Bacteriophage Isolation, Purification, and Characterization Techniques Against Ubiquitous Opportunistic Pathogens

Danielle L. Peters,[1,4] Greg Harris,[1] Carly M. Davis,[1,2] Jonathan J. Dennis,[2] and Wangxue Chen[1,3,4]

[1]Human Health Therapeutics (HHT), National Research Council Canada, Ottawa, Ontario, Canada
[2]Biological Sciences, University of Alberta, Edmonton, Alberta, Canada
[3]Department of Biology, Brock University, St. Catharines, Ontario, Canada
[4]Corresponding authors: *Danielle.Peters@nrc-cnrc.gc.ca*; *Wangxue.Chen@nrc-cnrc.gc.ca*

Published in the Microbiology section

Healthcare-associated infection with "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) is a global health crisis due to their extensive intrinsic antibiotic resistance and the ability to quickly acquire resistance determinants. Alternative treatment options are required to combat this crisis, and one possibility is the use of bacteriophages, or viruses that strictly infect the pathogenic bacteria. Currently, there is a renaissance in research and development into the use of phages to target multi-, extensively, and pan-resistant bacterial infections in humans, known as phage therapy. Using *A. baumannii* as an example, this article describes the isolation and purification of bacteriophages from sewage and soil samples, as well as general methods used in phage research such as precipitation of phages using polyethylene glycol, host range analysis, single-cell burst size determination, DNA extraction, and restriction fragment length polymorphism analysis. © 2022 National Research Council Canada. Current Protocols © 2022 Wiley Periodicals LLC. Reproduced with the permission of the Minister of Innovation, Science, and Industry.

Keywords: antimicrobial resistance • bacteriophage • characterization • host range • isolation

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols e594, Volume 2
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.594

How to cite this article:
Peters, D. L., Harris, G., Davis, C. M., Dennis, J. J., & Chen, W. (2022). Bacteriophage isolation, purification, and characterization techniques against ubiquitous opportunistic pathogens. *Current Protocols*, *2,* e594. doi: 10.1002/cpz1.594

INTRODUCTION

Antimicrobial resistance (AMR) is a major global public health catastrophe. The first comprehensive analysis of AMR impact from 204 countries estimates the total global burden of AMR in 2019 was 4.95 million deaths with 1.27 million of these deaths caused by bacterial AMR alone (Murray et al., 2022). The global COVID-19 pandemic is likely to further exacerbate the AMR crisis owing to the increased use of antibiotics for secondary bacterial infections and the delay in global action against AMR. As such, novel and distinct classes of antibiotics with improved potency and safety are urgently needed for patients infected with bacterial AMR pathogens. However, the antibiotic development pipeline, particularly for Gram-negative bacteria, is relatively dry with very few candidates in the advanced stage of clinical evaluation. Thus, innovative approaches are needed to develop alternatives to antimicrobials such as bacteriophages, antibodies, and vaccines.

Bacteriophages, or phages, are viruses that strictly infect and parasitize bacteria. Phages are highly abundant and ubiquitous in the environment and are readily detected in soil, water, sewage, and other environmental samples. There are two distinct lifestyles that phages can have: temperate or virulent. Temperate phages can lysogenize their host, whereby they integrate into the host chromosome or maintain themselves as a self-replicating episome that is passed on to progeny bacteria. When temperate phages lysogenize their host, they are referred to as a prophage and the host bacterium a lysogen. Prophages can be induced from the lysogenic cycle and switch to the lytic cycle. During the lytic cycle, the host bacterium turns into a phage factory, producing tens to hundreds of phages per cell that are released into the environment upon lysis of the host cell. Virulent phages can only undergo the lytic cycle and can be extremely effective at rapidly taking over the bacterial cell machinery to produce progeny phages. Some phages can infect a single bacterium, propagate, and lyse the cell within 15 min and produce more than 450 progeny per cell (Asif, Alvi, Tabassum, & Rehman, 2020).

The general principles of phage isolation involve incubating a sample with nutrient medium and the bacterial host of interest, which enables the phages that infect that bacterium to propagate (Fig. 1). Of note, because the host cell is lysed as the final step of a phage infection, the resulting liquid that contains the progeny phage is called a lysate and will be referred to as such throughout this article. The sample is then centrifuged, filter sterilized, and plated with the host again using a double-layer assay to observe plaques. Well-spaced plaques are collected and subjected to three rounds of plaque purification to generate an axenic stock for further characterization. Once a high-titer phage stock is obtained, it can be used for further characterization, including host range, burst size, and restriction profile following DNA extraction and treatment with a restriction enzyme panel. Once basic characterization of the phages has been completed, the phage DNA can be used for sequencing, and further studies on the virulence index of the phage and *in vitro* and *in vivo* efficacy can be carried out thereafter.

This article describes the isolation and purification of bacteriophages against *Acinetobacter baumannii* from sewage and soil samples to illustrate the general procedures and

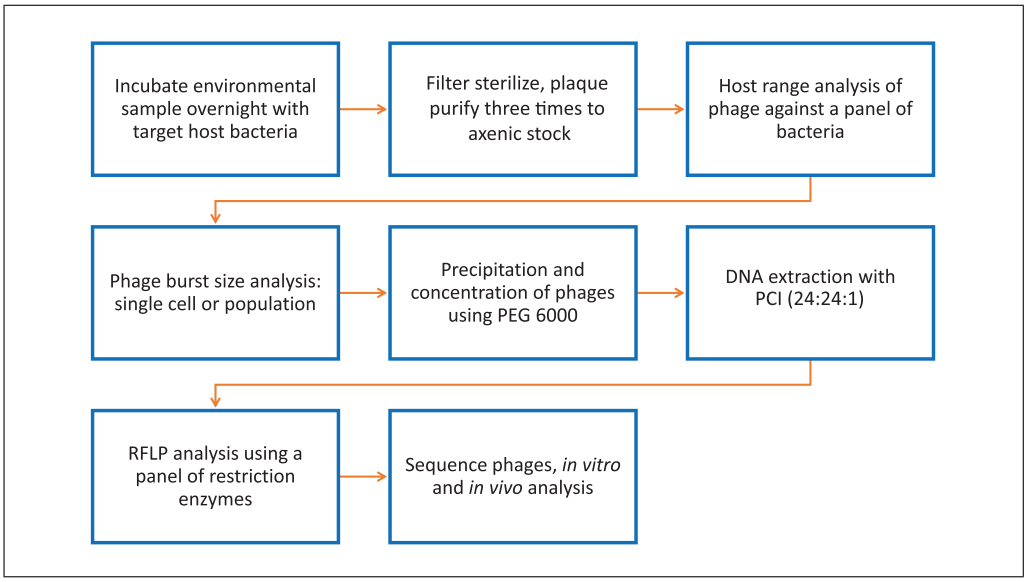

Figure 1 General phage isolation and characterization flowchart. PCI, phenol:chloroform:isoamyl alcohol; PEG, polyethylene glycol; RFLP, restriction fragment length polymorphism.

considerations in isolation and purification of phages against AMR bacterial pathogens. The article also includes the general methods used in phage characterization to include host range analysis, single-cell burst size determination, DNA extraction, and restriction fragment length polymorphism (RFLP) analysis.

A. baumannii is a Gram-negative bacillus ubiquitous in water, soil, and hospital environments (Eveillard, Kempf, Belmonte, Pailhoriès, & Joly-Guillou, 2013). The World Health Organization has ranked *A. baumannii* as a level one priority pathogen due to its extensive intrinsic antibiotic resistance mechanisms and ability to quickly acquire resistance determinants; thus, it is a great candidate for phage therapy (World Health Organization, 2017). The first step toward the therapeutic use of bacteriophages against *A. baumannii* is the isolation and characterization of phages against this pathogen.

CAUTION: A. baumannii and many other multidrug resistant bacteria are Biosafety Level 2 (BSL-2) pathogens. Any work with them must be carried out in a BSL-2 cabinet. All appropriate regulations and guidelines must be adhered to when handling pathogenic microorganisms.

STRATEGIC PLANNING

Use of Bacterial Strain and Phage Sample Identification Keys

The consistent use of a bacterial strain and phage sample identification key is highly recommended to reduce potential labeling mistakes and enable faster experimental setup. Each bacterial strain should be assigned a single letter for labeling, whereas each phage sample should be assigned a number. It is not recommended to phage hunt with more than five strains at a time to reduce experimental load. If the plaques appear uniform, a total of three plaques can be picked from the plate. If the plaques appear different in size or shape or if the presence of a halo is observed on only some plaques, then three plaques of each phenotype must be picked. Plaque isolates must be properly labeled with their purification round and isolate number. For example, if plaques appear on the lawn of strain "A" following enrichment with phage sample "3," the first collected plaque would be labeled "A3 P1.1" (Fig. 2). Two additional plaques picked from that plate would be labeled "A3 P1.2" and "A3 P1.3." Three plaques picked from a second round of purification of an "A3 P1.1" lineage would be labeled "A3 P21.1," "A3 P22.1," and "A3 P23.1" (Fig. 2).

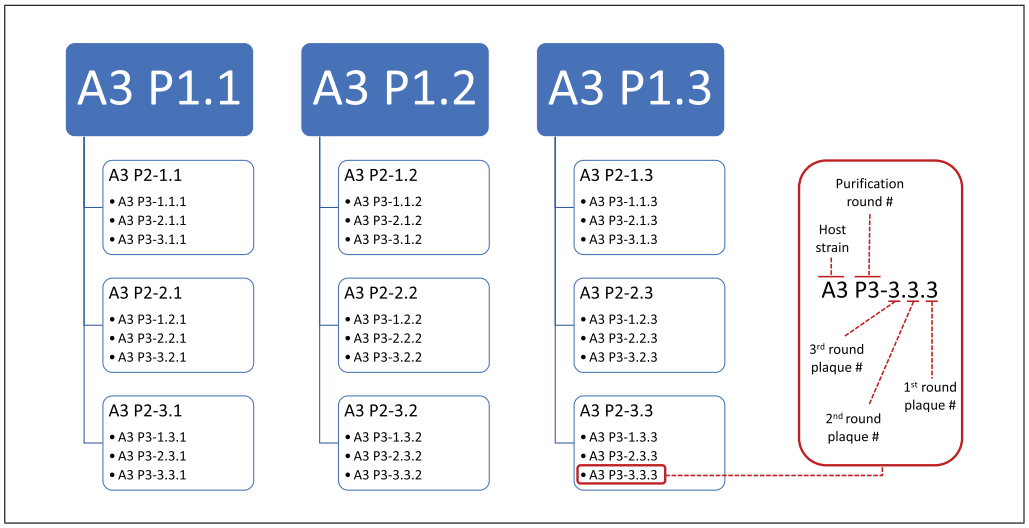

Figure 2 Labeling suggestions for each lineage of plaque collection from the phage "A3." Large blue boxes indicate the first round of plaque isolation with three plaques picked (P1.1, P1.2, P1.3). The second round of purification is indicated in the blue outlined boxes, and the third round of purification is shown as the bullet points beneath each purification round two.

Bacteria Considerations

All procedures involving growth or propagation of phages will require freshly grown overnight bacterial cultures. This is described in detail in Basic Protocol 1 step 1. In further protocols the step is omitted, but the overnight cultures are still required. Overnight bacterial cultures should be made in 50-ml conical tubes with a single fresh colony inoculated into 10 ml culture medium. The cultures are generally shaken at 200 rpm at 37°C overnight (16 to 18 hr), and the conical tube lids must be loose but taped down to ensure sufficient aeration. Single-colony plates can be stored at 4°C and reused for up to 2 weeks.

BASIC PROTOCOL 1

ISOLATION OF BACTERIOPHAGES AGAINST *A. baumannii* FROM SEWAGE SAMPLES

This protocol is designed to isolate *A. baumannii* bacteriophages from clarified sewage samples, though the protocol can be adapted to other Gram-negative pathogens given the correct growth medium and conditions are optimized for the new host system. The *A. baumannii* panel we used for phage hunting comprised the following strains: LAC-4 (NCBI:txid1455315), AB5075 (NCBI:txid1116234), AB0057 (ATCC 43498), 19606 (ATCC 19606), and 17978 (ATCC 17978). Influent sewage samples are collected from a water treatment plant, stored at 4°C, and used within a week of collection. The sewage samples are incubated overnight with the target host strain of interest to enable propagation of phages present in the sewage sample. Samples are clarified, filter sterilized, and used in a top agarose overlay technique. If phages capable of infecting that host strain are present in the sewage sample, there will be plaques or complete plate lysis observed on the top agarose overlay plates (Fig. 3). Plates with active phages can exhibit complete clearing, as shown in Figure 3A, or decreased turbidity compared with the control plate with plaques only visible when backlit by natural light (Fig. 3B).

Materials

Single-colony *A. baumannii* plates for each strain made by the quadrant streaking method
Lennox Luria Bertani (LLB; e.g., Sigma-Aldrich, cat. no. L3022) agar plates
50 ml influent sewage sample, collected within 1 week of use
2× LLB (e.g., Sigma-Aldrich, cat. no. L3022)

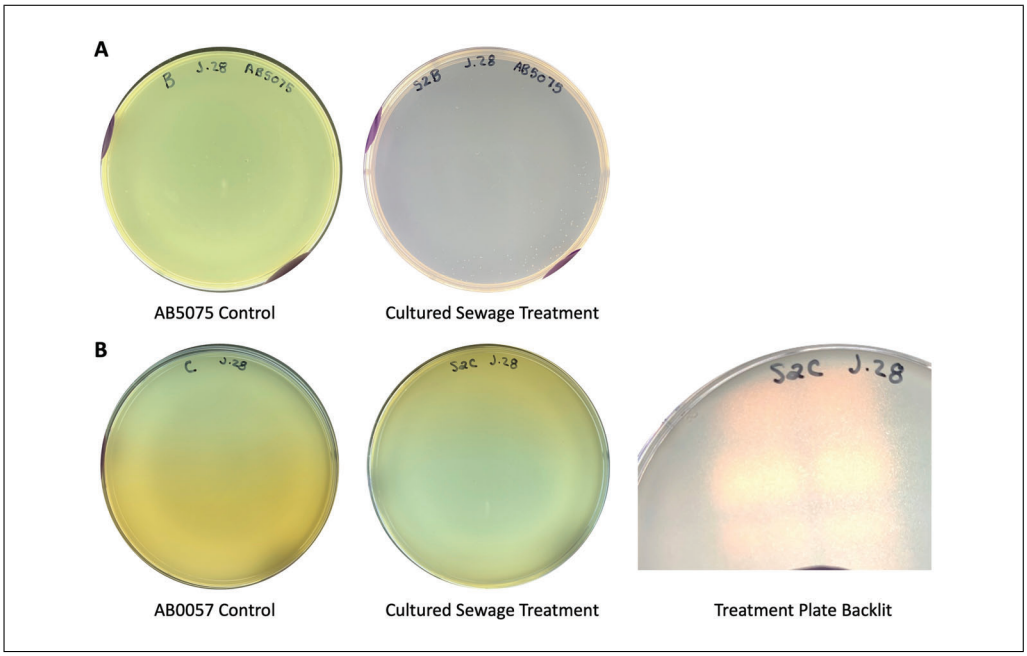

Figure 3 Successful phage isolation plates compared with controls. (**A**) Obvious plate clearing of *Acinetobacter baumannii* strain AB5075, designated strain "B," when cultured with lysate propagated on sewage sample two with AB5075. (**B**) Thinning of bacterial lawn on *A. baumannii* strain AB0057, designated strain "C," when cultured with lysate propagated on sewage sample two with AB0057. Plaques are only visible when plate is backlit with natural light.

Suspension medium (SM), sterile (see recipe)
1 M $CaCl_2$, sterile (e.g., Sigma-Aldrich, cat. no. C7902)
LLB top agarose (TALLB) at 50°C (see recipe)

37°C incubator with variable shaking
Refrigerated centrifuge
0.22-μm polyethersulfone (PES) syringe filters (e.g., Millipore Sigma, cat. no. SLGPR33RB)
50-ml flat-top conical tubes
20-, 200-, and 1000-μl pipettes and filter tips
10-ml Luer-lock syringes
5-ml culture tubes
1.5-ml low-bind microcentrifuge tubes (e.g., VWR, cat. no. 20170-038)
Plate imager (e.g., Fisher Scientific, cat. no. UVP 97053901)
Pasteur pipettes and bulbs, sterile
Vortex mixer
Refrigerated microcentrifuge
Cell scrapers, sterile (e.g., VWR, cat. no. 10062-904)

CAUTION: Ensure any work with sewage samples is conducted in accordance with BSL-2 protocols. The samples are contaminated with other human pathogens; thus, any work with them must be carried out in a BLS-2 cabinet. All appropriate regulations and guidelines must be adhered to.

NOTE: Prepare all solutions using deionized water.

Propagation of phages from sewage

1. Set up 10 ml LLB overnight cultures for each strain using a single-colony isolate grown on an LLB agar plate. Incubate cultures at 37°C for 16 to 18 hr with 200 rpm shaking. Ensure lids are taped down loosely, not screwed on, to allow airflow.

2. Centrifuge 50 ml sewage influent sample 10 min at 12,000 × *g*, 4°C, to clarify the sample.
3. Filter sterilize sewage supernatant with a 0.22-µm PES filter into a sterile 50-ml conical tube.

   If there is significant backpressure experienced when filtering the supernatant, stop and repeat step 2, or switch to a new filter.

4. Label five sterile 50-ml conical tubes with each strain-designated letter and sample number.
5. To each conical tube, aseptically dispense the following into the designated conical tube for that strain:

   5 ml of 2× LLB
   5 ml filter-sterilized sewage sample
   500 µl SM
   50 µl of 1 M $CaCl_2$
   100 µl overnight culture.

6. Tape lids down, and incubate tubes for 16 to 18 hr with 200 rpm shaking at 37°C.
7. Remove tape and tighten lids. Centrifuge 10 min at 12,000 × *g*, 4°C.
8. Label five new 50-ml conical tubes with designated strain letter, sample number, date, and "filter sterilized 0.22 µm."
9. Working with one sample at a time, filter sterilize supernatant into the new conical tube using a 10-ml syringe attached to a sterile 0.22-µm PES filter. Repeat for all samples.

   Filtered supernatants are henceforth referred to as "lysate."

   If there is significant backpressure during the filtration, stop and repeat step 7 if the bacterial pellet has dislodged, or switch to a new filter.

Plating of propagated sewage samples

10. Label two LLB plates per lysate tube generated in step 9 with its alphanumeric code and one control plate per strain. Repeat with 5-ml culture tubes. Organize plates and tubes from A to E.

    An example plate label for strain A, sample 5, plates 1 and 2 would be A5-1 and A5-2, respectively.

11. Sequentially add each component to strain A lysate conical tubes:

    50 µl SM
    100 µl overnight culture of strain A
    100 µl filter-sterilized lysate cultured with strain A.

12. Repeat step 11 with the remaining strains.
13. Sequentially add each component to the control conical tubes:

    150 µl SM
    100 µl overnight culture of strains A, B, C, D, or E.

14. Incubate culture tubes with lids closed for 10 min at room temperature.
15. Using a serological pipette, aspirate 9 ml molten TALLB, and dispense 3 ml into each strain A tube, including the control tube.
16. Moving quickly, pour contents of each tube onto the center of their respective plate, and swirl TALLB to completely cover the plate.

Move quickly to spread the TALLB out before it solidifies to ensure a smooth surface. This will greatly enhance the ability to identify plaques. Use a new serological pipette between strain samples to avoid the presence of solidified pieces of agarose in the overlays.

17. Repeat steps 15 and 16 with the remaining strain tubes.

18. Allow plates to solidify for at least 20 min before placing plates upside down in a 37°C static incubator for 16 to 18 hr.

Examination of plates and collection of phages

19. Examine each lysate plate, and compare it against the control plate. If both lysate plates do not look identical in lawn density, appearance, and plaque formation, repeat experiment. If no plaques are observed, return plates to the incubator until they have incubated for 24 hr and reexamine.

    Use a backlit colony counter to fully examine the plates. Some plaques are hard to see without backlight examination. Holding the plates up to a room light or window also works. Some phages have shown delayed plaque development (24 hr), even on fast-growing hosts.

20. Image any plates that show plaquing or clearing and their control for future reference.

21a. Collect phages depending on the plate plaquing pattern: For uniform, well-spaced single plaques, pick plaques directly into 1.5-ml microcentrifuge tubes:

    The below steps are for the collection of three morphologically homogeneous plaques. If there are heterogeneous plaques, increase the number of plugs collected to three plugs per plaque morphology.

    a. Label and fill three 1.5-ml microcentrifuge tubes with 500 µl SM each.
    b. Use a new sterile Pasteur pipette to remove each plaque as a plug of agar, and transfer plug to a 1.5-ml microcentrifuge tube.

       Hold the Pasteur pipette perpendicular to the plaque, and stab the narrow end of the Pasteur pipette into the plate right to the bottom. Slightly move the pipette laterally to loosen the plug from the surrounding agar, and angle the pipette to 45° to retain the plug in the end of the tip while slowly withdrawing from the agar. Place the tip of the pipette with the plug just above the SM in the 1.5-ml microcentrifuge tube, and attach the rubber balloon to the end of the pipette to force out the plaque plug. Do not place the tip directly in the SM, or liquid may splash out of the tube when the plug dislodges.

    c. Vortex plaque sample for 30 s.
    d. Repeat substeps a to c for all plaques.
    e. Centrifuge tubes 2 min at 14,000 × *g*, 4°C.
    f. Remove most of the supernatant without disturbing the pellet, and transfer lysate to a new 1.5-ml microcentrifuge tube.
    g. Store 1.5-ml microcentrifuge tubes in the fridge until the purification process.

       These samples are all at their first of three purification rounds (see Basic Protocol 2).

       If the samples will be stored in the fridge without propagation for more than 2 days, filter sterilize them with a 0.22-µm PES filter. Contaminating bacteria from the plug will inactivate the phages over time, and the use of chloroform is not recommended until it is known if the phage is resistant to it.

21b. For confluent lysis of the plate without individually well-spaced plaques, scrape agarose top layer into a 50-ml conical tube (Fig. 3A):

    a. Label a new 50-ml conical tube with the alphanumeric code of the plate. Open lid and set it beside the tube.

b. Carefully scrape top agarose layer into the conical tube using a sterile cell scraper.
c. Add 4 ml SM to the conical tube.
d. Tightly close conical tube lid, and vortex sample for 2 to 3 min until the agarose has broken down into small pieces.
e. Centrifuge sample 10 min at 12,000 × *g*, 4°C.
f. Filter sterilize supernatant with a 0.22-µm PES filter into a new 50-ml labeled conical tube.
g. Store conical tube in the fridge until the purification step.

ALTERNATE PROTOCOL 1

ISOLATION OF BACTERIOPHAGES AGAINST *A. baumannii* FROM SOIL SAMPLES

Alternate Protocol 1 is designed to isolate phages from soil samples. There is a higher chance of success when using sewage to isolate phages of the pathogens of interest. However, obtaining influent sewage samples can be difficult, and working with sewage has its own safety considerations. If sewage samples are not available, soil can serve as an alternate if the bacteria are known to be prevalent in the environment. The major differences between isolating bacteriophages from soil samples and from sewage samples are the initial sample processing and overnight incubation with the target bacterial strains (i.e., Basic Protocol 1 steps 1 through 5).

Additional Materials *(also see Basic Protocol 1)*

~50 ml soil sample
1× LLB (e.g., Sigma-Aldrich, cat. no. L3022)
Scoopula

1. Label five 50-ml conical tubes with their alphanumeric codes according to strain letter and sample number.
2. Use a scoopula to dispense enough soil into each conical tube to reach the 10-ml line.

   It is ok if the sample has air pockets and is heterogeneous. Use enough sample to fill the bottom of each conical tube to the 10-ml mark without compressing the sample into the bottom of the tube.

3. Sequentially dispense the following into strain A tube:

   10 ml of 1× LLB
   500 µl SM
   50 µl of 1 M $CaCl_2$
   100 µl overnight culture strain A.

4. Repeat step 3 with the remaining strains.
5. Tightly close tubes, and vortex until samples appear relatively homogeneous. Loosen lids and tape them down.
6. Follow Basic Protocol 1 from step 6 onward.

SUPPORT PROTOCOL 1

TITERING A BACTERIOPHAGE STOCK

Determining the concentration of bacteriophages in the lysate generated from Basic Protocol 2 is important for downstream phage analysis and characterization, such as host range analysis, DNA extraction, and burst size determination. Although the approach to determining phage titer of a lysate is very similar to bacterial dilutions and plating for colony-forming unit (CFU) counts, there are some differences that are critical for obtaining consistent plaque-forming unit (PFU) counts.

Additional Materials *(also see Basic Protocol 1)*

Filter-sterilized phage lysate (see Basic Protocol 2)

Dilution series and plating of phage stock

1. Dispense 225 µl phage lysate into a labeled 1.5-ml microcentrifuge tube. Allow lysate to come to room temperature.

   Standardizing the phage lysate temperature can help minimize pipetting differences.

2. Label two sets of 1.5-ml microcentrifuge tubes from 10^{-1} to 10^{-9} and two LLB plates per dilution.

   Each dilution will be tested in duplicate.

3. Dispense 900 µl SM into each 1.5-ml microcentrifuge tube and close lids.

4. Using a 200-µl pipette set to 100 µl, charge pipette tip in the phage lysate by aspirating and dispensing the volume 10 times.

   Ensure that the pipette tip is submerged in the lysate while charging to decrease aerosol generation.

5. Using the charged tip, dispense 100 µl phage lysate into the 10^{-1} 1.5-ml microcentrifuge tube. Discard pipette tip.

6. Repeat steps 4 and 5 for all subsequent dilutions, charging a fresh pipette tip each time.

7. For each dilution being tested, set up two 5-ml culture tubes with 100 µl host strain overnight culture.

   If there is a general idea of where the titer may be, the dilution range can be reduced to one dilution step above and one below the expected target to reduce the number of plates consumed.

8. For each dilution being tested, charge a 200-µl pipette tip with 100 µl diluted lysate, and then dispense it into the corresponding culture tube.

9. Allow tubes to incubate at room temperature for 10 min.

10. Working quickly, dispense 3 ml TALLB into the first conical tube, and pour mixture onto the center of the corresponding plate. Swirl plate to spread the TALLB into a confluent layer, and put plate aside to allow the TALLB to solidify. Repeat for all remaining tubes.

11. Incubate plates inverted at 37°C for 16 to 18 hr or longer depending on the phage's specific plaque development time.

Observation of plates and calculation of PFUs

12. Observe plates and count number of plaques on plates showing 20 to 200 plaques per plate. Note plaque morphology.

13. Determine concentration of phage stock by calculating the PFU/ml. Start by summing the number of plaques on both dilution plates, and divide by two to obtain the average. Use this value in the following formula:

$$\text{PFU/ml} = \frac{\text{average PFU}}{\text{amount plated (ml)}} \times \text{inverse of dilution factor.}$$

   Thus, if two plates had an average of 53 plaques for a 10^{-6} dilution where 100 µl was plated, the concentration calculation would be:

$$\frac{53 \text{ PFU}}{0.1 \text{ ml}} \times 10^6 = 5.3 \times 10^8 \text{ PFU/ml.}$$

PURIFICATION OF PHAGE TO AN AXENIC WORKING STOCK

This protocol is used to purify phage lysate generated from Basic Protocol 1 to an axenic stock for further characterization. A modified streak-for-isolation technique (Fig. 4) removes the need to perform a full dilution and plating series, which reduces time and medium requirements. It is important to work quickly once the impregnated agarose is poured onto the plate to ensure the agarose does not start to solidify before being streaked through with the phage lysate. If performed correctly, single-plaque isolates will be present on the plate and can be easily picked into SM using a Pasteur pipette.

Additional Materials *(also see Basic Protocol 1)*

Phage lysate (see Basic Protocol 1 or Alternate Protocol 1)
Inoculating loops (e.g., VWR, cat. no. 12000-810)
15-ml conical tubes
Serological pipettes

Production of single-plaque phage plates

1. Label one plate for each phage lysate produced using Basic Protocol 1 or Alternate Protocol 1. Prewarm plates at 37°C.

   Prewarming the plates is optional, but it can help to slow the gelling time of the TALLB.

2. Set up one culture tube per lysate sample, and dispense 100 µl host overnight culture into the tube.

3. Process one culture tube at a time by adding 4 ml TALLB and quickly pouring it onto its corresponding prewarmed plate. Swirl plate to get a confluent TALLB layer (Fig. 4A).

   Work very quickly from this point to ensure the TALLB does not solidify before the phage lysate is streaked through the molten agarose. A larger volume of TALLB is used in this protocol, so the streak-for-isolation technique can be performed before the agarose begins gelling.

4. Dip a sterile inoculating loop into the lysate, and quickly streak loop through the molten TALLB on one side of the plate (Fig. 4B).

5. Use a new sterile loop to cross through the previously streaked area, and drag loop in wide zags through the untouched region of the plate (Fig. 4C).

6. Repeat steps 3 to 5 with the remaining phage lysates being purified.

7. Let plates sit undisturbed for 20 min to allow the TALLB to solidify into a confluent layer.

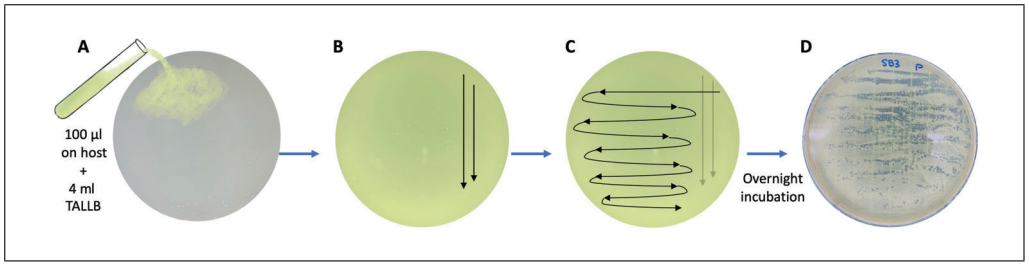

Figure 4 Streak-for-isolation plating method. (**A**) Host-impregnated TALLB is poured onto an LLB agar plate. (**B**) An inoculating loop dipped in phage lysate is streaked through the molten agarose along the side of the plate to produce a high-concentration area. (**C**) A second inoculating loop is streaked through the high-concentration zone into the untouched center of the plate and struck out in a back-and-forth motion across the plate to further dilute the phage into the molten agarose. (**D**) The plate is incubated for 16 to 18 hr and observed for single, well-spaced plaques to pick into SM. LLB, Lennox Luria Bertani; SM, suspension medium; TALLB, top agarose LLB.

8. Incubate plates inverted at 37°C for 16 to 18 hr or longer depending on the phage's specific plaque development time.

Plaque purification of novel phage

9. Compare plaques on each group of plates (Fig. 4D). Use a new sterile Pasteur pipette to remove each plaque as a plug of agar, and eject plug into its designated 1.5-ml microcentrifuge tube containing 500 µl SM. If there are plaque morphology differences between the same isolation source, pick three plugs per plaque morphology.
10. Vortex sample for 30 s.
11. Centrifuge tube 2 min at 14,000 × *g*, 4°C. Transfer supernatant to a new 1.5-ml microcentrifuge tube, or filter sterilize it first with a 0.22-µm PES filter if it will be stored longer than 2 days in the fridge without propagation.

    These samples are at purification round 1 (if generated using Basic Protocol 1 step 21b) or 2 (if generated using Basic Protocol 1 step 21a). It is important to keep track of the purification round each sample is at.

12. Repeat steps 2 to 11, processing each 1.5-ml microcentrifuge tube generated in step 9 until each isolate has undergone plaque purification three times.

    After the third round of purification, the phage stock is considered axenic.

Propagation of axenic phage for further characterization

13. Label three LLB plates for phage propagation.
14. Dispense 300 µl host overnight culture and 300 µl corresponding purified phage stock into a 15-ml conical tube, and incubate 10 min at room temperature.
15. Dispense 9 ml TALLB into the conical tube, and aspirate the entire amount with a serological pipette.
16. Dispense 3 ml impregnated TALLB per plate, and swirl to cover the plate.
17. Let plates sit undisturbed for 20 min to allow the TALLB to solidify
18. Incubate plates inverted for 16 to 18 hr at 37°C.
19. Check plates for confluent lysis, and scrape top agarose into a 50-ml conical tube, pooling top agarose from all three plates into one tube.
20. Add 10 ml SM to the conical tube, and vortex until there is a relatively homogeneous slurry.
21. Centrifuge sample 10 min at 12,000 × *g*, 4°C.
22. Filter sterilize supernatant with a 0.22-µm PES filter into a new labeled 50-ml conical tube.
23. Repeat steps 13 through 22, but scale amounts up to 15 plates and mix phage and bacteria (1.5 ml each) in a 50-ml conical tube. Add 42 ml TALLB to the conical tube after a 10 min incubation, and dispense 3 ml onto each plate.
24. After overnight incubation, scrape top agarose from all of the plates into three sterile 50-ml conical tubes.
25. Add 15 ml SM to each tube and tighten lid. Vortex until mixture is relatively homogeneous.
26. Centrifuge as described in step 21, and then filter sterilize all supernatants into two sterile 50-ml conical tubes.
27. Titer newly generated axenic phage working stock using Support Protocol 1, and store stock at 4°C.

SUPPORT PROTOCOL 2

LIQUID PROPAGATION OF BACTERIOPHAGE

Propagation of bacteriophages in liquid is quite easy in comparison to the double-layer plate method (Basic Protocol 2) as it requires less materials, has fewer steps, and eliminates a precarious transfer of the upper agarose layer from a double-layer plate into a conical tube for vortexing, centrifugation, and filter sterilization. It is important to note that not all phages propagate well in liquid culture.

Additional Materials *(also see Basic Protocol 1)*

Filter-sterilized phage lysate (see Basic Protocol 2)

1. Set up 10 ml LLB overnight culture for each phage host strain.
2. Dispense 9.90 ml LLB containing 5 mM $CaCl_2$ into a 50-ml conical tube.

   It is important to add $CaCl_2$ to the growth medium for phage propagation.

3. Aliquot 100 µl overnight host strain into the conical tube, and tape down lid. Incubate at 37°C for 2.5 hr.
4. Dispense 200 µl phage lysate into the subculture tube from step 3, and tape down lid. Incubate at 37°C overnight.
5. Close culture tube lid, and centrifuge 10 min at 12,000 × *g*, 4°C.
6. Filter sterilize supernatant into a new 50-ml conical tube using a 10-ml syringe fitted with a 0.22-µm PES filter.
7. Titer phage lysate from step 6 according to Support Protocol 1.
8. Compare phage titers obtained using the liquid propagation method with the double-layer assay to determine if the phage can propagate sufficiently in liquid cultures.

BASIC PROTOCOL 3

HOST RANGE ANALYSIS USING THE SPOT PLATE METHOD

Bacteriophages have notoriously narrow tropisms, often limited to a single strain of a host species or select host species of a genus. Determining a bacteriophage's host range is important because it can be used to distinguish between novel phages, identify more permissive hosts to propagate the phage on, or build a broad host range phage cocktail targeting a large number of species for potential polyphage therapies. It is highly advisable to confirm that the phage can plaque on the host strain with spot plating, as some phages cannot plaque with this method and full plates must be used instead (Support Protocol 1). The objective of this protocol is to enumerate the number of plaques at a dilution factor, which gives 2 to 20 plaques in all spot replicates for that phage and strain (Fig. 5A-C). By comparing the phage titer on one strain to the titer obtained with the

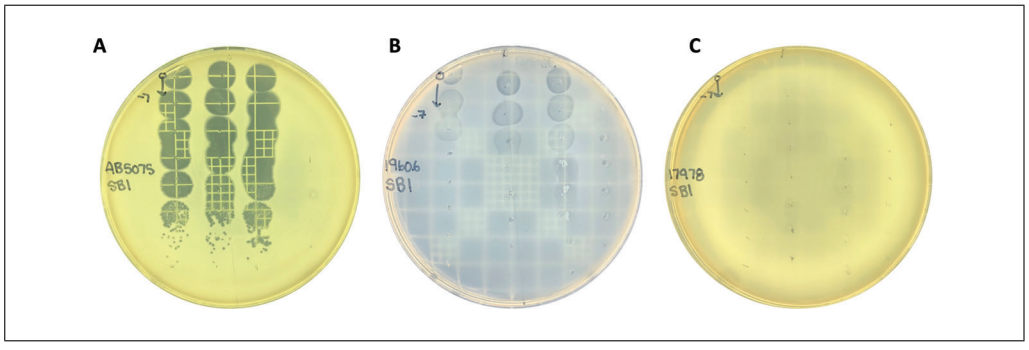

Figure 5 Representative host range plates for analysis and efficiency of plating. (**A**) SB1 serial dilution on host AB5075. (**B**) SB1 serial dilution on 19606 with decreased efficiency of plating. (**C**) SB1 on 17978 where no plaques or areas of clearing appear.

isolation host, the phage's efficiency of plating can be determined. This can be useful if a phage is struggling to propagate on the isolation host strain.

Additional Materials *(also see Basic Protocol 1)*

Filter-sterilized phage lysate with known titer ($>10^8$ PFU/ml recommended; see Support Protocol 1)
15-ml conical tube
Serological pipette
Pipette basin

Setting up a phage dilution series

1. Set up 10 ml LLB overnight culture for each bacterial strain to be tested against the phage, including the isolation host for a positive control.
2. Label eight 1.5-ml microcentrifuge tubes with the phage and a dilution factor from 10^0 to 10^{-7}.
3. Dispense 900 µl SM into each 1.5-ml microcentrifuge tube labeled 10^{-1} through 10^{-7}.
4. Dispense 1 ml phage lysate into the 10^0 1.5-ml microcentrifuge tube.
5. Charge pipette tip with 100 µl lysate, and dispense it into the 1.5-ml microcentrifuge tube labeled 10^{-1}. Discard tip.
6. Repeat step 5 until the phage lysate has been diluted to 10^{-7}.

Preparation of bacterial plates and controls

7. Label two plates per bacterial strain to be tested, one as a control and one as the lysate plate.
8. For each test strain and the host strain, combine 200 µl overnight culture with 6 ml TALLB in a 15-ml conical tube, and quickly dispense 3 ml mixture onto each agar plate using a serological pipette. Tilt and move plate to spread the agarose before it solidifies.

   Allow the plates to sit in the BSL-2 cabinet with the fan on for at least 1 hr to dry the top agarose before proceeding with spot plating, otherwise some of the phage suspension drops may run into each other.

9. While the plates are drying, centrifuge 5 ml phage host strain overnight culture for 10 min at 12,000 × *g*, 4°C. Filter sterilize supernatant into a new sterile tube.

   This step is required if the phage is being propagated on a bacterial strain with active prophages. If the prophage status of the test strain is unknown, this step must be used as a control.

10. Prepare pipette basin with the dilution series by dispensing 850 µl of each dilution into a separate well in the basin.
11. Add 850 µl filter-sterilized host lysate from step 10 into three consecutive basin wells for control spotting.

Spot plating procedure

12. Using a 20-µl eight-channel pipette set to 10 µl, charge tips with the dilution series, and then dispense 10 µl phage dilution series onto the treatment plate, leaving enough space on the plate to fit two more dilution lines. Discard tips.

    If the drops are spreading out and touching each other, leave the plates to dry for another hour or more. Discard the tips between every addition of the dilution series onto the plate to prevent bacterial contamination of the dilution series.

13. Repeat step 13 two more times onto the same plate to obtain a technical triplicate.
14. Carefully slide spotted plate out of the way to dry, and have strain control plate ready for host lysate control spotting.

Be very careful when moving the plates to prevent the drops from flooding together.

15. Charge a 20-µl pipette with 10 µl host supernatant from step 10, and spot one row onto the center of the control plate. Repeat twice more, and then gently slide plate out of the way to dry.
16. Repeat steps 13 through 16 for each test strain and the phage host strain.
17. Allow drop plates to dry for ~1 hr in the biosafety cabinet or until all the drop liquid is fully absorbed.
18. Invert plates and transfer to a 37°C static incubator for 16 to 18 hr. Ensure plates are well spaced to allow airflow.

Observation of plates and data collection

19. Remove plates from the incubator, and image to document the presence or absence of plaquing or clearing in the spotted areas. Document which dilution factor has clearing or plaquing on each bacterial strain.
20. Examine prophage control plate to ensure plaques are due to the phage of interest and not a contaminating temperate phage from the host strain.
21. Standardize PFU/ml results for each strain tested to those obtained with the phage host to determine efficiency of plating on each strain compared with the isolation host using the following equation:

$$\text{Relative efficiency of plating} = \frac{\text{average PFUs on test strain B}}{\text{average PFUs on host strain A}}.$$

BASIC PROTOCOL 4

SINGLE BURST SIZE ANALYSIS

Standard characterization of bacteriophages involves determining the burst size of a novel phage. The burst size refers to the number of phages produced when a bacterium is infected with a phage. This protocol uses a multiplicity of infection (MOI) of 1 to increase the likelihood that every bacterium is infected with a single phage. The protocol needs to be completed quickly once the bacterial subculture reaches target optical density at 600 nm (OD_{600}) to ensure the CFU count is as precise as possible to obtain reproducible results. Once the bacteria are infected with phages, the culture is diluted to 5 CFU/ml and dispensed into multiple culture tubes at 0.5 cells per tube. This ensures no tube has more than one bacterium and enables one to determine the exact number of phages produced from one replication cycle. It is vital to be very familiar with the protocol and have all the materials ready and appropriately labeled before commencing the experiment.

Additional Materials *(also see Basic Protocol 1)*

Filter-sterilized phage lysate, standardized to 1×10^7 PFU/ml
Microvolume spectrophotometer

Single-cell phage infection

1. Prewarm LLB to 37°C to use for dilutions in steps 8 and 9.
2. Subculture overnight grown host strain 1:100 in LLB in a 50-ml conical tube with the cap loosened and taped down. Culture at 37°C with 200 rpm shaking to an OD_{600} that corresponds to ~1×10^7 CFU/ml for the test strain.

The OD value must be determined and confirmed before starting the experiment.

3. While the bacterial culture is growing, charge a pipette tip with 500 µl standardized phage lysate, and then dispense 500 µl phage lysate into a 1.5-ml microcentrifuge tube.

   Ensure the phage lysate has come to room temperature before mixing with the host strain.

4. As soon as the bacteria has reached the target OD, add 500 µl bacterial subculture to the 1.5-ml microcentrifuge tube, and start a timer counting up.
5. Place tube in a centrifuge set to 4 min at 8000 × *g*, room temperature, and start centrifuge as soon as the timer hits 5 min.
6. Discard supernatant and wash cells with 1 ml SM.

   This step removes any unabsorbed phages from the bacteria.

7. Pellet cells again by centrifuging 4 min at 8000 × *g*, room temperature.
8. Remove supernatant and gently resuspend pellet in 1 ml prewarmed LLB.

   The bacterial concentration is now ~5 × 10^6 CFU/ml.

9. Dilute bacteria to 5 CFU/ml with prewarmed LLB by performing three 1:100 dilutions.
10. Dispense 100 µl suspension into 20 culture tubes, and incubate for 60 min at 37°C.

    At this point the theoretical concentration is 0.5 CFU/ml. Therefore half of the tubes should contain 1 CFU, and half of the tubes should contain 0 CFU.

Plating of lysate for phage enumeration

11. Dispense 100 µl overnight host culture into four culture tubes, and add 3 ml TALLB to each tube. Pour culture tube contents onto an agar plate, and swirl plates to spread the mixture into a confluent layer. Leave plates to solidify for 20 min at room temperature.
12. Repeat step 11 with the remaining culture tubes.
13. Incubate plates inverted at 37°C for 16 to 18 hr.
14. Determine number of phages produced per cell by counting the plaques present on each plate.

    If the experiment was performed correctly, approximately half of the plates will contain plaques.

ONE-STEP GROWTH CURVE

ALTERNATE PROTOCOL 2

Alternate Protocol 2 is more forgiving with respect to pipetting technique and is designed to obtain the average burst size of a phage, as well as other aspects of phage infection characteristics including adsorption rate, latency period, and total length of the infection cycle. This protocol uses an MOI of ~3 to ensure every bacterium is infected with a phage. The protocol requires a bacterial subculture of known CFU/ml and an accurately titered phage stock (>10^9 PFU/ml). The phage stock is added directly to the bacterial subculture and mixed well by swirling; this becomes time point 0 (T_0). Aliquots are taken in triplicate at every desired time point and serially diluted. The dilutions are then spotted onto bacterial overlays of the strain used in the one-step growth curve. The length of time needed to obtain a plateau for a one-step growth curve will usually be determined by how fast the bacteria grows. The range can vary but should normally fall between 2 and 8 hr. As with the previous protocol, it is vital to be very familiar with the protocol and have all the materials ready and appropriately labeled before commencing the experiment.

Additional Materials *(also see Basic Protocol 1)*

Phosphate-buffered saline (PBS; e.g., Fisher Scientific, cat. no. 10010023)
Filter-sterilized phage lysate of known titer ($>10^9$ PFU/ml)

Spectrophotometer
96-well plate reader (e.g., BioTek LogPhase 600)
96-well flat-bottom plates (e.g., Fisher Scientific, cat. no. 267578)

Preparation of bacteria and plates

1. Set up 10 ml LLB overnight culture of desired strain, and grow for 18 hr. Measure OD_{600} before subculturing to ensure accuracy and consistency between biological replicates.

2. Subculture overnight host strain 1:100 in LLB in a 50-ml conical tube with the cap loosened and taped down. Culture at 37°C with 200 rpm shaking to an OD_{600} that corresponds to $\sim 1 \times 10^7$ CFU/ml for the test strain.

   The OD value must be determined and confirmed before starting the experiment.

   Use a 96-well plate reader to measure the OD_{600} of the subculture. This will limit the change in volume significantly. Different CFU/ml can be used for this assay if desired, but ensure the amount of phage added is modified to reach an MOI of ∼3.

3. While the bacterial culture is growing, set up and label double agar overlay plates for every time point being tested, including T_0, and enough 96-well plates to dilute three samples for every time point:

   a. Dispense 100 µl overnight host culture into each culture tube. Dispense 3 ml TALLB into culture tubes, and pour contents onto an agar plate. Swirl plates to spread the mixture into a confluent layer. Leave plates to solidify for 20 min at room temperature.

      If plaques come out fuzzy or hard to see, try diluting the overnight culture 1:10 for the double agar overlays. Allow the plates to sit in the biological safety cabinet with the fan on for at least 1 hr to dry the top agarose before proceeding with spot plating to reduce the chance of phage suspension drops running into each other.

   b. Using a 200-µl eight-channel pipette set to 90 µl, dispense columns of 90 µl desired diluent (i.e., SM or PBS) into three times the number of time points being taken plus one. Label each column with the respective $T_\#$ on the 96-well plate lid.

      The extra column is to serially dilute the subculture to confirm the CFU/ml used in the experiment.

      For example, if 10 time points are being tested, 31 columns (three 96-well plates) will be needed.

      If the bacterial strain is fast growing, the plates can be stored in the fridge to help slow down the infection while diluting the samples.

4. Once bacteria reach the target OD, remove 10 µl and dispense it into well A of the extra column. Discard tip.

   This is the 10^{-1} dilution.

5. Mix dilution by pipetting up and down 10 times. Remove 10 µl from well A with the same tip, and dispense into the respective well B. Discard tip. Repeat this until the dilution has reached well H.

6. Using a 20-µl eight-channel pipette set to 10 µl, mix dilution series 10 times, and then dispense 10 µl dilution series onto an agar plate leaving enough space on the plate to fit two more dilution lines. Discard tips.

7. Repeat step 6 two more times for a technical triplicate.

Phage infection and time points

8. Charge a pipette tip with 100 µl of 6×10^9 PFU/ml phage stock, and then add 100 µl lysate to the subculture for a final concentration of 6×10^7 PFU/ml. Mix well by swirling.

   If a different starting CFU/ml was used, use the formula $C_1V_1 = C_2V_2$ (where C is concentration in PFU/ml and V is volume in ml) to determine how much of the phage stock to add to the 10 ml subculture.

   A high titer phage stock is required to ensure the volume of the subculture is not being changed significantly by adding the phage.

9. Using a 20-µl pipette set to 10 µl, charge a pipette tip with the subculture, and dispense 10 µl into well A for each of the three columns labeled with T_0 in the 96-well plate. Discard tip. Start a timer and return subculture to the shaker.

   These are the 10^{-1} dilutions.

10. With a 20-µl eight-channel pipette set to 10 µl, perform a serial dilution of the T_0 aliquots. Charge three tips with the 10^1 dilutions for T_0 from well A, and dispense 10 µl into their respective well B. Discard tips. Repeat this until dilutions have reached well H.

11. Using the same 20-µl eight-channel pipette set to 10 µl, charge tips with the phage dilution series, and dispense 10 µl onto the agar overlay plate labeled T_0, leaving enough space on the plate to fit two more dilution lines. Discard tips.

    If the drops are spreading out and touching each other, spot 5 µl instead of 10 µl. In this case, continue to spot 5 µl for all the time points and the other biological replicates.

12. Repeat step 11 for the other two columns onto the same plate to obtain a technical triplicate.

13. Carefully slide spotted plate to the side to dry.

14. Repeat steps 9 to 13 for each time point in the experiment.

15. Allow each drop plate to dry for ~1 hr in the biosafety cabinet or until all the drop liquid is fully absorbed.

16. Invert plates and transfer to a 37°C static incubator for 16 to 18 hr. Ensure plates are well spaced to allow airflow.

Data collection and analysis

17. Determine PFU/ml of the subculture at each time point according to Support Protocol 1, and plot data for each time point to obtain a one-step growth curve.

    If the slope of the curve during the latent period is negative, the rate of phage adsorption can be calculated using the following equation:

$$m = \frac{\text{rise}}{\text{run}} = \frac{y_2 - y_1}{x_2 - x_1}$$

    where y_1 is the starting phage concentration (PFU/ml), y_2 is the phage concentration at the end of the latency period (PFU/ml), x_1 is time zero (min), and x_2 is the time at the end of the latency period (min).

    The latent period is defined by the time it takes for the release of new viral progeny by host cell lysis.

    Not all phages will have a negative slope during their latent period; in this case no adsorption rate can be calculated.

18. Calculate burst size using the formula:

$$\text{burst size} = \frac{P - x}{I - x}$$

where P is the number of phages after lysis (PFU/ml), I is the initial phage concentration, and x is the unadsorbed phages.

If the one-step growth curve lacks a negative slope during the latent period, then unadsorbed phages cannot be used in the equation. Set x = 0, and solve for burst size with the modified equation.

BASIC PROTOCOL 5

PRECIPITATION OF BACTERIOPHAGE USING PEG 6000

Precipitation of bacteriophages with polyethylene glycol (PEG) is useful to concentrate the phages and remove bacterial contaminants before transmission electron microscopy or DNA extraction. This procedure is significantly easier to perform than a CsCl gradient, but it does not remove as much bacterial debris as a CsCl gradient would. It is also very important to note this procedure can inactivate some phages and result in reduced titers.

Additional Materials *(also see Basic Protocol 1)*

Filter-sterilized phage lysate ($>10^8$ PFU/ml)
1 M $MgCl_2$, sterile (e.g., Sigma-Aldrich, cat. no. M4880)
1 M $CaCl_2$, sterile
2000 U/ml DNase I (e.g., Fisher Scientific, cat. no. AM2222)
100 mg/ml RNase A (e.g., Qiagen, cat. no. 19101)
1 M NaCl, sterile (e.g., Sigma-Aldrich, cat. no. S5886)
PEG 6000 (e.g., Sigma-Aldrich, cat. no. 528877)

Precipitation of phages with PEG

1. Dispense 10 ml phage lysate into a 50 ml conical tube.
2. Add 25 µl of 1 M $MgCl_2$ and 5 µl of 1 M $CaCl_2$. Close tube lid, and vortex briefly to mix.
3. Add 4 µl DNase I and 10 µl RNase A. Close tube lid, and gently mix by inverting the tube by hand.

   The nuclease treatment is important to allow the phage pellet to form. Do not vortex the lysate once DNase I is added because this enzyme is quite sensitive to mechanical inactivation.

4. Incubate mixture at room temperature for 1 hr with occasional rocking.
5. Add 4 µl of 1 M NaCl and 1 g PEG 6000 to the sample, and vortex until PEG has dissolved.
6. Store at 4°C overnight.

Recovery of precipitated phages

7. Centrifuge tube 30 min at 20,000 × *g*, 4°C.

   Mark where the pellet should be based on its position in the centrifuge to aid in identifying the pellet because it will be translucent.

8. Carefully remove and discard supernatant. Do not disturb pellet.
9. Reposition tube in the centrifuge so that the pellet is in the same position as in step 7. Centrifuge tube again 1 min at 12,500 × *g*, 4°C, to bring down any residual liquid.
10. Use a 200-µl pipette to remove any remaining supernatant from the pellet.

11. Approximate pellet volume, and dispense the same volume of SM onto the pellet. Gently swirl tube every 20 min for 1 hr at room temperature.

    Do not pipette to resuspend the pellet. Be very gentle with the pellet to prevent phage inactivation during resuspension. If the pellet is still large and turbid after the incubation period, let it slowly dissolve overnight at 4°C.

12. Centrifuge sample 10 min at 5000 × *g*, 4°C, to remove any coprecipitated bacterial debris. Transfer supernatant to a 1.5-ml microcentrifuge tube, and store at 4°C.

    This step is not required for some larger phages, as it may result in the phages pelleting with the bacterial debris.

DNA EXTRACTION FROM dsDNA BACTERIOPHAGES

BASIC PROTOCOL 6

Extraction of DNA from phage lysate is a routine method when characterizing novel phages. The DNA produced by this protocol is of high purity and concentration. The DNA can be used for RFLP analysis, sequencing, and PCR.

Materials

Filter-sterilized phage lysate ($>10^8$ PFU/ml; see Basic Protocol 2 or 5)
100 mM $MgCl_2$, sterile (e.g., Sigma-Aldrich, cat. no. M4880)
100 mM $CaCl_2 \cdot 2H_2O$, sterile (e.g., Sigma-Aldrich, cat. no. C7902)
2000 U/ml DNase I (e.g., Fisher Scientific, cat. no. AM2222)
10 mg/ml RNase A (e.g., Fisher Scientific, cat. no. FEREN0531)
0.5 M EDTA, pH 8.0, sterile (e.g., Fisher Scientific, cat. no. AAJ62786A)
20 mg/ml proteinase K (e.g., Fisher Scientific, cat. no. AM2546)
10% (w/v) sodium dodecyl sulfate (SDS; e.g., Fisher Scientific, cat. no. 15553027)
24:24:1 phenol:chloroform:isoamyl alcohol (PCI), pH 5.2 (e.g., Fisher Scientific, cat. no. AAJ62336AE)
Chloroform (e.g., Sigma-Aldrich, cat. no. C7559)
70% and 99% ethanol, chilled to −20°C (e.g., Fisher Scientific, cat. no. 6590)
3 M sodium acetate, pH 5.2 (see recipe)
Nuclease-free water or Tris-EDTA (TE) buffer
0.7% Tris-acetate EDTA (TAE) agarose gel
High-molecular-weight DNA ladder
6× Tri-track loading dye
TAE running buffer (see recipe)
GelRed Nucleic Acid Stain (e.g., Biotium, cat. no. 41003)

1.5-ml microcentrifuge tubes (e.g., VWR, cat. no. 20170-038)
Variable temperature thermomixer
2-ml tubes with Phase Lock Gel Heavy
Vortex mixer
15-ml conical tubes
Microcentrifuge
Microvolume spectrophotometer (e.g., NanoDrop)
Electrophoresis equipment
Gel imaging equipment

CAUTION: All work with phenol and chloroform must be performed in a suitable chemical hood with appropriate personal protective equipment (PPE). Care must be taken when working with these reagents to prevent injury. Appropriate disposal of waste is required.

Removal of contaminating bacterial DNA and digestion of phage capsids

1. Transfer 970 µl phage lysate to a 1.5-ml microcentrifuge tube.

Phage lysate can be concentrated before DNA extraction with PEG 6000 (see Basic Protocol 5).

2. Dispense 25 µl of 100 mM $MgCl_2$ and 5 µl of 100 mM $CaCl_2$ into the phage lysate. Invert several times to mix.
3. Add 1 µl DNase I and 1 µl RNase A, and invert several times to mix.

   DNase I is sensitive to physical denaturation and must not be vortexed.

4. Incubate mixture for 1 hr at 37°C with 300 rpm shaking.
5. Add 40 µl of 0.5 M EDTA to the tube, and vortex briefly for 10 s.
6. Add 2.5 µl proteinase K and 50 µl of 10 % (w/v) SDS.
7. Vortex mixture vigorously for 1 min.
8. Incubate mixture at 55°C for 60 min with 300 rpm shaking.
9. Prepare six Phase Lock tubes per sample by centrifuging tubes 1 min at maximum speed to ensure gel is pelleted before adding samples.

   This step is important to avoid any incomplete separation of the phases.

DNA isolation using Phase Lock tubes

10. Transfer 500 µl sample mixture into two Phase Lock tubes.
11. Determine volume of PCI required for all samples, with each sample requiring 2 ml PCI total. Dispense enough PCI into a conical tube for the extraction.
12. Add 500 µl PCI to each Phase Lock tube. Invert multiple times to mix well.

    The sample is appropriately mixed if it turns cloudy white and if no layer separation is observed.

13. Centrifuge 10 min at 16,000 × *g*, room temperature.
14. Transfer upper aqueous layer into a second Phase Lock tube, and gently mix again with 500 µl PCI.
15. Centrifuge 10 min at 16,000 × *g*, room temperature.
16. Transfer upper aqueous layer into a third Phase Lock tube.
17. Add 500 µl chloroform to the Phase Lock tube, and gently invert several times to mix.
18. Centrifuge 10 min at 16,000 × *g*, room temperature.
19. Transfer upper aqueous layer to a 1.5-ml microcentrifuge tube.

DNA purification and precipitation

20. Add 1 ml ice-cold 99% ethanol and 50 µl of 3 M sodium acetate to each sample, and invert several times to mix.

    A cotton ball–like precipitate may form immediately upon adding the ethanol and sodium acetate, although this is not always the case, and a precipitate may only be apparent after the –20°C overnight incubation.

21. Store samples at −20°C overnight.
22. Centrifuge tubes 10 min at 16,000 × *g*, 4°C, to pellet DNA.
23. Remove supernatant from DNA pellet, and dispense 1 ml ice-cold 70% ethanol. Do not disturb the pellet.

24. Centrifuge 10 min at 16,000 × *g*, 4°C.

25. Remove supernatant from tubes with a 200-µl pipette, taking care not to disturb the pellet.

26. Centrifuge tubes 30 s at 16,000 × *g*, 4°C, and remove any remaining supernatant with a 20-µl pipette.

    At this stage the DNA pellet should appear as a glassy gel-like pellet.

27. Air dry pellet in a biological safety cabinet with the fan on for 10 to 20 min.

    Determine the amount of time required by keeping a close eye on the DNA sample throughout the drying step. A dry DNA pellet is white, with no glassy appearance. Do not over dry the pellet or it will be hard to dissolve and can damage the DNA.

DNA resuspension and quality analysis

28. Add either 50 µl nuclease-free water or TE buffer to the pellet, and incubate at 37°C for 10 min.

    DNA is considerably more stable over time in TE buffer because it protects the DNA from acid hydrolysis. The TE buffer can inhibit some downstream reactions, such as PCR, so it is important to determine which resuspension buffer is appropriate.

    Check the samples to make sure they have fully dissolved by flicking the tube to gently mix. Do not resuspend the samples by pipetting, which can damage the DNA. If the pellet is not dissolved after the 10 min incubation, slowly resuspend the pellet overnight at 4°C.

29. Ensure DNA mixture is homogeneous by repeatedly flicking the tube, and then measure DNA concentration in duplicate using a microvolume spectrophotometer.

    If the phage DNA is highly concentrated (>1000 ng/µl), dilute the sample to 500 ng/µl or less to ensure accurate measurements.

30. Make a 0.7% agarose gel with 1× TAE buffer.

    GelRed stain can be added directly to the molten agarose mixture before casting, but this is not recommended with novel phage DNA. Some DNA modifications can interact with the stain and cause irregular DNA migration through the gel, so it is best to stain the gel after the sample has run to avoid any complications.

31. Prepare high-molecular-weight DNA ladder according to manufacturer's instructions, and add 7 µl DNA ladder into the first well of the gel.

32. Determine volume of sample required to run a 100-ng aliquot of phage DNA on the gel.

    If the DNA sample is highly concentrated, it is best to dilute an aliquot of the sample to ensure pipetting accuracy.

33. Cut a piece of Parafilm, and dispense an appropriate amount of 6× Tri-track loading dye onto the surface of the Parafilm for every sample to be run.

    The amount of loading dye used is dictated by the volume of phage DNA equating to 100 ng. For example, if the phage DNA sample is 10 ng/µl, a 10-µl aliquot will equate to 100 ng and will require 2 µl loading dye.

34. Mix phage DNA sample with 6× Tri-track loading dye directly on the Parafilm. Add this to a designated well.

35. Repeat steps 32 to 34 with any remaining samples.

36. Once all samples have been loaded, run gel in TEA running buffer at 80 V until the orange tracking dye is 1 cm from the bottom of the gel.

Running the samples at a lower voltage is recommended to help ensure the phage DNA enters the gel instead of remaining in the well.

37. Remove gel from the electrophoresis chamber, and stain with GelRed for 15 min.

38. Check DNA integrity.

    The DNA should form a tight band with minimal streaking.

39. Adjust stock DNA concentration to what is required in further experiments, and aliquot DNA into 1.5-ml microcentrifuge tubes for storage at −20°C.

    Standardization of the phage DNA and aliquoting of samples for freezing are highly recommended to limit the number of freeze-thaw cycles the DNA is subjected to, as this damages DNA. In general, a phage DNA sample can be stored at 4°C for up to 1 month and at −20°C for up to 1 year. It is important to note that some phage DNA is less stable than others, potentially due to different DNA modifications; therefore, freshly extracted DNA should be used for any vital experiment.

BASIC PROTOCOL 7

RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF NOVEL PHAGE GENOMES

RFLP analysis is performed with the use of restriction enzymes that cleave DNA at specific sequences creating reproducible DNA banding following gel electrophoresis of the sample. The banding pattern will vary based on the genomic sequence of the phage, as observed with the novel *A. baumannii* phage isolates SA3 and SB5 digested with *Nde*I compared with the uncut and lambda DNA controls (Fig. 6). This technique enables even closely related phages to be distinguishable based on their banding pattern when treated with specific enzymes. It is important to note that many phages modify their DNA to protect it from degradation by bacterial restriction enzymes, and it is not uncommon for some phages to be completely resistant to all restriction enzymes tested in a panel.

Materials *(also see Basic Protocol 6)*

500 ng/μl purified DNA (see Basic Protocol 6)
Control lambda DNA (e.g., Thermo Fisher, cat. no. SD0011)
10× FastDigest Buffer
FastDigest restriction enzyme panel (e.g., Thermo Fisher, cat. no. K1991)
1.2% TAE agarose gel

–20°C benchtop cooler (e.g., Thermo Fisher, cat. no. DS5114-0012)
PCR tubes
Thermal cycler

RFLP reaction set up

1. Set up benchtop cooler with all enzymes and buffers. Obtain a bucket of ice and place the DNA samples in a rack on ice.

   Reaction mixtures must be kept on ice, and enzymes must stay in a benchtop cooler.

   It is easier to group the enzymes into screening panels according to their ability to thermally inactivate. For example, the restriction enzyme EcoRI can be thermally inactivated by incubating the reaction mixture at 80°C for 5 min. Once inactivated, the reaction mixture can be stored at 4°C and run at a later time. In contrast, the restriction enzyme PstI cannot be thermally inactivated, so the reaction mixture must be immediately run on an agarose gel following the digestion step to avoid star activity.

2. Determine how many reactions will be needed using the equation:

$$\text{number of reactions} = ([A \times 2] \times B) + (B + 1)$$

   where A is the number of restriction enzymes tested and B is the number of phage types tested.

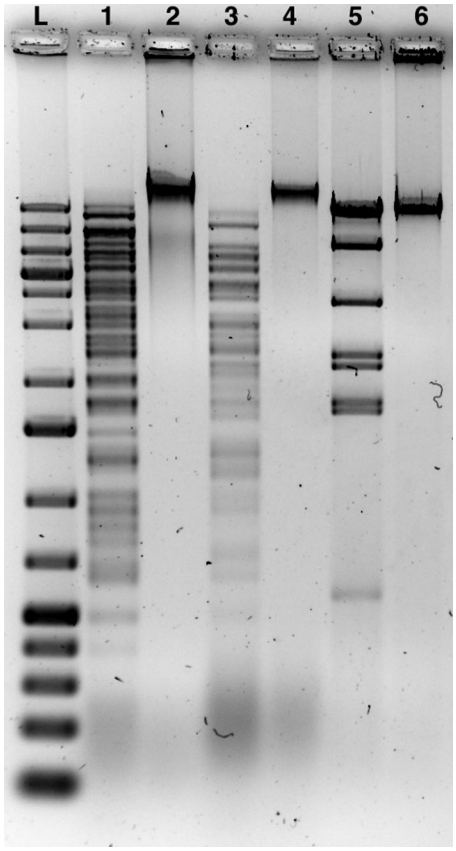

Figure 6 RFLP analysis of *Acinetobacter baumannii* phages SB5 and SA3 and *Escherichia coli* phage lambda. A 500-ng sample of phage genomic DNA was digested for 5 hr with *Nde*I, separated on a 1.2% TAE agarose gel, and stained with GelRed. Lanes are: L, 1-Kb Plus DNA Ladder (Invitrogen); 1, SB5 digested with *Nde*I; 2, SB5 uncut; 3, SA3 digested with *Nde*I; 4, SA3 uncut; 5, lambda digested with *Nde*I; 6, lambda uncut. RFLP, restriction fragment length polymorphism; TAE, Tris-acetate EDTA.

3. Determine amount of each component required to make the master mix by multiplying their volumes by the number of reactions required:

   15 µl nuclease-free water
   2 µl of 10× FastDigest buffer
   2 µl of 500 ng/µl DNA
   1 µl FastDigest enzyme.

4. Combine nuclease-free water and FastDigest buffer together. Briefly vortex and quickly spin in the centrifuge to collect the liquid.

5. Label 1.5-ml microcentrifuge tubes with each phage being tested plus one for the lambda control.

6. Determine number of reactions required per phage by setting $B = 1$ using the equation from step 2.

7. Multiply number of reactions required per phage by 17 µl, and dispense this volume of diluted FastDigest buffer from step 4 into each 1.5-ml microcentrifuge tube.

8. Multiply number of reactions required per phage by 2 µl, and dispense this volume of phage DNA into their respective tubes. Thoroughly flick each tube to mix, and quickly spin in the centrifuge to collect the liquid.

Mixing genomic DNA by flicking is recommended to avoid mechanical shearing of the DNA.

9. Set up PCR reaction tubes for each sample, and dispense 19 µl master mix from step 7 into their respective tubes.
10. Dispense 1 µl of each enzyme (or 1 µl nuclease-free water for negative controls) into their appropriate PCR tube, and flick tubes to mix. Then quickly spin in the centrifuge to collect the liquid.
11. Set up a thermal cycler to incubate the samples at their optimum temperature and time. Inactivate reactions according to the highest and longest inactivation requirement for the enzyme panel selected.

    Digest according to manufacturer's directions, ensuring complete digestion but no star activity.

Electrophoresis and analysis of RFLP reactions

12. Make a 1.2% agarose gel with 1× TAE buffer.

    As discussed in Basic Protocol 6, GelRed stain can be added directly to the molten agarose mixture before casting, but this is not recommended with novel phage DNA.

13. Prepare DNA ladder according to manufacturer's instructions, and add 7 µl DNA ladder into the first well of the gel.
14. Cut a piece of Parafilm, and dispense 2 µl of 6× Tri-track loading dye onto the surface of the Parafilm for every sample to be run.
15. For each reaction, add a 10-µl aliquot of the reaction mixture to one of the 6× Tri-track loading dye droplets, and mix it directly on the Parafilm. Add this to a designated well.
16. Repeat steps 9 to 15 with the remaining samples.
17. Once all samples have been loaded, run gel at 80 V until the orange tracking dye is 1 cm from the bottom of the gel.

    The gel can be run at a higher voltage (100 to 120 V), but it may prevent some of the uncut phage DNA from entering the gel. Depending on the banding pattern, better results might be obtained by running the gel at the lower voltage to get more distinct bands.

18. Remove gel from the electrophoresis chamber, and stain with GelRed for 15 min.
19. Image gel and look for any DNA banding of the phage samples.

    All of the positive controls should be fully digested and the uncut controls intact.

REAGENTS AND SOLUTIONS

SM

5.8 g NaCl (e.g., Sigma-Aldrich, cat. no. S9625)
2.0 g $MgSO_4 \cdot 6H_2O$ (e.g., Sigma-Aldrich, cat. no. M1880)
50 ml of 1 M Tris·HCl, pH 7.5 (e.g., Sigma-Aldrich, cat. no. T2319)
Bring volume to 1 L with deionized water
Stir well to dissolve all components
Autoclave 100-ml aliquots in bottles at 121°C on a standard liquid cycle
Store at room temperature for up to 1 year

Sodium acetate, 3 M, pH 5.2

24.61 g anhydrous sodium acetate (e.g., Sigma-Aldrich, cat. no. 7510-OP)
80 ml deionized water

Adjust pH to 5.2 with glacial acetic acid (e.g., Sigma-Aldrich, cat. no. 1.01830)
Bring volume to 100 ml with deionized water
Autoclave at 121°C on a standard liquid cycle
Store 1-ml aliquots at 4°C for up to 1 year

Preparation should be conducted in a fume hood with proper PPE because the glacial acetic acid is caustic and can burn the skin or lungs if exposed.

Aliquots should be prepared in sterile nuclease-free microcentrifuge tubes.

TAE running buffer, 50×

242 g Tris base (e.g., Sigma-Aldrich, cat. no. 77-86-1)
57.1 ml glacial acetic acid (e.g., Sigma-Aldrich, cat. no. 1.01830)
100 ml of 0.5 M EDTA, pH 8.0 (e.g., Sigma-Aldrich, cat. no. 03690)
500 ml deionized water
Stir until all Tris is dissolved
Bring to 1 L with deionized water
Store at room temperature for up to 1 year

Addition of glacial acetic acid should be conducted in a fume hood with proper PPE because it is caustic and can burn the skin or lungs if exposed.

TALLB

1. Add 500 ml deionized water to a 1.5-L beaker with a magnetic stir bar.
2. Weigh out 20 g LLB powder (e.g., Sigma-Aldrich, cat. no. L3022) and 3 g agarose.
3. Slowly add powders to actively stirring water until all of the reagents are suspended in solution without any aggregates.
4. Transfer liquid to a 1-L graduated cylinder, and bring volume to 700 ml with deionized water.
5. Swirl graduated cylinder until the reagents are suspended in the liquid, and quickly pour it back into the beaker.
6. Rinse sides of the graduated cylinder with 300 ml deionized water, and quickly swirl and dump it back into the beaker.

   The graduated cylinder should be relatively free of reagent particulate at this point. If there is significant particulate left in the graduated cylinder, stop the stir bar, and allow particulate to settle in the beaker. Dispense some of the upper liquid back into the graduated cylinder. Swirl the cylinder to resuspend the particulate and quickly pour the liquid back into the beaker before it settles.

7. Adjust speed of the stir bar until all of the reagent particulate is evenly suspended in the solution to ensure even distribution of agarose to all bottles.
8. Use a 25-ml serological pipette to aliquot 25 ml suspended solution (four aliquots) into each of the glass bottles.
9. Autoclave medium at 121°C for 20 min.
10. Allow medium to cool to ~60°C, and tightly close lids.
11. Place bottles in a 50°C bead bath.

    The medium is stable at this temperature for up to 3 weeks.

    Right before using the TALLB for the first time, aseptically dispense 500 µl of 1 M $CaCl_2$ into the bottle, and swiftly swirl to mix. Return medium to hot bead bath until just before it is required in the experiment.

    $CaCl_2$ is added just before use to prevent precipitates from forming.

COMMENTARY

Background Information

The ability to isolate bacteriophages and perform rudimentary characterization is desired for a variety of applications such as the detection of phage contamination in the dairy industry (Marcó, Moineau, &

Quiberoni, 2012), as an indicator of water quality (Toribio-Avedillo, Blanch, Muniesa, & Rodríguez-Rubio, 2021), or for use as a therapeutic to treat antibiotic-resistant bacterial infections (Hyman, 2019). Bacteriophage isolation is a relatively straightforward procedure where a sample is incubated with a host bacterium to enable phage propagation and detection. The isolation of phages from sewage samples is quite easy and straightforward in comparison to soil extraction techniques, which can be laborious and at times require specialty equipment like tangential flow filtration (Cross et al., 2015; Göller, Haro-Moreno, Rodriguez-Valera, Loessner, & Gómez-Sanz, 2020). Alternate Protocol 1 is straightforward and, if executed on a thoughtfully collected soil sample, is fruitful for the isolation of phages against opportunistic pathogens such as *Burkholderia* spp., *Escherichia coli*, *Bacillus cereus*, and *Stenotrophomonas maltophilia* (Gatedee et al., 2011; Lee, Puglisi, Erill, & Caruso, 2018; Lennon, Liao, Salvador, Lauzon, & Wu, 2020; Peters, McCutcheon, & Dennis, 2020; Peters, McCutcheon, Stothard, & Dennis, 2019; Peters, Stothard, & Dennis, 2017; Seed & Dennis, 2005; Yordpratum, Tattawasart, Wongratanacheewin, & Sermswan, 2011).

By providing a bacterial host, phages capable of infecting that bacterium will propagate in the sample, which is visualized using top agarose overlays. Each plaque formed on the bacterial lawn begins with one phage infecting one bacterium, ultimately leading to numerous rounds of phage propagation and bacterial cell lysis to form each plaque. It is important to note that not all phages form clear and distinct plaques. Some phages form turbid plaques, while others can simply cause thinning and a mottling appearance of the bacterial lawn compared with controls. The protocols presented here use 0.3% (w/v) LLB agarose with bacteria and phages to allow one to see zones of clearing on the bacterial lawn. The top layer of a double-layer assay is traditionally made with agar, but using agarose as the gelling agent instead has many advantages when hunting for novel phages. First, agarose is able to gel at lower concentrations with larger pore spaces compared with agar (Serwer & Wright, 2020). This is important as it allows for increased plaque size due to higher phage diffusion rates within the less-concentrated agarose matrix. Specialty agarose, such as SeaKem Gold by Lonza, has been shown form a stable gel at concentrations as low as 0.04%. This simple difference can be what enables one to identify a novel phage that would otherwise struggle to propagate in agar due to its size or aggregation characteristics (Serwer & Wright, 2020; Serwer, Hayes, Thomas, Demeler, & Hardies, 2009). Second, agar has been shown to inhibit some viruses from propagating in top agar overlays (Borden, Gary, & Murphy, 1970). Until a novel phage has been isolated and successfully purified, it is advisable to exclusively use agarose for the top layer. Once the phage has been successfully purified, comparison can be done of its behavior in agar and in agarose to decide if agar can be used with that phage-host pair moving forward, as agar is a less costly gelling agent.

Bacteriophages have notoriously narrow tropisms, often limited to infecting a single strain of a species or select species of a genus. Determining a bacteriophage's host range is important as it can be used to distinguish between novel phages, identify more permissive hosts to propagate the phage on, or build a broad host–range phage cocktail to target a number of species for therapeutic application. There are two standard approaches to host range studies, and they differ greatly on the amount of material that is consumed. Both methods use a dilution series to acquire efficiency of plating data of the study phage plaquing on a test strain verses the isolation host. The traditional method uses three plates per dilution plus an uninfected control and host lysate control plate. The spot plate method uses a single plate per strain and allows for technical triplicates to be performed on the single plate. There is also room to fit triplicate host lysate control spots on the test plate as well to have a complete analysis on a single plate. When deciding which host range analysis approach to be used, it is imperative to first verify that the phage can plaque on the host with the spot plate method, as some phages cannot plaque with this method and full plates must be used instead. Additionally, medium and reagent requirements can be further reduced when purifying a novel phage by using the modified streak-for-isolation protocol outlined here instead of the traditional full dilution series and plating.

The ability to concentrate phages and further remove contaminating bacterial debris is desirable when performing DNA extractions on the phage lysate or using the lysate for transmission electron microscopy. The use of PEG to concentrate phages is a very common technique due to its technical simplicity and

general success rates (Bourdin et al., 2014). The molecular weight of PEG used is important as this variable can drastically alter phage activity (Carroll-Portillo et al., 2021). The use of PEG 6000 is advisable over other molecular weights to decrease chances of the precipitation step affecting phage viability (Carroll-Portillo et al., 2021; Torii et al., 2022). An alternative to PEG precipitation is a 100-kDa Amicon Ultra-15 centrifugal filter unit to concentrate and wash the phages, although these units are costly to use for routine phage concentrations and can result in a small decrease in phage titer due to phages sticking to the filter (Bonilla et al., 2016).

DNA extraction from bacteriophages is required for RFLP analysis and whole-genome sequencing; thus, a robust and reliable protocol is necessary. There are few commercially available kits specific to phage DNA extraction, but often the resulting DNA is of low concentration and poor purity due to insufficient lysis of phage capsids or contaminating chaotropic salts. Although the protocol outlined here is laborious, time consuming, and uses dangerous chemicals, the resulting phage DNA is typically of high purity and concentration. It is important to note that some phages have heavily modified DNA, and their DNA extractions will always register as low purity due to the modifications present.

RFLP analysis can be a powerful screening tool when phage hunting from a single source at different points in time, as it is often possible to isolate a phage that has already been characterized. Without next-generation sequencing of the isolate, it is difficult to distinguish between two phages with the same phenotypic characteristics, especially if they share high homology. The novel phages SA3 and SB5 are easily distinguished with an *Nde*I digestion (Fig. 6). It is clear by the digestion profile that they are distinct phages. Unfortunately, this technique is not without limitations as it is only useful if the phage's DNA is not resistant to digestion by restriction enzymes. Phage DNA is notoriously modified, some to the extreme of replacing adenine with diaminopurine (Z) to create Z-DNA (Pezo et al., 2021; Sleiman et al., 2021; Zhou et al., 2021). Another example is the conical phage T4, which replaces cytosine with glucosyl-hydroxymethylcytosine (glc-HMC), thus blocking DNA cleavage by many restriction endonucleases and HMC-specific nucleases (Bryson et al., 2015). For these reasons, using a large panel of restriction enzymes for RFLP analysis can increase the chances of identifying an enzyme capable of digesting the phage genome.

An important disadvantage of these protocols is they are not favorable to the isolation of jumbo and aggregating phages, which can be removed from the lysate when using a 0.22-μm filter for the filter-sterilization step. A 0.45-μm filter could be used instead. However, this does not guarantee the lysate is not contaminated with bacteria, and it can also retain some jumbo phages. Filter sterilization can be eliminated in general with the use of chloroform, but there is a chance of inactivating chloroform-sensitive phages. If the isolation of jumbo or aggregating phages is of particular interest to the reader, please refer to the review by Serwer and Wright (2020) and the protocol describing the isolation of jumbo or aggregating phages (Serwer et al., 2009).

Critical Parameters

Bacteriophage considerations

Phages aerosolize very easily, and it can be difficult to detect a cross-contamination problem until it is too late. Ensure samples are protected from each other by keeping lids closed when not in use. Use filter tips exclusively when working with phage samples to prevent contamination of the pipettes and associated cross-contamination. Working with 50-ml conical tubes for phage stocks helps reduce risk of the pipette touching the inside of the sample tube, thus reducing risk of cross-contamination. When vortexing any phage sample, or removing samples from the fridge, centrifuging the sample can reduce the risk of phage contamination of the working area when opening the lids. Recommended centrifugation speed is 12,000 × *g* for 1 min. Care should be taken to prevent bacterial contamination of the phage sample as this can mask plaque development.

Bacteriophages can adhere to plastic or glass, such as pipette tips and flasks. This can be problematic when performing phage titrations or experiments where MOI is very important because it may lower the amount of phage particles transferred. To avoid this issue, it is important to first "charge" the pipette tip with phage sample. Tip charging is accomplished by mixing the sample with the pipette tip 10 times, then fully dispensing any liquid back into the sample. The tip walls are now coated with phages, and the sample is mixed. The sample can now be aspirated and then dispensed into the new tube. Discard the used tip, and repeat this approach for every transfer of

Table 1 Troubleshooting Guide

Problem	Possible cause	Solution
Inconsistent PFU values with titers	Phages sticking to inside of pipette tips	Charge tips; use low-bind tubes; ensure pipettes are calibrated
Picked plaques or plate lysates fail to propagate	Incubation temperature, agarose concentration, or lysis without issue	Incubate plates at 30°C or room temperature; decrease agarose concentration of TALLB; ensure $CaCl_2$ was added to TALLB; some phages simply fail to propagate past one or two rounds
Low DNA yield	Low phage titer, contaminating DNase	Ensure phage titer is $>10^8$ PFU/ml; work with gloves and nuclease-free tubes and water; ensure EDTA was added to tubes first following a DNase/RNase incubation step
Low purity	Contaminating phenol, salt, or carbohydrates	Complete another chloroform extraction and ethanol precipitation; if carbohydrates are suspected, run sample through a silica column
RFLP analysis reveals no digestion	Inactive enzymes or modified DNA	Ensure proper controls were used to determine if the problem is due to enzyme inactivation or DNA modification
Plaques are too small to pick	Agarose too concentrated for phage	Decrease agarose concentration from 0.3% to 0.2% or 0.15%
Spot plate drops flooding together	Agarose layer not sufficiently dried	Increase plate drying time before adding drops

PFU, plaque-forming unit; RFLP, restriction fragment length polymorphism; TALLB, top agarose Lennox Luria Bertani.

phages. This approach will help to standardize phage titration. Similarly, the use of low-binding microcentrifuge tubes will greatly reduce inaccuracies when preparing serial dilutions of phage samples, as well as prevent phage binding during long-term storage.

Lysogen considerations

Many opportunistic pathogens are lysogenized by one or more prophages that offer a fitness advantage to their host. The fitness advantage provided to the host bacterium by the prophages can be due to phage-encoded virulence factors, AMR proteins, and phage-resistance elements that prevent active infection by other closely related phages. Prophages can vary greatly in their ability to be induced from the lysogenic cycle into the lytic cycle. Some prophages undergo spontaneous induction to the lytic cycle in normal growth conditions, whereas others require mitomycin C, UV radiation, or other environmental cues for induction. Standardizing growth conditions for lysogens is extremely important, as even small changes in salt concentration or incubation temperatures can increase prophage induction for some lysogens. Identifying which strains have active prophages is important when isolating bacteriophages using that strain. Hosts with easily inducible prophages will often produce low levels of temperate phage that contaminate the axenic phage stock; the term "axenic" is used to describe a sample devoid of unwanted microorganisms. When plating a new phage on another strain for host range analysis, a negative control consisting of filtered overnight culture supernatant is required to account for background temperate phage activity.

Soil sample collection

If soil samples are to be used following Alternate Protocol 1, the collection location must be thoughtfully considered and selected based on the bacterium's characteristics. This will increase the likelihood of isolating phages against that bacterium. For example, if the pathogen of interest is associated with plant rhizospheres, then collection of soil around the roots of actively growing plants is advisable. If the pathogen is frequently identified in animal husbandry settings, the sample collection should be targeted to active farms featuring those animals.

Double-agarose overlays

It is critical to ensure $CaCl_2$ has been added to the TALLB bottle before commencing work because some phages are particularly sensitive to ion concentrations and require them as cofactors for infection. Additionally, promptly returning the TALLB bottle to the hot bead bath following a double-layer overlay is very important to avoid the formation of solidified

Table 2 Time Considerations

Protocol	Total time	Details
Basic Protocol 1	3 days	Day 1: 15 min (overnights) Day 2: 3 hr (experiment) + 15 min (overnights) Day 3: 2-4 hr (phage isolation)
Alternate Protocol 1	3 days	Day 1: 15 min (overnights) Day 2: 4 hr (experiment) Day 3: 2-4 hr (phage isolation)
Support Protocol 1	3 days	Day 1: 15 min (overnights) Day 2: 2 hr (experiment) Day 3: 30 min (counts)
Basic Protocol 2	5-7 days	Day 1: 15 min (overnights) Day 2: 15 min (per plaque or lysate tube) + 15 min (overnights) Day 3: 2 hr (pick plaques, plating) + 15 min (overnights) Day 4: 2 hr (pick plaques, plating) + 15 min (overnights) Day 5: 2 hr (pick plaques, plating) + 15 min (overnights) Day 6: 1 hr (propagation of axenic stock) Day 7: 1.5 hr (collection, filter sterilize)
Basic Protocol 3	3 days	Day 1: 15 min (overnights) Day 2: 4 hr (experiment) Day 3: 1 hr (counts)
Basic Protocol 4	3 days	Day 1: 15 min (overnights) Day 2: 4 hr (experiment) Day 3: 1 hr (counts)
Alternate Protocol 2	2 days	Day 1: 15 min (overnights) Day 2: 4 hr (experiment) Day 3: 1 hr (counts, analysis)
Basic Protocol 5	2 days	Day 1: 1.5 hr (nuclease treatment) Day 2: 3 hr (pellet, resuspend)
Basic Protocol 6	2 days	Day 1: 3 hr (nuclease, proteinase K) Day 2: 2 hr (pellet, wash, resuspend)
Basic Protocol 7	1 day	Day 1: 4 hr (set up, digest, run gel)

agarose pieces in the molten medium. One must act quickly once the TALLB has been dispensed into a culture tube to ensure the molten agarose does not begin to gel in the culture tube before being poured onto the plate, otherwise a confluent layer of TALLB will not form and it will be difficult to identify plaques. It is also important to avoid moving the double-layer plate once the TALLB has been poured until it has completely solidified to prevent disturbing the gelling agarose, which can make plaques difficult to see.

Basic Protocol 6

Using Phase Lock tubes significantly reduces the chances of having phenol contamination of the DNA samples. It is vital to prespin the Phase Lock tubes to ensure proper migration of the Phase Lock gel between the PCI and aqueous layer. Occasionally the final chloroform extraction can lead to incomplete migration of the Phase Lock gel, with part of the gel remaining at the bottom of the tube. If this occurs, care must be taken to not puncture the thinner gel layer when removing the upper aqueous layer. Additionally, it is important to prepare the ethanol and sodium acetate solutions in advance and ensure they have been in the fridge (sodium acetate) or freezer (ethanol solutions) overnight before use to aid in DNA precipitation.

Troubleshooting

See Table 1 for troubleshooting recommendations related to these protocols.

Time Considerations

See Table 2 for time considerations related to these protocols.

Acknowledgments

The authors would like to thank Hongyan Zhou for her support in making reagents. The bacteriophage research in our laboratories has been supported by a National Research Council of Canada (NRC) Ideation Small Team Project (National Program Office) and by NRC intramural (A base) program under the Vaccine and Emerging Infection Research Initiative (Human Health Therapeutics). Research conducted at the University of Alberta has been supported by a Natural Sciences and Engineering Research Council of Canada Discovery grant (238414-2018).

Author Contributions

Danielle Peters: conceptualization, data curation, formal analysis, investigation, methodology, project administration, supervision, visualization, writing–original draft, review, and editing; **Greg Harris:** methodology, writing–review and editing; **Carly Davis:** methodology; **Jonathan Dennis:** methodology, supervision; **Wangxue Chen:** conceptualization, funding acquisition, resources, supervision, writing–review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Literature Cited

Asif, M., Alvi, I. A., Tabassum, R., & Rehman, S. U. (2020). TAC1, an unclassified bacteriophage of the family *Myoviridae* infecting *Acinetobacter baumannii* with a large burst size and a short latent period. *Archives of Virology*, *165*, 419–424. doi: 10.1007/s00705-019-04483-8

Bonilla, N., Rojas, M. I., Netto Flores Cruz, G., Hung, S.-H., Rohwer, F., & Barr, J. J. (2016). Phage on tap–a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. *PeerJ*, *4*, e2261. doi: 10.7717/peerj.2261

Borden, E. C., Gary, G. W., & Murphy, F. A. (1970). Comparison of agar and agarose preparations for mengovirus plaque formation. *Applied Microbiology*, *20*, 289–291. doi: 10.1128/am.20.2.289-291.1970

Bourdin, G., Schmitt, B., Marvin Guy, L., Germond, J.-E., Zuber, S., Michot, L., … Brüssow, H. (2014). Amplification and purification of T4-like *Escherichia coli* phages for phage therapy: From laboratory to pilot scale. *Applied and Environmental Microbiology*, *80*, 1469–1476. doi: 10.1128/AEM.03357-13

Bryson, A. L., Hwang, Y., Sherrill-Mix, S., Wu, G. D., Lewis, J. D., Black, L., … Bushman, F. D. (2015). Covalent modification of bacteriophage T4 DNA inhibits CRISPR-Cas9. *mBio*, *6*, e00648–15. doi: 10.1128/mBio.00648-15

Carroll-Portillo, A., Coffman, C. N., Varga, M. G., Alcock, J., Singh, S. B., & Lin, H. C. (2021). Standard bacteriophage purification procedures cause loss in numbers and activity. *Viruses*, *13*, 328. doi: 10.3390/v13020328

Cross, T., Schoff, C., Chudoff, D., Graves, Li., Broomell, H., Terry, K., … Dunbar, D. (2015). An optimized enrichment technique for the isolation of arthrobacter bacteriophage species from soil sample isolates. *Journal of Visualized Experiments*, *98*, 52781. doi: 10.3791/52781

Eveillard, M., Kempf, M., Belmonte, O., Pailhoriès, H., & Joly-Guillou, M.-L. (2013). Reservoirs of *Acinetobacter baumannii* outside the hospital and potential involvement in emerging human community-acquired infections. *International Journal of Infectious Diseases*, *17*, e802–e805. doi: 10.1016/j.ijid.2013.03.021

Gatedee, J., Kritsiriwuthinan, K., Galyov, E. E., Shan, J., Dubinina, E., Intarak, N., … Korbsrisate, S. (2011). Isolation and characterization of a novel podovirus which infects *Burkholderia pseudomallei*. *Virology Journal*, *8*, 366. doi: 10.1186/1743-422X-8-366

Göller, P. C., Haro-Moreno, J. M., Rodriguez-Valera, F., Loessner, M. J., & Gómez-Sanz, E. (2020). Uncovering a hidden diversity: Optimized protocols for the extraction of dsDNA bacteriophages from soil. *Microbiome*, *8*, 17. doi: 10.1186/s40168-020-0795-2

Hyman, P. (2019). Phages for phage therapy: Isolation, characterization, and host range breadth. *Pharmaceuticals*, *12*, 35. doi: 10.3390/ph12010035

Lee, M., Puglisi, K. M., UMBC STEM-BUILD Cohort 1, Erill, I., & Caruso, S. M. (2018). Complete genome sequences of HonestAbe, Anthony, and Taffo16, three cluster C bacillus cereus group bacteriophages. *Genome Announcements*, *6*, e00493–18. doi: 10.1128/genomeA.00493-18

Lennon, M., Liao, Y.-T., Salvador, A., Lauzon, C. R., & Wu, V. C. H. (2020). Bacteriophages specific to Shiga toxin-producing *Escherichia coli* exist in goat feces and associated environments on an organic produce farm in Northern California, USA. *PLoS One*, *15*, e0234438. doi: 10.1371/journal.pone.0234438

Marcó, M. B., Moineau, S., & Quiberoni, A. (2012). Bacteriophages and dairy fermentations. *Bacteriophage*, *2*, 149–158. doi: 10.4161/bact.21868

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, *399*, 629–655. doi: 10.1016/S0140-6736(21)02724-0

Peters, D. L., McCutcheon, J. G., & Dennis, J. J. (2020). Characterization of novel broad-host-range bacteriophage DLP3 specific to

Stenotrophomonas maltophilia as a potential therapeutic agent. *Frontiers in Microbiology*, *11*, 1358. doi: 10.3389/fmicb.2020.01358

Peters, D. L., McCutcheon, J. G., Stothard, P., & Dennis, J. J. (2019). Novel *Stenotrophomonas maltophilia* temperate phage DLP4 is capable of lysogenic conversion. *BMC Genomics*, *20*, 300. doi: 10.1186/s12864-019-5674-5

Peters, D. L., Stothard, P., & Dennis, J. J. (2017). The isolation and characterization of *Stenotrophomonas maltophilia* T4-like bacteriophage DLP6. *PLoS One*, *12*, e0173341. doi: 10.1371/journal.pone.0173341

Pezo, V., Jaziri, F., Bourguignon, P.-Y., Louis, D., Jacobs-Sera, D., Rozenski, J., … Marliere, P. (2021). Noncanonical DNA polymerization by aminoadenine-based siphoviruses. *Science*, *372*, 520–524. doi: 10.1126/science.abe6542

Seed, K. D., & Dennis, J. J. (2005). Isolation and characterization of bacteriophages of the *Burkholderia cepacia* complex. *FEMS Microbiology Letters*, *251*, 273–280. doi: 10.1016/j.femsle.2005.08.011

Serwer, P., Hayes, S. J., Thomas, J. A., Demeler, B., & Hardies, S. C. (2009). Isolation of novel large and aggregating bacteriophages. In M. R. J. Clokie & A. Kropinski (Eds.), *Bacteriophages: Methods and protocols, Volume 1: Isolation, characterization, and interactions* (pp. 55–66). Humana Press. doi: 10.1007/978-1-60327-164-6_6

Serwer, P., & Wright, E. T. (2020). In-gel isolation and characterization of large (and other) phages. *Viruses*, *12*, 410. doi: 10.3390/v12040410

Sleiman, D., Garcia, P. S., Lagune, M., Loc'h, J., Haouz, A., Taib, N., … Kaminski, P. A. (2021). A third purine biosynthetic pathway encoded by aminoadenine-based viral DNA genomes. *Science*, *372*, 516–520. doi: 10.1126/science.abe6494

Toribio-Avedillo, D., Blanch, A. R., Muniesa, M., & Rodríguez-Rubio, L. (2021). Bacteriophages as fecal pollution indicators. *Viruses*, *13*, 1089. doi: 10.3390/v13061089

Torii, S., Oishi, W., Zhu, Y., Thakali, O., Malla, B., Yu, Z., … Katayama, H. (2022). Comparison of five polyethylene glycol precipitation procedures for the RT-qPCR based recovery of murine hepatitis virus, bacteriophage phi6, and pepper mild mottle virus as a surrogate for SARS-CoV-2 from wastewater. *Science of the Total Environment*, *807*, 150722. doi: 10.1016/j.scitotenv.2021.150722

World Health Organization. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics.

Yordpratum, U., Tattawasart, U., Wongratanacheewin, S., & Sermswan, R. W. (2011). Novel lytic bacteriophages from soil that lyse *Burkholderia pseudomallei*. *FEMS Microbiology Letters*, *314*, 81–88. doi: 10.1111/j.1574-6968.2010.02150.x

Zhou, Y., Xu, X., Wei, Y., Cheng, Y., Guo, Y., Khudyakov, I., … Zhao, S. (2021). A widespread pathway for substitution of adenine by diaminopurine in phage genomes. *Science*, *372*, 512–516. doi: 10.1126/science.abe4882