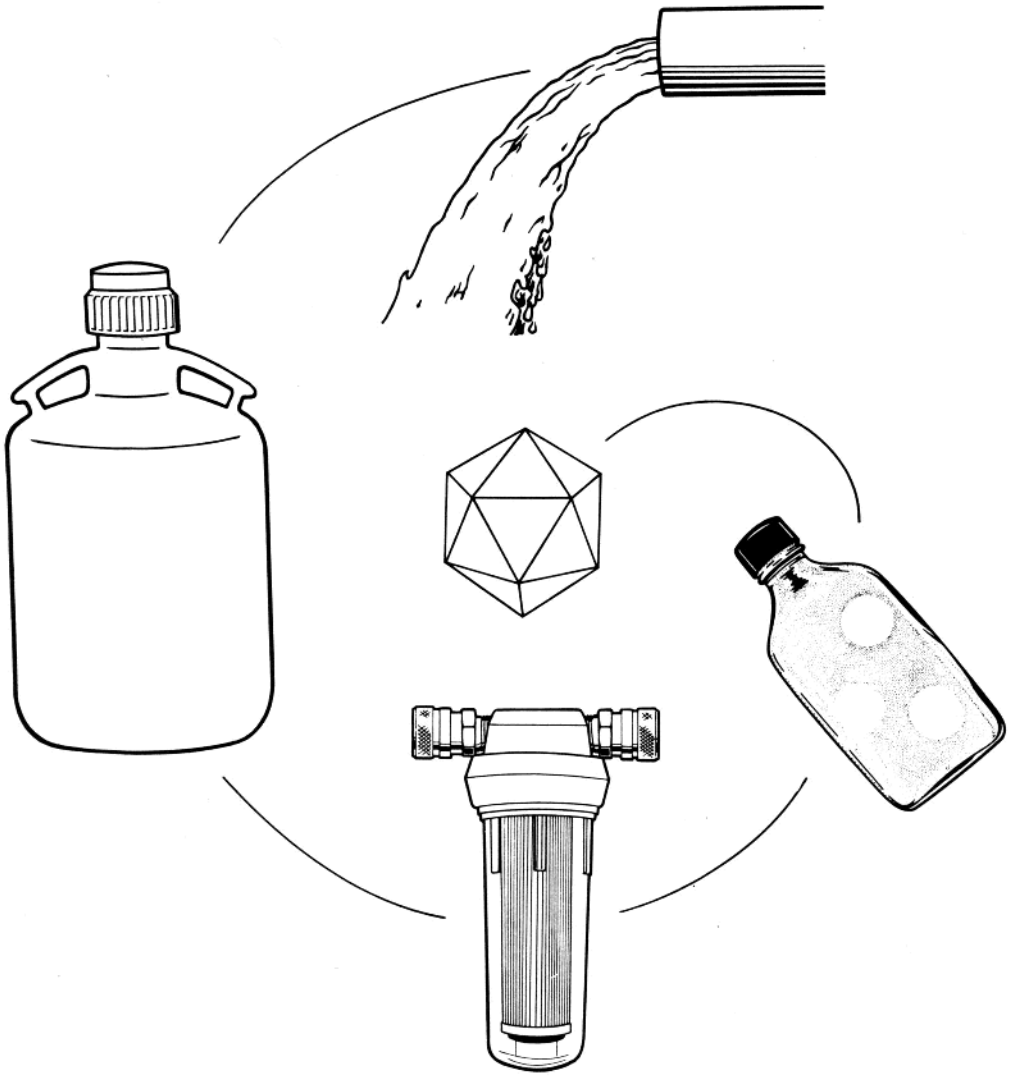

Chapter 16
PROCEDURES FOR DETECTING COLIPHAGES[1]

1. Introduction

1.1 Scope

This chapter describes procedures for the detection of coliphages in water matrices. These procedures are based on those presented in the Supplement to the 20th Edition of *Standard Methods for the Examination of Water and Wastewater* and *EPA Methods 1601 and 1602*. Two quantitative procedures and one qualitative, presence-absence procedure are presented. The procedures can be used, without supplementary methods, to assay small volumes of water (10 mL to 1L). For larger volumes (> 100 L), large-scale concentration methods such as described in Chapter 14 may be incorporated into the assay scheme. However, as some concentration procedures may result in appreciable loss or inactivation of coliphage, it is recommended that the suitability of any large volume concentration method be evaluated in measured recovery trials before implementation.

1.2 Significance

Coliphages are bacterial viruses that infect and replicate in *Escherichia coli*. They are found in human and animal feces. Coliphages are potentially important microorganisms for monitoring the microbial quality of waters because: 1) traditional bacterial monitoring does not accurately indicate the presence of nonbacterial organisms such as human pathogenic viruses, 2) human virus detection is beyond the capabilities of most water laboratories, and 3) coliphage detection is relatively inexpensive, easy to perform, and provides overnight results.

The procedures described below provide for the detection of two groups of coliphages commonly assayed for in water, the male-specific coliphages and the somatic coliphages. The male-specific coliphages infect male bacterial cells via the F-pilus, and the somatic phages infect bacterial cells via the cell surface. Male-specific coliphages have only limited replication in the environment. That is similar to human viruses which do not replicate outside the human body. The male-specific coliphages that possess an RNA genome are also similar to human enteroviruses in size, structure and resistance characteristics. Thus, male-specific RNA coliphages are promising candidate indicators of human viruses in waters. The somatic coliphages, on the other hand, are composed of a diverse group of bacterial viruses that are highly varied in size and structure and less resemble human viruses. They can replicate in the environment. While these factors make the somatic coliphages somewhat less suitable as specific indicators of human viruses in waters, they may be useful as a general indicator of water quality. Somatic coliphages are frequently found in greater abundance than male-specific RNA coliphages - an important consideration for water analyses where pollution indices are expected to be low.

1.3 Safety

Although coliphages are not pathogenic for humans, other microorganisms that are human pathogens may be present in waters impacted by untreated or inadequately treated domestic wastes. Laboratories performing virus analyses are responsible for establishing an adequate safety plan and must rigorously follow the guidelines on decontamination and waste disposal given in Chapter 2 (May 1991 Revision).

2. Apparatus, Materials, Media and Reagents

2.1 Apparatus and Materials

2.1.1 Centrifuge, 0.5-1.0 mL sample capacity, 5-10,000 x *g* performance capability

2.1.2 Cryovials, 2 mL

2.1.3 Erlenmeyer flasks, 125 mL, 250 mL and 2 L

2.1.4 Glass bottles, capped, 100 ml, 1L capacity

2.1.5 Graduated cylinders, 100 mL and 500 mL

2.1.6 Incubator, 36.5 ± 2°C

2.1.7 Inoculating loop

2.1.8 Laboratory balance

2.1.9 Pipets-Pipettors, 10 µL, 1mL, 5mL and 10 mL

2.1.10 Petri dishes, 100 x 15 mm, 150 x 15 mm

2.1.11 Filters, 0.22 µm, 0.45 µm — Note: When passing material containing phage, always pass about 10 mL of sterile 1.5% beef extract through the filter just prior to use to minimize phage adsorption to the filter.

2.1.12 Test tubes 16 × 150 mm, screw capped

2.1.13 Water bath set at 44.5 ± 1°C

2.2 Media and Reagents

[1]Prepared by F.P. Williams, R.E. Stetler and R.S. Safferman

The amount of media prepared may be increased proportionally to the number of samples to be analyzed.

2.2.1 Ampicillin solution — Dissolve 1.5 g of ampicillin in 100 mL of dH_2O and filter with the 0.22 µm filter (beef extract pretreatment not necessary). Store at 4°C. For use with male-specific coliphage assays that utilize *E. coli* Famp.

2.2.2 Beef extract — prepare buffered 1.5% beef extract by dissolving 1.5 g of beef extract powder and 0.375 g of glycine (final glycine concentration = 0.05 M) in 90 mL of dH_2O. Adjust the pH to 7.0 - 7.5, if necessary, and bring the final volume to 100 mL with dH_2O. Autoclave at 121°C for 15 min and use at room temperature. If beef extract solutions are prepared for use at a later date, store at 4°C.

2.2.3 Calcium chloride solution — Add 0.22 g of $CaCl_2$ to 50 mL of dH_2O and sterilize by autoclaving at 121°C for 15 min. Use at room temperature. *Note: divalent cations such as Ca^{++} or Mg^{++} are used to maintain phage stability and to facilitate efficient phage adsorption to host.*

2.2.4 Glycerol solution, 50% — Add equal volumes of distilled water and undiluted glycerol. Autoclave resulting 50% glycerol solution at 121°C for 15 min and use at room temperature.

2.2.5 Nalidixic acid solution — Dissolve 1.0 g of nalidixic acid sodium salt in 100 mL of dH_2O and filter with the 0.22 µm filter (beef extract pretreatment not necessary). Store at 4°C. For use with somatic coliphage assays that utilize *E. coli* CN-13. *Note: Nalidixic acid is listed as a carcinogen by the state of California. Review current Material Safety Data Sheet for appropriate handling of this chemical.*

2.2.6 Ribonuclease (RNase) solution — Dissolve 100 mg of RNase containing 50-100 Kunitz units/mg in 100 mL of dH_2O by heating to 100°C for 10 min. Store at -20°C in 0.5 mL aliquots. For use with male-specific coliphage assays.

2.2.7 Streptomycin solution — Dissolve 1.5 g of streptomycin sulfate in 100 mL of dH_2O and filter with the 0.22 µm filter (beef extract pretreatment not necessary). Store at 4°C. For use with male-specific coliphage assays that utilize *E. coli* Famp.

2.2.8 Tryptone agar slants — With gentle mixing, add 1.0 g tryptone, 0.1 g yeast extract, 0.1 g glucose, 0.8 g NaCl, 0.022 g $CaCl_2$, and 1.2 g of Bacto-agar to a total volume of 100 mL of dH_2O in a 250 mL flask. Further dissolve and sterilize by autoclaving at 121°C for 20 min. Proceed accordingly.

For somatic coliphage (using host *E. coli* C): After autoclaving, dispense 8 mL aliquots into 16 × 150 mm test tubes. Prepare slants by allowing the agar to solidify with the tubes at about a 20° angle. Slants may be stored at 4°C for up to three months.

For somatic coliphage (using host *E. coli* CN-13): After autoclaving, allow the agar to equilibrate in water bath set at 44.5 °C and then add 1.0 mL of filtered nalidixic acid solution. Dispense 8 mL aliquots into 16 × 150 mm test tubes. Prepare slants by allowing the agar to solidify with the tubes at about a 20° angle. Slants may be stored at 4°C for up to three months.

For male-specific coliphage (using host *E. coli* Famp): After autoclaving, allow the agar to equilibrate in water bath set at 44.5 °C and then add 0.1 mL of filtered ampicillin solution and 0.1 mL of filtered streptomycin solution. Dispense 8 mL aliquots into 16 × 150 mm test tubes with. Prepare slants by allowing the agar to solidify with the tubes at about a 20° angle. Slants may be stored at 4°C for up to three months.

2.2.9 Tryptone bottom agar — Prepare one day prior to sample analysis using the ingredients and concentrations listed for tryptone agar slants (Section 2.2.8), except use 1.5 g of Bacto-agar. Proceed accordingly.

For somatic coliphage (using host *E. coli* C): After autoclaving, pipet 15 mL aliquots aseptically into sterile 100 × 15 mm petri dishes and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.

For somatic coliphage (using host *E. coli* CN-13): After autoclaving, allow the agar to equilibrate in water bath set at 44.5 °C, and then add 1.0 mL of filtered nalidixic acid solution to the 100 mL volume of warm agar. Pipet 15 mL aliquots aseptically into sterile 100 × 15 mm petri dishes and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.

For male-specific coliphage (using host *E. coli* Famp): After autoclaving, allow the agar to equilibrate in water bath set at 44.5 °C, and then add 0.1 mL of filtered ampicillin solution and 0.1 mL of filtered streptomycin solution to the 100 mL volume of warm agar. Pipet 15 mL aliquots aseptically into sterile 100 × 15 mm petri dishes and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.

2.2.10 Tryptone broth — Add 1.0 g tryptone, 0.1 g yeast extract, 0.1 g glucose, 0.8 g NaCl, and 0.022 g $CaCl_2$ per each 100 mL of dH_2O. Sterilize by autoclaving at 121°C for 20 min. Proceed accordingly:

For somatic coliphage (using host *E. coli* C): After autoclaving, cool and store at 4°C.

For somatic coliphage (using host *E. coli* CN-13): After autoclaving, cool and add 1.0 mL of filtered nalidixic acid solution per 100 mL of broth. Store at 4°C.

For male-specific coliphage (using host *E. coli* Famp): After autoclaving, cool and add 0.1 mL of filtered ampicillin solution and 0.1 mL of streptomycin solution per 100 mL of broth. Store at 4°C.

2.2.11 Tryptone dilution tubes — Aseptically, dispense 9 mL aliquots of sterile tryptone broth into 16 × 150 mm screw-capped test tubes that have been

sterilized by autoclaving at 121°C for 15 min.

2.2.12 Tryptone enrichment broth — Add 10.0 g tryptone, 1.0 g yeast extract, 1.0 g glucose, 8.0 g NaCl, and 0.022 g $CaCl_2$ per each 100 mL of dH_2O. Sterilize by autoclaving at 121°C for 20 min. Proceed as described for tryptone broth in 2.2.10

2.2.13 Tryptone SAL agar — Add 2.0 g tryptone, 0.2 g yeast extract, 0.2 g glucose, 1.6 g NaCl, 0.022 g $CaCl_2$, and 1.2 g of Bacto-agar per each 100 mL of dH_2O. Sterilize by autoclaving at 121°C for 20 min.

2.3.14 Tryptone spot agar dishes — Prepare one day prior to sample analysis using the ingredients and concentrations listed for tryptone agar slants (Section 2.2.8), except use 0.75 g of Bacto-agar for each 100 mL. After autoclaving, place in water bath set at 44.5 °C and allow to equilibrate. Proceed accordingly.

For somatic coliphage (using host *E. coli* C): With gentle mixing, add 2 mL of a 4 h culture of host *E. coli* (Section 2.3.6). Pour the well mixed suspension into five sterile 100 × 15 mm petri dishes (approximately 20 mL per dish), swirl gently, and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.

For somatic coliphage (using host *E. coli* CN-13): Add 1.0 mL of filtered nalidixic acid solution to the 100 mL volume of warm agar. With gentle mixing, add 2 mL of a 4 h culture of host *E. coli* (Section 2.3.6). Pour the well mixed suspension into five sterile 100 × 15 mm petri dishes (approximately 20 mL per dish), swirl gently, and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.

For male-specific coliphage (using host *E. coli* Famp): Add 0.1 mL of filtered ampicillin solution and 0.1 mL of filtered streptomycin solution to the 100 mL volume of warm agar. With gentle mixing, add 2 mL of a 4 h culture of host *E. coli* (Section 2.3.6). Pour the well mixed suspension into five sterile 100 × 15 mm petri dishes (approximately 20 mL per dish), swirl gently, and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.

2.2.15 Tryptone top agar — Prepare the day of sample analysis using the ingredients and concentrations listed for tryptone agar slants (section 2.2.8), except use 0.7 g of Bacto-agar. Autoclave and place in the 44.5 ± 1°C water bath. *Note: nalidixic acid should be in the top agar used for somatic coliphages when host* E. coli *CN-13 is used; ampicillin and streptomycin should be in the top agar used for male-specific coliphages with host* E. coli *Famp.*

2.3 Host Bacteria and Coliphages

2.3.1 E. coli C, American Type Culture Collection (ATCC) #13706 — Bacterial host for somatic coliphages. *E. coli* CN-13 (ATCC #700609) can be used as an alternate host for somatic coliphages. CN-13 is a nalidixic acid resistant variant of *E. coli* C that appears to be comparable to *E. coli* C for coliphage detection. Use of CN-13 is indicated if indigenous bacteria in water samples interfere with the coliphage assay (using *E. coli* C). Use of CN-13 permits the addition of nalidixic acid to the assay media to suppress the interfering bacteria.

2.3.2 E.coli Famp (ATCC #700891) — Bacterial host for male-specific coliphages.

2.3.3 Coliphage ϕX174 (ATCC # 13706-B1) — Somatic coliphage to serve as positive control.

2.3.4 Coliphage MS2 (ATCC # 15597-B1) — Male-specific coliphage to serve as positive control.

2.3.5 Storage of host *E. coli* cultures: For short term storage, inoculate host culture onto tryptone agar slants with a sterile inoculating loop by spreading the inoculum evenly over entire slant surface. Incubate the culture overnight at 36.5 ± 2°C. Store at 4°C for up to two weeks. For long term storage inoculate a 5-10 mL tube of tryptone broth with the host culture. Incubate the broth culture overnight at 36.5 ± 2°C. Add 1/5th volume of 50% glycerol solution. Dispense into 1 mL aliquots in 2-mL cryovials and store at -70°C.

2.3.6 Preparation of host for coliphage assay: Inoculate 5 mL of tryptone broth with host *E. coli* from a slant using a sterile inoculating loop and incubate for 16 h at 36.5 ± 2°C. Transfer 1.5 mL of the 16 h culture to 30 mL of tryptone broth in a 125 mL flask and incubate for 4 h at 36.5 ± 2°C with gentle shaking. The amount of inoculum and broth used can be proportionally altered according to need.

2.3.7 Preparation of coliphage positive controls: Rehydrate the ATCC coliphage stock and store at 4°C. Prepare a 30 mL culture of the appropriate host (*E. coli* Famp for coliphage MS2; *E. coli* C or CN-13 for coliphage ϕX174) as described in Section *2.3.6*. Incubate culture for 2 h at 36.5 ± 2°C with shaking. Add 1 mL of rehydrated phage stock and incubate for an additional 4 h at 36.5 ± 2°C. Filter the culture through a beef extract-treated 0.22 µm filter (see 2.1.11). Prepare 10^{-7}, 10^{-8} and 10^{-9} dilutions of the filtrate using tryptone dilution tubes. (These three dilutions should be sufficient in most cases). Add 3 mL of melted tryptone top agar held in the 44.5 ± 1°C water bath to fifteen 16 × 150 mm test tubes. These test tubes should be kept in the heated water bath to avoid premature solidifying of the agar. Add 0.1 mL of the host culture to each of the 15 test tubes. Add 1 mL of the 10^{-9} dilution into each of five test tubes. Add 1 mL of the 10^{-8} dilution into five additional tubes and 1 mL of the 10^{-7} dilution into the remaining five tubes. Be sure the tubes are labeled with the appropriate dilution. For each tube, mix and immediately pour the contents over the bottom agar of a petri dish labeled with the dilution

assayed. Rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify. Invert and incubate the inoculated dishes at 36.5 ± 2°C overnight and examine for plaques the following day. Count the number of plaques on each of the 15 dishes. Five dishes from one of the assayed dilutions should yield plaque counts of 20 to 100 plaques. Average the plaque counts on these five dishes and multiply the result by the reciprocal of the dilution to obtain the titer of the undiluted stock. For use as a positive control in the coliphage assay, dilute the filtrate to 30 to 80 PFU/mL in tryptone broth. Store the original filtrate and the diluted positive control preparation at 4°C. Before using the positive control preparation for the first time, assay 10 mL by adding 1 mL volumes of the preparation to ten test tubes containing agar and host culture, and pouring their contents into ten petri dishes. Count the plaques on all dishes and divide by 10. If the result is not 30 to 80, adjust the dilution of the positive control sample and assay again.

3. Double Agar Layer (DAL) assay

This is a quantitative method that is based on the plaque assay. The procedure utilizes the traditional double agar layer technique described by Adams. The technique has been in widespread usage although the volumes assayed are typically small (0.5-1.0 mL per 100 mm assay dish). Refer to Figure 16-1.

3.1 Basic DAL Assay: Add 3 mL of melted tryptone top agar held in the 44.5 ± 1°C water bath to each of ten 16 × 150 mm test tubes for sample assay and to each of two additional test tubes that are to serve as negative and positive controls. The test tubes should be kept in the water bath to avoid premature solidifying of the agar. Add 0.1 mL of the host culture to each of the 12 test tubes. Add 1 mL of tryptone broth to the test tube serving as negative control. Add 1 mL of the appropriate coliphage preparation (30-80 PFU/mL) to the test tube serving as positive control. To each of the remaining ten test tubes, add 1mL of sample. For each tube, mix and immediately pour the contents over the bottom agar layer of a petri dish (see Section 2.2.9) that has been suitably labeled with identification information. Tilt and rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify. Invert and incubate the inoculated dishes at 36.5 ± 2°C overnight and examine for plaques the following day. Count the total number of plaques on the ten dishes receiving the sample. Calculate the coliphage concentration (C_a) in PFU per mL according to the formula:

$$C_a = (P \div 10) \times D \quad PFU/mL$$

where P is the total number of plaques from the ten dishes, D is the reciprocal of the dilution made on the inoculum before plating (D = 1 for undiluted samples). If the sample assayed is the product of a concentration procedure, such as an eluate from a large volume filter sampling of water or wastewater, then the coliphage concentration of the sampled water (C_b) can be calculated according to the formula:

$$C_b = \frac{C_a \times V_a}{V_b}$$

where C_a is the coliphage concentration of the concentrated material in PFU/mL, V_a the volume of that material in mL, and V_b is the volume of water processed in the sampling procedure in L. The resultant concentration is generally reported as PFU/L or PFU/100L. Count the plaques on the positive control dish. Maintain a record of the plaque count as a check on the virus sensitivity of the host. Assay any water samples again where the positive control counts are more than one log below their normal average. Should plaques be detected on the negative control dish, discard assay results and repeat the assay.

Note: With host E. coli *Famp, the basic DAL assay will detect both DNA and RNA male-specific coliphages as well as some somatic coliphages. For a procedure to selectively quantify the RNA male-specific coliphages, see the following section (3.2).*

3.2 DAL Assay with RNase: In this procedure an additional 10 mL of sample is assayed. The procedure is the same as that described above, except that RNase solution is added to the melted tryptone top agar in the assay of the additional 10 mL of sample. For the additional 10 tubes, the melted tryptone agar should contain RNase at a concentration of 40µg/mL. Before pouring tube contents to petri dishes, the dishes should be appropriately labeled so that the 10 dishes containing RNase are readily distinguished from the 10 dishes without RNase. Calculate the male-specific RNA coliphage (C_a) according to the formula:

$$C_a = [\,(P \div 10) - (P_R \div 10)\,] \times D \quad PFU/mL$$

where P is the total number of plaques from the 10 dishes without RNase, P_R is the total number of plaques from the 10 dishes with RNase, and D is the reciprocal of the dilution made on the inoculum before plating. If the sample assayed is the product of a concentration procedure, such as an eluate from a large volume filter sampling of water or wastewater, then the coliphage concentration of the sampled water (C_b) can be calculated as previously described for section *3.1*.

4. Single Agar Layer (SAL) assay

This is a quantitative method that is also based on the plaque assay. This procedure utilizes a single agar layer technique adapted from Grabow and Coubrough. It allows for the assay of larger (100 mL) sample volumes in 150 mm assay dishes. Refer to Figure 16-2.

4.1 Basic SAL Assay: Place a 100 mL volume of sample in the 44.5 ± 1°C water bath for 3 minutes. Add 5 mL of

calcium chloride solution to the warmed sample followed by 5 mL of the appropriate host bacterium preparation. Mix the host inoculated sample with 100 mL melted tryptone SAL agar also held at 44.5 ± 1°C. Distribute the 210 mL of material to eight 150 x 15mm petri dishes. For a positive control, add to 12.5 mL sterile water, 0.6 mL of calcium chloride solution. Then add 1 mL of the appropriate ϕX174 preparation or MS2 preparation (30-80 PFU/mL) and 0.6 mL of host bacterium. Mix the host inoculated control with 12.5 mL of melted tryptone SAL agar. Pour to a single 150 x 15mm petri dish. Repeat for a negative control but add 1 mL of sterile tryptone broth in place of the positive control phage preparation. Invert and incubate the inoculated dishes at 36.5 ± 2°C overnight and examine for plaques the following day. Count the total number of plaques on the eight dishes receiving the sample. That total is the coliphage concentration per 100 mL of sample.

Note: With host E. coli *Famp, the basic SAL assay will detect both DNA and RNA male-specific coliphages as well as some somatic coliphages. For a procedure to selectively quantify the RNA male-specific coliphages, see the following section (4.2).*

4.2 SAL Assay with RNase: In this procedure an additional 100 mL of sample are assayed. The procedure is the same as that described above, except that RNase solution is added to the melted tryptone agar in the assay of the additional 100 mL of sample. For the additional material, the melted tryptone agar should contain RNase at a concentration of 80μg/mL (before sample is added). Appropriately label dishes so that the dish containing RNase are readily distinguished from the dish without RNase. Calculate the male-specific coliphage concentration (C_a) according to the formula:

$$C_a = P - P_R \; PFU/100mL$$

where P is the total number of plaques from the dish without RNase, P_R is the total number of plaques from the dish with RNase.

5. Enrichment Spot assay

This is a qualitative, presence-absence method. It employs an initial enrichment step to amplify coliphage numbers in samples, prior to assay with a spot technique. The procedure is adapted from EPA Method 1601 and allows for the assay of 100 mL or 1 L sample volumes. The procedure is described for 100 mL samples, for 1L samples scale up the volumes described in Section 5.1 by a factor of 10. Spot assay volumes in Section 5.2 remain the same. Refer to Figure 16-3.

5.1 Enrichment: Place 100 mL of sample into sterile bottle. Add 1.25 mL of calcium chloride solution. Add 0.5 mL of a 4 h culture of host *E. coli* (Section 2.3.6). Then add 5 mL of tryptone enrichment broth. Repeat the procedure for the positive control, replacing the 100 mL of sample with 100 mL of sterile distilled water inoculated with 0.5 mL of coliphage positive control preparation (Section 2.3.7). Again repeat procedure for the negative control, replacing the 100 mL of sample with 100 mL of sterile distilled water and no coliphage inoculum. Proceed as follows:

For somatic coliphage (using host *E. coli* C): Mix well and incubate at 36.5 ± 2°C overnight.

For somatic coliphage (using host *E. coli* CN-13): Add 1.0 mL of filtered nalidixic acid solution and mix well. Incubate at 36.5 ± 2°C overnight.

For male-specific coliphage (using host *E. coli* Famp): Add 0.1 mL of filtered ampicillin solution and 0.1 mL of filtered streptomycin solution and mix well. Incubate at 36.5 ± 2°C overnight. *Note: As used in this presence-absence assay, both DNA and RNA male-specific coliphages as well as some somatic coliphages will be detected with host* E. coli *Famp*.

5.2 Spot assay: Remove enrichment cultures from incubator and again mix well. Remove 10 μL and carefully deposit the volume to a preselected area on a tryptone spot agar dish (Section 2.2.14). Repeat for positive and negative controls using the same spot agar dish and being careful to keep the deposited volumes, or spots, separate. Spot dish should be adequately labeled prior to spotting and it is advisable to mark a grid pattern on the spot dishes as a guide when multiple samples are assayed. Allow deposited volumes to absorb into agar for up to 60 min. Invert inoculated spot dish and incubate overnight at 36.5 ± 2°C. After overnight incubation, examine dish for lysis (clearing) at the spot where the enrichment inoculum was applied. Use the positive and negative control spots for comparison. A positive result is indicated where lysis is observed at the spot of application. The entire spot need not be lysed. In some cases, lysis will be observed as one or more small plaques within the spot of application.

5.3 Interference: Interfering bacterial growth may occasionally be observed at the application spot. This growth is noticeably different from host bacterial growth and can prevent accurate assessment of the spot assay. Such interference may be addressed by including filtration or centrifugation treatment at the end of the enrichment step. Proceed with either filtration or centrifugation treatments as follows:

Filtration: Take 0.5 to 1.0 mL of the incubated enrichment medium and place through a beef extract-treated 0.45 μm pore size sterile filter. Spot assay the filtered material as in 5.2.

Centrifugation: Take 0.5 to 1.0 mL of the incubated enrichment medium and centrifuge at 5,000 to 10,000 x *g* for 10 min. Recover the supernatant and spot assay as in 5.2.

6. References

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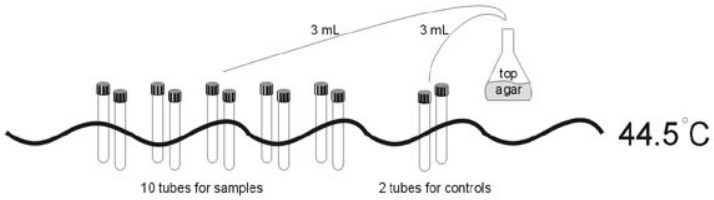
U.S. EPA. 2001. Method 1601: Male-specific (F^+) and somatic coliphage in water by two-step enrichment procedure. EPA 821-R-01-030.

U.S. EPA. 2001. Method 1602: Male-specific (F^+) and somatic coliphage in water by single agar layer (SAL) procedure. EPA 821-R-01-029.

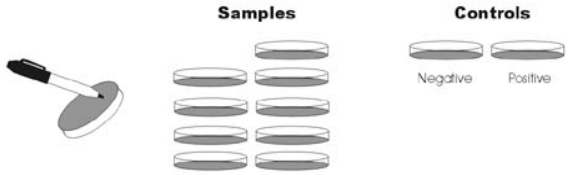
Disclaimer: Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Figure 16-1 basic double agar layer (DAL) procedure

1 Add 3.0 mL melted tryptone top agar to 12 test tubes and place in 44.5 ± 1°C water bath



2 Label 12 pre-poured bottom agar plates and keep at room temperature



3 For each top agar tube in water bath:

A. Add 0.1mL of a 4hr culture of host bacterium

B. Add 1.0mL of sample OR:

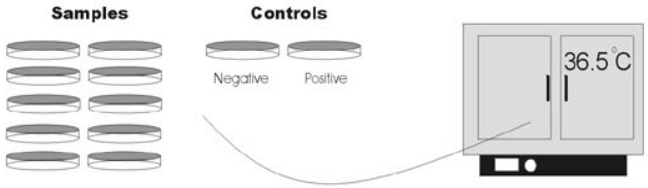
For negative control add 1.0 mL of tryptone broth

For positive control add 1.0 mL of coliphage prep (30-80 PFU/mL)

C. Gently mix tube in palm

D. Pour tube into pre-poured bottom agar plate

4 Invert and place in incubator at 36.5 ± 2°C for 16-24 hr



5 Examine for plaques and record results

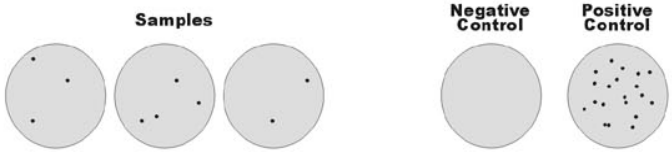


Figure 16-2 basic single agar layer (SAL) procedure

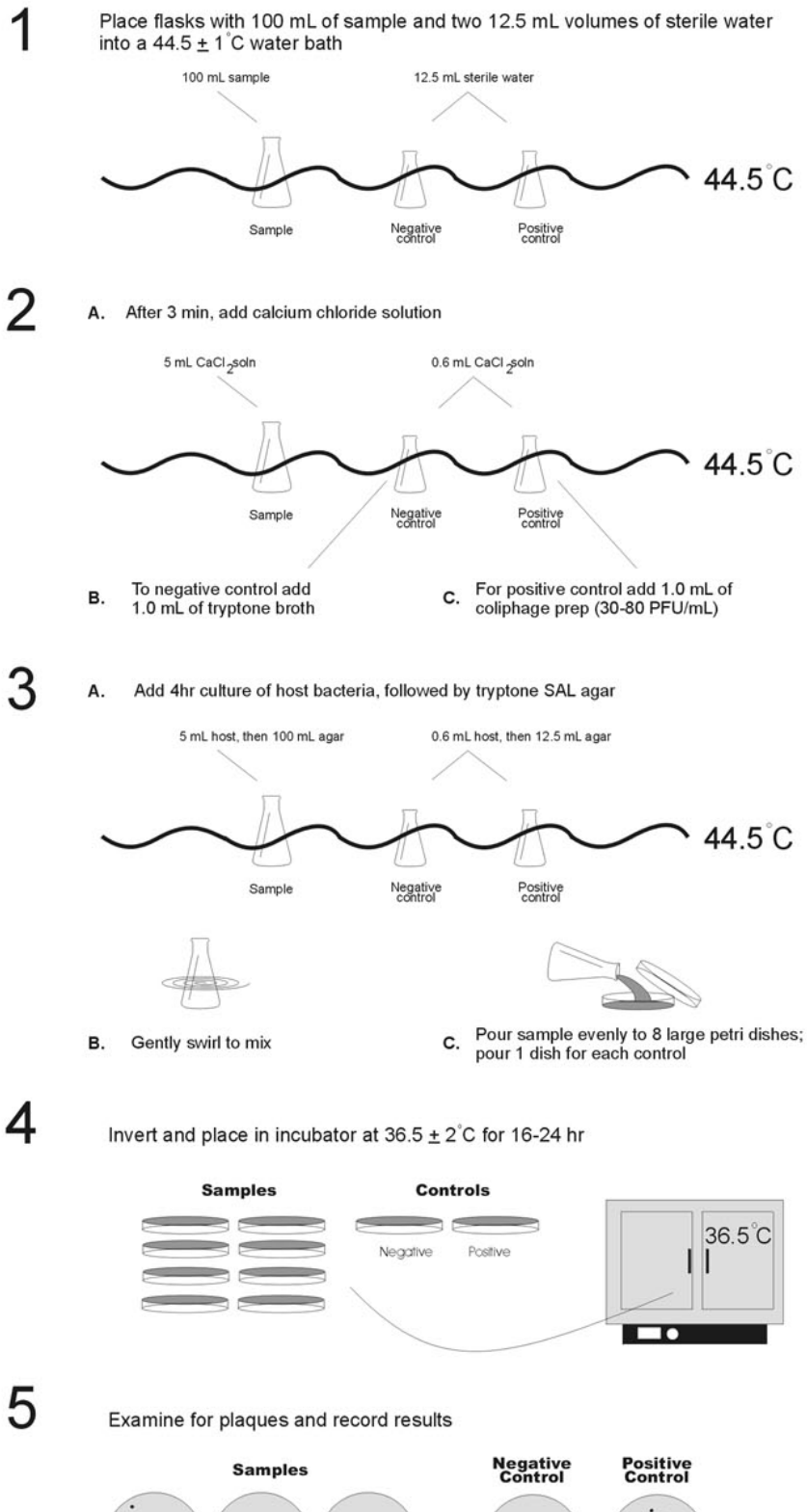
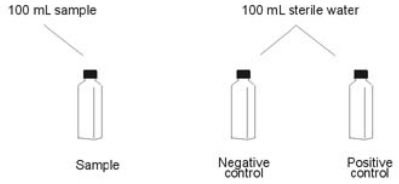
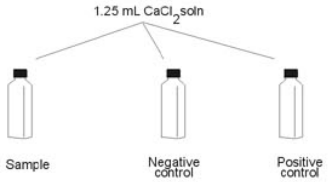


Figure 16-3 basic enrichment spot procedure

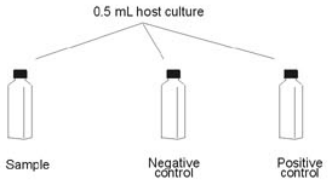
1 Place 100 mL of sample and two 100 mL volumes of sterile water into sterile bottles



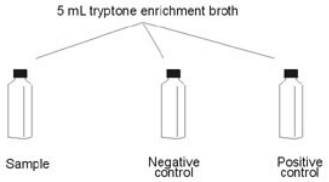
2 Add calcium chloride solution



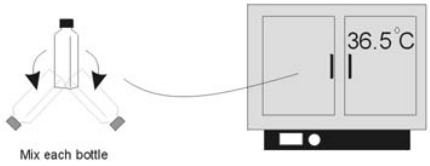
3 Add 4hr culture of host bacterium



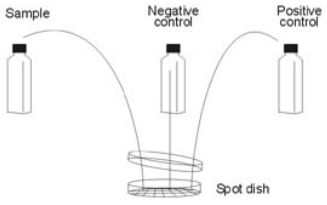
4 Add tryptone enrichment broth



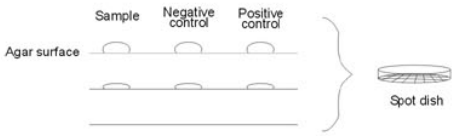
5 Mix each bottle well and incubate 16-24 hr at 36.5 ± 2°C



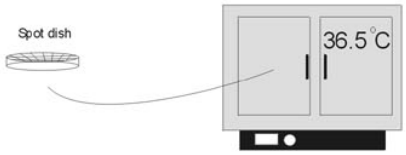
6 Remove 10µL from each bottle to labeled grid on spoti dish



7 Allow to absorb for up to 60 min



8 Invert and incubate 16-24 hr at 36.5 ± 2°C



9 Examine spots for zones of lysis and record results