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Comparative Analysis of Wastewater Influent Phage Profiles: Metagenomic Sequencing Versus Culture-Based Isolation V.1

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We use this protocol and it's working

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Abstract

Culture-based isolation of bacteriophages from wastewater relies on a limited set of laboratory-cultivable host strains, causing it to recover only a small fraction of the true viral diversity present in the influent (Chibani-Chennoufi et al., 2004). In contrast, metagenomic sequencing bypasses cultivation constraints and uncovers both known and novel phage genomes, providing a more comprehensive snapshot of viral community structure and genetic potential (Paez-Espino et al., 2016; Roux et al., 2016). By comparing metagenomic profiles with culture-derived isolates, researchers can validate in silico host predictions, recover functional phages for downstream characterization, and elucidate phage–host interactions that neither method alone can fully resolve (Brum et al., 2016).

Materials

1) Making a culture:

Materials required

- LB Agar Plate
- Inoculating Loops
- Bacterial Growth Media (LB Broth)
- Culture tube/15 mL Falcon tube
- Tube rack
- Pipetboy
- Serological pipette (5 mL)
- Biohazard Waste Bin
- 37°C Incubator and Incubator shaker
- Parafilm

2) Phage Enrichment:

- Overnight Host Bacteria Culture
- Environmental Sample
- 50 mL Centrifuge tubes
- P200 Pipette and pipette tips
- Pipetboy
- Serological pipette (10/25 mL)
- 2X LB broth spiked with 1M CaCl₂ and 1M MgSO₄
- Sterile 20 mL Syringes
- 0.45 µm Syringe Filter

3) Phage Discovery:

- Overnight Enrichment
- Overnight Host Bacteria Culture
- Top Agar spiked with 5mM CaCl₂ and 5mM MgSO₄
- LB agar plate
- Inoculating Loops
- 15 or 50 mL Centrifuge tubes
- P200 Pipette and pipette tips
- Pipetboy
- Serological pipette (5 and 10 or 25 mL)
- Culture tube
- 20 mL Syringes
- 0.45 µm Syringe Filter

4) Isolating Phage Plaque:

- Phage Discovery Agar Plate
- P200 Pipette and pipette tips

- P1000 Pipette and pipette tips
- SM Buffer
- Sterile 2 mL Microcentrifuge tube (Individually packed)
- Sharpie/marker

5) Scale-up of Phage Lysate*:

- Overnight Host Bacteria Culture
- 125 mL culture flask
- 50 mL centrifuge tubes
- 15 mL tube
- 1X LB Broth
- 20 mL Syringes
- 0.45 µm syringe filter

6) Titering Phage:

- Phage Lysate (from scaleup)
- Overnight Host Bacteria Culture
- Top Agar spiked with 5mM CaCl₂ and 5mM MgSO₄
- LB Agar Plates
- 2 mL Microcentrifuge tubes
- SM buffer
- Culture tube
- Pipetboy
- 5 mL serological pipette
- P1000 pipette and pipette tips
- P200 pipette and pipette tips
- P20 pipette and pipette tips

7) Phage Genome Extraction*:

- Promega wizard DNA cleanup kit
- Phage lysate
- 10 µg/mL DNase I
- 10 µg/mL RNase A
- Precipitant solution (30% w/v PEG-8000, 3M NaCl)
- 5 mM MgSO₄
- 70% isopropanol
- Sterile molecular grade water
- 50 mL centrifuge tubes
- 2 mL microcentrifuge tubes
- 3 mL syringes

8) Capture and Extract Microbes using Nanotrap Microbiome Particles:



- Environmental Water Sample
- Nanotrap Microbiome A Particles
- Nanotrap Microbiome B Particles
- Nanotrap Enhancement Reagent 3 (ER3)
- NucleoMag DNA/RNA Water Extraction Kit
- Mini Heat Block (fit for 2ml tubes)
- Mini Centrifuge (2000 x g)
- DynaMagTM-15 Magnet
- DynaMagTM-2 Magnet
- 15 mL Tubes
- 0.45µm Filter
- 20 mL Syringes
- Serological Pipette
- Pipetboy/Pipette Gun
- 2 mL Microcentrifuge tubes
- Vortex
- Molecular Grade Water

Basic Microbiology Techniques

- 1 We primarily work under BSL-2 conditions. Wearing appropriate personal protective equipment (PPE) is mandatory (lab coat, gloves, close-toed shoes, and eye protection & masks when needed).
Perform all experiments under the hood. If unable to do so, work near an open flame and properly clean the bench surface.

Steps to be followed:

- 2
 1. Wash hands thoroughly and put on required PPE.
 2. Spray 70% Ethanol on the bench surface/under the hood and wipe with a paper towel.
 3. Spray 70% Ethanol on the outer surface of pipettes and pipette tip boxes.
 4. Do not wave anything directly on top of sterile media, plates, and tip boxes when they are open.
 5. Caps/lids should be placed on the bench surface/under the hood face up.

Overview of the experimental steps for Making Host Culture, Phage Enrichment, Phage Discovery, Isolating Phage Plaque, Scale-up, and Titering Phage.

3

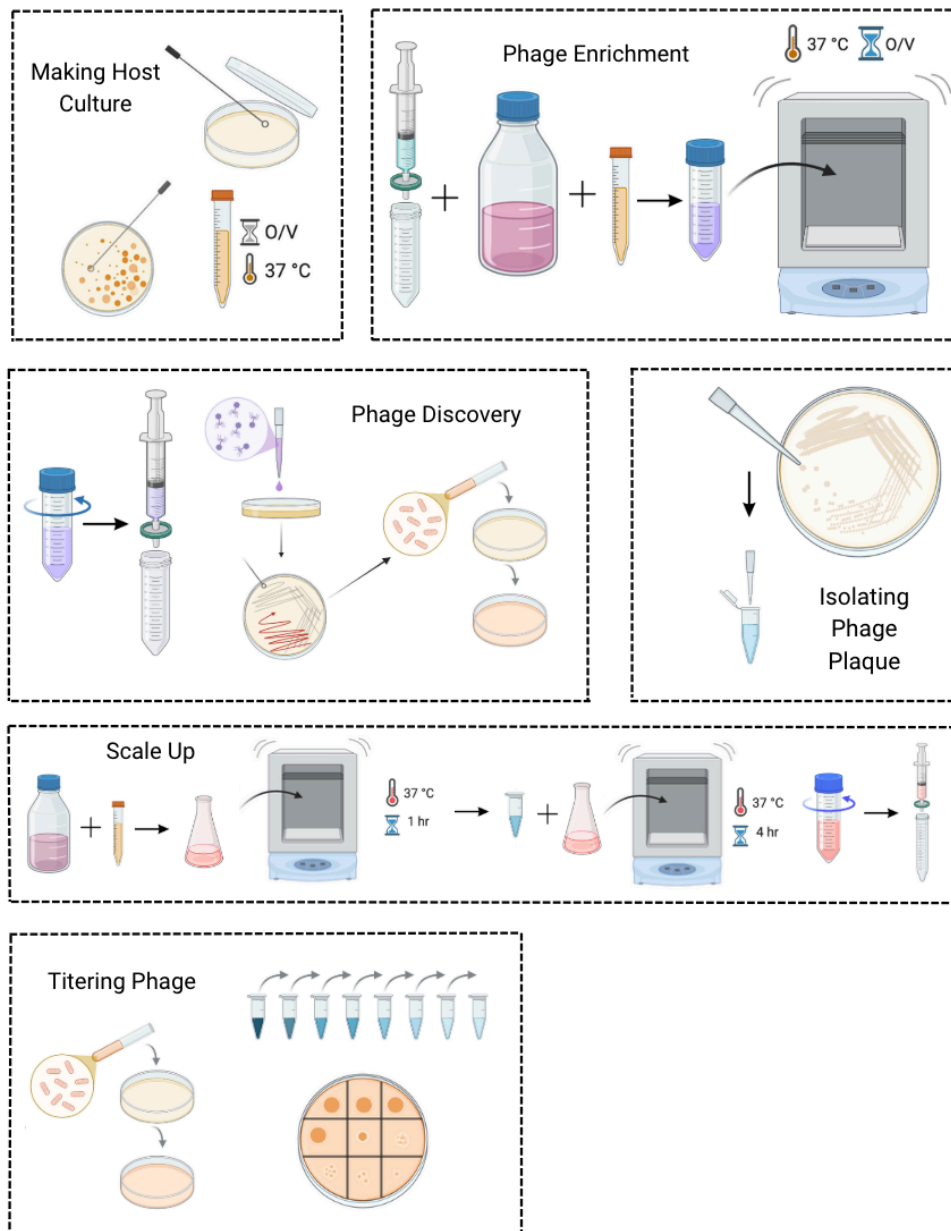


Diagram 1: Shows the steps for the phases of the protocol starting with 1) Making the culture, 2) Phage enrichment, 3) Phage discovery, 4) Isolating phage plaque, 5) Scale up, and 6) Titering phage.

1) Making the Host Culture

4 Materials required

- LB Agar Plate
- Inoculating Loops
- Bacterial Growth Media (LB Broth)



- Culture tube/15 mL Falcon tube
- Tube rack
- Pipetboy
- Serological pipette (5 mL)
- Biohazard Waste Bin
- 37°C Incubator and Incubator shaker
- Parafilm

Steps to be followed:

5 Day 1: Plating the host culture

1. Follow the steps outlined in "Basic microbiology techniques".
2. Warm an LB Agar plate by placing it inside the incubator for a few minutes with the agar side up and the lid facing down so there is no moisture on the agar surface.
3. Label the agar side of the plate with the date, your initials, and the host bacteria information. (If using a Kwik Stik, stick the label that comes with it on the plate, and write the date and your initials.)
4. The quadrant streaking method will be followed:

- a. If using a Kwik Stik, follow the instructions that come with it and streak in a zig-zag motion on the first quadrant of the plate.
- b. If using a glycerol stock from the -80°C freezer, do not let it thaw; grab a disposable loop (or flame sterilize a loop and let it cool, do not wave it around) and scrape the top surface and streak in a zig-zag motion on the first quadrant of the plate.
- c. Dispose of the loop in the biohazard bin, grab a new one (or flame the loop and let it cool), and go into the first quadrant a couple of times and streak into the second quadrant in the same manner.
- d. Dispose of the loop in the biohazard bin, grab a new one (or flame the loop and let it cool), and go into the second quadrant once or twice and streak into the third quadrant in the same manner.
- e. Dispose of the loop in the biohazard bin, grab a new one (or flame the loop and let it cool), and go into the third quadrant only ONCE and streak into the fourth quadrant in a zig-zag motion, make sure there is a wider gap between the streaking rows to get isolated colonies.
- f. Dispose of the loop in the biohazard bin or flame the loop.

5. Use parafilm to seal the plate, place it inverted (agar side up) in the incubator, and incubate overnight.

6 Day 2: Making the Overnight Culture

1. Follow the steps outlined in "Basic microbiology techniques".
2. Take the plate out of the incubator. Remove the parafilm and place it upside down (agar side up).
3. Use the pipetboy and attach a sterile 5 mL serological pipette to aliquot 5 mL of LB broth into a 15 mL tube (or a culture tube). Make sure the LB broth bottle is opened only under the hood.
4. Dispose of the serological pipette into the biohazard bin and close the LB broth bottle.
5. Grab a disposable loop (or flame-sterilize a loop and let it cool). Lift the part of the plate with the bacterial colonies on it with your other hand.
6. Touch a single isolated bacterial colony with the loop and insert it into the 15 mL tube (or culture tube), making sure the tip of the loop is fully submerged in the LB broth.
7. Gently shake the loop to ensure the colony is suspended in the media.
8. Dispose of the loop in the biohazard bin (or flame sterilize the loop and let it cool).
9. Transfer the 15 mL tube (or culture tube) into the 37°C incubator shaker and let it grow overnight (approximately 16 hours).

2) Phage Enrichment

7 Materials required

- Overnight Host Bacteria Culture
- Environmental Sample
- 50 mL Centrifuge tubes
- P200 Pipette and pipette tips
- Pipetboy
- Serological pipette (10/25 mL)
- 2X LB broth spiked with 1M CaCl₂ and 1M MgSO₄
- Sterile 20 mL Syringes
- 0.45 µm Syringe Filter

Steps to be followed:

- 8 1. Follow the steps outlined in "Basic microbiology techniques".
2. Grab an appropriate amount of environmental sample (amount will change depending on how many host bacteria are being used) and place it into a 50 mL centrifuge tube.
3. Centrifuge at 10,000 x g for 10 minutes.
4. After the centrifugation is complete, bring together a 0.45 µm syringe filter, a 20 mL syringe, and a 50 mL centrifuge tube.



5. Open a 50 mL centrifuge tube, leaving the cap facing up.
6. Open a sterile 20 mL syringe and attach a 0.45 μ m syringe filter to the syringe.
7. Rest the syringe with the attached syringe filter on the 50 mL centrifuge tube.
8. Remove the plunger of the syringe from the barrel.
9. Decant the supernatant into the syringe by holding the syringe barrel in one hand and the centrifuge tube in the other.
10. Slowly insert the plunger back into the barrel of the syringe, letting the sample pass through the filter. When all the sample have been filtered, discard the syringe and syringe filter into a biohazard waste bin.
11. Label another 50 mL centrifuge tube with your initials, the date, the host bacteria, and "phage enrichment" (label one 50 mL centrifuge tube per host)
12. Attach a 10 mL serological pipette to the pipetboy and transfer 10 mL of the filtered environmental sample into the labeled centrifuge tube.
13. Use another 10 mL serological pipette to transfer 10 mL of the 2X LB broth (spiked with 1M CaCl₂ and 1M MgSO₄) into the same centrifuge tube.
14. Use a P200 pipette to transfer 200 μ L of the overnight host bacteria into the same centrifuge tube.
15. Transfer the tube into the 37°C incubator shaker and incubate overnight.

3) Phage Discovery

- 9 Materials required
- Overnight Enrichment
 - Overnight Host Bacteria Culture
 - Top Agar spiked with 5mM CaCl₂ and 5mM MgSO₄
 - LB agar plate
 - Inoculating Loops
 - 15 or 50 mL Centrifuge tubes
 - P200 Pipette and pipette tips
 - Pipetboy
 - Serological pipette (5 and 10 or 25 mL)
 - Culture tube
 - 20 mL Syringes
 - 0.45 μ m Syringe Filter

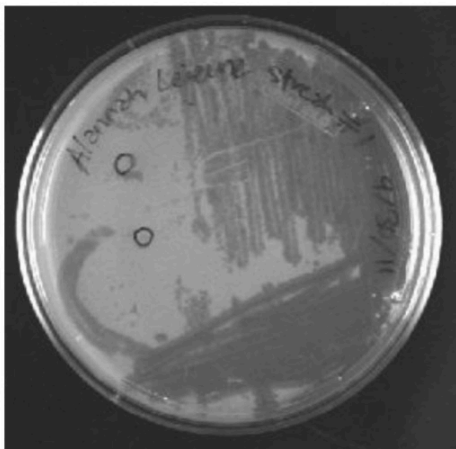
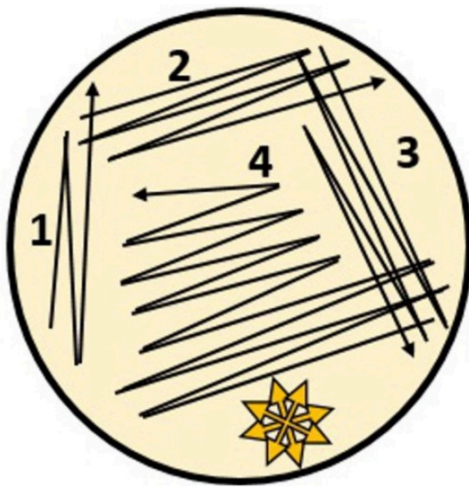
Steps to be followed:

- 10 Filtering Phage Enrichment, Making a lawn of bacterial host, and Streaking Phage Enrichment

1. Follow the steps outlined in "Basic microbiology techniques".
2. Turn the heat block on to 'low' and set the dial between 4 and 5. The goal is to reach 55°C, but anywhere between 50-60°C is okay.
3. Warm an LB agar plate in the incubator for a few minutes, so there is no moisture on the agar surface. (One plate per host)
4. Take the overnight phage enrichment tube out of the incubator shaker and centrifuge at 10,000 x g for 10 minutes.
5. While the centrifuge is running, heat the top agar in the microwave:
6. Loosen the cap of the bottle, but do not remove it.
7. Microwave in increments of 2 minutes, on power level 3.
8. Keep a close watch on the agar, making sure it doesn't boil over; if you start to see this happening, quickly stop the microwave and check on the agar.
9. If the agar isn't completely melted, keep heating, but watch it very closely to prevent any spills.
10. If the agar is completely melted, put on the oven mittens and carefully move the bottle (keeping it closed) and take it to the hood.
11. Attach a 5 mL serological pipette to a pipetboy and aliquot 4 mL of the top agar into a culture tube.
12. Place the culture tube in the heat block.
13. When centrifugation is complete, take a new 15 mL tube and label it with the date, your initials, 'phage discovery', and the host bacteria.
14. Attach a 0.45 µm syringe filter to a 20 mL syringe and rest it on the 15 mL tube. Remove the plunger.
15. Decant the supernatant of the phage enrichment into the syringe by holding the barrel of the syringe in one hand and the centrifuge tube in the other.
16. Slowly insert the plunger back into the barrel of the syringe, letting the phage enrichment pass through the filter. When all the sample have been filtered, discard the syringe and syringe filter into a biohazard waste bin.
17. After streaking, this tube can be kept in the 4°C fridge, to be used for the Nanotrap Extraction Protocol (see Diagram 2 below).
18. Take the LB agar plate out of the incubator and label it with the date, your initials, 'phage discovery', the host bacteria, and 10 µL (or the amount of filtered phage enrichment used).
19. Use a P20 pipette and pipette 10 µL of the filtered phage enrichment onto the LB agar plate.
20. Use a disposable loop (or flame-sterilize a loop and let it cool) and streak the drop as shown in the picture below: Diagram 1
21. Let it dry for about 5 minutes.
22. Use a P200 pipette to pipette 100 µL of the overnight host bacteria into the top agar in the culture tube.
23. Gently vortex the mixture, avoiding air bubbles.
24. Pour the mixture on the LB agar plate, making sure it is spread evenly throughout the entire surface.

25. Allow the top agar to solidify for about 5 minutes.

26. Seal the plate with parafilm and place it in the incubator. Leave overnight at 37°C.



From: Center for Phage Technology "Streaking out phage protocol"; Texas A&M University Agrilife Research

Diagram 2: Streaking culture on an agar plate.

4) Isolating Phage Plaque

11 Materials required

- Phage Discovery Agar Plate
- P200 Pipette and pipette tips
- P1000 Pipette and pipette tips
- SM Buffer
- Sterile 2 mL Microcentrifuge tube (Individually packed)
- Sharpie/marker



Steps to be followed:

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1. Follow the steps outlined in "Basic microbiology techniques".
2. Take out the phage discovery agar plate from the incubator.
3. Check for clear, isolated plaques.
4. Use a marker to circle the plaque you choose on the agar side of the plate.
5. Grab a sterile 2 mL microcentrifuge tube. Close the tube before taking it out of the plastic packaging. Only open the package under the hood.
6. Label the tube with the host bacteria, date, and "ori" (for "origin phage stock")
7. Pipette 500 μ L of SM buffer into the 2 mL microcentrifuge tube.
8. Attach a pipette tip to a P200 pipette and directly puncture the agar where the chosen plaque is. Make sure that the agar plug is visible in the pipette tip.
9. Release the agar plug into the SM buffer, pipetting up and down to ensure the phages are dispersed. Alternatively, you can vortex the tube gently.
10. To obtain a pure lysate of a single phage, the plaque picked needs to be streaked and isolated again at least once. Two times is preferred. This entails repeating the "Phage Discovery" protocol with suspended plaque in place of phage enrichment.

5) Scale-up of Phage Lysate*

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Materials required

- Overnight Host Bacteria Culture
- 125 mL culture flask
- 50 mL centrifuge tubes
- 15 mL tube
- 1X LB Broth
- 20 mL Syringes
- 0.45 μ m syringe filter

Steps to be followed:

14

1. Follow the steps outlined in "Basic microbiology techniques".
2. Add 15 mL LB broth to a 125 mL flask.
3. Add 150 μ L of overnigh host culture to the LB in the flask.
4. Incubate the flask in the incubator shaker for 1 hour at 37°C.
5. You should see cloudiness in the growth media, indicative of bacterial growth. Add 100 μ L of phage suspension from the "Isolating Phage Plaque" protocol into the culture.
6. Let it grow for 3-4 hours.
7. Transfer the culture into a 50 mL centrifuge tube.
8. Centrifuge the tube at 10,000 x g for 10 minutes.

9. When centrifugation is complete, take a new 15 mL tube and label it with the date, your initials, 'phage lysate', and the host bacteria.
10. Attach a 0.45 µm syringe filter to a 20 mL syringe and rest it on the 15 mL tube. Remove the plunger.
11. Decant the supernatant of the phage enrichment into the syringe by holding the barrel of the syringe in one hand and the centrifuge tube in the other.
12. Slowly insert the plunger back into the barrel of the syringe, letting the phage enrichment pass through the filter. When all the sample have been filtered, discard the syringe and syringe filter into a biohazard waste bin.
13. The phage lysate should be titered to confirm that the scaling up process worked. (This step can be omitted if there is time constraint)

6) Titering Phage

- 15 Materials required
- Phage Lysate (from scaleup)
 - Overnight Host Bacteria Culture
 - Top Agar spiked with 5mM CaCl₂ and 5mM MgSO₄
 - LB Agar Plates
 - 2 mL Microcentrifuge tubes
 - SM buffer
 - Culture tube
 - Pipetboy
 - 5 mL serological pipette
 - P1000 pipette and pipette tips
 - P200 pipette and pipette tips
 - P20 pipette and pipette tips

Steps to be followed:

- 16 Making a lawn of bacterial host and Spotting Phage dilutions on bacterial lawn
1. Follow the steps outlined in "Basic microbiology techniques".
 2. Turn the heat block on to 'low' and set the dial between 4 and 5. The goal is to reach 55°C, but anywhere between 50-60°C is okay.
 3. Warm an LB agar plate in the incubator for a few minutes, so there is no moisture on the agar surface. (One plate per host)
 4. Heat the top agar in the microwave:
 5. Loosen the cap of the bottle, but do not remove it.
 6. Microwave in increments of 2 minutes, on power level 3.
 7. Keep a close wathon the agar, making sure it doesn't boil over; if you start to see this happening, quickly stop the microwave and check on the agar.



8. If the agar isn't completely melted, keep heating, but watch it very closely to prevent any spills.
9. If the agar is completely melted, put on the oven mittens and carefully move the bottle (keeping it closed) and take it to the hood.
10. Attach a 5 mL serological pipette to a pipetboy and aliquot 4 mL of the top agar into a culture tube.
11. Place the culture tube in the heat block.
12. As the top agar reaches the desired temperature, take 8 microcentrifuge tubes and place them on a tube rack. Label them 1 through 8, to indicate a serial ten-fold dilutions of the phage lysate from 10⁻¹ to 10⁻⁸.
13. Using the P1000 pipette, pipet 450 µL of SM buffer into all tubes.
14. Using the P200 pipette, pipet 50 µL of phage lysate into the tube labeled "1". Close the lid and vortex the tube briefly at speed 5.
15. Take a new pipette tip and transfer 50 µL sample from tube "1" to tube "2". Close the lid and vortex briefly at speed 5.
16. Repeat step 10 until you have transferred 50 µL sample from tube "7" to tube "8".
17. Label the warmed LB agar plates with the date, your initials, and host bacteria. Divide the agar plate into 9 sections as shown in the Diagram 3 below.
18. Use the P200 pipette to pipet 100 µL of overnight host bacteria into the top agar in the culture tube.
19. Gently vortex the mixture, avoiding air bubbles.
20. Pour the mixture on the LB agar plate, making sure it is spread evenly throughout the entire surface.
21. Allow the agar to solidify for about 5 minutes.
22. When the agar has solidified, use the P20 pipette to pipet 5 µL of sample from tube "8" onto the LB agar plate. Position the pipette tip close to the lawn without directly touching the lawn. Press the pipette plunger slowly so that the phage solution forms a drop at the end of the pipette tip. Gently touch the lawn with this drop so that the phage sample is transferred to the lawn.
23. Using the same pipette tip, pipet 5 µL sample from tube "7" on the lawn. Keep doing this until you have pipetted all samples. Changing tips is not necessary when starting from the most dilute phage solution (tube "8") to the most concentrated phage solution (tube "1").
24. Let all the drops dry (you will know they are dry when they are no longer visible on the agar).
25. Seal the plate with parafilm and place it in the 37°C incubator agar side up and leave overnight.

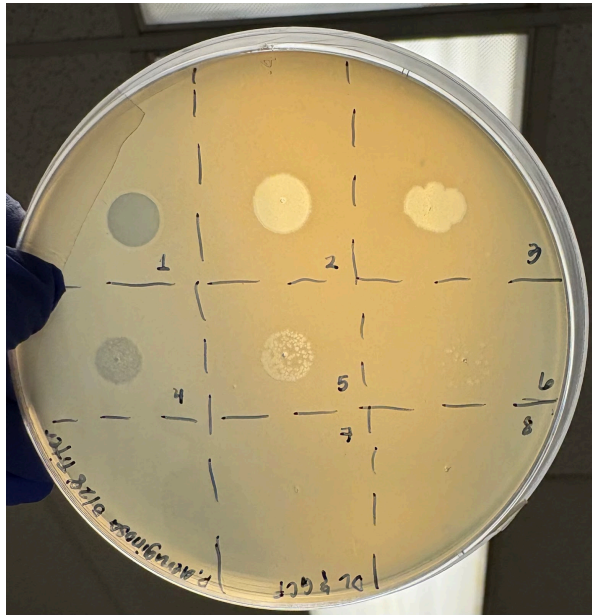


Diagram 3: Titering agar plate example

Overview of the experimental steps for Phage Genome Extractions Day 1 & Day 2

17

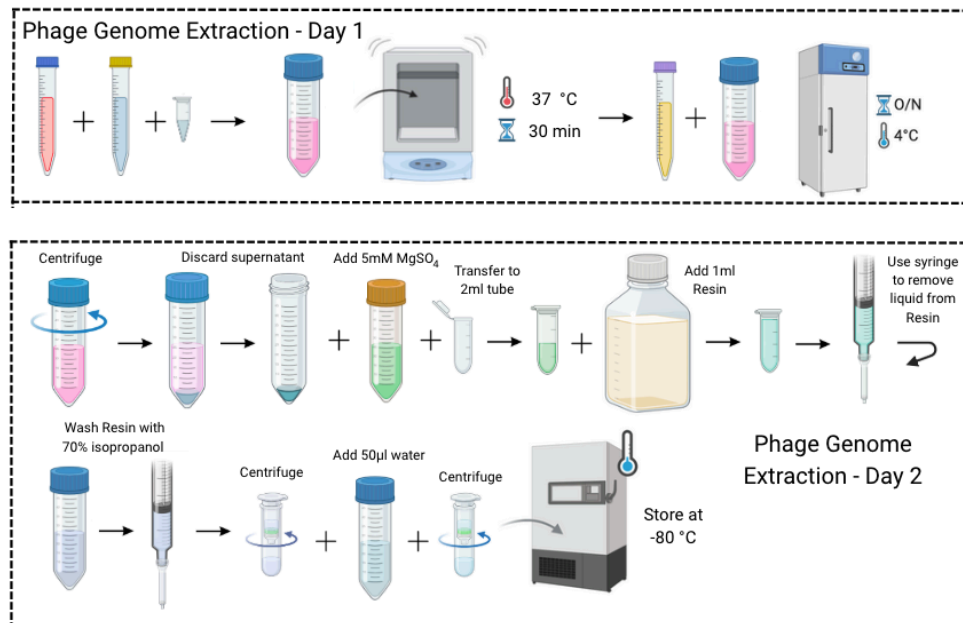


Diagram 4: Steps of Phage Genome Extraction Overview for Day 1 and 2.

7) Phage Genome Extraction*

18 Materials required

- Promega wizard DNA cleanup kit
- Phage lysate
- 10 µg/mL DNase I
- 10 µg/mL RNase A
- Precipitant solution (30% w/v PEG-8000, 3M NaCl)
- 5 mM MgSO₄
- 70% isopropanol
- Sterile molecular grade water
- 50 mL centrifuge tubes
- 2 mL microcentrifuge tubes
- 3 mL syringes

Steps to be followed:

19 Day 1

1. Pipet 4 mL of phage lysate into a 50 mL centrifuge tube.
2. Add 2 µL of DNase I and 2 µL of RNase A to the lysate (0.5 µL DNase I and 0.5 µL RNase A per mL of phage lysate used).
3. Incubate the lysate in the incubator shaker for 30 minutes at 37°C.
4. Add 2 mL precipitant solution to the lysate. Pipet up and down two times to ensure that the precipitant solution is not stuck to the pipette. Gently mix the solution by inverting the centrifuge tube. Incubate the tube at 4°C overnight.

Day 2

1. Centrifuge the lysate at 10,000 x g for 10 minutes at 4°C to pellet the phage particles. Precipitated phages might look translucent, so remember which side of the tube is facing outwards so that the phage pellet can be located.
2. Discard the supernatant by decanting into a waste bin.
3. Add 500 µL of 5 mM MgSO₄ into the centrifuge tube to resuspend the phage pellet by gentle pipeting.
4. Transfer the resuspended pellet into a 2 mL microcentrifuge tube.
5. Resuspend the resin in the Promega kit by gently swirling. Pipet 1 mL of the resin into the phage solution. Mix by inverting the tube 5-6 times.
6. Open up a 3 mL syringe. Remove the plunger and attach a minicolumn to the syringe.
7. Pipet the resin/lysate mix into the 3 mL syringe. Holding the syringe over a waste beaker, insert the plunger and push the resin into the minicolumn. Keep pressing until



all the liquid has been forced through the resin. A slow flow rate usually means a good DNA yield.

8. Remove the minicolumn and the plunger, then reattach the minicolumn.
9. Wash the column by adding 2 mL of 70% isopropanol to the syringe. Holding the syringe over a waste beaker, insert the plunger and push the isopropanol through the minicolumn. Keep pressing until all the liquid has been forced through the resin.
10. Remove the syringe from the minicolumn and discard the syringe.
11. Obtain a new 2 mL microcentrifuge tube, label it and place the minicolumn into it. Centrifuge at 13,000 x g for 2 minutes, RT to dry the resin.
12. Obtain a new 2 mL microcentrifuge tube, label it and place the minicolumn into it. Place the tube + minicolumn into the microcentrifuge. Pipet 50 µL of water into the top of each column and immediately centrifuge at 13,000 x g for 1 minute, RT to elute the DNA.
13. The eluted solution is the isolated DNA, store this in the -80°C.

Capture and Extract Microbes using Nanotrap Microbiome Particles

20 Materials required

- Environmental Water Sample
- Nanotrap Microbiome A Particles
- Nanotrap Microbiome B Particles
- Nanotrap Enhancement Reagent 3 (ER3)
- NucleoMag DNA/RNA Water Extraction Kit
- Mini Heat Block (fit for 2ml tubes)
- Mini Centrifuge (2000 x g)
- DynaMag™-15 Magnet
- DynaMag™-2 Magnet
- 15 mL Tubes
- 0.45µm Filter
- 20 mL Syringes
- Serological Pipette
- Pipetboy/Pipette Gun
- 2 mL Microcentrifuge tubes
- Vortex
- Molecular Grade Water

Nanotrap Combined NucleoMag Manual Procedure-Part 1

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1. Acquire an environmental water sample
2. Place 10 mL of raw environmental water sample into a clean 15 mL conical tube
3. Filter 10 mL of raw environmental water sample with a 0.45 µm filter and place into a clean 15 mL conical tube



4. Similar to phage enrichment steps 4-10
5. Place 10 mL filtered phage enrichment into a clean 15 mL conical tube (Repeat per host)
6. Turn on and set the mini heating block to 95°C

For Each Sample (at the same time):

1. Add 100 µL of Nanotrap Enhancement Reagent 3 (ER3) to the sample
2. Add 150 µL of Nanotrap Microbiome A Particles to the sample
3. Add 150 µL of Nanotrap Microbiome B Particles to the sample
4. Cap the tube and then invert 2 times to mix the particles
5. Incubate samples with Nanotrap particles at room temperature for 10 minutes. Note: Invert every 5 minutes or use a rotator.
6. Place the tube on a DynaMag-15 magnetic rack to separate the Nanotrap particles from the sample for 5 minutes.
7. Using a serological discard the supernatant carefully without disturbing the Nanotrap particle pellet.
8. Add 1 mL of Molecular Grade Water to the tube, pipetting onto the walls of the conical tube to resuspend the pellet. Note: Resuspend as much Nanotrap particles as possible. Rotate liquid around walls of tube while holding at an angle. Do not let the liquid touch the lid.
9. Transfer the Nanotrap particle suspension to a new 2 mL microcentrifuge tube.
10. Place the 2 mL microcentrifuge tube on a DynaMag-2 magnetic rack to separate the Nanotrap particles from the sample for 2 minutes.
11. Using a P-1000 pipette, discard the supernatant carefully without disturbing the Nanotrap particle pellet. Note: If any small amount of liquid is still present, use a smaller pipette to remove all the supernatant from the bottom of the tube.
12. Add 500 µL of Lysis buffer MWA1 to Nanotrap particle pellet. Vortex to resuspend pellet.
13. Incubate the samples on a heating block at 95°C for 10 minutes. Note: Briefly centrifuge tube in mini centrifuge (2000 x g) for 2-5 seconds to remove drops from lid before magnetic separation. Note: When done heating at 95°C, set mini heat block to 56°C
14. Place the 2 mL microcentrifuge tube on a DynaMag-2 magnetic rack to separate the Nanotrap particles from the sample for 2 minutes.
15. Transfer all supernatant/lysate to a new 2 mL collection tube (set P-1000 to ~550µL) and discard the Nanotrap particles pellet. Note: Do not discard supernatant/lysate.
16. Samples are now ready for Part 2.

Nanotrap Microbiome Combined NucleoMag Manual Procedure-Part 2

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1. Add 475 µL of Binding Buffer MWA2 to the sample/lysate.
2. Add 25 µL of NucleoMag B-beads to the sample/lysate.

3. Vortex to mix, then incubate at room temperature for 10 minutes. Note: Invert every 5 minutes or use a rotator.
4. Add 850 μ L of Wash Buffer MWA3 to sample. Vortex to resuspend.
5. Place the tube on a DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes, then discard the supernatant.
6. Repeat steps 5 and 6 (Pellet wash with MWA3).
7. Centrifuge the tube in mini centrifuge (2000 x g) for 5 seconds.
8. Place the tube on a DynaMag-2 magnetic rack, then remove excess Wash Buffer MWA3 using a smaller pipette (P-20).
9. Add 850 μ L of Wash Buffer MWA4 to sample. Vortex to resuspend.
10. Place the tube on a DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes, then discard the supernatant.
11. Centrifuge the tube in mini centrifuge (2000 x g) for 5 seconds.
12. Place the tube on a DynaMag-2 magnetic rack, then remove excess Wash Buffer MWA3 using a smaller pipette (P-20).
13. Take samples off magnetic rack, open caps, and allow samples to air dry at room temperature for 10 minutes.
14. Add 100 μ L of Rnase-free water to re-suspend the magnetic beads and then incubate at 56°C for 5 minutes on a heat block (close caps).
15. Place the tube in the DynaMag-2 magnetic rack to separate the magnetic beads from the sample for 2 minutes. Note: May need to briefly centrifuge tube in mini centrifuge (2000 x g) for 2-5 seconds to remove drops from lid before magnetic separation.
16. Transfer the supernatant to a new tube. The sample is now ready for downstream analysis or can be stored at -80°C. Note: Multiple freeze-thaw cycles may cause degradation.

*This portion is subject to change.



Protocol references

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Acknowledgements

Protocol Sources: **“BLDAP 2024; Isolation Of Novel Bacteriophages From Environmental Samples”** By: Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory; **Center For Phage Technology Phage Protocols**, From: Texas A&M University Agrilife Research; **Nanotrap® Microbiome A and B Enviro Water Protocol using NucleoMag Kit (APP-081)** By: Ceres Nanotrap; **Microbe/Phage Wastewater DNA/RNA Concentration and Extraction (Nanotrap® and NucleoMag® RNA Water)** By: Archana Anand, Michael Hajkowski, Katherine Dick, Brett Rasile, Kendra Maas – San Francisco State University & University of Connecticut