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Phage hunters: A CURE for retention in the sciences

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Abstract

This workshop, geared towards college freshmen, introduces participants to a course-based undergraduate research experience (CURE) that fosters student engagement and promotes excitement and retention in Biology and Biology research. The Howard Hughes Medical Institute (HHMI) Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) CURE begins with simple digging in the soil or collecting day-old grass clippings to find new viruses, but progresses through a variety of microbiology techniques and, in the second semester, to genome annotation and bioinformatic analyses. Compared to traditional labs, the SEA-PHAGES CURE increases student interest in Biology and has been shown to increase retention in STEM disciplines. This workshop will guide participants through an abbreviated version of the isolation stage that kicks off the semester-long SEA-PHAGES CURE. Participants will collect grass from on campus and then perform a direct isolation to extract phages from their grass clippings. Participants will then plate their extract to create a plaque assay which will be used to identify and characterize their phage.

Keywords: CURE, Bacteriophage, Microbiology, Molecular biology, HHMI, SEA-PHAGES

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INTRODUCTION

The Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) CURE is an exciting year-long discovery-based undergraduate research course supported by the Howard Hughes Medical Institute (HHMI) with a proven track record of increasing retention in the sciences (Jordan et al., 2014, Hanauer et al., 2017). The SEA-PHAGES course is designed for college freshmen Biology students, and can serve as a pipeline to any microbiology-focused upper-level course or independent research project.

The exercise described here is a modified version of the first step in the SEA-PHAGES program, where students isolate a novel bacteriophage from a grass sample. The exercise is divided into three sections. First, students are instructed to collect three appropriate grass samples, and record relevant environmental data about each one. Second, students are introduced to aseptic technique and are encouraged to apply it to liquid transfer using a serological pipette. Third, students isolate phage from their grass samples by incubating each grass sample in buffer, filtering the liquid to exclude bacteria, and plating the filtrate along with a bacterial host to encourage the growth of phage. After 24-48 hours of incubation, plaques should be apparent on plates derived from samples where phage are present.

After the isolation exercise described here, students in the HHMI SEA-PHAGES program will spend the rest of the first semester

purifying, amplifying, and characterizing their phage using a series of microbiology and molecular biology techniques. They also name their phage, produce an image of it through electron microscopy, and submit their isolate to the Actinobacteriophage database (www.phagesdb.org), all of which serve to increase feelings of ownership to their project. The second semester of SEA-PHAGES is spent delving into the genome of a single phage isolate, thereby giving students invaluable training in genome annotation and bioinformatics.

The authors would like to stress that they were not involved in the original creation of the SEA-PHAGES curriculum; rather they modified, with permission from HHMI, some of the materials from the Phage Discovery Guide (originally created by HHMI) to create this publication and the workshop materials from which it was derived. The authors hope that this publication serves as a good introduction to these materials, all of which are publicly available at seaphages.org.

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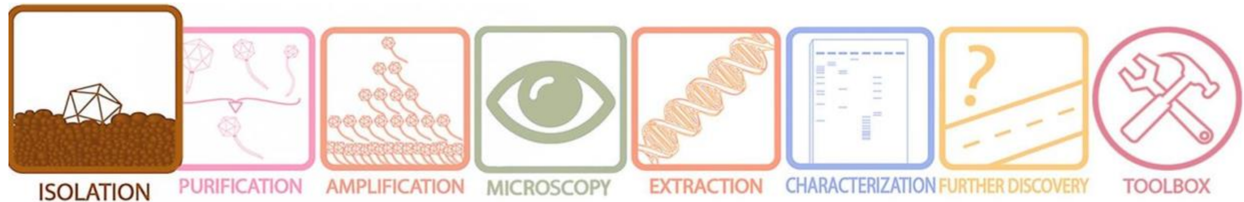
STUDENT OUTLINE

Student Laboratory Handout 1 - Collecting Environmental Samples

The following information was modified from the SEA PHAGES Discovery Guide for use at ABLE 2023



The following information was modified from the SEA PHAGES Discovery Guide for use at ABLE 2023.

Protocol 1: Collecting Environmental Samples

Objective: To obtain an environmental sample containing bacteriophage.

Rationale: By collecting grass clippings rich in bacteria (at least a day old and still green), you will aim to collect a bacteriophage that can infect those bacteria. To increase your chances of isolating phage that can infect your specific host bacteria, you should consider sampling environments where your host bacteria thrive. The host bacterium that we are using is well-suited to decaying grass.

Supplies:

- Plastic sandwich bags for collecting samples and a tool for digging.
- Data collection sheet
- Labeling pen
- Smartphone or tablet with GPS capabilities or computer

Procedure:

1. Collect three samples of day-old green grass clippings. For each sample perform the following steps:
    a. Turn a clean plastic sandwich bag inside out and insert your hand into the bag as if it were a glove. Grab a handful of grass clippings, keeping the plastic bag between your hand and the sample.
    b. Remove your hand, inverting the bag with the clippings to the inside, and seal the bag.
2. Label the sample bag appropriately (*e.g.*, initials, location) so you can identify where the sample was collected.

3. Record important aspects of the sample and collection site.
    a. Name the sample something that will identify the location where it was collected.
    b. Record the GPS coordinates of your sample collection site.

        If you have a smartphone or tablet during sample collection, determine the GPS coordinates and record this information.

        If you do not have a smartphone or tablet during sample collection, determine the GPS coordinates when you have access to a computer. Record this information.
    c. Record the physical characteristics of your sample.

        For grass samples: Was the grass wet or dry? Was the grass still green?

        What was the ambient temperature?
    d. Repeat steps 1 - 3 for each sample collected.

4. Bring your collected samples to the lab; your next step will be to process your environmental sample(s) using the Direct Isolation protocol (3a).

Helpful Tips:

- It is best to collect samples the same day.
- If you collect samples earlier, store them at 4˚C.

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Phage hunters: a CURE for retention in the sciences
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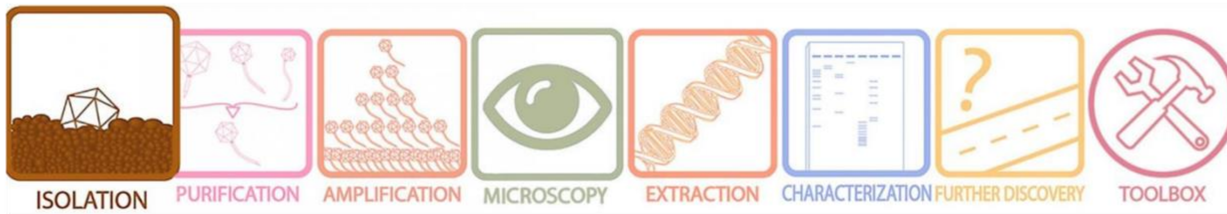
Environmental Sample Data Sheet

Sample	1	2	3
Name of Collector			
Date of Collection			
Description of Grass (type if known, wet or dry, fresh or old, pure or weedy)			
Location Descriptor (forest, field, compost, etc.)			
General Location (neighborhood, town, farm, adjacent to water, etc.)			
Specific Location (GPS coordinates)			
Ambient Temperature (°C)			

Student Laboratory Handout 2 – Aseptic Technique



The following information was modified from the SEA PHAGES Discovery Guide for use at ABLE 2023.

Protocol 2: Aseptic Technique

Objective: To prevent contamination in microbiology experiments.

Rationale: The growth of contaminating bacteria, fungi, and viruses can be avoided by disinfecting your bench, creating an updraft with a Bunsen burner, and moving with care and deliberation.

Supplies:

- 70% Ethanol (EtOH)
- Bunsen burner and striker
- paper towel, gauze pad, or Kimwipes™

Procedure:

1. Prepare your work area.
   a. Tidy up your workbench by removing clutter, papers, bottles, etc.
   b. Using a squeeze bottle containing EtOH, dispense enough disinfectant to dampen the entire work surface.
   c. Using a paper towel, gauze pad, or Kimwipes™, wipe the entire surface, starting at the back, spread EtOH across the entire work surface.
   d. Let the disinfectant evaporate—do not wipe dry.

2. Ignite the Bunsen burner.
   a. After the disinfectant has completely dried, light the Bunsen burner.
   b. Adjust the flame so that a blue "cone" can be seen in the flame. (The tip of this cone is the hottest part of the flame.)

3. The instructor will now demonstrate aseptic use of serological pipets (Figure 2.1) Later, you will use this technique to transfer filtrate to the host bacteria. For reference, the steps from the demonstration are outlined below.
   a. Arrange your necessary supplies in your aseptic field.
   b. Loosen (but do not remove) the tube and bottle caps.
   c. Peel down the wrapper on the 5 ml serological pipette (as you would a banana) from the top (opposite the tip). Hold the flaps against the pipette in your nondominant hand. (If you write with your right hand, that hand is your dominant hand.)
   d. Hold the pipettor in your dominant hand, place the pipette on the pipettor and remove the wrapper. Do not let the tip of the pipette—which is sterile—touch any surface! Using your nondominant hand, unscrew the top of the bottle/tube and leave it between your forefinger and middle finger (Figure 2.1G).

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e. Briefly flame the lip of the bottle, then place the serological pipette into the bottle/tube, and using the pipettor, draw out 1.5 ml of filtrate.
f. Flame the lip of the bottle, replace the cap and set the bottle/tube down.
g. Using your nondominant hand, pick up the culture tube. Using the smallest finger on the hand holding the pipette, remove the top of the culture tube. (Alternatively, lift the cap off the tube with the fourth and middle fingers of your nondominant hand and then pick up the open tube with your index finger and thumb).
h. Dispense the liquid from the pipette into the tube.
i. Replace the cap of the culture tube and set the tube down.
j. Place the pipette back in its wrapper, remove from the pipettor, and then discard the pipette.

4. Turn off your Bunsen burner and tidy up your area as directed by your instructor.

Helpful tips:

- Never pass your hands or fingers over the tops of open containers (such as open bottles or flasks, the inside of tubes and bottle caps, and agar plates) within the work area near the flame.
- Plastic conical tubes and microcentrifuge tubes cannot be flamed, so it is doubly important to work with an open flame and take care not to pass fingers or hands over any tube openings.
- Never wear gloves when working with an open flame to avoid the risk of injury due to the material melting.
- Never set a bottle or tube cap or Petri dish lid on a bench top.
- Never go into a sterile solution with a used pipette or tip. In other words, never reuse a pipette, even if great care has been taken to keep it sterile.
- Never leave an open flame unattended.
- Always work with an open flame when opening sterile tubes or bottles.
- Hold open tubes and bottles in the aseptic field at an angle to reduce the chance of airborne contamination.
- Never have more than one tube, bottle, or flask open on the bench at one time.
- Even if someone else has recently used the bench and the bench top has been wiped down with disinfectant, always begin your laboratory time by wiping down the bench top.

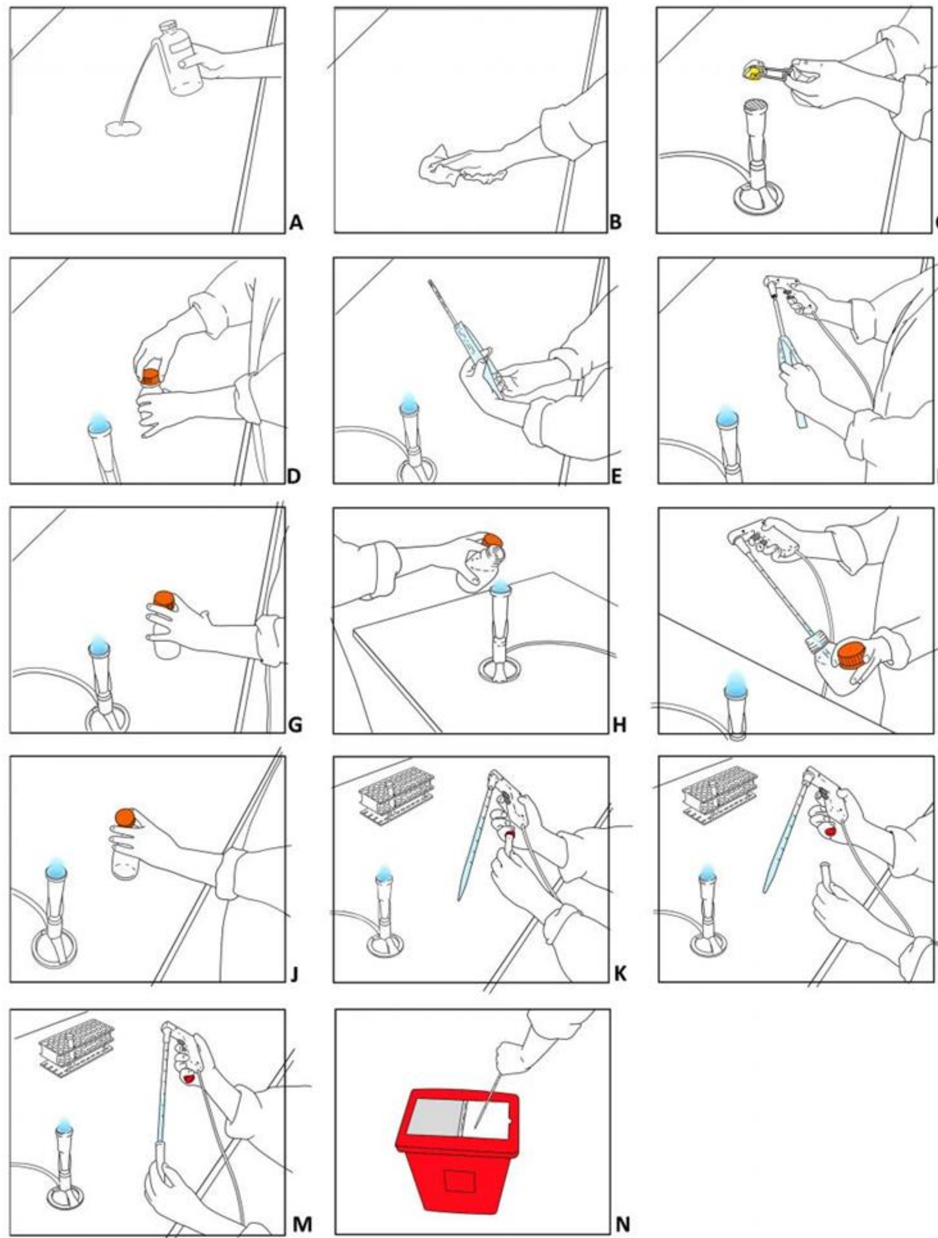

Figure 2.1. Transferring liquid samples using aseptic technique. Before beginning, dispense ethanol or other disinfectant onto the surface of your bench (A) and wipe from back to front (B). Light your Bunsen burner (C). Prepare your materials and loosen all bottle and tube caps (D). Peel down both sides of the pipette package (E) and hold both flaps while attaching the pipettor (F). Using your first and second fingers, remove the bottle top (G) and pick up the bottle with the same hand and "flame" (H). Remove your sample (I) and re-flame and recap the bottle (J). Using the smallest finger of your pipettor hand, remove the top of the tube (K and L) and add your sample to the tube (M). Recap the tube before discarding the pipette (N). When finished, turn off your Bunsen burner, tidy up, and wipe down your bench.

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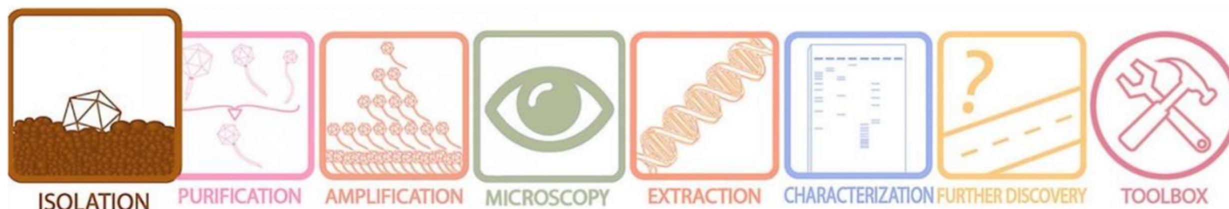
Student Laboratory Handout 3 - Direct Isolation and Plaque Assay



The following information was modified from the SEA PHAGES Discovery Guide for use at ABLE 2023

Protocol 3a: Direct Isolation

Objective: To extract phages from an environmental sample.

Rationale: This method extracts phages from microbes and particulate matter in a solid environmental sample. The extracted sample is then used to infect your host bacteria by using the plaque assay. This method offers a snapshot of all the phages present in your environmental sample that may infect your host. Indeed, using direct isolation, you may discover many different phages with distinct plaque morphologies. However, since not every phage in your sample will succeed at infecting your host bacteria, you may find very few plaques on your plate after incubation.

Supplies:
- Environmental sample
- Liquid media ~35 ml/sample
- Sterile 3 ml or 5 ml syringe
- 0.22 µm syringe filter
- 5 ml serological pipettes
- Microcentrifuge tubes
- 50 ml conical tubes

Procedure:

1. Prepare your bench for aseptic work and assemble your supplies.

2. You will need an environmental sample collected using the protocol Collecting Environmental Samples (Protocol 1).

3. Extract phage from solid environmental samples, such as soil or grass.
    a. Label each of three 50 mL conical tubes with your sample name, date, and initials.
    b. Light your Bunsen burner.
    c. Fill each tube ~1/3 full with grass clippings from a single sample.
    d. Add liquid media to the 35 mL mark of each tube and vortex briefly.
    e. Cap the tubes and shake all three by hand for 3-5 minutes to mix thoroughly.
4. Prepare a phage filtrate using aseptic technique.
    a. Label two new 50 mL conical tubes per sample as “direct filtrate” and your sample name, date, and initials.
    b. Working aseptically under a flame to complete the following steps.
    c. Open the package of a syringe filter (0.22 µm), leaving the filter in the packaging.
    d. Using a syringe, remove at least 2 ml of liquid from the first grass sample.

i. Avoid withdrawing solid material to prevent clogging the filter during filtration.

e. Attach the syringe to the top of the filter, and then remove the filter from the package. Be careful not to contaminate the filter in the process.
   i. Make sure the filter is screwed firmly into place.
f. Depressing the syringe plunger, dispense a minimum of 2 ml of filtrate into the appropriately labeled 50 ml conical tube.
   i. Because debris can clog the filter, you may encounter resistance. Do not continue to force liquid through the filter or it will break. If your filter clogs, remove the clogged filter, replace it with a new one, and continue filtering.
   ii. Cap the tube immediately.
   iii. Remove the filter and place it back in its packaging.
   iv. Repeat this process if you need to collect more filtrate.
g. Discard the syringe and filter.
h. Repeat steps c-g for your second and third samples. These three tubes contain your direct isolates.
i. Proceed directly to the Plaque Assay (3b) protocol.

Protocol 3b: Plaque Assay

Objective: To detect the presence of phage on bacterial lawns.

Rationale: The plaque assay allows you to visually confirm the presence of phage particles in a sample by exposing phage to bacteria and looking for evidence of bacterial cell death. It is a versatile assay that can be used for phage isolation, purification, and titering. In a plaque assay, host bacteria are mixed with a phage sample and grown as a lawn on agar. If phages are present, they will infect and replicate within the bacterial host, killing the host in the process. Newly replicated phages diffuse within the agar and repeat the process of infection, replication, and lysis of nearby host bacteria. As a result, a visible circular zone of clearing/killing called a plaque will become apparent on the bacterial lawn. Note that each plaque arises from a single phage particle in the original phage sample.

Supplies:
- Direct isolates from procedure 3a
- Host bacteria (500 µl/plate)
- PYCa Agar plates
- PYCa Top agar, molten (between 55 - 60 ˚C)
- 5 ml serological pipettes
- Serological pipette pump

Procedure:
1. Assemble your supplies and ensure you are working aseptically near an open flame.

2. Inoculate the host bacteria with your direct isolates
   a. Obtain three aliquots of 500 µl host bacterial cultures; label these tubes with your sample names.
   b. As demonstrated earlier, use a serological pipette and aseptic technique to dispense 1.5 mL of each direct isolate into the appropriate culture tube containing 500 µl of host bacteria.
   c. Mix each inoculated host culture by gently tapping the tube. (do not invert tubes)
   d. Let your sample sit undisturbed for 5–10 minutes to allow for attachment.

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3. Plate the samples with top agar. For this part of the experiment, you will need 3 ml of molten top agar per sample.
    a. Obtain three agar plates. Label the bottom perimeter of the plates with your sample name, date, and initials.
    b. Remove a bottle of top agar from the 55 °C bath.
        i. Important: You want to keep the top agar in the 55 °C bath for as long as possible to prevent it from prematurely solidifying on your bench.
    c. For each sample, aseptically transfer 3 ml of top agar to an inoculated host tube (*i.e.*, the tube containing bacterial host and phage sample) using a sterile 5 mL pipette.
        i. Important: Try to avoid making or withdrawing bubbles, as they can look like plaques on plates.
    d. Immediately aspirate (suck-up) the mixture back into the same pipette. Dispense the mixture onto the appropriate plate and discard the pipette.
        i. Important: The top agar should not sit in the pipette for more than a few seconds because the agar will begin to solidify.
    e. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats the agar plate.
    f. Repeat this process for each of your samples.

4. Incubate plates to allow bacterial growth and phage infection.
    a. Let the plates sit undisturbed for ~20 minutes until the top agar solidifies.
    b. After the top agar has solidified, gently invert the plates.
    c. Incubate the plates at 30ºC.
5. Check the plates for plaques after 24-48 hours (note: plates will be available to view either electronically or in person at a later date).
    a. Record your results. Be thorough. What do you see on your plates? Count the number of plaques and take note of the size, shape, and other distinctive features of the plaques. Remember, negative results are important too.
        i. Important: The morphology of a plaque is an important characteristic. Simply noting “small” or “round” is not an adequate descriptor of plaque morphology. Try to be specific in your plaque descriptions. A good description will include size, turbidity, margin type, etc.

Helpful Tips:

- When you pipette hot top agar onto the plate, avoid introducing bubbles because they will look like plaques later.
- It is important that the top agar is neither too hot (it will kill bacteria and phages) nor too cold (it sets too quickly).
- A common problem is inverting the plate before the top agar has properly set, resulting in top agar and bacteria sliding off the plate onto the lid! How quickly the top agar sets depends on many factors, including room temperature and humidity. You can check the top agar by gently tapping the side of the dish and seeing if it moves. Also, when you pick up the plates, watch what happens when you start to tilt the plate.
- When observing plates after incubating, you may see as few as one plaque and it may be as tiny as a pinpoint.
- If your plates have excessive condensation on the lid, be mindful of drips when viewing your plates.

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MATERIALS

Sample Collection Kit (to be handed out at the beginning of the conference) – 1/participant

- 1 gallon zip top bag containing the following:
  - printed sample collection instructions and environmental sample data sheet (protocol 1)
  - 3 sandwich bags
  - 1 sharpie (also used for labeling during the workshop)
  - 1 pen (for filling out environmental sample data sheet)
  - tool for digging (spoon/metal scoops)

Equipment needed in lab:

- Gas
- Biohazardous waste receptacles
- Data projector (for demonstration purposes)
- Water bath set to 55 deg. C

Materials required for lab:

- 70% EtOH (spray or squirt) bottle - 1 per pair of students
- paper towels, gauze pads, or Kimwipes™ - 1 pack per pair of students
- Bunsen burner and striker - 1 per student
- sterile 5 ml syringe - 3 per student
- sterile 0.22 µm syringe filter – 3 per student
- sterile PYCa liquid media (store at room temp) - ~125 ml aliquot per student
- 5 ml sterile serological pipettes - 6 per student
- Serological pipette pump - 1 per student
- 50 ml conical tubes – 6 per student
- Small rack for holding 50 ml conical tubes - 1 per student
- Host bacteria – 500 µl aliquots in culture tubes with vented caps – 3 per student
- Small racks for holding culture tubes – 1 per student
- Sterile PYCa plates – 3 per student (additional plates may be required for host prep)
- Sterile top agar, molten (between 55 – 60°C) - 6 m per student

Figure 1. Example cultures, media (left), and materials (right) required for this workshop.

NOTES FOR THE INSTRUCTOR

To participate in the SEA-PHAGES program, an institution needs two or more willing faculty members, funding to purchase reagents and consumables, and a complement of basic lab equipment, much of which may already be available for other lab courses (e.g. centrifuges, shakers, incubators, water baths, gel boxes, gel imaging equipment, and refrigerators). Once approved to join the program, institutions receive an unparalleled level of support from HHMI, the privately-run philanthropic funding agency that administers SEA-PHAGES as part of its mission to "advance basic biomedical research and science education for the benefit of humanity." (hhmi.org)

Faculty are invited to attend week-long training workshops focused on running through streamlined versions of the lab protocols for a given semester. Lab manuals and video demonstrations are all freely-available on the SEA-PHAGES website, which also includes information for faculty (e.g. ordering lists) and forums that foster information exchange between SEA-PHAGES institutions. The Science Education Alliance (SEA) staff, who faculty interact with during training workshops, respond to email and phone inquiries quickly and also maintain and distribute all of the cultures necessary for phage isolation and propagation.

The SEA-PHAGES program is continually updated each year with the goals of increasing instructional clarity and adjusting protocols to incorporate the latest available techniques. Furthermore, HHMI has recently launched the SEA-GENES project, a sequel to SEA-PHAGES where students "generate expression libraries for phage genes, test for the ability of phage gene products to modulate bacterial host phenotypes, and screen phage gene products against the bacterial host proteome to identify putative host target(s)." (www.hhmi.org)

Finally, HHMI runs the annual SEA symposium (https://seaphages.org/meetings) at which students from both SEA-PHAGES and SEA-GENES are encouraged to present their findings, thereby fostering interactions among SEA-PHAGES institutions and providing students with the invaluable experience of participating in a scientific meeting. For more information about the SEA-PHAGES course please contact the organizers of this workshop, visit the web resources listed in the references section of this document, or email info@seaphages.org

CITED REFERENCES

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About the Authors

John Drummond is the General Biology Laboratory Coordinator at Lafayette College where he teaches all the introductory Biology Laboratories and coordinates the teaching assistant program. He has been teaching the discovery semester of the HHMI SEA-PHAGES course since 2020. He also teaches courses at Cedar Crest College in the areas of “Environmental Science” and “Human Biology.”

Jeff Norman has been the Teaching and Research Support Specialist in the Biology Department at Lafayette College where he helps develop protocols and prepare materials for laboratory classes, manages access to scientific equipment, and assists students with independent research projects. He also teaches seminar classes in the areas of “Microbial Ecology and Evolution” and “Biodiversity and Ecosystem Function.”

Michèle Barmoy is a Professor of Biology at Allegany College of Maryland where she teaches a variety of General Biology classes. She has been teaching both the discovery and bioinformatics semesters of the HHMI SEA-PHAGES course since 2019.

APPENDIX A – Sample Results

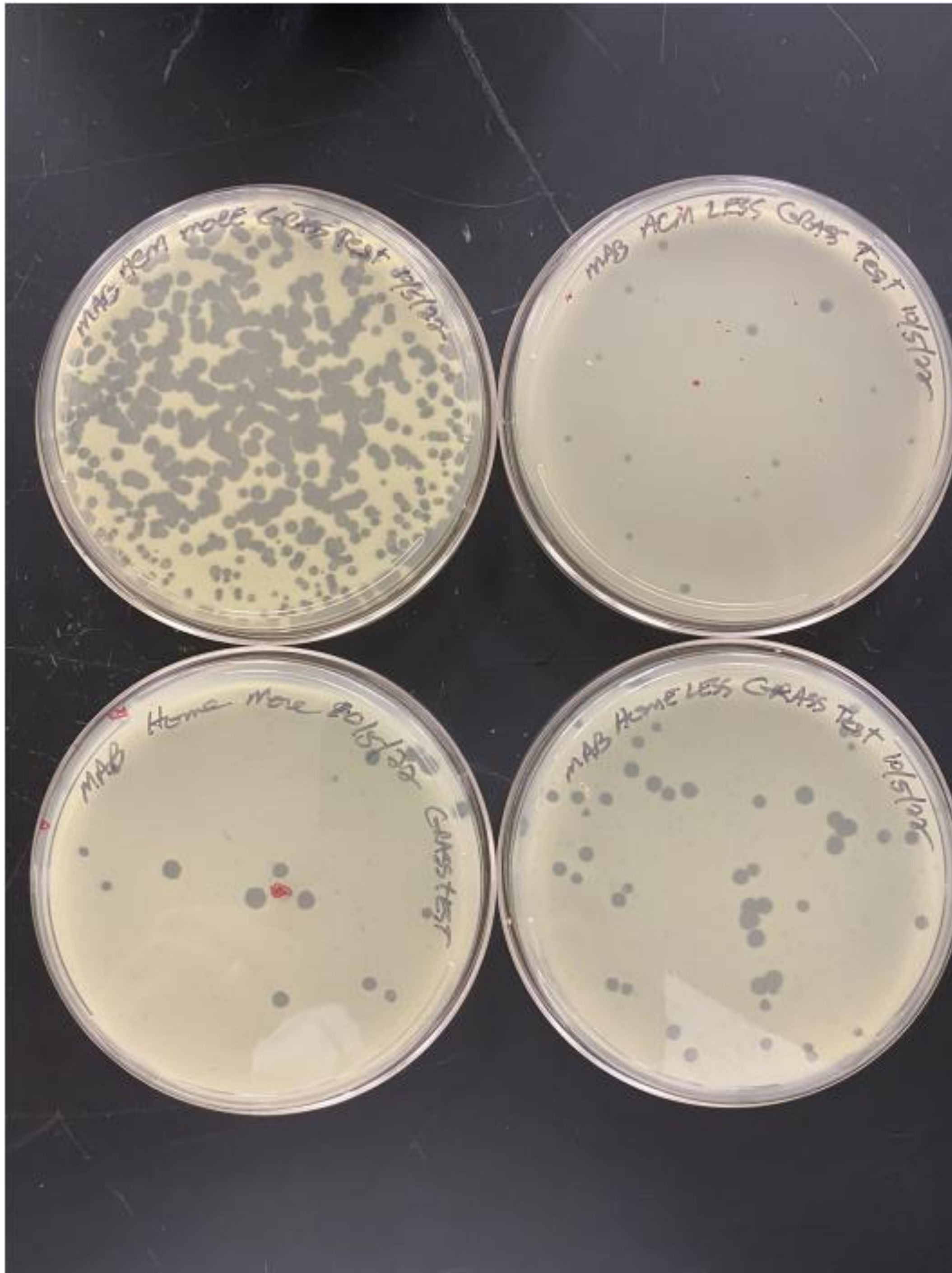

Figure 2. Four sample direct isolation plates as prepared by the protocols listed above. Plates were incubated for ~48 hours at 30°C prior to pictures being taken. Clear zones on plates are plaques formed by phage lysis of the bacterial host.

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APPENDIX B – Relevant Websites

The SEA-PHAGES website (specific resources highlighted below): https://seaphages.org

Fall 2022 ordering guide:
https://seaphages.org/media/docs/Ordering_Guide_Fall_2022.pdf

Phage discovery guide (lab instructions for first semester of SEA-PHAGES):
https://seaphagesphagediscoveryguide.helpdocsonline.com/home

Software overview (for second semester of SEA-PHAGES):
https://seaphages.org/software/

Forums:
https://seaphages.org/forums/

Information on the SEA-GENES program:
https://seaphages.org/faculty/genes-information/

The Actinobacteriophage database, where students submit information on their phage isolates: https://phagesdb.org

APPENDIX C – Prep Notes

Prep Notes:

Make Ahead:

Sample Collection Kits: hand out to participants at conference registration

Microbacterium foliorum liquid culture; see the following recipe cards in the Phage Discovery guide (seaphages.org):

Growing liquid cultures of bacteria from a frozen stock: https://seaphagesphagediscoveryguide.helpdocsonline.com/growing-bacteria-from-a-frozen-stock

Growing liquid cultures of bacteria from a single colony: https://seaphagesphagediscoveryguide.helpdocsonline.com/growing-liquid-stock-of-cultures-from-a-single-colony

Liquid and solid media; see the following recipe cards in the Phage Discovery guide (seaphages.org):

PYCa liquid media
PYCa Agar plates
PYCa Top Agar

Tips

- Start streak plates 7 days before workshop and start liquid cultures 2 days before workshop.
- For liquid cultures, use sterile 250 ml baffled glass flasks with caps or flask with a few sterile micropipette tips in the bottom
- Growth temperature: optimal at 28 ˚C - 30 ˚C; grows well at room temperature (~ 22 ˚C); this host does not grow at 37˚C.
- Liquid culture preparation video demonstration: https://seaphages.org/video/56/
- PYCa plate preparation Video demonstration: https://seaphages.org/video/55

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