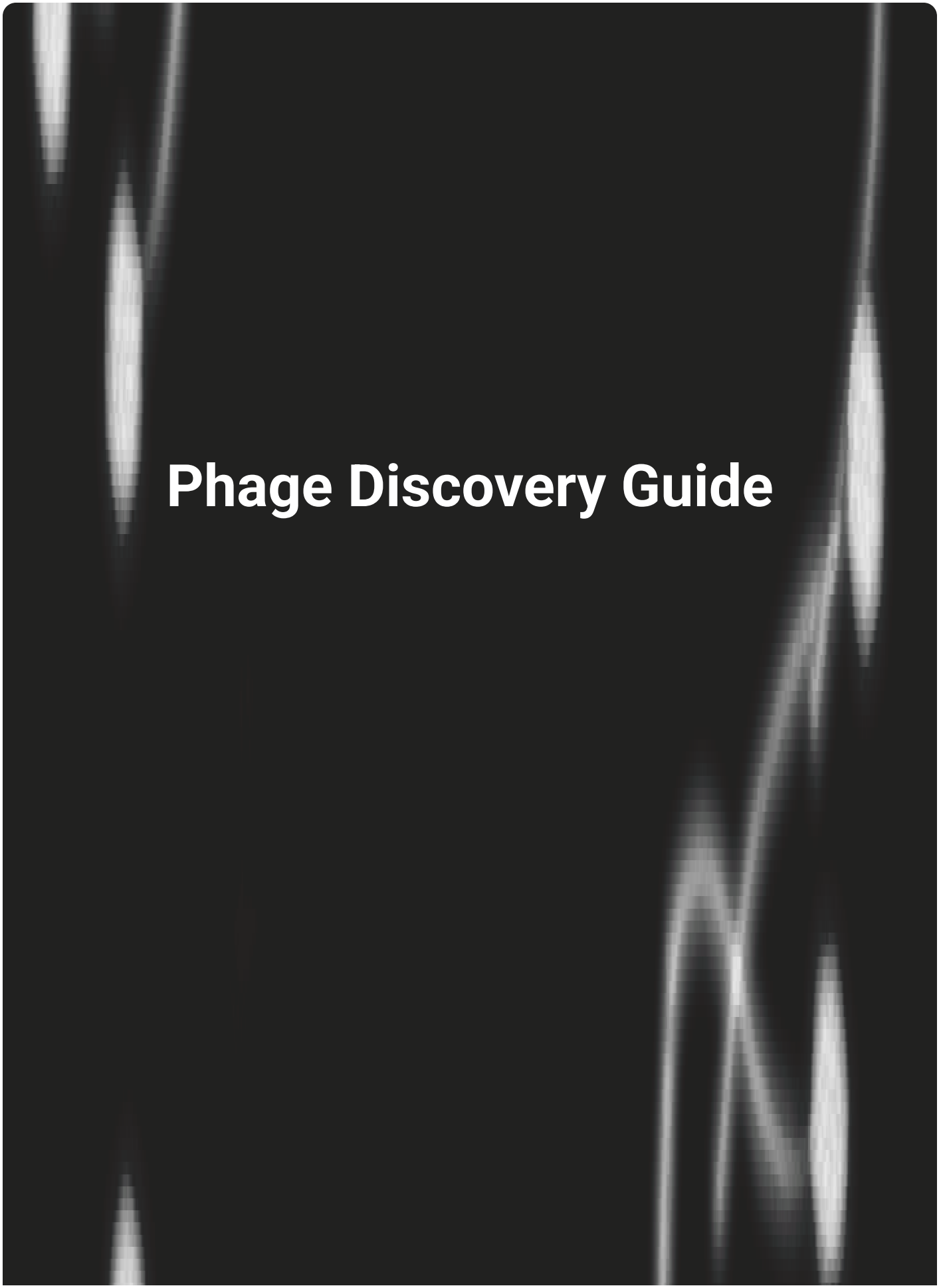

Phage Discovery Guide

Chapter1: Welcome

Welcome to SEA-PHAGES, an innovative research collaboration dedicated to making breakthroughs in the field of bacteriophage biology. You are joining a diverse international community of student researchers, faculty, and support staff from more than 100 colleges and universities. SEA-PHAGES (Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science) is supported by the Howard Hughes Medical Institute, in collaboration with lead scientist Professor Graham Hatfull from the University of Pittsburgh.

As a SEA-PHAGES student researcher, you will help discover new bacteriophages and analyze their genomes. In this way, you are supporting the scientific objective of the SEA-PHAGES program: to discover new insights about bacteriophages by exploring their diversity, evolution, and genetics. SEA-PHAGES research is published in peer-reviewed resources every year, frequently with students as co-authors.

Another important goal of the SEA-PHAGES program is to increase undergraduate students' interest and retention in biology through participation in authentic and valuable research. Thus, we hope that your participation in this collaborative research community will enrich your college studies and assist you with your academic and professional aspirations.

Bacteriophages, known simply as phages, are viruses that infect bacteria, often killing the bacteria in the process. Bacteriophages therefore have many practical applications. For example, phages are gaining more attention as potential therapeutic agents for treating patients battling infections caused by antibiotic-resistant bacteria. In several circumstances, phages have cleared infections that were unresponsive to conventional antimicrobial compounds. The growing concern about antibiotic-resistant bacteria has also catalyzed the use of phages in other ways, including veterinary and food safety applications.

Overview of Scientific Workflow

The SEA-PHAGES program has two primary research components: phage discovery and genome analysis. During phage discovery, student researchers first isolate, purify, and then amplify new phages collected from environmental samples. This is then coupled with electron microscopy to visualize the phage, followed by DNA isolation and genome sequencing. The second main component is the analysis of phage genomes using bioinformatic software programs to identify genes, regulatory elements, and other genomic features. The ensuing insights typically present a variety of hypotheses that can be tested computationally or with molecular genetic approaches.

This Phage Discovery Guide contains a complete set of detailed protocols for each of the approaches required for the *de novo* isolation and characterization of bacteriophages, along with supporting material explaining the experimental approaches and biological reasoning.

Expanding Scientific Knowledge

The bacteriophage population is vast, dynamic, and old, so it is not surprising that it is so diverse. There are estimated to be 10^{31} phage particles in the biosphere, but fewer than approximately 5,000 have been characterized genomically. As you venture to discover new

phages, you will experience one of the hallmarks of authentic scientific research: scientists don't know the answer before doing the experiment! Therefore, as a scientist, you will need to formulate hypotheses, design experiments, and interpret the data. You will learn that not every experiment works the first time. Sometimes they never work, and you need to practice your problem-solving skills to advance your research.

This work may sound daunting, but the SEA-PHAGES program provides many resources to help you, including this detailed guide. Other resources include databases, websites, scientific articles and reviews, access to experienced scientists and, of course, the thousands of other faculty and students in the SEA-PHAGES community who are sharing in this journey of discovery.

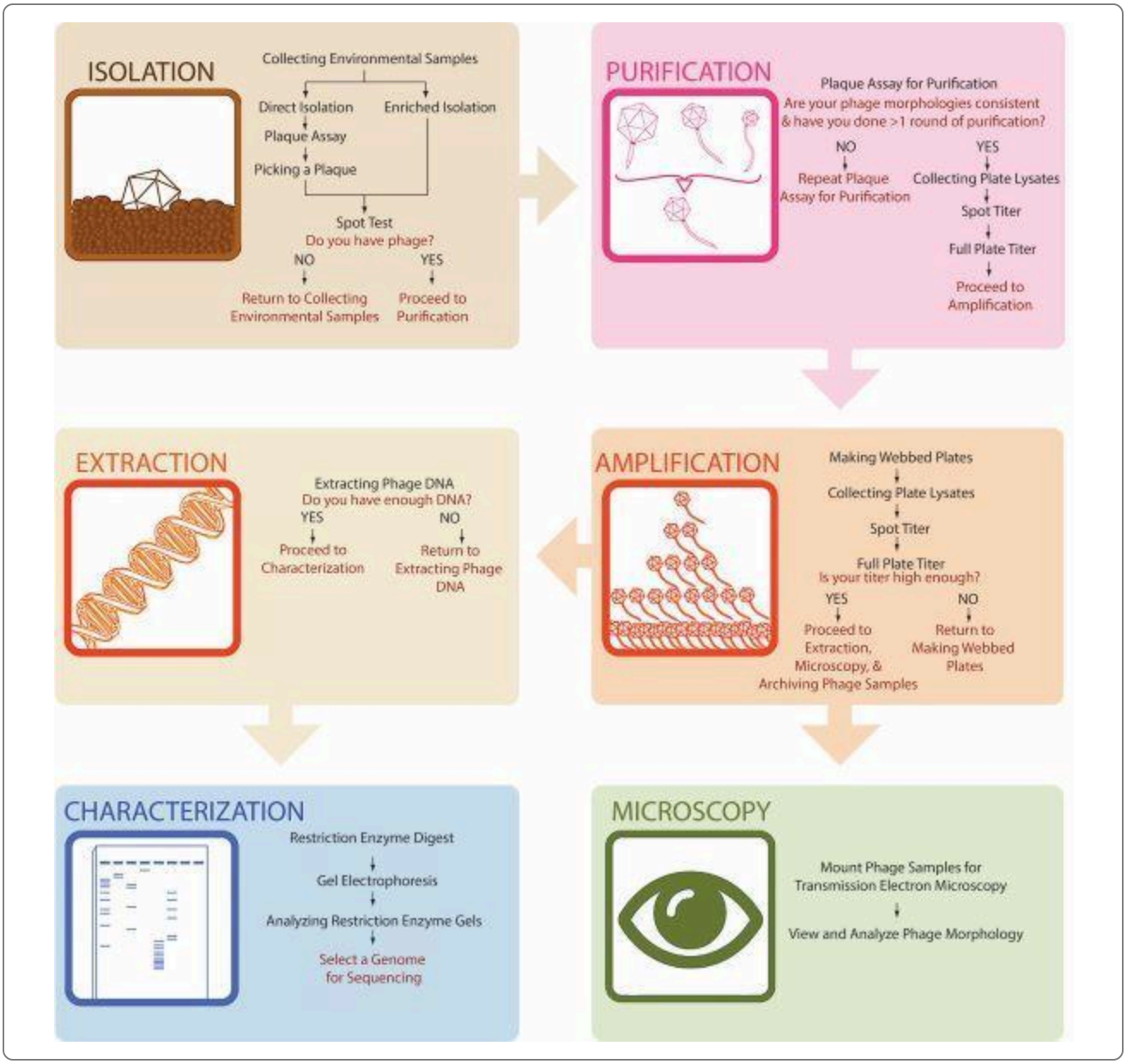
How to Use This Phage Discovery Guide

This guide is organized into chapters that focus on individual stages of your quest to isolate and characterize new bacteriophages. Each chapter contains step-by-step protocols, lists of required reagents, and troubleshooting information. The chapters and protocols are organized in the order that you will commonly use them. Because each protocol corresponds to a stand-alone module, you may find it useful to sometimes pick and choose among the protocols most pertinent to the task you are undertaking. You will use some protocols more than once. In these cases, the protocol is in the chapter where it is first encountered, and you will have to return to it when needed. Chapter 12, the Toolbox, contains some additional protocols you may or may not need. When used as an online document, the protocols are connected by hyperlinks in the text, in the table of contents, and in the flow charts.

We strongly recommend that when you start a protocol for the first time you read the *entire* protocol before you begin. There are helpful tips and troubleshooting options at the end of each protocol.

Getting Started

Now it's time to get started with your phage isolation! We wish you luck and hope that you embrace the inevitable challenges and enjoy the thrill of discovery and contributing to the body of scientific knowledge. The flow chart below provides a framework for how the component pieces of the program fit together, and we encourage you to refer to this frequently as a reminder of where you are in the scientific workflow.


ISOLATION
Collecting Environmental Samples
Direct Isolation
Enriched Isolation
Plaque Assay
Picking a Plaque
Spot Test
Do you have phage?
NO
YES
Return to Collecting Environmental Samples
Proceed to Purification
PURIFICATION
Plaque Assay for Purification
Are your phage morphologies consistent & have you done >1 round of purification?
NO
YES
Repeat Plaque Assay for Purification
Collecting Plate Lysates
Spot Titer
Full Plate Titer
Proceed to Amplification
EXTRACTION
Extracting Phage DNA
Do you have enough DNA?
YES
NO
Proceed to Characterization
Return to Extracting Phage DNA
AMPLIFICATION
Making Webbed Plates
Collecting Plate Lysates
Spot Titer
Full Plate Titer
Is your titer high enough?
YES
NO
Proceed to Extraction, Microscopy, & Archiving Phage Samples
Return to Making Webbed Plates
CHARACTERIZATION
Restriction Enzyme Digest
Gel Electrophoresis
Analyzing Restriction Enzyme Gels
Select a Genome for Sequencing
MICROSCOPY
Mount Phage Samples for Transmission Electron Microscopy
View and Analyze Phage Morphology

Lab Basics

Chapter 2: Lab Basics

Chapter 2: An Overview

Performing scientific research in a laboratory can be an exciting and rewarding experience. As a research scientist in this program, you will learn scientific techniques and protocols that are often used in microbiology and molecular biology research. Before you begin, however, you will need to understand the fundamentals of working in a lab that are critical to your safety and success.

Lab Safety

While every laboratory has its own specific rules and regulations, there are some universal guidelines that you should always follow. They are designed to keep you and your lab mates safe and to comply with state and federal regulations. Please read the list below carefully and talk to your instructor about any specific rules that must be adhered to in your workspace.

General Rules

- No eating or drinking.
- No open-toe shoes.
- Never work alone in a lab.
- Tie back long hair.
- Avoid wearing baggy clothing.
- Never leave a lit Bunsen burner unattended.
- Keep workspace clear of clutter.
- Minimize loud talking and distractions.
- Know the location of fire extinguishers, emergency eye wash stations, and emergency showers.
- Properly dispose of waste generated from an experiment. Not everything can go down the drain or in a garbage can.
- No mouth pipetting!
- Wear safety glasses at all times.
- Wear gloves when working with bacteria.
- Wash your hands before leaving lab.

- If you are not sure, ask first!

Guidelines for working with bacteria

Exposure to the Actinobacteria species recommended for use in this course do not pose health risks to individuals with healthy immune systems. If an individual is immuno-compromised or pregnant, the individual should consult a physician to determine the appropriate level of participation in lab activities. The primary hazard of working with the Actinobacteria used in this course is from exposure via mucosal membrane routes (eyes, mouth and nose), broken skin, or ingestion. Therefore, you should wear safety glasses at all times, wear gloves when handling bacteria, avoid generating aerosols of bacterial cultures, and avoid touching your face, applying cosmetics, adjusting contact lenses, biting your nails etc., while in lab. Consult your instructor if you have health concerns about working with bacteria.

Most importantly, work responsibly! There is no way to predict all dangerous lab situations, but most can be avoided simply by using common sense, being thoughtful and deliberate, and paying attention to your surroundings. In the event of an accident, be sure to alert your instructor immediately so the proper course of action can be followed.

Record Keeping

Laboratory Notebooks

When performing scientific research it is important to keep a record of your work that follows the principle of autonomous replication. Basically, anyone should be able to repeat your experiments by using your notes and obtain the same results. Therefore, maintaining a detailed and descriptive laboratory notebook is a key part of laboratory research. To fulfill the requirement for the principle of autonomous replication, your notebook should follow these basic guidelines:

A. The level of detail should be high enough that you can go back at a later time and troubleshoot your procedures if, for some reason, a procedure does not work or yields questionable results.

B. Jargon, personal shorthand, and personal abbreviations should be kept to a minimum.

C. Drawings, figures, and tables are encouraged. They must contain enough information so that another scientist can interpret them without extensive reference to the text.

There are endless ways to format a good scientific notebook and different disciplines have different conventions. In fact, every professional scientist develops a personal notebook style over time. Below is an outline of the minimum information required in a laboratory notebook. You may be asked to follow your instructor's stylistic preferences, or you may be able to organize your notebook in your own way. Regardless of the format you follow, it is imperative that you write in your lab notebook as you perform the procedure every time you are in the lab. Memory is not reliable, and experimental data and results without sufficient documentation are not trustworthy and must be discarded.

Laboratory Notebook Outline

A. **Table of Contents.** You should leave 1–3 pages at the beginning of the notebook blank to accommodate a table of contents.

B. **Date.** Date each page of the notebook at the beginning of the entry. It is imperative that you record everything in your notebook as you do it.

C. **Title.** This should explain, in three or four words, the focus of the experiment. The title of the experiment is not necessarily the title of the protocol being followed, since multiple procedures may be used to complete a single experiment.

D. **Aims and Purpose.** This is a thumbnail sketch of the reason you are performing the protocols and experiments for the day. It should stand alone as your rationale. In other words, anyone should be able to open your notebook to any experiment and understand the "why" of the laboratory work performed.

E. **Procedures and protocols.** This should be a comprehensive, accurate, and detailed step-by-step accounting of your procedures. Even if you are repeating a protocol without any changes, write out the exact steps. Use a bulleted list that outlines the action items used to complete the experiment. It is recommended that you write out this list before beginning the procedure so you know what steps you will be doing, and so you can check them off as you proceed and note any changes.

F. **Results.**

1. Primary data (e.g., numerical values, photographs, printouts, observations, drawings) are results of an experiment.

2. Negative results are results too! Be sure to include them.

3. All results must be described comprehensively and accurately.

4. Any loose data (e.g., photographs, printouts) must be securely taped into the notebook, dated, and properly labeled.

5. Anyone should be able to read the results of an experiment and know exactly what happened.

6. Data tables are labeled with a descriptive legend above the table.

7. Figures are accompanied by a descriptive legend below the figure.

G. **Analysis and interpretation.**

1. The results of an experiment must be analyzed and an objective interpretation documented in the notebook. Even if the results indicate a simple "yes" or "no" answer, it is important to state that explicitly in this section.

2. Report any unexpected findings or problems during the course of the experiments. This can act as a rationale for additional experiments, for changing the protocol or materials, or for altering the path your research has been following.

3. Interpretation of data can sometimes be subjective, but your reasons for a particular interpretation *must* be stated here.

H. **Future plans.** On the basis of your interpretation of the data, you should end each experiment or day of recording in your notebook with a short outline of your next steps. The rationale for this should be clear in your "analysis" section and doesn't need to be restated. This section will lead you and the reader to the next procedure with a clear idea of "what's next," and why you are proceeding in that direction.

As you keep your lab notebook for this course, it is important to note that when the scientific data (such as where your phage was found and how it was purified) are published, the source of the data (namely, your notebook) belongs to and must be retained by the institution for at least seven years. Your notebook should, therefore, be a document that can be read and understood for years to come, especially if the science surrounding your phage progresses from the point of discovery outlined in your notebook.

Labeling in the Laboratory

Maintaining a good laboratory notebook isn't the only important record keeping that happens in a lab. You also need to be able to identify your experimental samples when they are placed in an incubator or freezer with hundreds of other identical-looking samples. Just as with keeping a notebook, every scientist has his or her preferred way of labeling things like Petri plates, microcentrifuge tubes, and reagents—but here are some general strategies to follow.

A. Each set of tubes, plates, and bottles should be labeled with the following (examples in Figure 2.0-1)

   - Your name or initials
   - The date
   - The contents (i.e., DNA)

B. Any prepared bottles of media or other solutions, tubes, cultures, or stocks that are exclusively yours should be labeled with

   - Your name or initials
   - The contents
   - The date(s) received, prepared, and opened

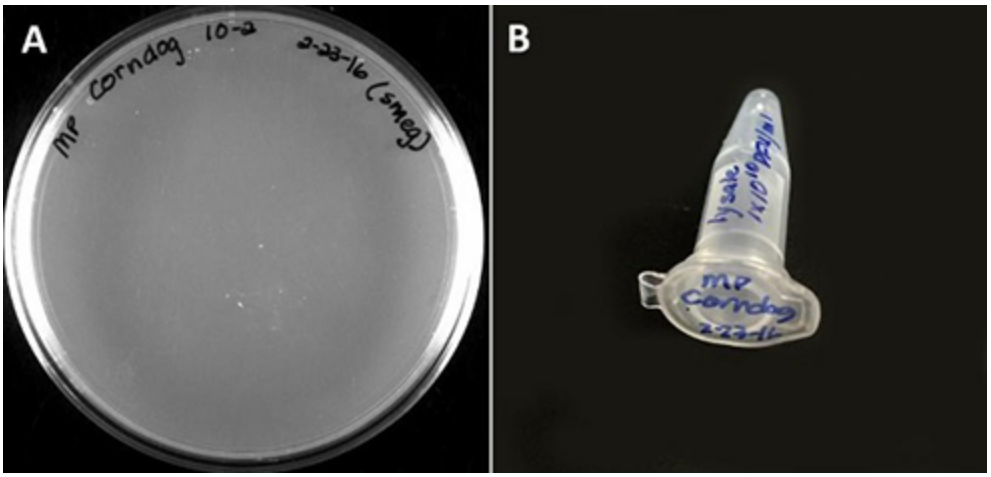


Figure 2.0-1. Examples of proper labeling. (A) Petri plates are labeled on the bottom (agar-side) to ensure that the label accompanies the experiment even if the lid is lost, displaced, or swapped with another plate. By labeling around the edge of the plate you can see the results without looking through the label. (B) Microcentrifuge tubes are labeled with an identifier on the top of the tube so it can be located when it is in a tube rack. Additional information should be written on the side of the tube, in the designated area, where the label will not rub off.

Preventing Contamination

Microbes are everywhere: on lab surfaces and equipment, on human skin and hair, and in the air. In a microbiology laboratory we use materials and procedures to ensure that we grow only the bacteria and phages we are interested in. If we pick up other bacteria, fungi, or phages from our surrounding environment we say that we have “contamination.”Sterilization and aseptic technique help avoid contamination.

“Sterile” materials have been rendered free of biological organisms—including phages,bacteria, and fungi. The most common method for sterilizing commonlab materials and reagents, such as phage buffer, bacterial growth media, plastic tips, and glass tubes, is to use an autoclave, a machine that uses pressure and steam to heat materials to 121 °C and 18 psi. Materials that have been autoclaved are frequently marked with special tape that appears white before autoclaving, and striped afterward. Alternatively, some liquids that do not tolerate extreme heat can be passed through a filter (“filter-sterilized”) to physically remove cellular organisms.

Sterilization of larger or sensitive surfaces (such as you!) requires alternative methods. Lab benches and equipment are routinely treated with “disinfectants”—chemicals that kill or inhibit growth of contaminants on surfaces. Common lab disinfectants used to remove bacteria are 70 % alcohol, 10 % bleach, or a phenolic like CiDecon™. Many phages are not sensitive to 70 % ethanol, so for disinfection of phage-contaminated materials bleach and phenolics are better choices. In addition, you should wash your hands thoroughly with hot water and soap before and after each lab class since you are probably the biggest source of contamination in the lab!

Aseptic technique refers to the best lab practices that minimize contamination of your sterile working materials and experiments. Many of these practices are just common sense: for example, make sure you declutter and disinfect your working space before you start an

experiment. The classic key components of aseptic technique are described below and can be practiced in the Using Aseptic Technique exercise in protocol 2.1. Read this section and then do the exercise to learn the specific techniques.

Work Area Preparation

When preparing for an experiment requiring aseptic technique, you want to organize your bench first. Remove any unnecessary clutter, and disinfect your workspace with 70 % ethanol. As the ethanol dries it dehydrates bacteria on the bench surface the same way hand sanitizer kills bacteria on your skin. Be mindful not to try to speed up the drying process by waving your hands around or blowing on the surface because this will only introduce additional contaminants. When working with certain bacteria and viruses, it is a good idea to use a second disinfectant containing phenol or quaternary ammonia compounds as well. Because the active ingredients in these supplementary disinfectants denature proteins, it is important to always wear gloves when handling them.

Bunsen Burner Use

Classic aseptic technique requires the use of a Bunsen burner. Once lit, the flame should be adjusted so that blue “cone” can be seen in the flame. The tip of this cone is the hottest part of the flame. The function of the flame is to provide an updraft by heating the air (Figure 2.0-2). Because heat rises, airborne bacteria, spores, fungi, and dust particles are forced upward and away from your work area. Therefore, it is important to work inside this “zone,” being careful not to disturb the updraft with rapid movements that disturb the air around your bench. You also need to arrange your supplies to be close enough to the Bunsen burner to take advantage of the updraft. Until you are familiar with using a Bunsen burner, it is important to constantly be mindful of the flame. Even with an apparent blue cone the flame is hard to see and can be overlooked. Never leave a flame unattended in case a lab mate or instructor doesn’t see it and inadvertently reaches over or through it.

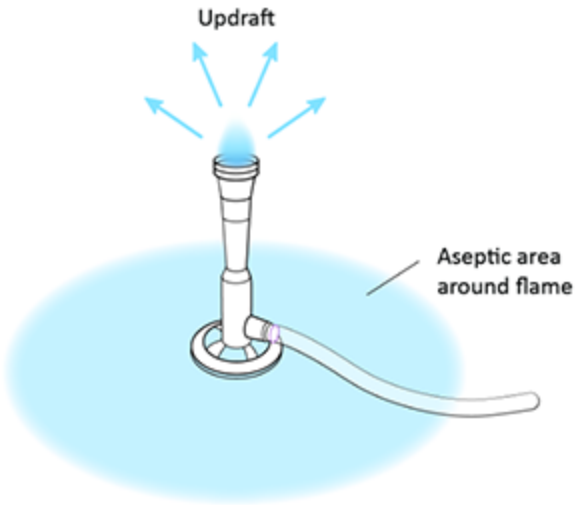


Figure 2.0-2. How a Bunsen burner creates an updraft to keep the area aseptic

Figure 2.0-2. How a Bunsen burner creates an updraft to keep the area aseptic

Aseptic Transfer of Materials and Reagents

When you move liquids from one vessel to another, follow three simple rules to maintain an aseptic environment. First, always flame the opening of glass bottles and test tubes every time you open them and again before you close them. The goal of flaming a glass vessel is not to heat the glass itself, but to heat the air inside the neck of the vessel. This will cause the air to rise and prevent airborne contaminants from falling inside. This requires one or two quick passes through the flame only because extended exposure to the flame can cause the glass to break. This is an example of classic aseptic technique. With today's plastic components, however, flaming is not in widespread use.

The second rule is to never lay down a lid or a cap on the bench top. Because you will most likely be holding a pipette in your dominant hand, you will have to figure out how to hold the bottle and the lid in one hand, or some other arrangement. This is a feat of dexterity that has nothing to do with the size of your hands, and it comes with practice. Finally, the third rule to good aseptic technique is to never use a pipette for more than one transfer. After you pipette from one sterile vessel into another, ALWAYS get a new pipette or pipette tip, regardless of how sure you are that you didn't contaminate it the first time.

Bench Top Management

As noted above, you want to keep the supplies you are using within the aseptic field created by the Bunsen burner. In addition, you want to minimize clutter and keep your workspace well organized. If you have supplies you know you will need, keep them close to the aseptic zone, but out of the way until needed. Keep pipette tip boxes and microcentrifuge tubes closed at all times to prevent contamination. Wipe up any spills immediately with disinfectant.

Disposal, and Cleanup

After you finish your work for the day and start cleaning your lab bench, you need to follow proper disposal protocols. Bacteria, either in liquid or on plates, should not be discarded in the sewer or garbage until treated properly. Follow the protocol outlined by your instructor for the disposal of old plates and culture tubes. When working with microorganisms, always disinfect your workstation with 70 % ethanol and a disinfectant like CiDecon, before leaving and wash your hands thoroughly.

Protocol 2.1: Aseptic Technique Exercise

Objective :

Practicing aseptic technique .

Rationale:

Aseptic technique is used to prevent contamination in microbiology experiments. 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 % EtOH
- CiDecon™ or other disinfectant
- Bunsen burner and striker
- 10 ml serological pipettes and pipettor
- Bottles, culture tubes, conical tubes, microcentrifuge tubes, and water to practice sterile transfers

Procedure:

A. Prepare your work area.

- Tidy up your workbench by removing clutter, papers, bottles, etc.
- Using a squeeze bottle containing CiDecon™, dispense enough disinfectant to dampen the entire work surface. Be certain to wear gloves if the disinfectant is known to denature proteins, as it can be hazardous to your health.
- Using a paper towel, gauze pad, or Kimwipes™, wipe the entire surface, starting at the back, to moisten the entire work surface.
- Let the disinfectant evaporate—do not wipe dry.
- Repeat steps 2–4 with 70 % EtOH. There is no need to wear gloves when handling EtOH.

B. Ignite the Bunsen burner.

- Remove your gloves before working with an open flame.
  Important: It is generally recommended that gloves not be worn while working with an open flame. This is because gloves, when worn and accidentally placed

too close to or within an open flame, may melt and cause severe burns to the skin.
Important: Note that your gloves are never sterile.

- Light the Bunsen burner, but only after the disinfectant has completely dried.
- 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.)

C. Transfer 10 ml from a small sterile bottle to a sterile culture tube (Figure 2.1-1)

- Arrange a 50 ml or 100 ml bottle containing sterile water and a sterile culture tube in your aseptic field.
- Loosen (but do not remove) the tube and bottle caps.
- Peel down the wrapper on the 10 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.)
- Using your other (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 and leave it between your forefinger and middle finger (Figure 2.1-1G).
- Using your nondominant hand, hold the bottle close to the flame. You can also leave the bottle on the table as long as it remains close to the flame.
- Immediately place the serological pipette into the bottle, and using the pipettor, draw up 10 ml.
- Replace the cap, and set the bottle down.
- 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).
- Dispense the liquid from the pipette into the tube.
- Replace the cap, and set the tube down.
- Remove the pipette from the pipettor, and then discard the pipette.
-

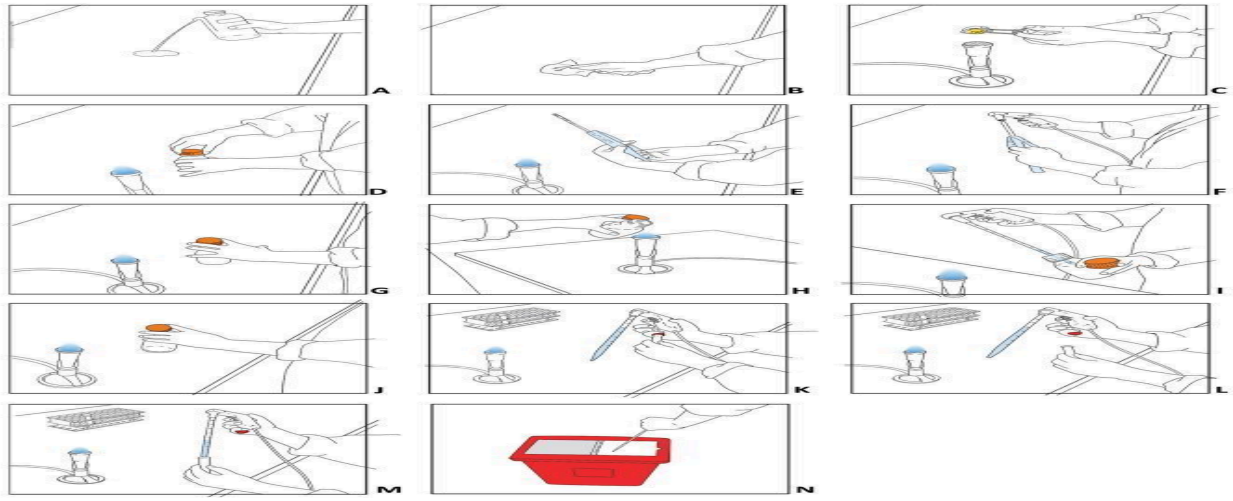


Figure 2.1-1. Transferring liquid samples using aseptic technique. Before beginning, dispense ethanol or other disinfectant onto the surface of your bench (A) and wipe from back to front (B). Light your Bunsen burner (C). Prepare your materials, and loosen all bottle and tube caps (D). Peel down both 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 keep the bottle close to the flame (H). Remove your sample (I) 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.

D. Transfer 100 µl from a microcentrifuge tube into a culture tube.

- Arrange a microfuge tube filled with water and a culture tube in your aseptic field.
- Adjust the p200 pipettor to transfer 100 µl. Then, holding your p200 in your dominant hand, open the pipette tip box and retrieve a sterile pipette tip.
- Using your nondominant hand, pick up the microcentrifuge tube and open it.
- Insert the pipette into the microfuge tube and draw 100 µl into the pipette (to the first stop).
- Close the microfuge tube and return it to the rack.
- Using your nondominant hand, remove the cap from the culture tube and pick up the tube. (This can be done a number of different ways, practice with what works for you.) Be careful not to invert the pipettor or touch the tip of the pipette to anything in the process.
- Insert the pipettor into the culture tube only as far as the sterile tip. Touch the tip to the side of the tube and expel the 100 µl (depressing the plunger to the second stop) allowing the liquid to run down the side of the tube.
- Replace the culture tube cap.
- Eject the tip into the proper receptacle.

E. Practice the techniques above with a partner. Things to watch:

- Hand and finger placement
- Movement of pipette and tips
- Aseptic field location
- Placement and handling of tube and bottle caps

Helpful Tips:

- Never pass your hands or fingers over the tops of open containers within a sterile field (such as open bottles or flasks, the inside of tubes and bottle caps, and agar plates).
- 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.
- The microcentrifuge tube should be kept closed until liquid is transferred and it should be closed immediately when you are done.
- 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.

Guidelines for working with bacteria

The following are recommendations that should be used to supplement general lab practices and established safety rules at your institution when working with bacteria. Please review these guidelines carefully and adhere to them to ensure a safe laboratory environment for everyone.Before working with bacteria:

Before working with bacteria:

1. Ensure that you are aware of lab practices and safety rules, which should include the recommendations listed in this document.

2. Make sure you are aware that:

    - Actinobacteria recommended for use in this project are safe for those with a normal immune system. If an individual has any health concerns related to exposure to bacteria, including being immuno-compromised or pregnant, the individual should consult a physician to determine the appropriate level of participation in lab activities.

    - The primary hazards of working with the Actinobacteria used in this course are from exposure via mucosal membrane routes (eyes, mouth and nose), parenteral inoculation (broken skin), or ingestion.

    While working with bacteria:

    Gloves and lab coats should be used. Change gloves when contaminated or when integrity is compromised. Do not touch face, apply cosmetics, adjust contact lenses, bite nails, etc.

3. Care should be taken to avoid generating aerosols of cultures or suspensions of bacteria; do not sonicate or vortex bacterial cultures or suspensions; transfer bacterial cultures and suspensions with care and deliberation. Safety glasses and surgical masks, or a biosafety cabinet, are recommended if there is risk of generating aerosols.

4. Label cultures, tubes and plates containing bacteria appropriately.

5. Lab work surfaces should be wiped-down with an appropriate phenolic disinfectant before and after use. An example of an appropriate disinfectant for any Actinobacterial strain is CiDecon Detergent Disinfectant Concentrate, available from multiple vendors including Fisher Scientific and VWR. Disinfectants should be used according to the manufacturer instructions. Once cleaned, the work surface should then be wiped-down with 70 % ethanol to remove residual disinfectant from the surface.

6. Materials that have come in contact with bacteria and that are intended for reuse (e.g. glass flasks) should be treated with CiDecon before washing. Spent CiDecon can be disposed of in sinks with tap-water running.

7. Materials for disposal that have come in contact with bacteria (e.g. microcentrifuge tubes or agar plates) should be disposed of in biohazard bins.

8. Sticks or loops used for streaking or inoculating bacteria should not be flamed after use. Instead, sticks should be disposed of in a biohazard bin, while metal loops should be treated with CiDecon followed by 70 % ethanol.

After working with bacteria:

9. Wash hands thoroughly.

Contamination

- As a disinfectant, 70 % EtOH is sufficient to kill most bacterial contaminants on surfaces. However, it is not effective on Mycobacteria because of the mycolic acid wall. Use of an additional disinfectant like CiDecon will remove mycobacterial contaminants.

- Many phages are resistant to 70 % EtOH, so daily decontamination of lab spaces and equipment with CiDecon or 10 % bleach will help prevent cross contamination of phages.

- Cross contamination of phages within a classroom can be a big problem. To reduce phage contamination, each researcher should have their own small aliquots of phage buffer, clean up phage spills immediately, and keep pipette barrels clean.

Disposal

- Dispose of all unused bacterial cultures appropriately by mixing the contents with CiDecon, bleach, or autoclaving.

- Materials for disposal that have come in contact with bacteria (e.g. microcentrifuge tubes or agar plates) should be disposed of in biohazard bins.

- Waste collected in biohazard bins should be inactivated using an autoclave, or removed by a licensed waste removal service provider.

- Materials that have come in contact with bacteria and that are intended for reuse (e.g. glass flasks) should be treated with bleach or CiDecon before washing. Spent bleach and CiDecon can be disposed of in sinks with tap water running.

- Unused soil samples can be returned to the outside, and the plastic bags they were collected in can be discarded in the regular trash.

- Soil that has been incubated during enriched isolation experiments should be autoclaved in their flasks or conical tubes prior to discarding.

Chapter 3: Phage Basics

Phages Are Viruses

Bacteriophages (also known as phages) are viruses that infect bacterial hosts and have properties shared by all viruses. First, they are unable to replicate by themselves. They require a bacterial host to reproduce, taking advantage of the host's cellular machinery and redirecting it toward viral reproduction. Second, like all viruses, phages are specific for particular hosts. The host range can be restricted to a single bacterial strain, or it can span different bacterial species, even genera. However, there are few—if any—phages with a host range that spans different bacterial orders, and having a host range that spans different phyla is even less likely.

The Bacteriophage World

Bacteriophage particles comprise the majority of all biological entities in the biosphere. The global phage population is not only vast (an estimated 1031 particles) but also highly dynamic and very old. It is also highly genetically diverse. We understand a few phages in great detail, but our knowledge of the phage population at large is murky at best. So, three general questions about phages emerge: What are they? How are they related to each other? How did they get to be that way? Exploring their viral diversity and the evolutionary mechanisms that give rise to this diversity are the central scientific issues addressed by the SEA-PHAGES program.

100 Years and Counting

Bacteriophages were discovered about 100 years ago (1915–1917), and two of the major contributors to that discovery were Frederick Twort and Felix D'Herrelle. Others may have observed bacteriophage behavior before this, and even Twort did not fully recognize the nature of the viral properties he witnessed. D'Herrelle characterized the particulate nature of bacteriophages in part through the plaque assay he developed, which is still in widespread use today.

This assay provides a simple way to visualize the presence of phage particles in a sample. In brief, a single phage particle present within a thin layer, or "lawn," of bacteria growing in a petri dish can propagate in the bacteria to form a clearing, called a plaque (Figure 3.0-1). In essence, a plaque is caused by the destruction of bacteria cells by phage particles. Plaque formation requires multiple rounds of phage infection and growth, and each plaque can contain 10^8 or more particles by the time the bacterial cells have formed a confluent lawn. These early studies by D'Herrelle and colleagues were abstract but experimentally sound, and in the late 1940s the morphologies of various phage types were finally visualized with the invention of the electron microscope.

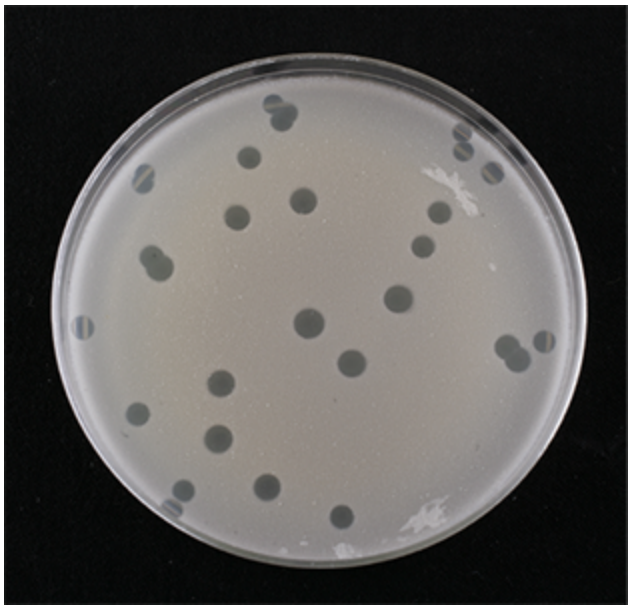


Figure 3.0-1. Bacterial lawn with visible plaques.

Figure 3.0-1. Bacterial lawn with visible plaques.

Simple Is Beautiful

Bacteriophage particles come in many shapes and sizes, but the majority are tailed viruses containing double-stranded DNA (dsDNA) genomes (the order Caudovirales). In these phages the linear DNA genome is contained within a protein shell (the **head** or **capsid**), which is attached to a **tail** (Figure 3.0-2). Phages in other orders may have either single-stranded DNA (ssDNA) or RNA and can be enveloped with a lipid membrane, have one or more than one chromosome, or have a variety of other configurations. Phages may have as few as a handful of genes or more than 500, but the dsDNA-tailed phages typically have 50–250 genes. (Note that this is much fewer than their bacterial hosts, which may have more than 6,000 genes.)

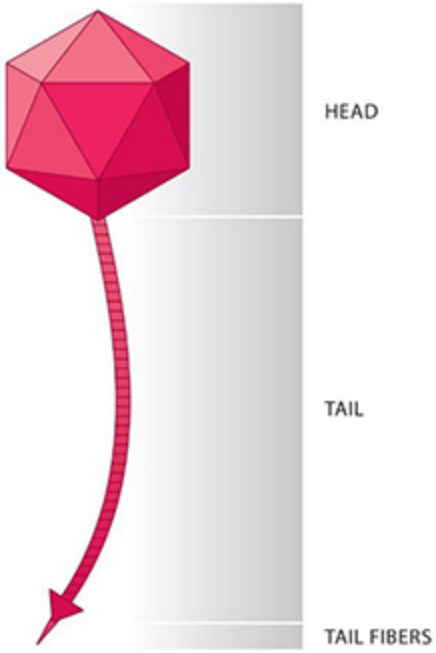


Figure 3.0-2. Phage structure. Phages are composed of a head and tail, with tail fibers that are used for host cell attachment.

Eating Away at Bacteria?

The term “bacteriophage” was coined from the Greek word phaegin (φαγεῖν), meaning “to eat,” because phages appear to “eat” bacteria when they infect them. This is not strictly true, although many phages do repurpose and recycle cellular components and use them to make more of themselves. Typically, infection of a single bacterial cell by a single phage results in the generation of many progeny phage particles. This is accompanied by a complete breakdown of cell integrity, called lysis, that releases the new phage so they can disperse and find new cells to infect. The process by which a phage generates new phage particles via infection and lysis of the host cell is called lytic growth and is explained in greater detail below.

Lytic Growth of Bacteriophages

The process of lytic growth can be broken down into several phases (see Figure 3.0-3). The first phase is adsorption, in which the phage recognizes the bacterial host cell and attaches to its surface using its tail fibers. Next, the phage’s DNA is injected into the host cell while the phage’s protein structure (head and tail) remains attached to the outside of the cell. The phage’s DNA passes from the head, through the tail, and across the host’s cell wall and cell membrane into the cytoplasm. After the linear phage DNA has completely entered the cytoplasm, it circularizes to avoid the host cell’s defenses against linear DNA.

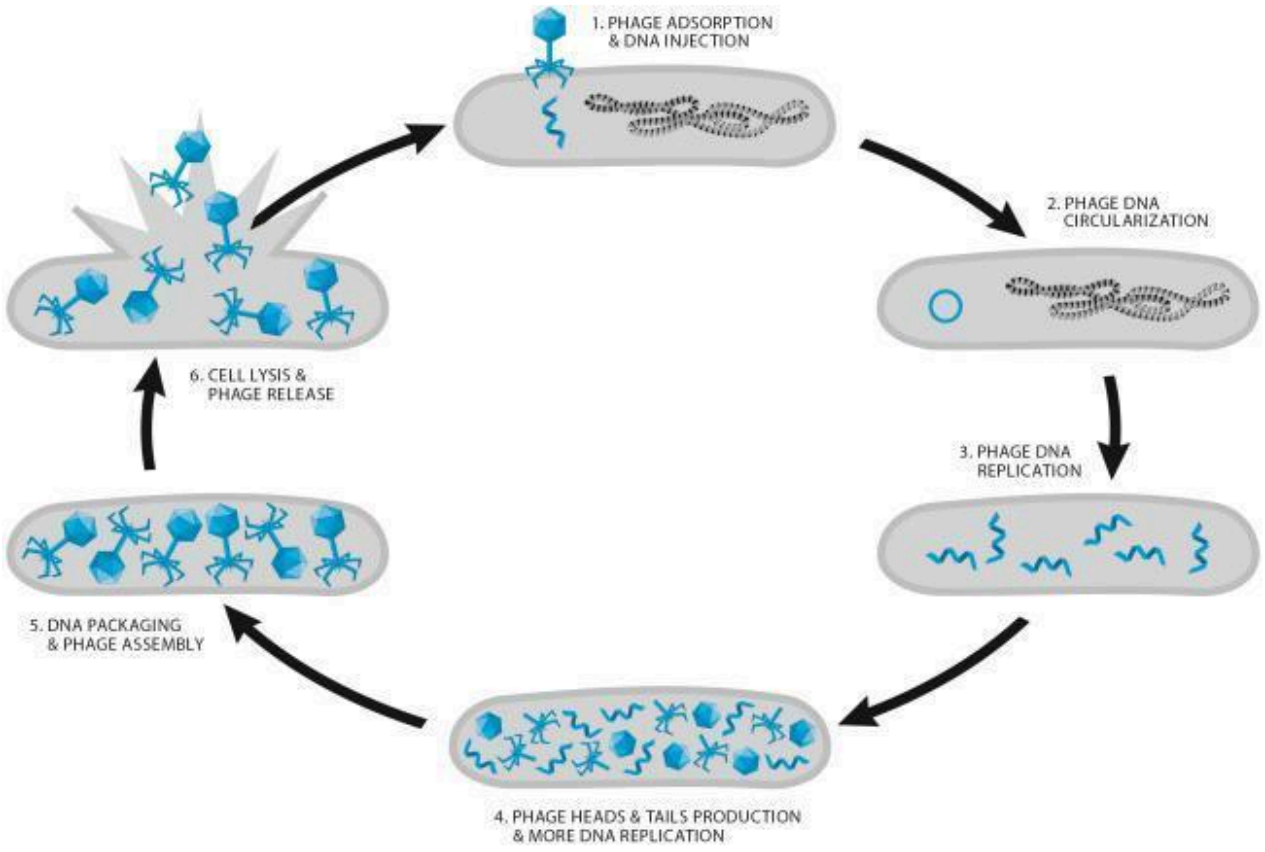


Figure 3.0-3. The lytic life cycle of phage. (1) A phage attaches and adsorbs to a host cell using its tail fibers and injects its linear chromosome into the bacterium. (2) The phage chromosome circularizes inside the host cell. (3) The phage DNA is replicated. (4) Phage heads and tails are produced. (5) New virions are assembled. (6) The bacterial host cell is lysed and the new virions released.

Once the phage DNA reaches the cytoplasm, expression of some of the phage genes begins. In some phages, this can happen before the genome has even fully entered the cell! These genes that are transcribed and translated first (see Preview: Introduction to Bioinformatics for a detailed description of these processes) are called “early” genes. Proteins expressed from early genes may include polymerases necessary for phage DNA replication, transcription factors necessary for the expression of other phage genes, and enzymes that degrade the host cell’s DNA.

Following the early gene expression, a new set of phage genes is expressed. These are known as “late” genes. Late-gene products include the structural proteins that comprise the new phage particles, or **virions**. The protein composition of each phage particle includes approximately 20 different types of proteins, each of which is incorporated into the virion particle in a specific order and copy-number. The copy-number is the total number of molecules of the same protein found in a single virion particle. Phage capsid proteins have a very high copy number. A phage with a genome size of 50 kb will have ~400 copies of the capsid protein per head, while the copy-number of the tail fiber protein can be quite low, with only 3–18 per tail. Usually, phage heads and tails are assembled as separate substructures, then DNA is packaged into the heads, and tails are attached (Figure 3.0-3). Finally, cell lysis occurs when phage-encoded enzymes degrade the cell wall and the cell membrane breaks. A single infected cell may yield 100 or more phage particles upon lysis, a parameter defined as the ”burst size” of the phage.

Virulent Phages Versus Temperate Phages

Virulent phages (also known as lytic phages) are those that use the lytic cycle, and only the lytic cycle, to reproduce. However, some phages have the ability to choose a different life cycle—the lysogenic cycle. These phages are called **temperate phages**. Temperate phages differ from lytic phages in that following DNA injection there are two possible outcomes of infection (Figure 3.0-4). In approximately 80–90 % of single-cell infections, the phage will proceed through the lytic cycle and create many progeny phages. However, in the other 10–20 % of infections, the phage will enter the lysogenic cycle (Figure 3.0-5). In the lysogenic cycle, the genes required for the lytic cycle are repressed, rather than expressed, and the phage genome becomes stably maintained within the cell, even through many subsequent generations of cell division. The phage genome within the cell is called a **prophage**, and a cell that carries a prophage is called a **lysogen**. A common mechanism used by a prophage to ensure its stability is by integrating into the host chromosome through a process known as homologous recombination.

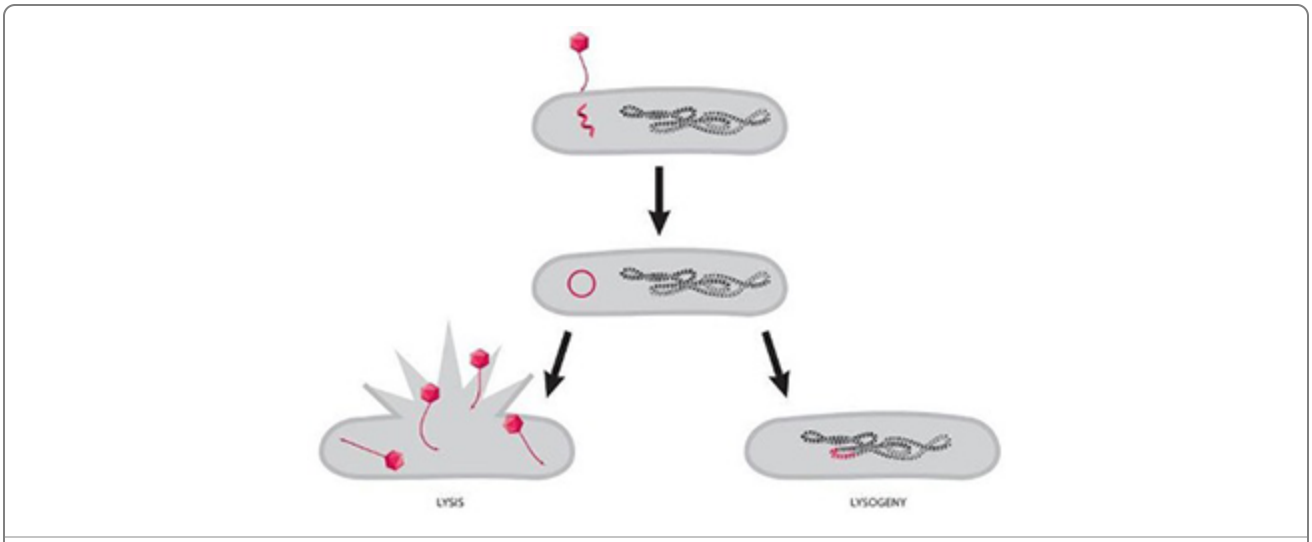


Figure 3.0-4. Decision of a temperate phage. Following DNA injection into a host cell a temperate phage may enter the lytic cycle or become a prophage (shown in red) and repress its lytic functions. This decision is controlled through a set of genes and regulatory sequences that are collectively called a “genetic switch”.

Once integrated, the prophage is replicated by the host DNA polymerases and passed to both daughter cells during normal cell division. In some temperate phages, the prophage does not integrate into the host genome but instead is established as a large extrachromosomal plasmid. These prophages encode and express several “partitioning” proteins that ensure each daughter cell receives a copy of the prophage plasmid.

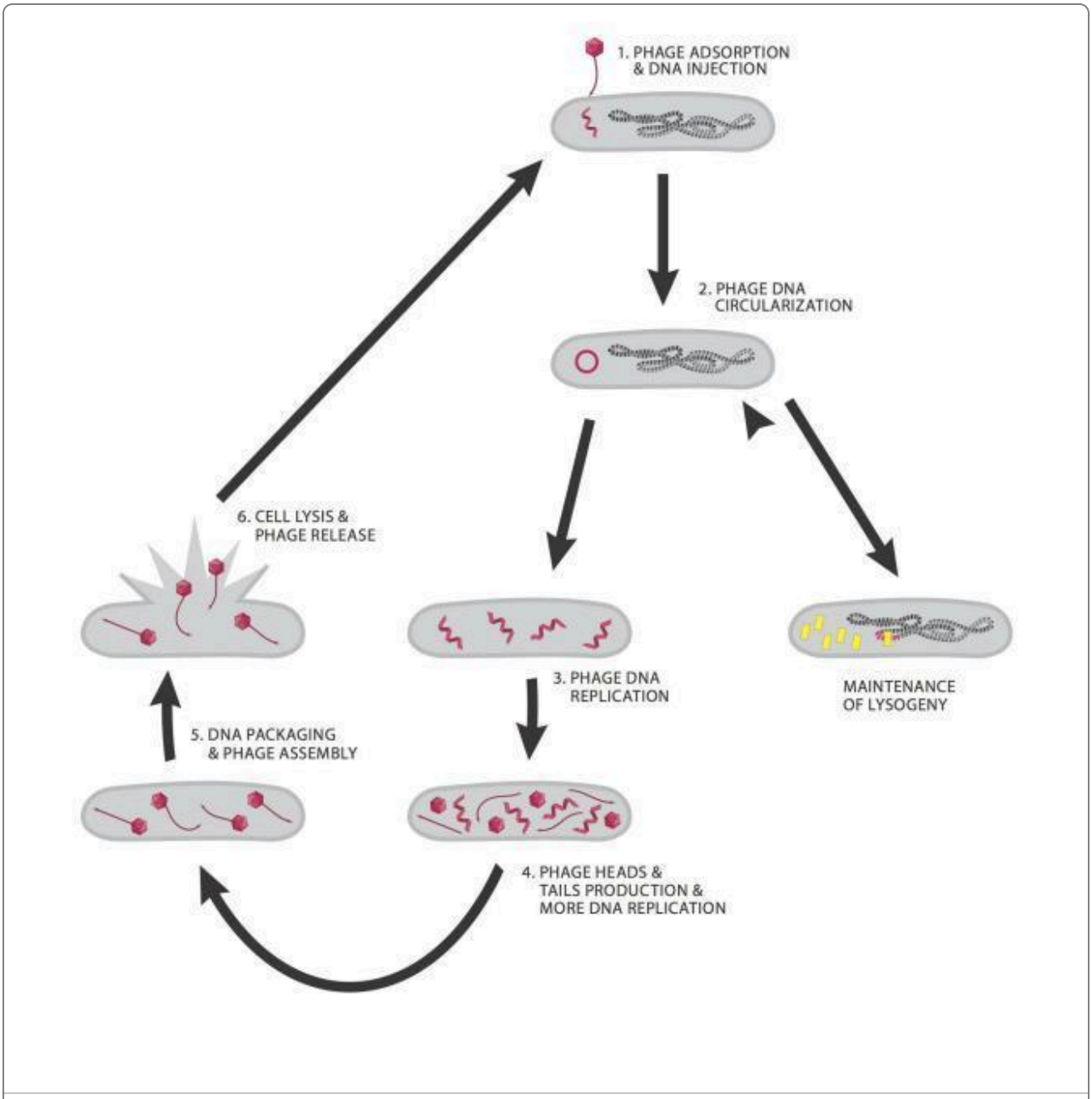


Figure 3.0-5 Life cycle of a temperate phage. (1) A phage attaches to a host bacterium using its tail fibers and injects its linear chromosome. (2) The phage chromosome circularizes and it is either maintained in the host as a prophage (shown here integrated into the bacterial chromosome in red) or enters the lytic cycle. The expression of the lytic genes is prevented through constant expression of the immunity repressor protein (shown as yellow rectangles). When the lysogen is stressed, the prophage may excise and begin the lytic cycle. (3) The phage DNA is replicated. (4) New tail and capsids are produced. (5) New virions are assembled. (6) The bacterial cell is lysed and the new virions are released.

In the lab, it is sometimes possible to distinguish between lytic and temperate phages by examining the differences in the appearance of the plaques that the phages form on bacterial lawns. Lytic phages form clear plaques (Figure 3.0-6 A) as a consequence of all of the bacterial cells being infected and killed. In contrast, temperate phages form cloudy or “turbid” plaques (Figure 3.0-6 B) that contain a mixture of killed cells and lysogens. However, it is important to note that other factors play a role in the appearance or “morphology” of a plaque, and there are phages that are NOT temperate that also produce cloudy plaques.

Likewise, mutants of temperate phages sometimes lose their ability to form lysogens and will only produce clear plaques. Without genomic information, it can be difficult to distinguish between all these possibilities. See Chapter 11 for more detailed information.

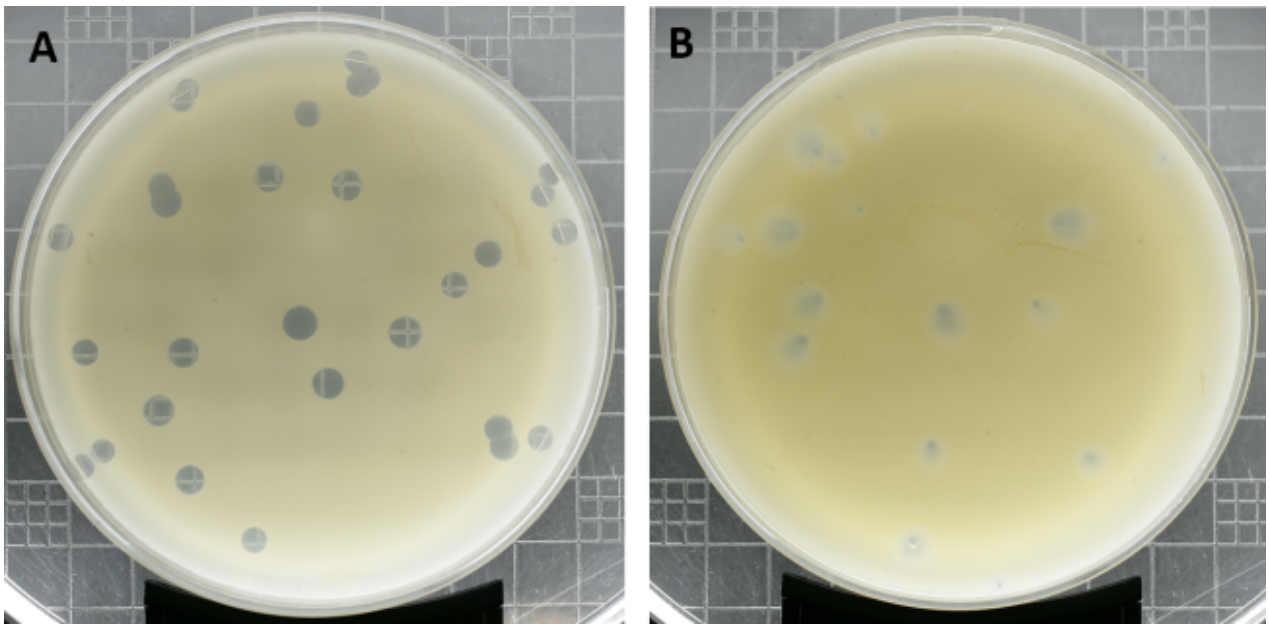


Figure 3.0-6. Two plaque morphologies, one clear (A) and the other cloudy (B). Frequently, lytic phages will produce clear plaques (as in A) while temperate phages produce cloudy or "turbid" plaques (as in B). Note that plaque appearance or "morphology" is influenced by many factors, including media type, number of cells and phage present, and incubation time and temperature. In the mycobacteriophage collection there are many examples of phages that produce plaques that are not clear and are not able to form lysogens.

While only a fraction of the global phage population has been sequenced and examined, genomic analysis of thousands of bacterial host strains shows that many carry prophages. And some strains contain dozens of different prophages simultaneously, all integrated at different locations in the chromosome. Some of these prophages encode toxins or other genes that confer pathogenicity to the host bacterium (cholera is a good example). This evidence suggests that the temperate phage life cycle is possibly the "norm."

Phage Host Range

The host range of a particular phage is a description of the range of bacterial types that can be infected by that phage. Many phages have narrow host ranges and can infect only a single strain of bacteria and no other strains in the same bacterial species. Other phages have broad host ranges and can infect bacteria of different species, or even different genera.

There are many factors that influence whether a particular phage can infect a particular bacterium. These determinants of host specificity include the presence of a receptor in the host's cell wall that the phage can recognize and adsorb to. The phage also has to be able to interact with the host's cellular machinery, including the host transcription and translation systems. Furthermore, it is relatively easy for phages to alter their host ranges, sometimes with as little as a single base mutation in their genome sequence. So unsurprisingly, host

range mutants occur frequently in some phages (as often as 1 in 100,000 or 1 in 1,000,000 particles in a lysate). There is usually no easy way to predict with confidence the host range from a phage's genomic sequence alone, so the host range needs to be determined experimentally.

The Great War

The phage population is not only vast but also highly dynamic, with the entire global phage population predicted to turn over in a matter of days. The phage-bacteria relationship involves constant selective pressure for bacterial hosts to become insensitive to phage infection, along with the necessity for phages to coevolve so they always have hosts that can support their replication. It is this struggle, perhaps going on for several billion years, that gives rise to such a diverse population. There are many known ways in which bacteria can become insensitive to infection, and there are likely many more ways to be discovered. The known ways include loss or alteration of the host receptor, host restriction–modification systems, superinfection immunity, CRISPR systems, and surface exclusion. There are also many examples of abortive infection systems in which a bacterial host cell sacrifices itself to prevent a phage particle from reproducing, thus sparing other bacterial cells nearby. These are described in more detail below and in Chapter 11.

Mechanisms of Insensitivity: Immunity, Exclusion, and Resistance

Immunity and Exclusion

Superinfection **immunity** refers to the ability of a lysogen to resist infection from an invading phage by repressing the invader's lytic genes. A lysogen's ability to resist infection is mediated through three processes: the constitutive expression of the immunity repressor protein from the prophage, its subsequent binding to the prophage DNA, and the repression of transcription of the lytic genes. The immunity repressor protein can also bind to the DNA of phages that attempt to infect the lysogen ("superinfection") and repress expression of the lytic genes from the invader's injected DNA, granting the lysogen "immunity."

In addition to repressor-mediated superinfection immunity, a prophage in a host cell can confer insensitivity to phage infection though exclusion. **Exclusion** refers to when prophage-encoded and expressed proteins alter the host cell surface in such a way that other phages are "excluded" from absorption and DNA injection.

Resistance

Bacterial resistance to phage infection describes the insensitivity of a host cell to infection due to mutation or loss of the phage receptor or through the action of one or more bacterial defense systems. The best characterized systems are restriction, abortive infection, and CRISPR, however, there are likely many more yet to be discovered.

Bacterial restriction systems were first discovered through the observation that a particular phage was "restricted" from infecting a related strain of its preferred host. Upon investigation, it was discovered that the host encoded an endonuclease that cut double-stranded DNA at a specific base pair sequence. The host's DNA was protected from digestion at these sequences by the addition of methyl groups to its DNA. Subsequent investigations revealed that there are a vast number of different restriction systems that

recognize different sequences; each system contains both an endonuclease and a DNA modification enzyme that recognize the same DNA sequence. Typically, these sequences are 4 to 6 bp in length. The nucleases have since been dubbed “restriction endonucleases” or just “restriction enzymes,” and they are commonly used tools in molecular biology labs.

Abortive-infection mechanisms allow cells to deliberately sacrifice themselves when infected by a phage. One example is the toxin-antitoxin system. During normal cell growth, both the toxin and the antitoxin are expressed, and the antitoxin prevents the toxin from killing the cell, usually by binding to it and down-regulating its expression. Phage infection can lead to cessation of host cell gene expression, including that of the toxin and antitoxin. However, in many of these systems, the antitoxin is less stable than the toxin and degrades more rapidly. The degradation of the antitoxin releases the toxin and causes the death of the cell. In this way the cell sacrifices itself to prevent the phage from multiplying, sparing other bacterial cells in the population.

Clustered Regularly-Interspaced Palindromic Repeats (CRISPRs) and their Cas proteins (Cas, for CRISPR– associated) are found in prokaryotic genomes and are a means of phage defense through acquired immunity. The CRISPR array is constructed of short (30–40 bp), repeated palindromic sequences that are interspersed with short (~40 bp) “‘spacer’” pieces of DNA. These spacer pieces of DNA are identical to short DNA segments present in bacteriophage genomes. Through the actions of the CRISPR-Cas system, a host can use the library of spacer sequences present in the CRISPR array to prevent infection from a bacteriophage whose genome contains one of the protospacer sequences. Recently, CRISPR-Cas systems have been used for easy, specific editing of eukaryotic and prokaryotic gene sequences. Consequently, they have enormous potential as molecular tools in areas such as human health and food production.

Prophage genomes can also carry restriction systems, toxin-antitoxin genes, exclusion genes, and even CRISPR arrays. Because phages also have to counteract resistance mechanisms, they can encode antirestriction genes and anti-CRISPR genes, among many others.

All the World’s a Phage

When we compare the genomes of two phages that infect phylogenetically distant bacterial hosts, we find they typically share little or no sequence information. As more phage genomes are sequenced, the genetic connections between phages begin to emerge, suggesting that phages have shared, but have had unequal access to, a common gene pool. But our knowledge of phage genomics remains scant. Currently, (as of summer 2016) the sequences of only about 2,000 dsDNA phage genomes are available. This number is dwarfed by the more than 50,000 sequenced bacterial genomes.

Nonetheless, the genetic connections among phages can be elucidated by deepening our knowledge of the phages that infect particular hosts and extending this knowledge to phylogenetically-proximal hosts. This is the strategy employed by the SEA-PHAGES program, which has dug deeply into the phages that infect Mycobacterium smegmatis mc2155 and is now exploring the phages of other hosts within the phylum Actinobacteria.

Is Any of This Useful?

Bacteriophages have played central roles in what we know today about the fundamental basics of molecular biology. Because bacteriophages are simple, both structurally and genetically, and propagate quickly to large numbers, mutants with specific phenotypes can be isolated, mapped, and characterized. Then using the awesome power of phage genetics, we can address fundamental questions about gene structure, organization, regulation, and function. Because of the extensive biological novelty associated with this great genetic diversity, phage genetics continues to uncover new, interesting, and useful insights.

Phages have many potential applications, either as whole particles or as individual genetic components. For example, the therapeutic use of phages—which has captured the imagination of scientists for the past 95 years or so—remains an area of interest, especially in light of the emergence of antibiotic resistance among bacterial pathogens. Phages have also proved useful in disease diagnosis—from the early studies in phage typing (using phage host range to identify clinical isolates) to the use of reporter phages for rapid drug-susceptibility testing of clinical samples. The use of phage-derived tools in bacterial genetics is also extensive, including in the development of vector systems, the development of microbial computers, mutant construction, and transposon delivery.

The Road Ahead

The phage population is vast (10^{31} particles) and its genetic diversity is huge. With only about 2×10^{3} genomes sequenced to date, we've yet to make significant progress in defining viral diversity. And because of its extensive genetic novelty and number of genomes, the phage population harbors the greatest reservoir of undiscovered genetic information in the biosphere. Elucidating the functions of all these yet-to-be-discovered genes remains a substantial challenge, but their potential for advances in biotechnology and medicine is beyond question. Get to work!

Host Basics

Chapter 4: Host Basics

Chapter 4: An Overview

An Introduction to Bacteria

Bacteria are prokaryotes and among the oldest organisms on the planet. They are also among the simplest free-living life-forms known. Like all prokaryotes, they are single-cell organisms and lack a nuclear membrane and other internal membrane-bound organelles. Bacteria are small, usually in the range of 1 to 5 microns (μm) across (a human red blood cell is 8 μm).

All bacteria have a cytoplasmic plasma membrane made of lipids that enclose the inner workings of the bacterial cell. A cell wall constructed of sugars and proteins surrounds the plasma membrane and gives the bacteria structural strength. Bacteria can have an additional lipid membrane and a slimy capsule that protects them from harsh environments or performs other functions. Some bacteria have appendages called “pili” and “flagella” (singular are “pilus” and “flagellum,” respectively). These hair-like structures protrude from the bacteria and perform specialized functions. Inside the plasma membrane all bacteria contain a DNA genome (typically 2–10 megabase pairs Mbp). But unlike the genome in eukaryotic cells, the bacterial genome is not enclosed by a lipid membrane (Figure 4.0-1). Genes are transcribed by RNA polymerase to make messenger RNAs (mRNA), which are used by ribosomes as substrates for protein synthesis. Because bacteria lack a nuclear membrane, the processes of transcription (mRNA synthesis) and translation (protein synthesis) are tightly coupled events.

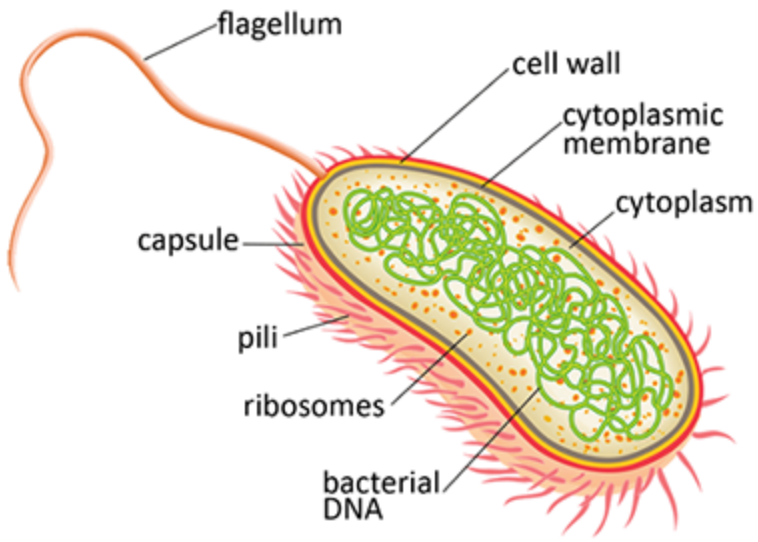


Figure 4.0-1. Bacterial structure.

The Bacterial World

Bacteria are extremely abundant and by mass outweigh all plants and animals on the planet, combined. They are present in almost all habitats and naturally reside in and on you.

Bacteria are also tremendously diverse, and different bacteria thrive in different environments. In each environment, bacteria have rich interactions with other forms of life. For example, the many different bacteria that live within the human gastrointestinal tract contribute to our health by helping us digest food and build our immune system. There are, however, bacteria that cause devastating human diseases like cholera (Vibrio cholerae) and tuberculosis (Mycobacterium tuberculosis). Wherever bacteria live, their environment is hardly ever theirs alone and can be a hostile place. They must deal with toxins and antibiotics naturally produced by fungi and other bacteria, as well as bacteriophages that can infect them. Over time, bacteria have evolved a variety of strategies to combat these assaults, making them incredibly successful organisms despite their simplicity.

All free-living organisms contain ribosomes for protein synthesis, and these ribosomes contain large RNA molecules that contribute to catalytic function. Different bacterial species have slightly different ribosomal RNA (rRNA) sequences, and these can be used to construct models—or a phylogeny—of their evolutionary relationships (Figure 4.0-2).

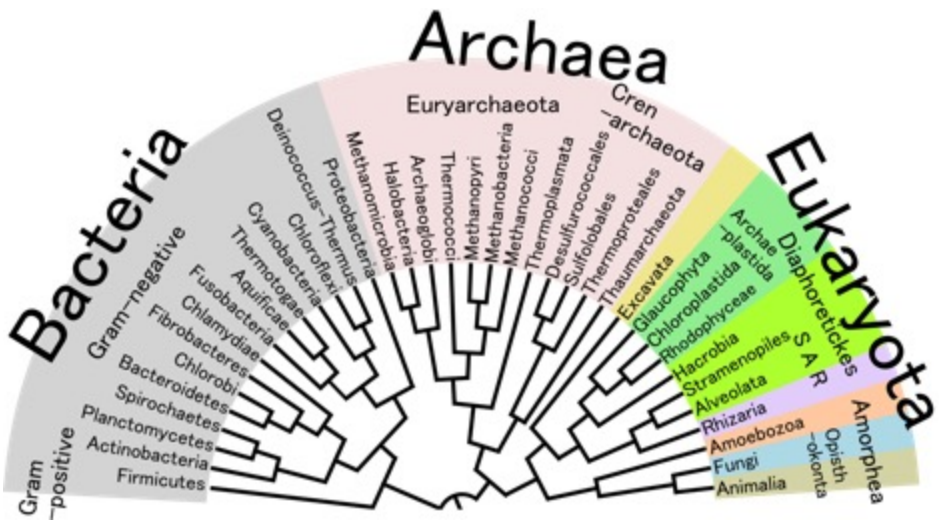


Fig. 4.0-2. Phylogeny showing evolutionary relationships between the three phyla. (Photo courtesy of Wikipedia Commons, https://commons.wikimedia.org/wiki/File:Phylogenetic_Tree_of_Life.png_)

The Bacterial Life Cycle

It is common for bacteria to reproduce asexually by dividing in half through a process known as binary fission. Bacterial growth is thus exponential, with one cell dividing to produce two cells, each of these dividing to produce four cells, then each of those dividing to produce eight cells, and so on. Fortunately, various influences, including resource limitations, lead to growth cessation. Otherwise, the world soon would be engulfed in bacteria! In the lab, when bacteria are growing in a liquid medium, this stage of growth is referred to as “stationary phase” (Figure 4.0-3) and the culture is sometimes referred to as being “saturated.” The number of bacterial cells in a saturated cultured is typically 2–4 x 109 cells/ml.

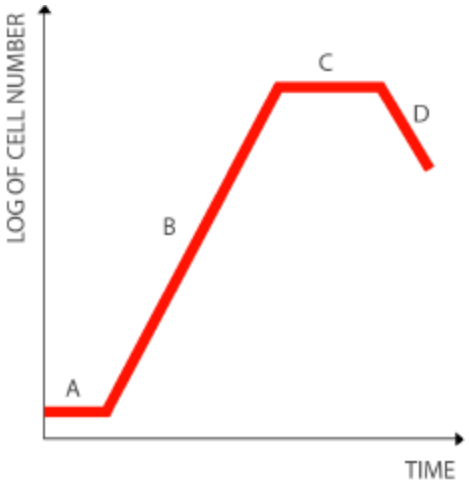


Figure 4.0-3. Bacterial growth over time. When inoculated into fresh media, bacteria will take some time recover without growth. This is called the “lag phase” (A). Bacteria will then enter a logarithmic (or exponential) growth phase (B), where the number of bacteria doubles once every time period (the period depends on the bacterial species). Once nutrients are used up, the bacterial number will remain constant in the stationary phase (C), and eventually decline in the death phase (D).

The growth rate of bacteria can be measured as the “doubling time,” that is, the time it takes for the number of bacterial cells to double. The doubling time varies greatly among different bacteria, and it also fluctuates widely depending on environmental aspects such as temperature and nutrient availability. Some bacteria can grow with doubling times as fast as 15 –20 minutes, whereas others may take days or even weeks to double.

In the lab we normally study a bacterial strain in isolation from other types of bacteria. We provide the bacteria with growth media, either in the form of a solid (agar) or a liquid, that is rich in nutrients to encourage their growth. This is called “culturing bacteria.” When a bacterial sample is streaked onto solid media, we can observe individual bacterial colonies. All the bacteria in a colony are genetically identical (apart from any in which rare mutations have occurred) because they arose from a single bacterium. The number of cells in a bacterial colony varies enormously depending on how large the colony is, but it is generally in range of 10^{7}–10^{8} cells.

The morphology of bacterial colonies—that is, their color, size, and shape—differs greatly from species to species, ranging from yellow to purple and from smooth to rough. If many bacterial cells (e.g., 10^{5}) are added to solid media, individual colonies do not form. Instead, the bacteria will grow as a lawn that encompasses the entire surface area of the plate. When bacteria are cultured in liquid, they diffuse in the solution, and as they accumulate, the liquid becomes increasingly cloudy as it forms a saturated culture.

Chapter 4: Tips and Hints

Growing and maintaining cultures.

- Always determine the temperature range within which your bacteria will grow. Once you have selected a growth temperature to use, for reproducibility of experiments, it is important that cultures used for any given phage or set of experiments be prepared at the same temperature.

- Use freshly prepared cultures, as older cultures do not form bacterial lawns reproducibly. In order to maintain a supply of fresh cultures for experiments, it is best to start new cultures **weekly or biweekly** throughout the semester.

- It is a good idea to check your working cultures by performing a spot test with a phage known to infect this host. A nice smooth lawn is indicative of a good working culture, and a clearing where the known phage was spotted is a good indicator that the culture remains susceptible to phage infection. If the control phage does not form the expected clearing, you might not be growing your bacterial host, but a contaminant instead. Note that the quality of a lawn often deteriorates as a culture ages, with older or dying cultures often producing embedded colonies in the top agar that can make it difficult to observe plaques.

- Try to have a dedicated space with dedicated pipettes where you prepare media and cultures. Avoid working with phage in this area, and with these pipettes.

Arthrobacter globiformis

An Introduction to the Host Bacterium, Arthrobacter globiformis

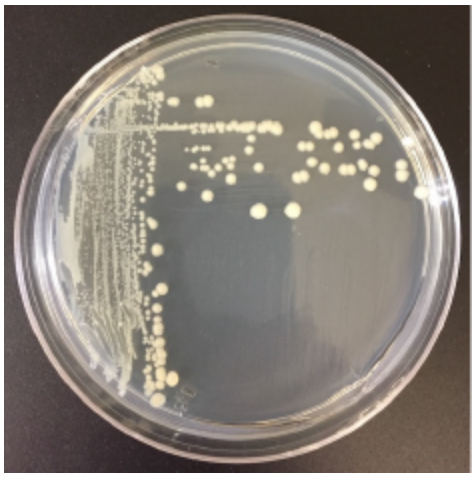
As a network of researchers, SEA-PHAGES explores the genetic diversity and relationships of populations of bacteriophages that infect Actinobacteria, incluidng the bacterium Arthrobacter globiformis. There are many different strains of Arthrobacter globiformis. (isolated from different environmental samples). The Arthrobacter globiformis strain widely used in SEA-PHAGES is called Arthrobacter globiformis B2979-SEA, and is made available to you via the USDA culture collection. If you intend to isolate phages using this bacterium, then this will be your 'host' for phage isolation.

Arthrobacter globiformis is one of over 70 species that make up the genus Arthrobacter, classified within the phylum Actinobacteria. Arthrobacter globiformis is a gram-positive soil organism, whose relatives are well equipped to breakdown various hydrocarbons. Arthrobacter arilaitensis, for instance, grows on the surface of cheese and catabolizes the fatty acids, amino acids, and lactic acid present, contributing to the color and flavor of the cheese. Other Arthrobacter species are capable of breaking down compounds such as hexavalent chromium, which is a carcinogen, suggesting a potential use in bioremediation (i. e. the breakdown of pollutants). The specific Arthrobacter strain you will use, alongside its relatives, has been found to produce penicillin derivatives and aspartic decarboxylase.

Arthrobacter globiformis will grow and divide on many nutrient-rich media. In your research, you will grow Arthrobacter sp. on PYCa media and at 30 - 37 ˚C. Under these conditions, Arthrobacter globiformis grows rapidly, dividing approximately once every 2 - 3 hours. This means that it typically takes about 3 days for an individual cell to form a colony on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g. 10^7 or more) so that a lush lawn grows within 1 – 2 days. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature. Arthrobacter globiformis colonies are white to tan in color, smooth and glossy. A liquid culture of Arthrobacter globiformis inoculated from a single colony will take 2 days to form a saturated liquid culture, exhibiting a tan color. The strain you will work with is not inhibited by the antifungal cyclohexamide. Therefore, this can be added to the growth media to prevent other microorganisms from growing in your cultures.

Arthrobacter globiformis colonies are white to tan in color, smooth and glossy. A liquid culture of Arthrobacter globiformis inoculated from a single colony will take 2 days to form a saturated liquid culture, exhibiting a tan color. The strain you will work with is not inhibited by the antifungal cyclohexamide. Therefore, this can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 30 % of attempts to isolate phage from soil by enrichment with Arthrobacter globiformis yield phage.



trial|Figure 4.0-4. Arthrobacter globiformis. growing on an agar plate.

If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth media: PYCa

Growth temperature: 22 - 30 ˚C.

Antimicrobials: cycloheximide (10 µg/ml)

Phage Buffer: 10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: BamHI, HaeIII, MseI, NspI, and SacII.

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Arthrobacter sp.

An Introduction to the Host Bacterium, Arthrobacter sp.

As a network of researchers, SEA-PHAGES explores the genetic diversity and relationships of populations of bacteriophages that infect Actinobacteria, incluidng the bacterium Arthrobacter sp. There are many different strains of Arthrobacter sp. (isolated from different environmental samples). The Arthrobacter sp. strain widely used in SEA-PHAGES is called Arthrobacter sp. KY3901. This strain is from the American Type Culture Collection (catalog # 21022). If you intend to isolate phages using this bacterium, then this will be your 'host' for phage isolation.

Arthrobacter sp. is one of over 70 species that make up the genus Arthrobacter, classified within the phylum Actinobacteria. Arthrobacter sp. is a gram-positive soil organism, whose relatives are well equipped to breakdown various hydrocarbons. Arthrobacter arilaitensis, for instance, grows on the surface of cheese and catabolizes the fatty acids, amino acids, and lactic acid present, contributing to the color and flavor of the cheese. Other Arthrobacter species are capable of breaking down compounds such as hexavalent chromium, which is a carcinogen, suggesting a potential use in bioremediation (i. e. the breakdown of pollutants). The specific Arthrobacter strain you will use, alongside its relatives, has been found to produce penicillin derivatives and aspartic decarboxylase.

Arthrobacter sp. will grow and divide on many nutrient-rich media. In your research, you will grow Arthrobacter sp on PYCa media and at 22 - 28˚C. Under these conditions, Arthrobacter sp grows rapidly, dividing approximately once every 2 - 3 hours. This means that it typically takes about 3 days for an individual cell to form a colony on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g. 10^7 or more) so that a lush lawn grows within 1 – 2 days. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature.

Arthrobacter sp colonies are tan to yellow in color, smooth and glossy (Figure 4.0-4). When incubated for prolonged periods, the color of the colonies intensifies. A liquid culture of Arthrobacter sp inoculated from a single colony will take 1 – 2 days to form a saturated liquid culture, exhibiting a tan color. The strain you will work with, Arthrobacter sp. KY3901, is not inhibited by the antifungal cyclohexamide or the antibiotic spectinomycin. Therefore, this can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 40 % of attempts to isolate phage from soil by enrichment with Arthrobacter sp. yield phage

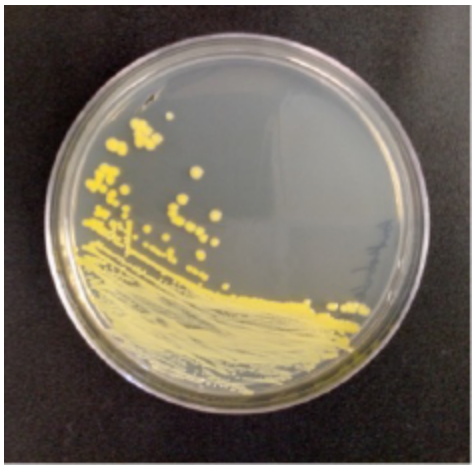


Figure 4.0-4. Arthrobacter sp. growing on an agar plate.

If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth media: PYCa

Growth temperature:22˚C - 28˚C (this host does not grow at 37˚C)

Antimicrobials: cycloheximide (10 µg/ml), spectinomycin (optional)

Phage Buffer:10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: BamHI, ClaI, EcoRI, HaeIII, HindIII, MseI.

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Gordonia rubripertincta

An Introduction to the Host Bacterium, Gordonia rubripertincta

As a network of researchers, SEA-PHAGES explores the genetic diversity and relationships of populations of bacteriophages that infect Actinobacteria, incluidng the bacterium Gordonia rubripertincta. There are many different strains of Gordonia rubripertincta (isolated from different samples). The Gordonia rubripertincta strain widely used in SEA-PHAGES is called Gordonia rubripertincta B16540-SEA, and is made available to you via the USDA culture collection. If you intend to isolate phages using this bacterium, then this will be your 'host' for phage isolation.

G. rubripertincta is one of over 19 species that make up the genus Gordonia, classified within the phylum Actinobacteria. G. rubripertincta is a gram-positive soil organism and, like its relatives, is well equipped to breakdown various hydrocarbons. This ability makes Gordonia attractive bacteria for use in bioremediation (i. e. the breakdown of pollutants) and in industrial biotechnology. For example, Gordonia sp. MTCC 4818 is able to breakdown phthalic acid esters, which can act as hormones that disrupt normal body function. Another Gordonia species, CYSK-1, is able to remove sulfur from fossil fuels, which will allow for the production of cleaner fuels, such as ultra-low-sulfur diesel.

G. rubripertincta will grow and divide on many nutrient-rich media. In your research, you will grow G. rubripertincta on PYCa media and at 30 ˚C. Under these conditions G. rubripertincta grows rapidly, and it typically takes about 2 - 3 days for an individual cell to form a colony on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g. 10^7 or more) so that a lush lawn grows within 1 – 2 days. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature.

G. rubripertincta colonies are tan to orange in color, glossy and smooth in texture, and round or slighly irregular in shape (Figure 4.0-4). When incubated for prolonged periods, the color of the colonies intensifies to a brilliant orange. A liquid culture of G. rubripertincta inoculated from a single colony will take 2 – 3 days to form a saturated liquid culture, exhibiting a tan to orange color. The strain you will work with, G. rubripertincta B16540-SEA, is not inhibited by the antifungal cyclohexamide, or the antibiotic carbenicillin at low concentrations. Therefore, this can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 20 % of attempts to isolate phage from soil by enrichment with G. rubripertincta yield phage.

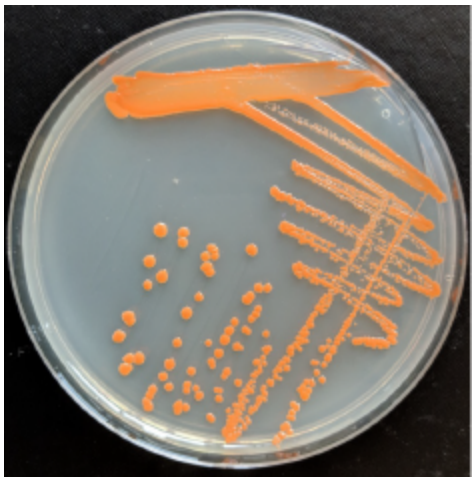


Figure 4.0-4. Gordonia rubripertincta growing on an agar plate.

If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth media: PYCa, supplemented with Tween80 as necessary (see instructions for culturing bacteria that require Tween80)

Growth temperature:30 ˚C

Antimicrobials: cycloheximide (10 µg/ml), carbenicillin (not recommended)

Phage Buffer:10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: BamHI, HaeIII, HindIII, MseI, NspI, and SacII.

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Gordonia terrae

An Introduction to the Host Bacterium, Gordonia terrae

As a network of researchers, SEA-PHAGES explores the genetic diversity and relationships of populations of bacteriophages that infect Actinobacteria, incluidng the bacterium Gordonia terrae. There are many different strains of Gordonia terrae (isolated from different samples). The Gordonia terrae strain widely used in SEA-PHAGES is called Gordonia terrae CAG3 that was originally isolated from oil-contaminated soil in China. If you intend to isolate phages using this bacterium, then this will be your 'host' for phage isolation.

G. terrae. is one of over 19 species that make up the genus Gordonia, classified within the phylum Actinobacteria. G. terrae. is a gram-positive soil organism, whose relatives are well equipped to breakdown various hydrocarbons. This ability makes Gordonia attractive bacteria for use in bioremediation (i. e. the breakdown of pollutants) and in industrial biotechnology. For example, Gordonia sp. MTCC 4818 is able to breakdown phthalic acid esters, which can act as hormones that disrupt normal body function. Another Gordonia species, CYSK-1, is able to remove sulfur from fossil fuels, which will allow for the production of cleaner fuels, such as ultra-low-sulfur diesel.

G. terrae. will grow and divide on many nutrient-rich media. In your research, you will grow G. terrae on PYCa media and at 30 ˚C. Under these conditions G. terrae grows rapidly, dividing approximately once every 2 - 3 hours. This means that it typically takes about 3 days for an individual cell to form a colony on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g. 10^7 or more) so that a lush lawn grows within 2 – 3 days. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature.

G. terrae colonies are tan to orange in color, round, rough and dry in texture (Figure 4.0-4). When incubated for prolonged periods, the color of the colonies intensifies. A liquid culture of G. terrae inoculated from a single colony will take 2 – 3 days to form a saturated liquid culture, exhibiting a tan to orange color. The strain you will work with, G. terrae CAG3, is not inhibited by the antifungal cyclohexamide, or the antibiotic ampicillin at low concentrations. Therefore, this can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 33 % of attempts to isolate phage from soil by enrichment with Gordonia terrae yield phage.

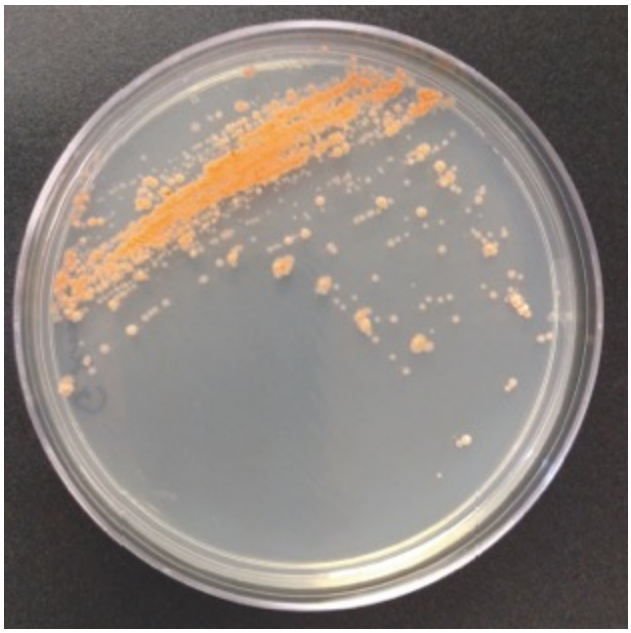


Figure 4.0-4. Gordonia terrae growing on an agar plate.

If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth media: PYCa, supplemented with Tween80 (see instructions for culturing bacteria that require Tween80)

Growth temperature:30 ˚C

Antimicrobials: cycloheximide (10 µg/ml), ampicillin (optional, and up to 10 mg/ml)

Phage Buffer:10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: BamHI, ClaI, EcoRI, HaeIII, HindIII, MseI.

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Microbacterium foliorum

An Introduction to the Host Bacterium, Microbacterium foliorum

As a network of researchers, SEA-PHAGES explores the genetic diversity and relationships of populations of bacteriophages that infect Actinobacteria, incluidng the bacterium Microbacterium foliorum (M. foliorum). There are many different strains of M. foliorum (isolated from different environmental samples). The M. foliorum strain widely used in SEA-PHAGES is called M. foliorum SEA B-24224 that was isolated from grass in Germany, and made available to you from the Agricultural Research Service of USDA. If you intend to isolate phages using this bacterium, then this will be your 'host' for phage isolation.

M. foliorum is one of more than 90 species that make up the genus Microbacterium, classified within the phylum Actinobacteria. Microbacteria are gram-positive rod-shaped bacteria that are slightly smaller than typical rod-shaped bacteria, hence the name microbacteria. Microbacteria have been isolated from soil, water, and plants. Microbacteria have also been isolated from cheese rinds, where it may contribute to the flavor profile.

M. foliorum will grow and divide on many nutrient-rich media. In your research, you will grow M. foliorum on PYCa media at 28 – 30 ˚C. Under these conditions, M. foliorum grows rapidly, dividing approximately once every 90 minutes. This means that it typically takes about 2 – 3 days for an individual cell to form a colony on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g., 10^7 or more) so that a lush lawn grows within 24 hours. M. foliorum will also grow at room temperature (~ 22 ˚C), but it divides more slowly and therefore requires extra time to form either a colony or a lawn, typically an additional 24 hours. M. foliorum does not grow at 37 ˚C. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature.

M. foliorum colonies are tan to yellow in color, smooth and glossy (Figure 4.0-4). When incubated for prolonged periods, or when grown at room temperature, the color of the colonies are a brighter yellow. A liquid culture of M. foliorum inoculated from a single colony will take 24 hours to form a saturated liquid culture, exhibiting a tan to yellow color, which intensifies with prolonged incubation or when grown at room temperature. The strain you will work with, M. foliorum, is not inhibited by the antifungal cyclohexamide. Therefore, cycloheximide can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 20 % of attempts to isolate phage from soil by enrichment with M. foliorum yield phage.

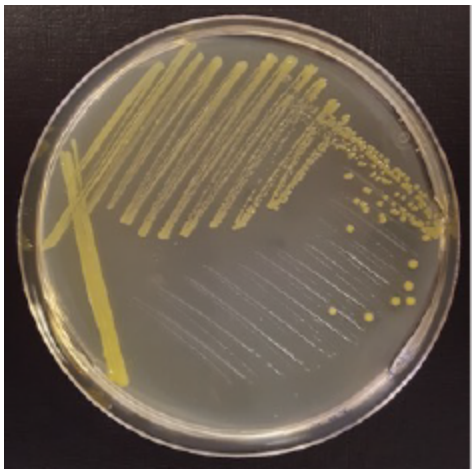


Figure 4.0-4. Microbacterium foliorum growing on an agar plate.

If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth media:PYCa

Growth temperature:optimal at 28 ˚C - 30 ˚C; grows well at room temperature (~ 22 ˚C); this host does not grow at 37˚C.

Antimicrobials: cycloheximide (10 µg/ml)

Phage Buffer:10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: HaeIII, MseI, NspI, SacII, and SalI.

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Microbacterium testaceum

An Introduction to the Host Bacterium, Microbacterium testaceum

In this course, you will be isolating bacteriophage that infect the bacterium Microbacterium testaceum (M. testaceum). You will use a strain called M. testaceum SEA B-24232 that is made available to you from the Agricultural Research Service of USDA. This is your “host” for phage isolation.

M. testaceum is one of more than 90 species that make up the genus Microbacterium, classified within the phylum Actinobacteria. Microbacteria are gram-positive rod-shaped bacteria that are slightly smaller than typical rod-shaped bacteria, hence the name microbacteria. Microbacteria have been isolated from soil, water, and plants. Microbacteria have also been isolated from cheese rinds, where it may contribute to the flavor profile.

M. testaceum will grow and divide on many nutrient-rich media, and at temperatures ranging from room temperature (22 ˚C) to 37 ˚C. In your research, you will grow M. testaceum on PYCa media at 30 ˚C. Under these conditions, M. testaceum grows rapidly and it typically takes about 2 – 3 days for an individual cell to form a colony on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g., 10^7 or more) so that a lush lawn grows within 24 hours. At room temperature (~ 22 ˚C), M. testaceum divides more slowly and typically requires an additional 24 hours to form either a colony or a lawn on plates. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature.

M. testaceum colonies are tan to yellow in color, smooth and glossy (Figure 4.0-4). When incubated for prolonged periods, or when grown at room temperature, the color of the colonies are a brighter yellow. A liquid culture of M. testaceum inoculated from a single colony will take 24 - 48 hours to form a saturated liquid culture, exhibiting a tan to yellow color, which intensifies with prolonged incubation or when grown at room temperature. The strain you will work with, M. testaceum SEA B-24232, is not inhibited by the antifungal cyclohexamide. Therefore, cycloheximide can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 20 % of attempts to isolate phage from soil by enrichment with M. testaceum yield phage.

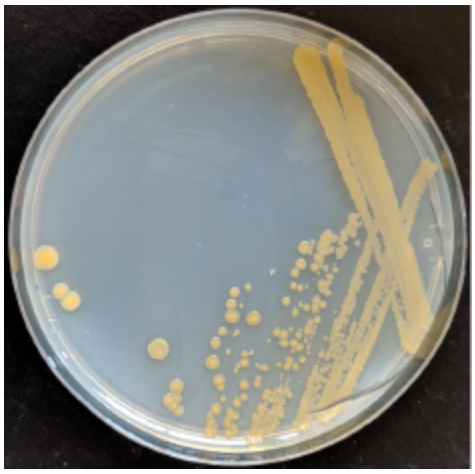


Figure 4.0-4. Microbacterium testaceum growing on an agar plate

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If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth media:PYCa

Growth temperature:30 ˚C

Antimicrobials: cycloheximide (10 µg/ml)

Phage Buffer:10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: HaeIII, MseI, NspI, and SacII.

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Mycobacterium smegmatis

An Introduction to the Host Bacterium, Mycobacterium smegmatis

As a network of researchers, SEA-PHAGES explores the genetic diversity and relationships of populations of bacteriophages that infect Actinobacteria, incluidng the bacterium Mycobacterium smegmatis (M. smegmatis, or colloquially known as “smeg”). There are many different strains of M. smegmatis (isolated from different environmental or clinical samples). The M. smegmatis strain widely used in SEA-PHAGES is called M. smegmatis mc2155. If you intend to isolate phages using this bacterium, then this will be your ‘host’ for phage isolation.

M. smegmatis is one of more than 100 species that make up the genus Mycobacterium, classified within the phylum Actinobacteria. Mycobacteria were named this because they sometimes have a fungus-like appearance under the microscope—although they are definitely not fungi! The Mycobacterium genus contains many species that affect human health, including M. tuberculosis and M. leprae, which are the causative infectious agents of tuberculosis and leprosy, respectively. M. smegmatis, however, is a saprophyte and is found in environmental samples, including soil and compost. Although they share the same genus, M. smegmatis and M. tuberculosis have many other differences, including vastly different doubling times: approximately 3 hours for M. smegmatis and 24 hours for M. tuberculosis. Some bacteriophages are capable of infecting both species, whereas others infect only one or the other.

M. smegmatis will grow and divide on many nutrient-rich media. In your research, you will grow M. smegmatis on 7H9 media supplemented with albumin and dextrose (AD) at 37 ˚C. Under these conditions, M. smegmatis grows at its fastest, dividing approximately once every 3 hours. This means that it typically takes about 3–4 days for an individual colony growing from a single cell on an agar plate. However, when preparing a bacterial lawn, you can start by adding many bacterial cells (e. g., 10^7 or more) so that a lush lawn grows within 20–24 hours. M. smegmatis will also grow at 30 ˚C, but it divides more slowly and therefore requires more time to form either a colony or a lawn. For reproducibility of experiments, it is important that cultures used for a given set of experiments be prepared at the same temperature.

M. smegmatis mc2155 colonies are tan-white in color and dry, rough, and wrinkled in texture (Figure 4.0-4). A liquid culture of M. smegmatis inoculated from a single colony will take 3–5 days to form a saturated liquid culture, exhibiting a tan-white color. The strain you will work with, M. smegmatis mc2155, is not inhibited by the antibiotic ampicillin or the antifungal cyclohexamide. Therefore, these can be added to the growth media to prevent other microorganisms from growing in your cultures.

On average, 60 % of attempts to isolate phage from soil by enrichment with M. smegmatis yield phage.

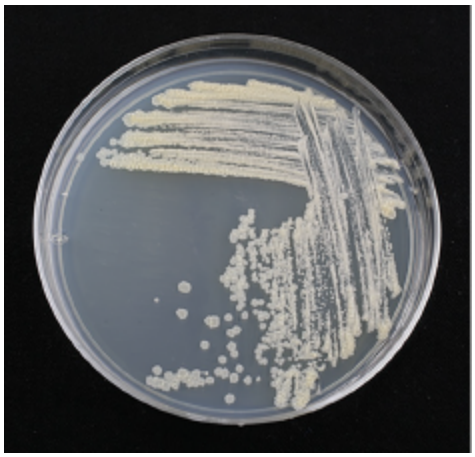

Figure 4.0-4. Mycobacterium smegmatis growing on an agar plate.

If you are using this bacterium as your host for phage isolation, refer back to the list below for the specific growth and culture requirements for your experiments.

Growth Media:

- Liquid: 7H9, supplemented with albumin dextrose (AD) and Tween80 (see instructions for culturing bacteria that require Tween80)
- Agar: L-agar plates
- Top Agar: 7H9

Growth temperature:37˚C, (or 30˚C)

Antimicrobials: carbenicillin (50 µg/ml), cycloheximide (10 µg/ml)

Phage Buffer:10 mM Tris (pH 7.5), 10 mM MgSO4, 68 mM NaCl, 1 mM CaCl2, (10% glycerol, optional)

Restriction Enzymes: BamHI, ClaI, EcoRI, HaeIII, HindIII, MseI..

Note: Isoschizomers, which are different restriction enzymes that recognize the same DNA sequence, may be used in place of any of the enzymes listed above.

Isolation

Chapter 5: Isolation

Chapter 5: An Overview

Identifying and characterizing new bacteriophages continues to be an important and rewarding avenue of research. Bacteriophages are the most abundant life forms on the planet and therefore are a rich source of novel genetic information. Although several thousand unique bacteriophages have been discovered that infect various bacterial hosts, new phages are continually being found that are remarkably different genetically from any previously discovered. Sequencing the genomes of these new phages may reveal similarities to previously identified phages, but even small differences can reveal important insights about the biology and evolution of viruses.

The road to discovering a new phage begins with collecting an environmental sample. All environmental samples, including soil, compost, and water, contain phages. But not all samples contain phages specific to your bacterial host. A good strategy to use for finding new phages is to learn about your host and where it lives in the environment. Where you find host bacteria, you will find phages.

In the lab, you will extract phages present in your environmental sample and test for their ability to infect your bacterial host by using one of two techniques: direct isolation or enriched isolation (or maybe both). The steps you use to detect phage in these samples take advantage of the bacteriophage infection cycle.

Direct Isolation

Direct Isolation (protocol 5.2) performed in combination with a Plaque Assay(protocol 5.3) is a quick method to detect phages in both solid and liquid environmental samples. This method provides a snapshot of all of the phages present in your 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 as few as one plaque on your plate, or you may not find any plaques at all.

Enriched Isolation

The Enriched Isolation (protocol 5.5) method involves amplifying the number of phages in your environmental sample before identifying plaques on solid media with a Spot Test (protocol 5.6). An enriched isolation is accomplished by mixing the soil sample with bacterial growth media to extract phages, followed by filtering out unwanted soil microbes. Bacterial host cells are then added to the filtrate. Any phage particles present that recognize the specific host can then grow and amplify through multiple rounds of lytic growth. A single phage particle can multiply to become many millions of particles. It is important to note that one particular type of phage may outcompete other phages present, so that phages you recover at the end are not necessarily representative of those present in your starting sample.

Plaque Assay

Because individual phage particles are too small to be seen directly with the naked eye, you have to look for evidence of phage growth in the form of plaques, which are the result of

bacterial cell death. **The Plaque Assay** (protocol 5.3) allows you to detect plaques on a "lawn" of host bacteria. Lawns of bacteria are made by mixing bacteria with liquid media and agar in a concentration which allows bacteria to form a smooth opaque layer. As the bacteria grow, phages present in your sample will infect and replicate within the bacterial host, killing the bacteria at the end of each lytic cycle. Each phage infection can produce tens to hundreds of new phage particles. The new phage that escape from each lysed bacterium diffuse through the agar and infect nearby bacteria, repeating the process of replication and lysis. Given enough time, a circular zone of cleared bacteria called a plaque will be visible, indicating the presence of phage!

Whereas some plaques will be large and clear and therefore easy to identify, some may be difficult to see because they are small or turbid. What's more, air bubbles or improperly set top agar can often be mistaken for plaques. Therefore, ambiguous-looking plaques should always be tested to confirm that they were caused by phages. The methods presented—Picking a Plaque (protocol 5.4) followed by the Spot Test (protocol 5.6 and also described below)—allow you to test multiple putative plaques quickly by using a single agar plate. If you are sure that your plaques are genuine, your instructor may encourage you to skip the spot test. However, it can be gratifying to test multiple plaques that differ in morphology by using the spot test to see how many different phages you may have isolated.

Spot Test

Enriched isolation cultures are screened for the presence of phages by spreading a thin layer of bacteria, media, and agar on a plate and pipetting small aliquots of the sample to be tested on top. As the lawn of bacteria grows, phage present in the sample will complete the infection and lysis cycle, creating large cleared areas or "spots" composed of multiple overlapping plaques. Consequently, Spot tests are a way to screen multiple samples for phage on the same plate.

Congratulations! Once you have a positive spot test you have successfully isolated at least one phage and you are ready to move on to Chapter 6, Phage Purification.

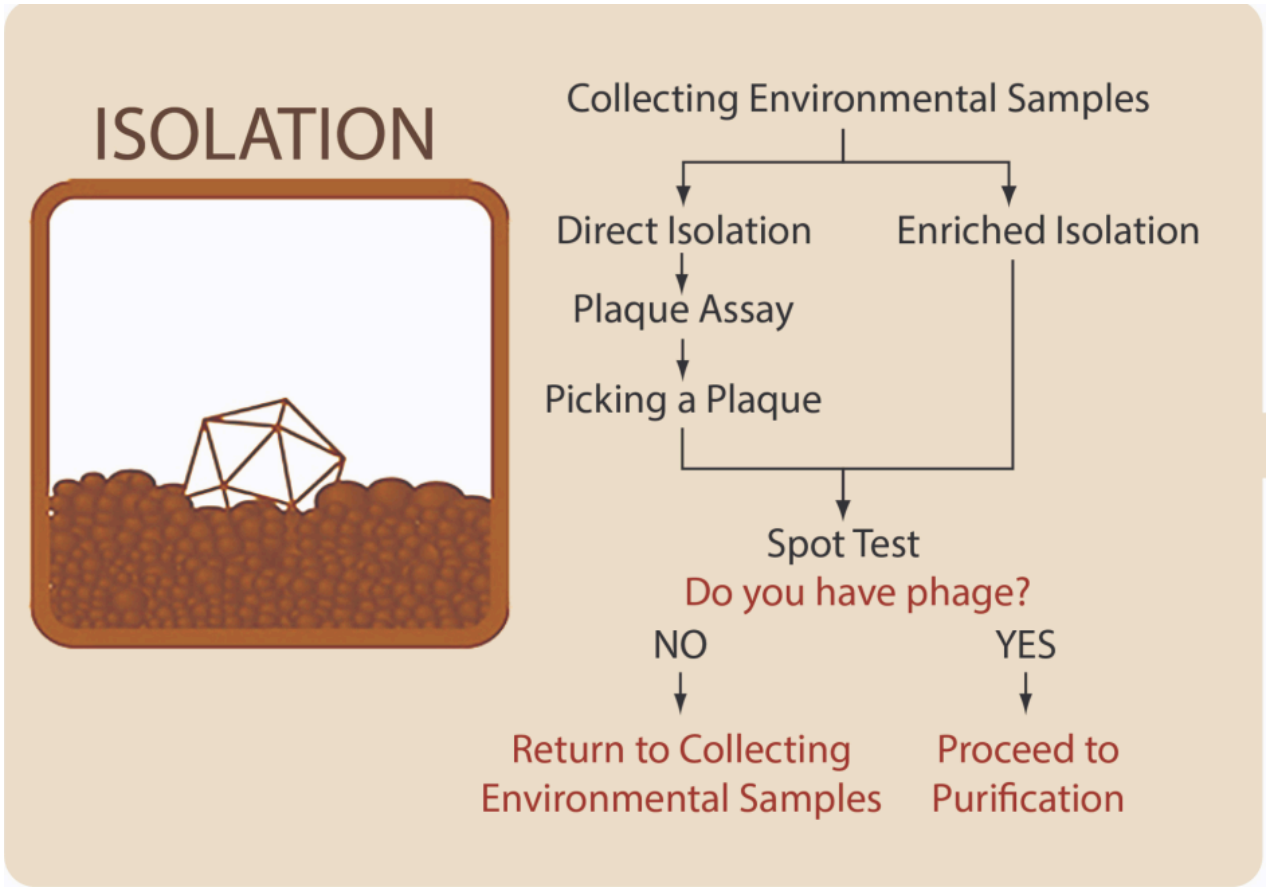

Figure 5.0-1. Flow chart of experiments for Phage Isolation.

Chapter 5: Tips and Hints

Enriched and Direct Isolation

- To extract phage from soil, the initial soil “washing” step is critical in overcoming electrostatic interactions between phages and the soil matrix. While one hour of shaking at 250 rpm may be okay, two to three hours is better, and substantially increases the phage yield per sample.
- Because phage yields may be lower with the new enrichment protocol, you may want to set up multiple enriched isolation cultures at once.
- The phage extraction step (washing) and enriched isolation can be performed in 50 ml conical tubes. This allows for additional samples to be incubated at once (Figure 1.0). In such instances, it may be necessary to use vented conical tubes as some bacteria can be sensitive to low oxygen levels (Figure 1.1).

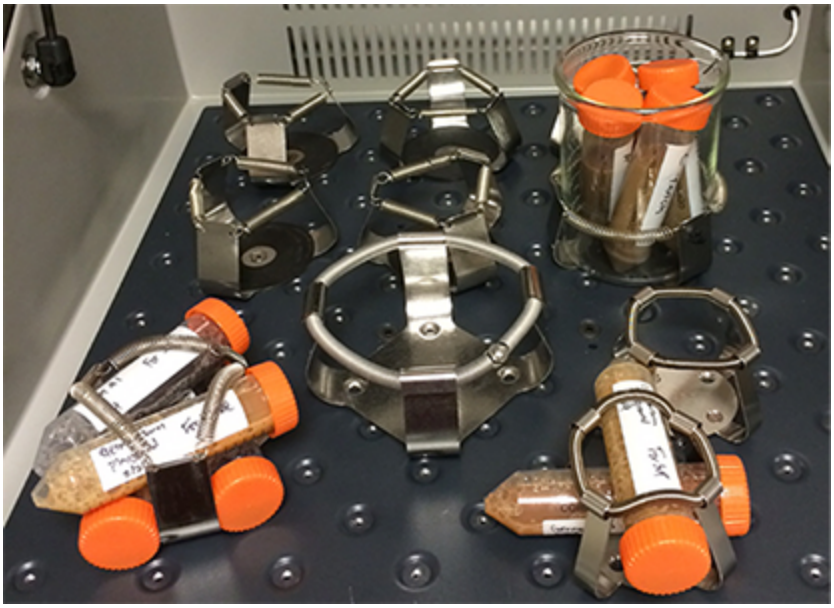

Figure 1.0. Arrangement of 50 ml conical tubes in a shaker equipped for flasks

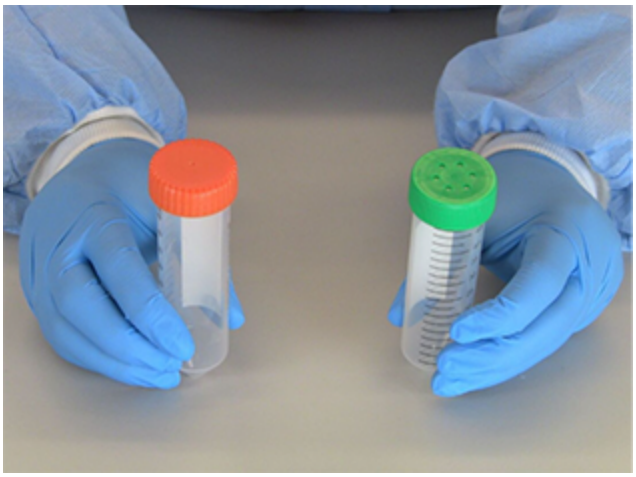

Fig 1.1. Right: A 50 ml conical tube with a vented cap. Left: A 50 ml conical tube without a vented cap, shown for comparison.

- Direct plating/isolation is used to avoid the bias introduced by enrichment cultures, where fast-replicating phages quickly outnumber/outcompete slower-replicating phages. Although yields from direct isolation might be lower than with enriched isolation, the direct isolation procedure is relatively fast.

- The greater the volume of direct isolation sample assayed for plaques, the better your chances of finding a phage. As such, you can vary the total volume you plate by varying the concentration of agar in your top agar.
  Example:
  If you washed your soil in medium, you can plate 5 ml total on your plate by using 2.5 ml direct filtrate and 2.5 ml 2X top agar. By increasing the agar concentration in your top agar to 4x (without altering the concentration of other media components), you can still plate 5 ml total on your plate, but now using 4 ml of direct filtrate and 1 ml of your new top agar.

- It is possible to vary the length of time and the temperature for incubating enrichment cultures. Peak yield of phages from cultures seems to be between 24 - 48 hours, though on occasion this can extend out to 4 days. If you want to test an enriched isolation multiple times over a long incubation period, a 1.5 ml sample can be removed using a sterile serological pipette and the culture returned to the incubator. Phage yields will change as the cultures are allowed to incubate longer. Incubations can also occur between room temperature and the highest growth temperature recommended for a given bacterial host. In addition, the phage profile tends to change with time, as well as with different temperatures.

- Excess enriched cultures should be treated as a saturated bacterial culture for disposal by treating with bleach or autoclaving.

- You may want to incubate the spot plates without inverting initially, and return after 24 hours to invert. This will prevent slippage of the top agar.

Plaque Assay

- If your plaques are very small, try reducing the amount of cells used in plating, decreasing the temperature of plate incubation, and/or decreasing the percentage of agar in the top agar. You can go as low as 0.2 % top agar and your plates will still set. For example, if you are using 2X top agar, you can use 1 part 2X and 3 parts liquid media. You will want to empirically test that your plates will solidify in your atmospheric conditions.
  To increase plaque size:

  1. Temperature: decrease incubation temperature below 37 °C to room temperature (or 30°C to room temperature for non-smegmatis hosts.)

  2. Cell concentration: decrease number of host cells to as low as 100 ml of saturated cell culture (~10^7 to 10^8 cfu/ml)

  3. Top agar concentration: decrease to 0.35% to 0.18% (you can go lower if you use agarose but that gets expensive).

- Time of pre-adsorption of phage to cells prior to mixing with top agar and plating can be varied. Some phages absolutely require at least 15 minutes of pre-adsorption with host cells prior to mixing with top agar and some do not. In a pinch, this step can be shortened or skipped—usually the result is an apparently lower titer (rather than no infection), and more irregularly sized plaques. However, lengthening the time of pre-absorption beyond 15 minutes does not really change any experimental outcomes unless you go past one infection cycle, (~ three hours in mycobacteriophages).

Protocol 5.1: Collecting Environment Samples

Objective:

To obtain an environmental sample containing bacteriophage

Rationale:

By collecting soil, compost, water, or other samples rich in bacteria, you will aim to collect a bacteriophage that can infect those bacteria. To increase your chances of gathering a variety of phages that can infect your specific host bacteria, you should consider environments where your host bacteria thrive. Therefore, collecting samples from a variety of environments is ideal.

Supplies:

- Plastic sandwich bags for collecting soil samples and a tool for digging
- Clean plastic bottle for liquid samples
- Labeling pen
- Smart phone or tablet with GPS capabilities or computer

Procedure:

A. Collect the specified number of samples as directed by your instructor. For each sample perform the following steps:

   a. For solid samples, 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 soil, keeping the plastic bag between your hand and the sample. Remove your hand, inverting the bag with the soil to the inside, and seal the bag.

   b. For liquid samples rinse the plastic bottle with your sample water by filling it one-third full, capping it, shaking it vigorously, and then dumping the sample water back out. After doing this three times, fill the bottle with your sample and cap the bottle.

B. Label the sample bag or bottle appropriately (e.g., initials, location) so you can identify where the sample was collected.

C. 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 soil samples: Was the soil wet or dry? Was it sandy or full of organic matter? Approximately how far below the surface was the soil collected?
- What was the ambient temperature?

d. Repeat steps 1 - 3 for each sample collected.

D. Back in the lab, make certain that all the sample information you collected, including collection site and physical charatceristics, are recorded in your lab notebook.

a. Be as specific as possible when recording this information. You will need to log this information on the program database.

E. As directed by your instructor, your next step will be to process your environmental sample(s) using the Direct Isolation (5.2) or Enriched Isolation (5.5) protocol, or both.

Helpful Tips:

- While soil samples can be collected several days ahead of processing, it is best to use the freshest samples possible.
- If you collect soil samples ahead of time, store the soil samples in a cool place and do not let them dry out.
- Aerobic bacteria are often most abundant at a depth of 3 – 8 cm from the surface of the ground.
-

Protocol 5.2: Direct Isolation

Objective:

To extract phages from an environmental sample Rationale: This method extracts phages from microbes and particulate matter in a solid or liquid 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.

Supplies:

- Environmental sample
- Liquid media* (5 ml/sample)
- Sterile 3 ml or 5 ml syringe
- 0.22 µm syringe filter
- 5 ml serological pipettes
- Microcentrifuge tubes
- 15 ml conical tube

Procedure:

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

B. You will need an environmental sample collected using the protocol Collecting Environmental Samples (5.1).

C. Extract phage from solid environmental samples, such as soil or compost. If you collected a liquid sample, proceed directly to Step D.

1. Using a 15 ml conical tube, fill it approximately one-third to one-half full with soil.
2. Add liquid media until the sample is submerged beneath 2–3 ml of liquid.
3. Cap the tube and invert several times to mix thoroughly.
4. Incubate the tube while shaking vigorously in a shaking incubator at 250 rpm for 1–2 hours.
5. Allow the sample to sit until particulate matter has mostly settled. This may take as few as 2 minutes or as many as 20 minutes. Alternatively, you can centrifuge these samples to hasten this process. To do this, balance the tubes and

centrifuge at 2,000 x g for 10 minutes to pellet (i.e., force to the bottom of the tube) most of the soil.

D. Prepare a phage filtrate using aseptic technique.

1. Open the package of a syringe filter (0.22 µm), leaving the filter in the packaging.

2. Using a syringe, remove approximately 2 ml of liquid from the top of the flooded sample.

    a. Avoid withdrawing any solid material from the bottom of the tube to prevent clogging the filter during filtration.

3. 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.

    a. Make sure the filter is screwed firmly into place.

4. Depressing the syringe plunger, dispense a minimum of 0.5 ml of filtrate into a labeled microcentrifuge tube.

    a. 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.

    b. Cap the tube immediately.

5. Discard the syringe and filter.

6. Proceed directly to the Plaque Assay (5.3) protocol.

Helpful Tips:

- Soils are composed of many different types of particles, each with its own surface charge. Phages stick to these charged soil particles through electrostatic interactions. Vigorously shaking your soil sample in media for 1-2 hours will help break these interactions, releasing the phages from the soil. If you have your sample shaken overnight, make sure you use phage buffer instead of media so as not to accidentally grow pathogenic soil microbes.

- This method will also work well with liquid samples.

- After being shaken, some parts of your soil sample may never settle (bark floats!). Avoid adding the floating particles to your syringe before filtering.

- These samples cannot be stored longer than 24 hours at 4 °C. The concentration of infectious phage decreases rapidly when stored at low concentrations.

- You can do a direct isolation at the same time as an enriched isolation without having to set up a separate experiment. See the Enriched Isolation (5.5) protocol for details.

- A brief video demonstrating this protocol is available here.

*If you are using M. smegmatis as the host for phage isolation, you may use Enrichment Broth as the liquid media in this experiment, as opposed to 7H9 media.

Protocol 5.3: Plaque Assay

Objective:

Detecting the presence of phages on bacterial lawns

Rationale:

The plaque assay allows you to visually confirm the presence of phage particles in a sample. 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:

- Phage samples for isolation, purification, or titering
- Host bacteria (250 µl/plate)
- Agar plates
- Phage buffer
- Top agar, molten (between 55 - 60 ˚C)
- Microcentrifuge tubes
- 5 ml serological pipettes
- Positive control phage (optional)

Procedure:

Day 1

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

B. Assemble the samples you want to assay.
Important: Your phage samples could be taken from your direct isolation, your filtered enriched isolation, or a picked plaque. It is probable that your samples include serial dilutions.

C. Inoculate the host bacteria with your phage sample.

1. Obtain the same number of aliquots of 250 µl host bacterial cultures as you have phage samples, plus one for a positive control and one for a negative control.

    a. Label the culture tubes accordingly.

2. Use a micropipettor and aseptic technique to

    a. Dispense each phage sample into the appropriate culture tube containing 250 µl of host bacteria according to Table 5.3-1.

3. Mix each inoculated host culture by gently tapping the tube.
   Important: Make sure your sample makes contact with the bacteria. When you pipette a volume as small as 10 µl sometimes your sample may stick to the side of the tube.

4. Let your sample sit undisturbed for 5–10 minutes to allow for attachment.

Sample Type	Sample Volume
Direct isolation sample	500 µl
Enriched culture	10 µl
Serial dilutions of picked plaques	10 µl
Lysates for titering	10 µl
Negative control	10 µl phage buffer
Positive control	10 µl provided phage sample

D. Plate the samples with top agar. For this part of the experiment, you will need 3 ml of molten top agar per sample. Your instructor may provide this for you or you may need to make it according to the protocol found in the Toolbox.

1. Obtain the same number of agar plates as you have samples. (Don't forget your positive and negative control samples.) Label these plates accordingly.

2. Remove a bottle of top agar from the 55 °C bath.
   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.

3. For each sample, including controls

    a. Using a sterile 5 ml pipette, aseptically transfer 3 ml of top agar to an inoculated host tube (i.e., the tube containing bacterial host and phage sample).
    Important: Try to avoid making or withdrawing bubbles, as they can look like plaques on plates.

b. Immediately aspirate (suck-up) the mixture back into the pipette and transfer it to the appropriate plate and discard the pipette.
Important: The top agar should not sit in the pipette for more than a few seconds because the agar will begin to solidify.

c. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats the agar plate.

E. Repeat this process for each of your samples.

F. Incubate plates to allow bacterial growth and phage infection.

1. Let the plates sit undisturbed for ~20 minutes until the top agar solidifies.

2. After the top agar has solidified, gently invertthe plates and place them in the proper incubator.

3. Incubate the plates at the specified temperature for 24–48 hours.

4. Record the incubation time and temperature in your notebook.

Day 2

A. Check the plates for plaques.

1. Remove the plates from the incubator and hold them up to a light source to look for plaques. (It is easier to see them if you remove the lid.)

a. If you do not see plaques, return the plates to the incubator for further incubation and check again.

2. Record your results in your lab notebook.

a. 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.
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 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.
- A brief video demonstrating this protocol is available **here.**

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Protocol 5.4: Picking a Plaque

Objective:

To retrieve phage particles from a plaque and create a liquid sample

Rationale:

A plaque is a zone of clearing on a bacterial lawn that is formed when a single phage particle infects, replicates, and lyses bacteria. As a result, a plaque is filled with millions of identical phage particles. To retrieve phage from a plaque, a plaque is “picked” and phage particles from the plaque are resuspended in phage buffer. You can use the resulting liquid sample of phage for multiple follow-up experiments, including a spot test and plaque purification.

Supplies:

- Agar plates with plaques of interest
- Phage buffer
- Microcentrifuge tubes

Procedure:

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

B. Label the plaques.

1. Using a labeling pen, mark the plaques you intend to pick by drawing a small circle around the plaque on the bottom of the plate. If picking multiple plaques, label each plaque with a unique letter or number, or with some other identifier.

    a. It is possible that you will be picking plaques from more than one agar plate. Be sure to label plaques in a way that will allow you to keep track of them and record the details in your lab notebook.

C. Record the detailed morphology of each plaque (e.g., size, cloudy or clear, margin type) you have circled.

D. Label and prepare microcentrifuge tubes.

1. Obtain as many tubes as the number of plaques you intend to pick.

2. Label each tube according to the identifier you used for each plaque.

3. Using aseptic technique, aliquot 100 µl of phage buffer into each microcentrifuge tube.

E. “Pick” a plaque.

1. Place a sterile tip onto a p200 micropipettor.
2. Holding the pipettor perpendicular to the agar surface, stab the top agar in the center of the plaque (Figure 5.4-1).
    a. Avoid touching the bacteria surrounding the plaque.
3. Place the end of the tip into the phage buffer in the corresponding microcentrifuge tube. Then tap the tip on the wall of the tube and pipette up and down to dislodge phage particles. Discard the tip.
4. Mix well by vortexing.
5. Repeat Steps 1–4 for each plaque you are picking.

F. As directed by your instructor, your next step will be to perform the Serial Dilutions (6.2) protocol or the Spot Test (5.6) protocol.

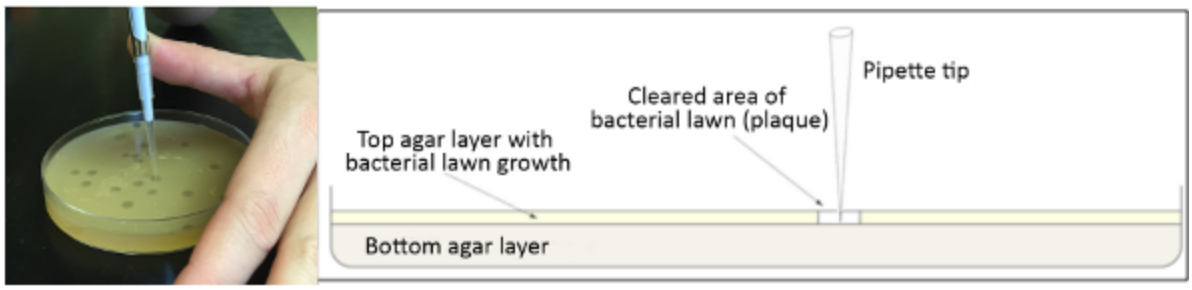


Figure 5.4-1. Picking a plaque from an agar plate.

Helpful Tips:

- During incubation of the plate, the phage particles are located primarily within the visible plaque, which stops enlarging once the cells stop growing. But after the cells stop growing, phage particles can continue to diffuse into the surrounding areas. Therefore, it is helpful to pick from reasonably fresh plates. Also, remember that the farther apart plaques are, the more likely they are to be free of contaminating phages from neighboring plaques.
- When you purify a phage, pick from a plate with a low number (2–30) of plaques. The farther away a plaque is from adjacent plaques, the better your chance of picking only one type of phage.
- These samples should be used immediately because concentration of infectious particles decreases rapidly in phage buffer.
- A brief video demonstrating this protocol is available here.

Protocol 5.5: Enriched Isolation

Objective:

To amplify phages present in your environmental samples

Rationale:

Enriched cultures are used to amplify phages present in samples by providing favorable conditions for phage replication. Phages are first extracted from a solid environmental sample into bacterial growth media. Bacteria, including those that are potentially dangerous, are removed via filtration. The filtered sample is seeded with host bacteria and the cultures are incubated to encourage growth and replication. As bacteria replicate, phages present in the sample go through multiple rounds of infection and lysis. The result is a culture with an exponentially larger concentration of phages specific to your bacterial host than your initial environmental sample.

Supplies:

- Solid environmental sample
- 0.22 µm Corning ® Tube-Top Vacuum Filter Systems or syringe filters
- Liquid media*
- 10X liquid media (if using liquid environmental samples)
- Baffled Erlenmeyer flask, Erlenmeyer flask autoclaved with pipette tips in the bottom, or 50 ml sterile conical tubes
- Sterile 5 ml syringes (if needed)
- 0.22 µm syringe filters (if needed)
- Microcentrifuge tubes
- Host bacteria (500 µl)

Procedure:

Day 1

A. You will need the solid environmental samples you collected using the protocol Collecting Environmental Samples (5.1).

B. Extract phages from a soil sample.

1. Fill a 50 ml conical tube with your sample to the 15 ml mark.

2. Add liquid media to the 35 ml mark and vortex.

3. Shake the sample at ~250 rpm for 1–2 hour[1].

4. Balance the tubes and centrifuge at 2,000 x g for 10 minutes to pellet (i.e., force to the bottom of the tube) most of the soil[2].

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

1. Filter the supernatant through a 0.22 µm filter to remove unwanted bacteria and soil particles[3].

   a. Collect the flow through in a sterile baffled Erlenmeyer flask or a 50 ml sterile conical tube.

   b. Recovered volumes will range between 20 and 25 ml.

D. Seed the culture with host bacteria.

1. Add 0.5 ml of bacterial host culture to the flask or conical tube.

2. Incubate the flask or conical tube at the proper temperature, shaking at 220 rpm for 2–5 days.

   a. If you are using a 50 ml conical tube, you must ensure that the culture will be properly aerated. To do so, screw the cap on one-quarter of a turn so that the conical tube is only loosely capped, and then secure the cap with a short piece of lab tape to ensure it does not fall off. Check to make sure that the conical tube remains only loosely capped. Tubes must remain upright while being shaken, and care taken to avoid spillage.

   b. If using a liquid environmental sample, you must add the appropriate volume of 10X liquid media as a source of nutrients for your host bacteria.

Day 2

After the enriched culture has been allowed to incubate for 1–5 days, you can continue with the protocol.

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

B. Filter the enriched culture.

1. Using an appropriate pipette, transfer 1.4 ml of your enriched culture from the Erlenmeyer flask to a microcentrifuge tube.

2. Repeat this procedure so that you have two microcentrifuge tubes, each with 1.4 ml of enriched culture.

3. Spin the tubes at high speed in the microcentrifuge for 1 minute to pellet the bacteria.

4. Filter the supernatant through a 0.22 µm filter as described below.

    a. Remove the plunger from a syringe.

    b. Open a sterile filter and attach it to the barrel of the syringe.

    c. Pipette 1 ml of supernatant from each microcentrifuge tube into the syringe barrel (for a total of 2 ml).

    d. Place the tip of the filter/syringe over a sterile microcentrifuge tube and insert the plunger into the syringe.

    e. Depress the plunger and collect the sterile filtrate.

5. Transfer the supernatant into a clean microcentrifuge tube, avoiding the bacterial pellet.

6. Immediately cap the microfuge tube containing your supernatant or filtrate and label it appropriately. It should be stored at 4 °C.

7. Either return your culture to the incubator, or dispose of your enriched culture as directed by your instructor.

C. As directed by your instructor, your next step will be to test your supernatant for phages by using a Spot Test (5.6).

Helpful Tips:

- If you want to do a direct isolation at the same time as your enriched isolation you can remove 500 µl of filtered sample prior to Step D and set up a plaque assay directly. Be sure to work aseptically.

- If you want to test your culture after 24 or 48 hours of shaking, feel free to remove a 1.4 ml aliquot with a serological pipette, filter it, and proceed with a spot test. Be sure to work aseptically and replace your culture on the shaker while you await your results.

- This protocol is designed for solid soil samples only. If you want to isolate phages from a liquid sample use the Direct Isolation protocol.

- 50 ml conical tubes can be incubated with the lids tightly sealed as long they are opened every 24 hours to allow access to oxygen. Alternatively, large sized culture tubes with loose fitting lids can be used when incubating enriched isolations.

References:

1. If you do not have a shaker that accommodates 50 ml conical tubes this can be done in an Erlenmeyer flask.

2. If you do not have a centrifuge to accommodate 50 ml conical tubes, the wetted soil can be filtered through Whatman filter paper folded to fit into the top of a conical tube before sterile filtering.

3. Corning ® Tube-Top Vacuum Filter Systems or sterile syringes with syringe filters can be used.

4. * If you are using*M. smegmatis* as the host for phage isolation, you may use Enrichment Broth as the liquid media in this experiment, as opposed to 7H9 media.

5. If syringe filters are not limited, filtering the enrichment in Step B4 is highly recommended.

6. A brief video demonstrating this protocol is available here..

Protocol 5.6: Spot Test

Objective:

To test a sample for the presence of phage

Rationale:

Plaques are zones of clearing on a bacterial lawn that are formed when phages infect, replicate, and lyse bacteria. Spot tests can be used to screen enriched cultures or verify the presence of phage in putative plaques. By “spotting” a sample onto a fresh lawn of bacteria, you can confirm the presence of phage if a zone of clearing occurs following incubation.

Supplies:

- Liquid phage sample (either a picked plaque from a direct isolation, or an enriched isolation)
- Agar plates
- Host bacteria (250 µl/plate)
- Top agar, molten (between 55 - 60 ˚C)
- Phage buffer
- 5 ml serological pipette

Procedure:

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

B. Collect the liquid phage samples that need testing.

C. Prepare a bacterial lawn by using aseptic technique.

1. Label the bottom of an agar plate.

    a. Divide the plate into as many sections as you have samples by drawing on the bottom of the plate. (See Figure 5.6-1 for examples of how to best divide the plate into sections.) Label each section according to your samples.

2. Obtain a 250 µl culture of host bacteria.

3. For this part of the experiment, you will need 3 ml of molten top agar per plate. Your instructor may provide this for you, or you may need to make it according to the protocol found in the **Toolbox**.

a. Using a sterile 5 ml pipette, transfer 3 ml of molten top agar to a culture tube containing host bacteria and then immediately draw the solution back into the same pipette.
Important: Try to avoid making or withdrawing bubbles, as they can look like plaques on plates.

4. Dispense the top agar-bacteria mixture onto an agar plate.

    a. The top agar should not sit in the pipette for more than a few seconds because the agar will begin to solidify.

    b. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats agar plate.

5. Allow the plate to sit undisturbed for 20 minutes or until the top agar solidifies completely.

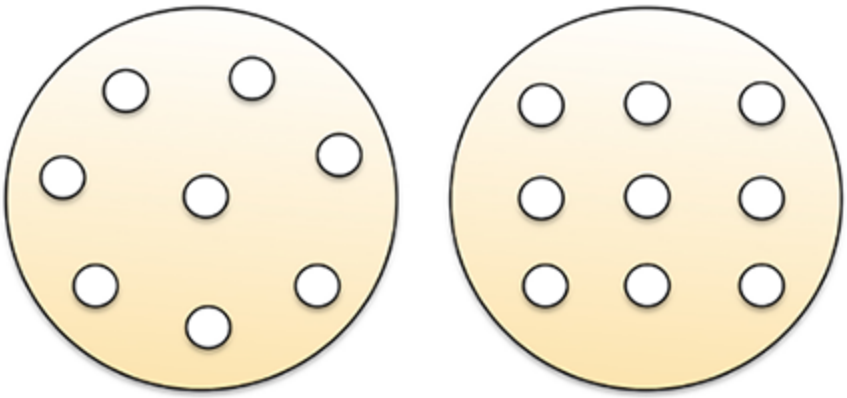


Figure 5.6-1. Possible spot plate patterns.

D. Spot the liquid phage samples and controls on the prepared bacterial lawn.

1. Aseptically transfer 10 µl of each sample, one at a time, onto the proper location on the bacterial lawn

    a. Hold the tip slightly above the agar and expel the drop slowly to avoid splattering.

    b. Be sure to spot your samples in the right place! Remember that labels on the bottom of a plate are mirror images (i.e., they will appear backward) of your labeling scheme when the plate is turned over.

2. Spot 10 µl of sterile phage buffer on the plate as a negative control.

3. Allow the liquid from the spots to absorb into the agar (generally 10–15 minutes).

4. Without inverting the plates, incubate them at the proper temperature for 24–48 hours.

E. Check spot plates for clearing.

1. After at least 24 hours, check each spot on the agar plate. If you see a zone of clearing for any of your spotted samples, congratulations! Your original sample contained phage!

2. Make sure that your negative control does NOT show signs of phages.

F. Record the details of your spot plate in your laboratory notebook.

G. You can now proceed to Chapter 6, Purifying Your Phage!

Helpful Tips:

- You should include no more than 10 samples on a single plate.
- A zone of clearing in a spot test is NOT a plaque; rather, it is many overlapping plaques that appear as a single large zone of clearing.
- It is not recommended to pick a phage for purification from a spot if multiple phages were spotted on the same plate.
- A brief video demonstrating this protocol is available here.

Purification

Chapter 6: Phage Purification

Chapter 6: An Overview

Now that you have isolated phages from your environmental sample, you are ready to start the purification process. It is possible that your environmental sample yielded more than one kind of phage (Figure 6.0-1). The goal of phage purification is to ensure that you end up with a phage sample that contains only one kind of phage. In other words, once purified, all the phage particles in your sample should be genetic clones of each other. This is called a "homogenous population" or a "clonal population" of phage.

Having a pure phage population is very important because it will enable you to characterize your phage and discover its biological properties, including morphology, plaque size, and genome sequence. Imagine how difficult it would be to ascribe these properties to your phage if your sample contained many kinds of phages, each with its own morphologies and traits!

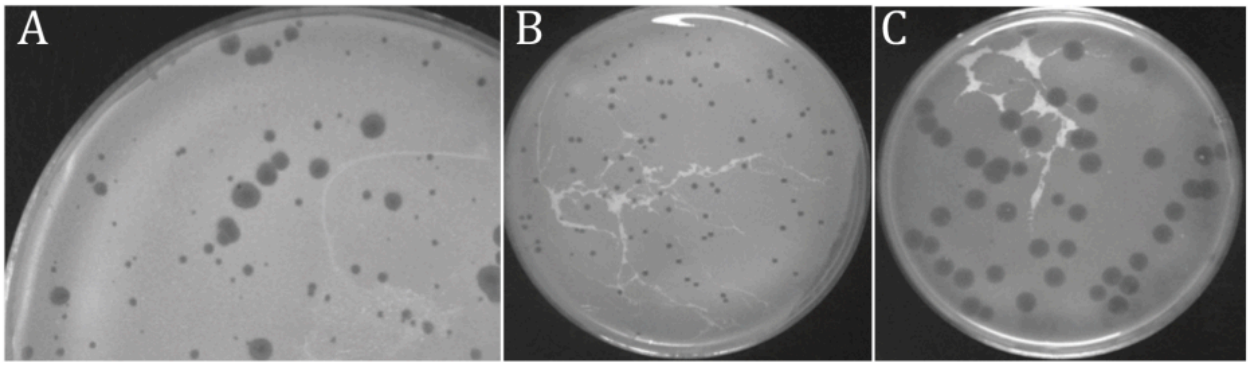


Figure 6.0-1. Phage purification. Before purification a heterogeneous phage sample may have more than one plaque morphology (A). Following purification, the phage with the small (B) and large (C) plaques are homogeneous populations.

Picking a Plaque

The best method to ensure that your phage sample is a single clonal population is with plaque purification. This is accomplished by separating the phages from each other by diluting the samples so that only a few phage particles are placed on a plate. This will enable you to obtain well-isolated plaques.

Because each plaque on a plate originated from a single phage particle, a sample of genetically identical phage can be prepared by touching a single plaque with a stick or pipette tip to pick up phage and then swirling the tip in phage buffer. This is called "picking a plaque". This liquid sample of phage particles can then be serially diluted and examined by plaque assay for consistency of plaque morphology according to the Plaque Assay for Purification protocol 6.1. This process is repeated until all plaque morphologies are consistent. Usually, no more than three rounds of purification are needed.

When purifying a phage it is important to understand that phage particles diffuse within the agar plate; therefore, phages from a nearby plaque could diffuse into your selected plaque. To minimize the presence of contaminating phages, you should pick from a well-isolated

plaque taken from a highly dilute phage sample. Likewise, if multiple positive spot tests are present on the same agar plate, phages can diffuse from one spot to another. For this reason, you should not pick from a spot plate that has multiple positive spots (unless they all came from the same direct isolation plate). You would not have any way to guarantee that the phage you eventually isolate originated from the spot that you picked from, rather than from an adjacent spot. Therefore, when you perform phage purification, use the original direct isolation plaque or the enriched culture instead of picking phages from the positive spots.

Collecting a Lysate and Calculating Titer

The last steps in phage purification are collecting a lysate and calculating the concentration, or titer, of that lysate. To do this, collect a lysate from a plate densely packed with plaques from your last round of purification (protocol 6.3, Collecting Plate Lysates). The best lysates come from plates that are so densely packed that the plaques cover the entire plate, leaving remnants of bacteria in a pattern that looks like a spiderweb. Plates like this, with confluent plaques, are called “webbed” plates. When phage buffer is added directly to the plate surface, phage particles from the plaques diffuse into the buffer, which can then be collected and filtered to separate out the phage. Finally, the titer, or concentration, of phage particles can be calculated.

Calculating the Titer

Spot Titer (protocol 6.4) followed by a Full Plate Titer (protocol 6.5). The spot titer is typically used to provide a quick titer estimate by spotting multiple dilutions of a phage sample on a single plate. The titer is calculated by counting the number of plaques in a specific spot. This can be tricky and inaccurate, therefore providing only an estimate. The full plate titer requires separate plates for each dilution. Although this method uses more plates, it allows you to accurately count plaques and calculate more precise titer values.

By performing a spot titer and a full plate titer consecutively, you can use the estimate from the spot titer to predict which dilutions (i.e., how many phage) will yield a countable number of plaques, thereby minimizing the number of plates needed for a full plate titer. Because the titer of phage lysates is determined by counting plaques, a titer is typically presented as the number of plaque-forming units, or pfu, in every milliliter of lysate, or pfu/ml. In practice, phage lysates typically have titers in the range of 10^{8} pfu/ml to 10^{11} pfu/ml.

Once you have an initial lysate you can amplify your phage to generate enough lysate for collecting DNA and archiving as described in Chapter 7: Phage Amplification.

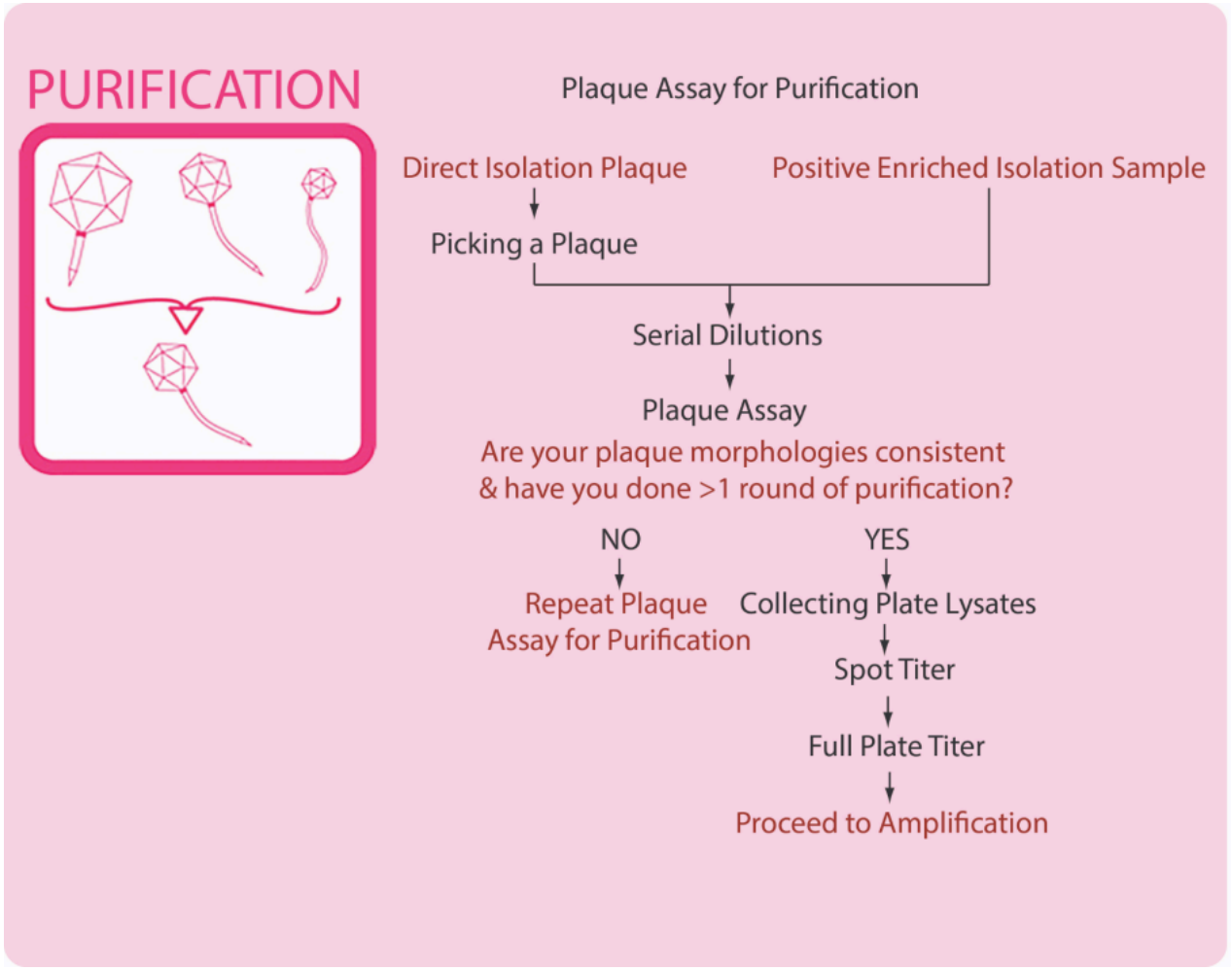


Figure 6.0-2. Flow chart of phage purification experiments.

Chapter 6: Tips and Hints

How many rounds of purification?

- First, it is important to note that phage will continue to diffuse across an agar plate beyond the plaque, diffusing further with time. It is therefore important to pick plaques for purification soon after plaques are visible. Don't allow diffusion of phages from surrounding plaques to complicate the purification process.

- According to classic microbiology techniques, three rounds of purification should be performed to ensure a single clonal phage population.

- Additional rounds of purification are discouraged because they provide your phage with the opportunity to accumulate mutations when its genome is replicated within the bacterial host. This leads to genome truncations and other issues that are problematic during annotation.

- The chances of a well-isolated plaque from a direct isolation experiment containing multiple types of phages is extremely low. Therefore, one or two rounds of purification may be sufficient, especially if there is only a single plaque on the plate. Regardless, always take into account the consistency of plaques formed on plates.

- Some phages can have plaques with different morphologies, typically varying in plaque size, and no amount of purification will change this. Should you encounter a phage that forms different plaque morphologies even after 3 rounds of purification, consider picking a plaque for each morphology, serially dilute each sample, and plate dilutions for each sample using a plaque assay to see if these morphologies persist to can be separated. If the morphologies separate (i.e. each plate only has plaques with one morphology), you've shown that there are two phages. If the multiple morphologies persist (i.e. each plate continues to contain all the morphologies), it indicates that your phage likely has multiple plaque morphologies and you have reciprocal replicates confirming this.

- The process of phage isolation is complete when a lysate is collected and its titer calculated. This initial lysate is collected from the last round of purification, and once plaque morphologies are consistent on all of the dilution plates. The plate used to collect this lysate will probably not be a perfect webbed plate, which is okay. Note: this is no longer called a Medium Titer Lysate since it could easily have as high a titer as the final lysate.

Warning about mutations.

- When picking a plaque, make sure you are picking a plaque that is representative of your phage sample. If you have thirty turbid plaques on a plate and one clear plaque. Pick a turbid plaque.

- Make sure that your plaque morphology remains consistent over time. If you have a cloudy plaque that suddenly turns clear you may have isolated a mutant and should go

back and pick another turbid plaque.

- If you have a dilution series that has two morphologies on a low dilution plate, you should make an attempt to separate the plaque morphologies as described above, but do not continue to do so for many rounds of purification.

Changes from the Resource Guide

- We are now recommending no more than three rounds of plaque purification to prevent the accidental creation of mutants in the lab.

- Using plaque streaks for purification is strongly discouraged as it seems to contribute to mixed phage samples.

Protocol 6.1: Plaque Assay for Purification

Objective:

To generate well-isolated plaques

Rationale:

Before you begin characterizing a phage, you need to ensure that you have a clonal phage population. The process involves serially diluting either a positive enriched culture or after rounds of purification, a picked plaque, and then performing a plaque assay with the diluted samples. By repeating this process one or two times, and always picking a plaque that is well isolated from adjacent plaques, you will obtain a clonal phage population.

Supplies:

- Phage samples for purification
- Phage buffer
- Microcentrifuge tubes
- Host bacteria
- Agar plates
- Top agar
- 5 ml serological pipettes

Procedure:

This is not actually a new procedure, but a combination of protocols that you have already used. The protocols referenced here are found in previous chapters as well as the Toolbox.

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

B. Gather your phage samples.

1. If you are starting from an enriched isolation that tested positive for phage, skip to Step D.

2. If your phage sample is a plaque, draw a circle around the plaque of interest on the bottom of the plate and label it. Record the details in your lab notebook.

C. Follow the Picking a Plaque(5.4) protocol to pick the plaque(s) you want purify.

1. It is best to pick only well-isolated plaques (at least 1.5 cm apart) from a fresh plate.

2. At the end of the “Picking a Plaque” protocol, you will have liquid phage samples.

D. Dilute your liquid phage samples according to the Serial Dilutions (6.2) protocol.

E. Prepare your agar plates.

1. Obtain as many agar plates as you have phage samples to purify.

2. Label the bottom of the plate with your name, the date, and a reference to the sample.

F. Plate your dilutions according to the Plaque Assay (5.3) protocol.

G. After incubating the plates for 24–48 hours, record your results in your lab notebook. Consider the following questions:

1. How many plaques are on each plate? Do the number of plaques on your plates follow the expected pattern of your serial dilutions?

2. Do all your plaques share the same morphology?

3. If you have more than one morphology, how do they differ?

4. If you think you still have more than one kind of phage in your sample, what should you do next?

H. Once you are certain you have a single phage population, you can move on to the Collecting Plate Lysates (6.3) protocol as directed by your instructor.

1. To collect plate lysates, use the best webbed plate from your final round of phage purification.

Helpful Tips:

- It is not uncommon for a phage to form plaques that have more than one morphology. If you continue to observe plaques with multiple morphologies, even after carefully performing one or two rounds of purification, you may have such a phage.

- Phage morphologies are also known to change based on concentration. It is not uncommon for plaques to look smaller when they are closer together at higher concentrations.

- When purifying a phage, its size is not the most reliable morphological trait since the size can sometimes vary. Instead, the presence or absence of haloes or defined borders, as well as the turbidity of plaques, is more dependable.

Protocol 6.2: Serial Dilutions

Objective:

To prepare liquid phage samples of decreasing concentrations

Rationale:

Serial dilution is a method used to manipulate the number of phages in a sample. You will use serial dilutions to purify, amplify, and titer your phage. This protocol uses 10-fold serial dilutions, meaning that the concentration of phage in each tube is 10 times less than the previous tube, allowing for easy mathematical calculations.

Supplies:

- Phage buffer
- Samples requiring diluting (e.g., picked plaques or lysate)
- Microcentrifuge tubes

Procedure:

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

B. Set up 10-fold serial dilutions.

1. Arrange the proper number of microcentrifuge tubes in a rack and label them 10^{-1}, 10^{-2}, 10^{-3},....10^{-8}.
   Important: See “Helpful Tips” to determine how many dilutions to make.

2. Add 90 µl of phage buffer to each of the tubes.

C. Perform 10-fold serial dilutions (Figure 6.2-1).

1. Add 10 µl of your undiluted phage sample to the “10^{-1}” tube and vortex well.

   a. The solution in this “10^{-1}” tube contains $1/10^{th}$ the number of phage particles as your undiluted sample. It is also referred to as a 1:10 dilution.

   b. Make sure to use a clean pipette tip for each transfer and pipette carefully, vortexing your sample well before making each dilution. Otherwise, you will not make accurate 10-fold dilutions.

2. Transfer 10 µl of the “10^{-1}” sample to the “10^{-2}” tube and vortex well.

   a. This solution contains $1/100^{th}$ as many phage particles as your undiluted sample. It can also be referred to as your 1:100 dilution.

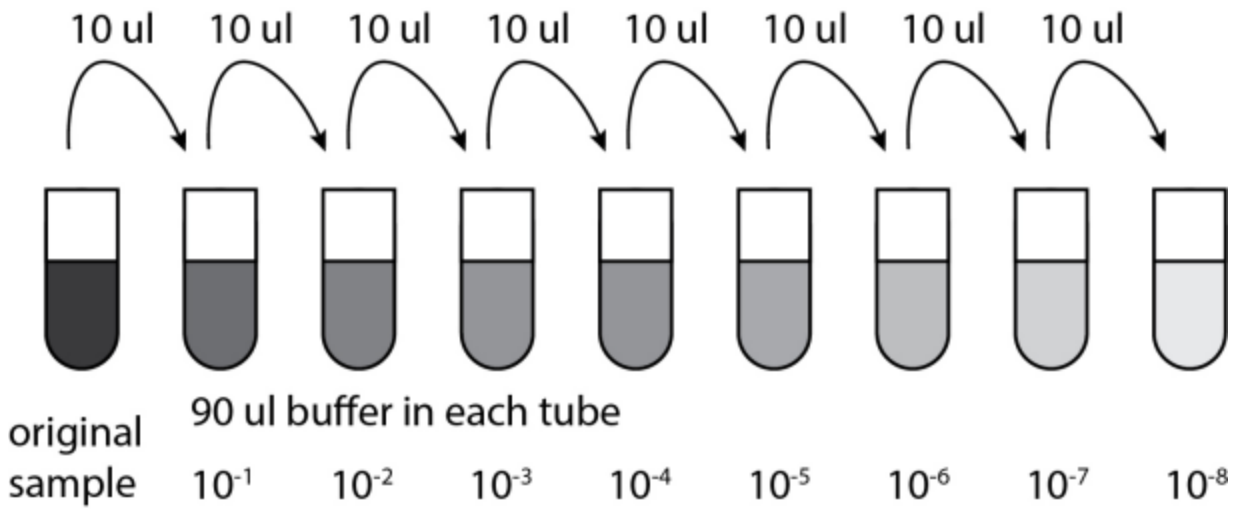


Figure 6.2-1. Serial dilutions. The serial dilution of phage in preparation for a plaque assay. A volume of the undiluted phage sample (10 µl) is transferred into 90 µl of phage buffer in the 10-1 tube. After the tube is mixed, 10 µl of the 10-1 sample is transferred into the 10-2 tube, and the tube is mixed. This serial dilution continues until the desired dilutions are obtained. The last tube will contain a final volume of 100 µl.

3. Continue each successive dilution until you get to your last tube.

D. Return to the protocol requiring the diluted samples; for example, Plaque Assay (5.3), Spot Titer (6.4) , Full Plate Titer (6.5), and Making Webbed Plates (7.1).

Helpful Tips:

- The number of dilutions you make depends on the experiment you are doing and the phage you are working with.
- If you are diluting an enriched culture for the plaque assay, diluting to 10^{-5} is usually sufficient.
- If you are diluting to titer a plate lysate, you may need to dilute to 10^{-8} to obtain a countable number of plaques. You want to plate a dilution that can produce 20–200 plaques.
- Phages at low concentration tend to lose viability over time when they are stored in phage buffer. Therefore, storing serial dilutions is not recommended.
- A brief video demonstrating this protocol is available here.

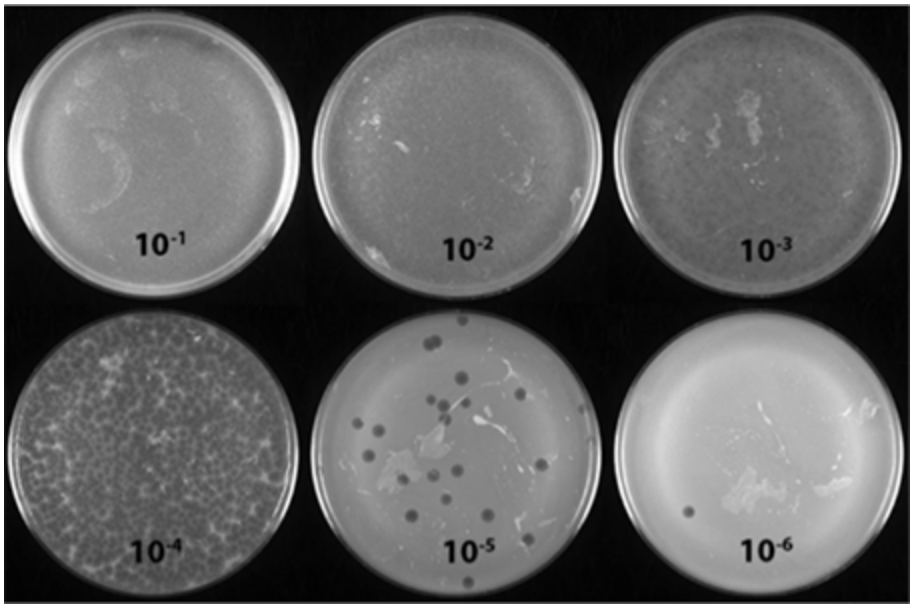


Figure 6.2-2. Plaque assay of dilution series. The phage StarStuff was serially diluted and plated for a plaque assay. The most concentrated samples (10-1 and 10-2) create cleared plates where no bacteria are visible. The 10-3 plate shows a very high concentration of plaques, but individual plaques are not visible until the 10-4 plate, which is webbed plate. The 10-5 and 10-6 plates have countable numbers of plaques, but the 10-5 plate offers the most accurate number for counting.

Protocol 6.3: Collecting Plate Lysates

.**Objective:**

To generate a highly concentrated liquid phage sample

Rationale:

Depending on the phage, each plaque on a plate may contain between 10^3 and 10^7 phage particles. These phage are collected to generate a concentrated phage solution called a lysate. Lysates are collected by “flooding” a plate with a high concentration of phage, such as a webbed plate (Figure 6.3-1). Flooding is accomplished by adding phage buffer to cover the surface of the plate and allowing the phage to diffuse into the buffer. The buffer is then harvested (removed from the plate by syringe) and filtered to remove agar particles and bacteria. Lysates are used for many basic phage biology experiments, including phage DNA extraction and transmission electron microscopy, as well as for long-term phage storage.

Supplies:

- Webbed plate(s) with clonal phage population
- Phage buffer (8 ml/plate)
- 0.22 µm filter
- 5 ml syringe
- 15 ml sterile conical tube for lysate storage

Procedure:

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

B. Identify one or more plates for lysate collection.

1. Your webbed plate may be one prepared from your last round of plaque purification or from the protocol Making Webbed Plates (7.1).

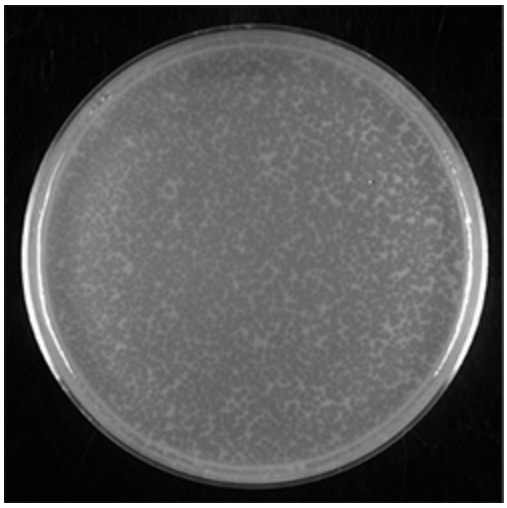


Figure 6.3-1. Webbed plate with confluent plaques.

C. Flood the webbed plate(s).

1. Apply 8 ml of sterile phage buffer to the webbed plate.
2. Let the plate(s) sit at room temperature for 2–4 hours OR store the plates overnight (12–14 hours) at 4 °C.
3. Swirl the phage buffer gently, taking care not to splash.

D. Harvest a plate lysate

1. When the incubation time is complete, remove the lid from the plate and place it on the bench. Tilt the plate slightly by placing one edge of the plate on the lid, allowing the lysate to pool to one side (Figure 6.3-2).
2. Prepare a 0.22 µm filter by opening the packaging but not removing the filter. Set aside.
3. Using a 5 ml syringe aspirate (suck up) the lysate from the plate.
4. Carefully attach the syringe to the filter. Depress the syringe plunger and collect the filtrate in a 15 ml sterile conical tube.
5. If you still have unfiltered lysate remaining on your plate, remove the filter and store it in its plastic packaging to maintain sterility. Aspirate the remaining lysate, reattach the used filter, and filter the remaining lysate, collecting the filtrate in the same sterile conical tube.
6. Label the tube appropriately.

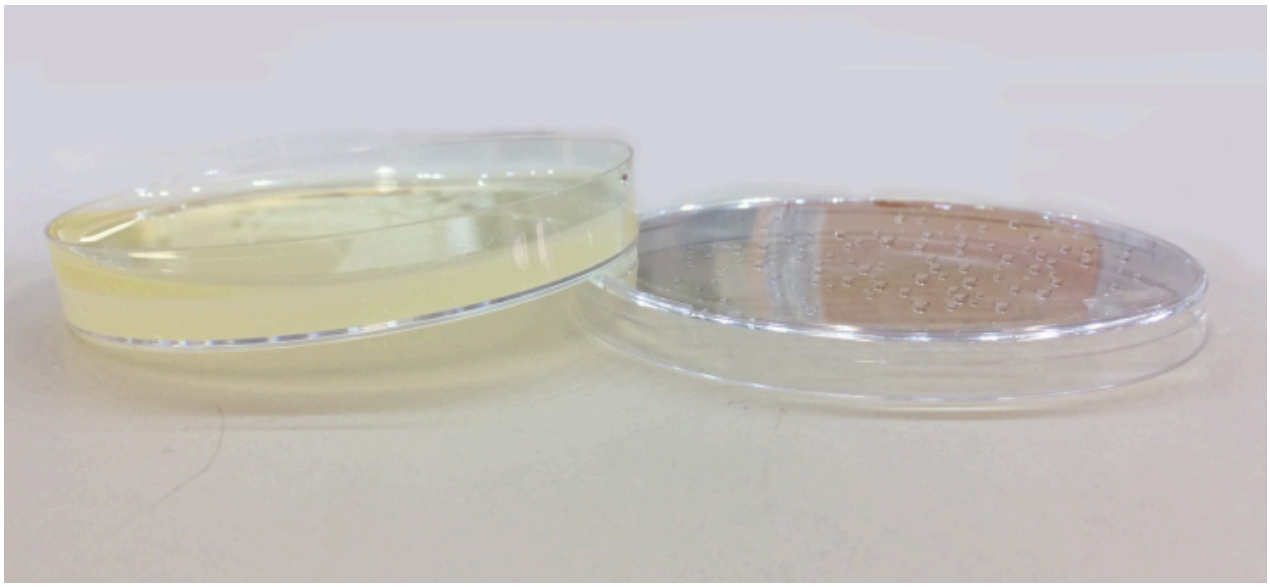


Figure 6.3-2. Collecting a plate lysate. After flooding a plate with phage buffer, the lysate can be collected by tipping the plate and allowing the liquid to pool to one side. It can then be aspirated into a syringe.

E. Pool the lysates.

1. If you have multiple webbed plates for a single phage sample, repeat Step D for each plate. The same filter can be used for all plates if it remains sterile and unclogged. However, if it has been contaminated or becomes clogged, use a new filter.

2. Combine all of the filtered lysates into the same sterile conical tube.

3. Record the final volume of lysate collected.

F. Store the lysates at 4 °C.

1. This lysate can be stored for months at 4 °C. For longer-term storage, follow the protocol Archiving Your Phage Sample (7.3).

G. According to your instructor's directions, calculate the titer of your lysate using the Spot Titer (6.4) protocol, the Full Plate Titer (6.5) protocol, or both.

Helpful Tips:

- Phages remain viable in lysates because compounds like albumin and glycerol diffuse into the phage buffer and stabilize the capsid and tail proteins.
- It is OK if your last round of purification did not yield a perfect webbed plate. Use the plate that contains the highest number of phage.
- A brief video demonstrating this protocol is available here.

Protocol 6.4: Spot Titer

.**Objective:**

To determine the concentration of phage particles in a lysate using a spot test

Rationale:

The total number of phage particles in your lysate can be calculated by performing serial dilutions, followed by a spot test for each dilution and then counting the number of plaques formed. By taking into account the volume and dilution used to generate those plaques, you can calculate the concentration, or titer, of your phage lysate. Because each plaque is formed from a single phage particle, that original phage particle is called a plaque-forming unit (pfu). The concentration of a lysate is presented as the number of plaque-forming units in every milliliter of lysate, or pfu/ml. The spot titer is a modified plaque assay that uses just one agar plate for as many as eight lysate dilutions, which allows you to calculate the titer of your lysate. The drawback of using a spot titer is that the number of countable plaques per spot is small, introducing a large margin of error. Therefore, a spot titer is used to predict which dilutions you should use when performing a full plate titer.

Supplies:

- Lysate for titering
- Agar plate
- Host bacterial culture
- Top agar, molten (between 55 - 60 ˚C)
- Phage buffer
- Microcentrifuge tubes
- 5 ml serological pipettes

Procedure:

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

B. Label one agar plate for a spot titer.

1. Label the bottom of an agar plate with your name, the date, and the notation “spot titer.”
2. Using a labeling pen, divide the plate into as many sections as you have samples. Remember to include a section for a negative control. See Figure 6.4-1 for examples of how to best divide the plate into sections. Label each section according to your samples.

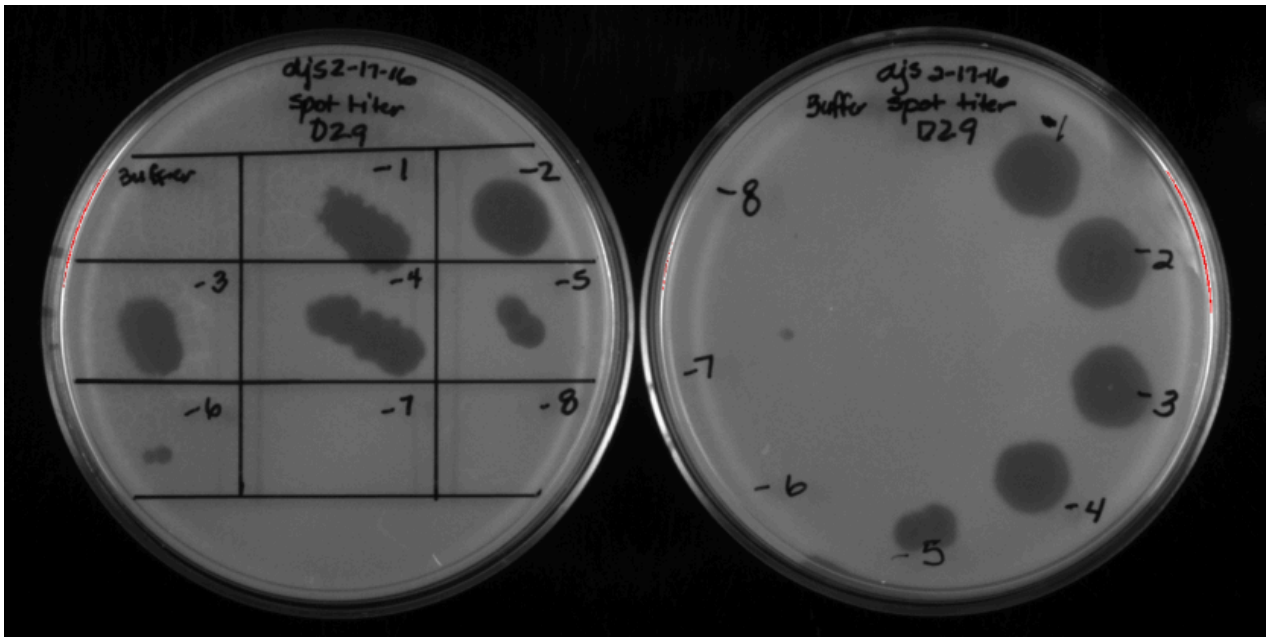


Figure 6.4-1. Two spot plate examples.

C. Prepare a bacterial lawn using aseptic technique. (For this part of the experiment you will need 3 ml of molten top agar per plate. Your instructor may provide this for you or you may need to make it according to the protocol found in the Toolbox).

1. Using a sterile 5 ml pipette, transfer 3 ml of molten top agar into a 5 ml sterile pipette.
2. Transfer the top agar to a culture tube containing 250 µl of host bacteria and immediately draw the solution back into the same pipette.
3. Dispense the top agar-bacteria mixture onto an agar plate.
4. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats the agar plate.
5. Allow the plate to sit undisturbed until it solidifies completely. This will take at least 10 minutes.

D. Perform the Serial Dilutions (6.2) protocol on the sample you wish to titer.

1. Lysates should be diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} in phage buffer.

E. Spot the dilutions and controls on the prepared bacterial lawn.

1. One at a time, aseptically transfer 3 µl of all samples onto the proper location on the bacterial lawn.
2. Use 3 µl of sterile phage buffer as the negative control.

3. Allow the liquid from the spots to absorb into the agar for 30 minutes or longer.

4. Incubate plates (right-side up, not inverted) at the proper temperature for 24– 48 hours.

F. After incubating the plates, count the number of plaques.

1. After at least 24 hours, check each spot on the agar plate.

2. Do the numbers of plaques in each spot make sense? For example, is there a 10-fold reduction in number of plaques as you move through the spots? If so, choose the dilution(s) that contain a countable number of plaques.

G. Calculate the titer in pfu/ml using the formula:

Titer (pfu/ml) = (# pfu/ volume used in µl) x (10^3 µl/ml) x dilution factor*
*The dilution factor is the reciprocal of the dilution used. For a 10^{-7} dilution, the dilution factor is 10^7.

Example: If 12 plaques were observed on a spot created by 3 µl of a 10^{-7} dilution.
Titer (pfu/ml) = (12 pfu/3 µl) x (10^3 µl/ ml) x (10^7)
= (4 x 10^3 x 10^7) pfu/ml
= 4 x 10^{10} pfu/ml

Essentially, you are dividing the number of plaques by the volume of sample plated. Then you multiply that number by the reciprocal of the dilutions used to make that plate and convert µl to ml to obtain the titer in pfu/ml.

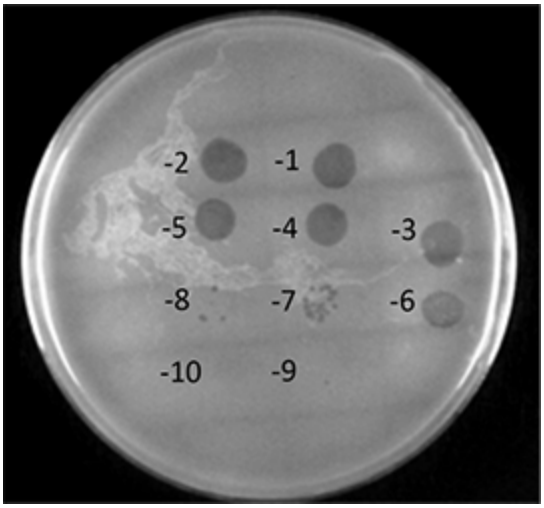
H. See the Full Plate Titer (6.5) protocol for how to use your spot titer to decide which dilutions to use to determine a more accurate titer.

Helpful Tips:

- When exponential numbers are multiplied, the exponents are added together. For example, 10^5 x 10^4 = 10^9.
- Bacterial lawns can be spread in advance, refrigerated overnight, and “spotted” the next day.
- When making your spots avoid touching the agar with the pipette tip. Hold the tip slightly above the agar and expel the drop slowly to avoid splattering.
- Remember that labels on the bottom of a plate are mirror images (i.e., they will appear backward) of your labeling scheme when the plate is turned over. Be sure to spot your samples in the right place!
- A high-titer lysate has a titer of at least 5 x10^9 pfu/ml. (If you have at least three plaques in your 10^{-7} spot, you have a high enough titer to isolate DNA and archive your lysate.)

Sample Problem:

Calculation of titer from a spot test.



The above spot plate contains spots from 10 µl of 10^{-1} through 10^{-10} dilutions. The 10^{-1} through 10^{-5} dilutions form cleared spots, and no plaques are evident on the 10^{-9} or the 10^{-10} spots. However, individual plaques are visible on the 10^{-6}, 10^{-7}, and 10^{-8} dilution spots. The 10^{-7} dilution spot above shows 28 plaques and the 10^{-8} dilution shows 3. That both spots yield approximately the same titer as the difference between 2.8×10^{10} and 3×10^{10} is a reflection of the inherent error between counting 28 vs 3 plaques.

28 pfu/10 µl x 1000 µl/1 ml x 10^{7} = 2.8×10^{10} pfu/ml

or

3 pfu/10 µl x 1000 µl/1 ml x 10^{8} = 3.0×10^{10} pfu/ml

Protocol 6.5: Full Plate Titer

.**Objective:**

To determine the concentration of phage particles in a lysate by using a plaque assay

Rationale:

The total number of phage particles in your lysate can be calculated by performing serial dilutions, followed by a plaque assay and then counting the number of plaques formed. By taking into account the volume and dilution used to generate these plaques, you can calculate the concentration, or titer, of your phage lysate. Because each plaque is formed from a single phage particle, that original phage particle is called a plaque-forming unit (pfu). The concentration of a lysate is presented as the number of plaque-forming units in every milliliter of lysate, or pfu/ml. The full plate titer is the most accurate method of determining the number of infectious phage in a liquid sample.

Supplies:

- Liquid phage sample requiring titering
- Phage buffer
- Agar plates for plating dilutions
- Top agar, molten (between 55 - 60 ˚C)
- Host bacteria
- Microcentrifuge tubes
- 5 ml serological pipettes

Procedure:

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

B. Perform the Serial Dilutions (6.2) protocol on the sample you wish to titer and set up a Plaque Assay (5.3) for the dilutions.

1. If you are titering a highly concentrated phage sample, like a plate lysate, dilute and plate the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} dilutions according to the protocols for Serial Dilutions (6.2) and the Plaque Assay(5.3).
   OR

2. If you have already performed a Spot Titer (6.4) on the sample and determined its estimated titer, plate only the dilutions predicted to yield a countable number of plaques (typically 20 –200) on a plate from a Plaque Assay (5.3).

a. For example, if the 10^{-7} dilution from your spot titer resulted in 12 plaques, you should "bracket" this dilution by plating one dilution on either side to ensure you obtain a countable number of plaques on at least one plate. This means you should plate the 10^{-6}, 10^{-7}, and 10^{-8} dilutions. If the spot titer is ambiguous, plate additional dilutions; for example, the 10^{-5} 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} dilutions.

C. The next day check the plates and confirm that your dilutions are valid (i.e., a 10-fold decrease in plaques).

1. Use a plate with 20–200 isolated plaques and count the number of plaques.

D. Calculate the titer in pfu/ml using the formula:
Titer (pfu/ml) = (# pfu/ volume used in µl) x (10^{3} µl/ml) x dilution factor*
**For a 10^{-7} dilution, the dilution factor is 10^{7}.*
Example: If 24 plaques were observed on a plate from a culture infected with 10 µl of a 10^{-5} dilution (Figure 6.5-1), the calculations look like this:
Titer (pfu/ml) = (24 pfu/10 µl) x (10^{3} µl/ ml) x (10^{5})
= ($2.4 \times 10^{3} \times 10^{5}$) pfu/ml
= 2.4×10^{8} pfu/ml
Essentially, you are dividing the number of plaques by the volume of sample plated. That number is multiplied by the reciprocal of the dilutions used to make that plate and then converted from µl to ml to obtain the titer in pfu/ml.

Helpful Tips:

- *When exponential numbers are multiplied, the exponents are added together. For example, $10^{5} \times 10^{4} = 10^{9}$.*
- *A high-titer lysate has a titer of 5×10^{9} pfu/ml or more. We recommend that you try to attain at least this titer for future experiments and long-term storage.*
- *If there are 24 plaques on the 10^{-7} plate, you should expect 240 plaques on the 10^{-6} plate. However, if when counting the plaques you count 200 plaques, the titer equals 2×10^{10} pfu/ml. Realize that there is no practical difference between the two titers, but counting more plaques is more accurate.*

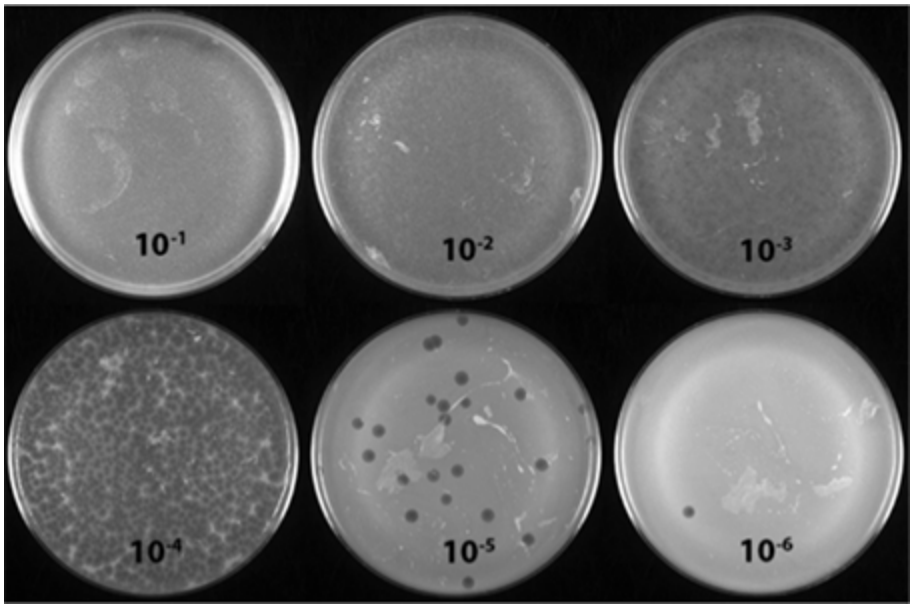


Figure 6.5-1. Plaque assay of dilution series. The phage StarStuff was serially diluted and plated for a plaque assay. The most concentrated samples (10-1 and 10-2) create cleared plates where no bacteria are visible. The 10-3 plate shows a very high concentration of plaques, but individual plaques are not visible until the 10-4 plate, which is a webbed plate. The 10-5 and 10-6 plates have countable numbers of plaques, but the 10-5 plate offers the most accurate number for counting

Amplification

Chapter 7: Phage Amplification

Chapter 7: An Overview

You completed the process of phage isolation when you made and titered a lysate. If you collected your initial lysate from a single plate with a high density of plaques during your last round of plaque purification, you probably have only five milliliters of lysate, which is not a sufficient volume for DNA extraction, transmission electron microscopy (TEM), and archiving. Therefore, you need to make more lysate to increase your phage stock. Indeed, your will need to collect 8–10 ml of lysate with a titer of 5×10^9 pfu/ml or higher. This can be accomplished by making additional plates that have a very high density of plaques, known as webbed plates (Figure 7.0-1), and flooding these plates to harvest a lysate. A lysate prepared from webbed plates is often of a high titer and is called a high-titer lysate (HTL). HTLs are the starting point for many basic phage biology experiments and long-term storage.

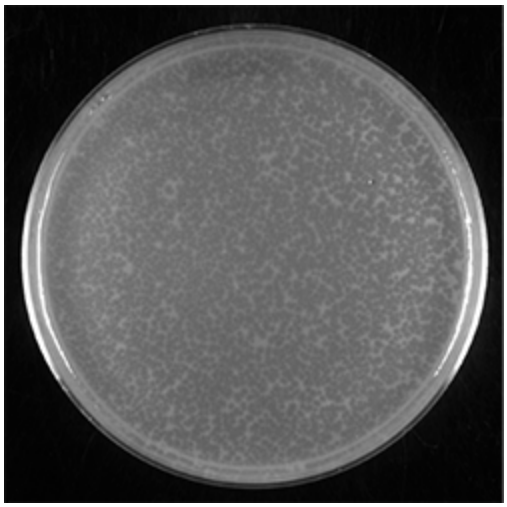


Figure 7.0-1 Webbed plate with confluent plaques.

The trick to making webbed plates that will yield a lysate with a titer of 5×10^9 or above is getting the right number of phage on the plate to start with. If too few phages are added to the top agar before incubation, the plate will be sparsely covered with plaques and yield too few phage when flooded and the lysate harvested. Likewise, if too many phages are added to the top agar before incubation, they will kill all of the bacteria in an early round of infection and the resulting phage yield will be low. Therefore, you have to start with the number of phages that will allow for maximum rounds of infection and lysis before running out of available host bacteria. This can be accomplished by determining the number of plaques formed by your phage that will result in a webbed plate, as described below.

Plaque size varies from phage to phage (Figure 7.0-2), and so will the number of plaques needed to form a webbed plate. The smaller the plaque size, the more plaques you will need to make a webbed plate. The larger the plaque size, the fewer plaques you will need to make a webbed plate.

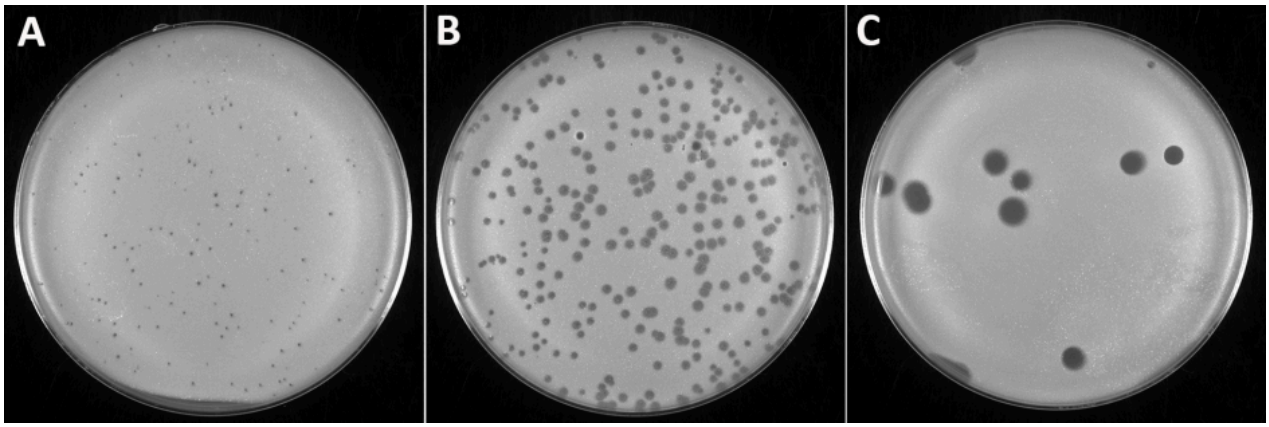


Figure 7.0-2. Examples of small (A), medium (C), and large (C) plaques.

You can calculate the number of plaques needed to make a webbed plate based on data you already have. One way to do this is to refer back to plates from your multiples rounds of plaque assays for purification. It is very likley that one of your dilutions yielded a webbed plate. You can easily calculate the number of plaques that were on that webbed plate because you also performed serial dilutions and obtained plates in that same experiment that yielded a plate with a countable number of plaques. (An example of how to perform this calculation is provided later in protocol 7.1, Making Webbed Plates from Lysates of Known Titer.) If your previous plaque assays never generated a plate that resembles a webbed plate, you could still use information from plates that yielded a high density of plaques to estimate the number of plaques needed to generate a webbed plate. For example, doubling the number of plaques on a plate may make a webbed plate. Alternatively, you could calculate the number of plaques needed to cover a plate based on plaque diameter and plate surface area.

Once you know how many plaques are needed for a webbed plate, you know how many phage particles to use in a plaque assay to generate a webbed plate. Remember, each plaque on a plate originates from a single plaque. Using the titer of your lysate, calculate the volume of your lysate that will contain the desired amount of phage particles.

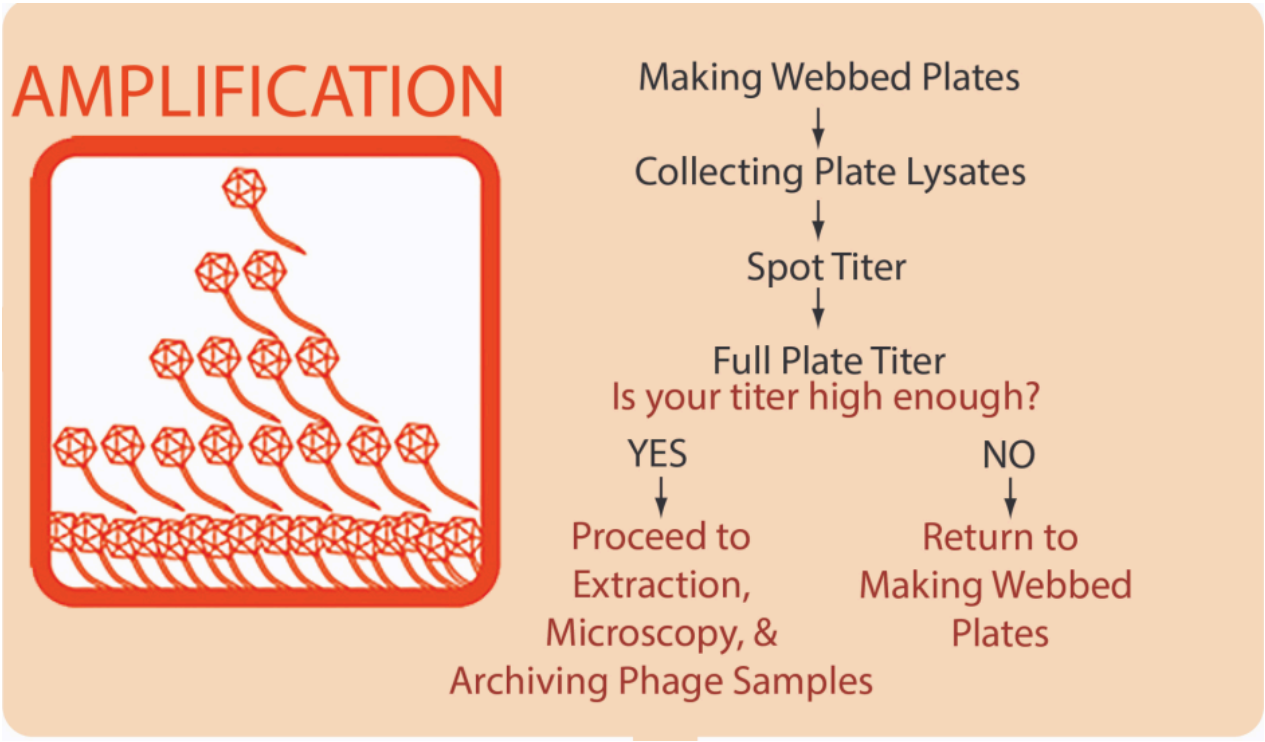


Figure 7.0-3. Flow chart of experiments in phage amplification.

Protocol 7.1: Making Webbed Plates from a Lysate of Known Titer

Objective:

To create a plate with a very high density of plaques from a lysate of known titer

Even if you have a small volume of titered lysate, it can be used to make additional lysate for experiments like DNA extraction and transmission electron microscopy (TEM) and for long-term storage. Lysates of high titer can be collected by flooding plates with as many plaques as possible. These plates are called “webbed” plates because they have densely packed confluent plaques with only wisps, or a “web” of bacteria, left between them.There are many approaches to determining how many phages (i.e., how much phage lysate) need to be added to a plate to obtain a web. The method presented below relies on information from the plaque assay you used to create your initial lysate. With this method you can calculate the volume of lysate needed to generate a webbed plate. Typically, a single webbed plate allows you to harvest ~ 4 ml of a highly concentrated phage lysate. It is recommended that you harvest 8–10 ml of lysate; therefore, you will need two or three webbed plates.

Supplies:

- Lysate of known titer
- Agar plates
- Host bacterial cultures
- Phage buffer
- Top agar, molten (between 55 - 60 ˚C)
- Microcentrifuge tubes
- 5 ml serological pipettes

Procedure:

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

B. Obtain the titered lysate you prepared from your last round of phage purification using the Collecting Plate Lysates (6.3) protocol and consult the protocols in chapter 6 for calculating titer.

C. Estimate the number of plaques you need and calculate the volume of lysate necessary to generate a webbed plate.

1. Using data from a previous plaque assay, for example from your most recent full-plate titer of your lysate, estimate how many plaques would result in a webbed

plate.

2. Calculate how many phage were on the webbed plate used to make the lysate. (See Helpful Tips for sample calculations.)
   OR

3. Choose a plate with a high density of plaques and count how many plaques are in a quadrant. Use a sharpie to mark each plaque on the bottom of the plate as you count it so you don't count it twice.

4. Estimate how many more plaques you need to make a webbed plate.

5. Calculate the volume of lysate needed to generate a webbed plate.

   Example: Vince has medium-sized plaques and estimates that he needs approximately 3,000 plaques to create a webbed plate. His lysate has a titer of 5 x 10^{9} pfu/ml. To find the volume of lysate needed to create a webbed plate, divide the number of plaques needed by the titer of the lysate and convert to microliters.

   Volume of lysate needed (ml)
   = 3.0 x 10^{3} pfu/ 5 x 10^{9} pfu/ml
   = 6.0 x 10^{-7} ml
   = (6.0 x 10^{-7} ml) x (1000 µl/ml)
   = 6.0 x 10^{-4} µl

   Although you cannot pipette 6.0 x 10^{-4} ul of lysate, you can pipette 6.0 µl of a 10^{-4} dilution, or 60 µl of a 10^{-5} dilution.

D. Perform the dilutions according to the protocols for Serial Dilutions(6.2).

1. Plaque size often decreases the closer the plaques are on a plate. To ensure that you obtain proper webbed plates plates, it is a good idea to "bracket" the number of phage particles you put on the plate. This means you should also prepare plates with fewer phage and with more phage than the optimum number estimated. For larger plaques you may want to double and halve the number of phage. For smaller plaques you may want to use 10 times more and 10 times less. This is phage-dependent.

   Example: After Vince looked at his plates, he decided that for his medium-sized plaques his brackets should be 5 times fewer and 5 times greater than his estimate of 3,000 (i.e., 600 and 15,000) plaques. Therefore, in addition to plating 60 µl of a 10^{-5} dilution, he will also plate 12 µl of a 10^{-5} dilution and 30 µl of a 10^{-4} dilution.

E. Plate your dilutions to create webbed plates.

1. Retrieve the proper number of agar plates and host bacterial cultures needed to plate your dilutions, including your bracket dilutions and replicates.

2. Infect the bacterial host cells with the appropriate volume and dilutions of your lysate and and incubate the mixture for 5–10 minutes for phage attachment.

Then add 3 ml of molten top agar to the mixture and plate. (If you need additional direction, refer to the Plaque Assay(5.3) protocol.)

3. Remember that each webbed plate will yield ~ 3–5 ml of lysate and that you will need a minimum of 8–10 ml for subsequent experiments and long-term storage. Therefore, you may need to plate each dilution two or three times to make additional webbed plates. Check with your instructor.

F. Incubate (without inverting) for at least 24 hours at the proper temperature.

1. Choose the best webbed plate and collect a lysate according to the Collecting Plate Lysates (6.3) protocol.

Helpful Tips:

- Making webbed plates that will yield lysates with titers above 5 x 10^{9} is highly phage-dependent. Therefore, you need to understand *your* phage, including its plaque morphology and behavior to be successful.

  Example: Priscilla collected her initial lysate for phage "Axelito" from the 10^{-2} dilution plate generated during the last round of purification of phage "Axelito." Her notebook says that the 10^{-4} dilution plate had 40 plaques on it and was made by infecting the host culture with 10 µl of the 10^{-4} dilution of a picked plaque.

  She can directly determine the number of plaques on the webbed plate from the number of plaques on the 10^{-4} plate because both plates were generated as part of a serial dilution series. Since 10^{-2} is 100-fold more concentrated than 10^{-4} she can multiply the number of plaques (40) on the 10^{-4} plate by 100 to for a value of 4.0 x 10^{3} plaques.

  When she made her lysate she noted that the 10^{-2} plate was not quite a perfect webbed plate. From the picture of the plate in her notebook she estimates that a perfect webbed plate would have twice as many plaques on it, or about 8.0 x 10^{3} pfu.

  Next, Priscilla calculates how much of Axelito's initial lysate she will need to create new webbed plates, so that she can collect enough lysate for DNA isolation and TEM. She calculated Axelito's lysate titer to be 5.0 x 10^{10} pfu/ml. If she needs 8.0 x 10^{3} pfu per plate:

  (8.0 x 10^{3} pfu/ml) / (5.0 x10^{10} pfu/ml) = 1.6 x 10^{-7} ml

  Priscilla converts 1.6 x 10^{-7} ml to microliters by multiplying by 10^{3} µl/ml, so she knows that she needs 1.6 x 10^{-4} µl of Axelito's lysate per plate to create a good webbed plate. She can't pipette that small a volume, but she can pipette 1.6 ul of a 10^{-4} dilution, or 16 ul of a 10^{-5} dilution.

  Because Pricilla needs 8 ml of lysate, she decides to make two webbed plates by infecting two host cell aliquots with 16 µl of a 10^{-5} dilution. To make sure she has a good webbed plate tomorrow when she comes to the lab, and because she had tiny plaques, she decides to make plates with 10 times fewer and 10 times more phage on

them as well. Therefore, she makes four more plates, two with 16 µl of the 10^{-4} dilution, and two with 16 µl of the 10^{-6} dilution.

Protocol 7.2: Entering a Phage into the Actinobacteriophage Database

Objective:

To include your phage in the Actinobacteriophage Database to make it available for other scientists

Rationale:

The Actinobacteriophage Database is a permanent online record of actinobacteriophage isolates and their characteristics. It provides a means of quickly identifying and sorting through the thousands of phages in the SEA-PHAGES archives as a first step toward further and more extensive analyses. Once a new record for a phage isolate is started, information about the phage can be contributed by both the isolator and by the SEA-PHAGES team. The more information you can provide, the better!

You can begin creating an entry for a phage as soon as you have a positive spot test, though it is recommended that you wait until you have a high titer lysate for that phage. At the latest, you must add your phage to the database to receive its genome sequence. As the isolator, you are responsible for collecting all the information for the phage you will "add" to the database, including your phage's name and conditions of the collection site—especially GPS coordinates, temperature of the isolation, host strain, naming notes, and whether your phage is from direct or enriched isolation. As you progress through the characterization process, you can add additional information, such as plaque pictures, genomic restriction digest pictures, and an electron micrograph.

Procedure:

A. Name your phage according to the rules found in the "Phages" dropdown menu and "Naming Rules" on phagesdb.org.

B. Log in to phagesdb.org or create an active PhagesDB account by clicking on the "Sign in or Register" button at the bottom of the left column on the page.

C. Enter your phage on PhagesDB.

1. Once you have logged in to PhagesDB, add a phage by selecting the "Data" dropdown menu and clicking on 'Add Phage.'

2. Fill in as many fields as possible, paying special attention to the following:

1. GPS coordinates: If your GPS fields were not prepopulated by the SEA-PHAGES App, you can find the GPS coordinates by using a web mapping service application. One such service can be found via this link: http://www.heywhatsthat.com/profiler.html OR http://bit.ly/yqKB . Simply drag the map to the location where the environmental sample was found, zoom in as much as possible, and click on the map to obtain the GPS

coordinates of that location. The output should be to the right of the map and in the N/W Decimal Degree format.

2. Program

3. Institution

4. Bacterial host

5. Name

    - Convert the GPS coordinates to the correct format if necessary. For example, the coordinates *40° 26′ 46″ N, 79° 57′ 11″ W* from the iPhone's "Compass" app will be converted into *40.446111 N, 79.953056 W*.
      Important: Phages must have GPS coordinates to be submitted to GenBank after sequencing and annotation.

6. If you found your phage using an enriched culture, click "Yes" to "From Enriched Soil" or "No" if you used a direct isolation.

7. Fill in the Discovery Notes to explain where the sample was collected and the soil conditions, etc.

8. Complete the Naming Notes to explain where your phage name came from and why you chose the name.

9. Enter the details about your phage's plaque morphology, including size, turbidity, margins, etc.

10. The "Morphotype" of your phage is determined from the TEM data. Details about the different morphotypes can be found in Chapter 8, Viewing Phage Particles by Transmission Electron Microscopy.

11. The Cluster, Subcluster, Sequencing Facility and Method, and GenBank Accession Number can be filled in only after a phage's genome has been sequenced. Therefore, you should not fill in these fields.

12. If you know the titer of the sample to be archived at the University of Pittsburgh, enter the titer here (or return later and enter the data).

13. Upload unaltered images of your phage where indicated. If you do not have a particular image at this time, you will need to return and add it at a later date.

3. Click "Submit" to submit your request to PhagesDB. Your submission will be reviewed and approved by a scientist at the University of Pittsburgh; therefore, your submission will not be visible immediately. Please submit your phage only ONCE!

    1. Once approved, information is publically available for everyone to see and use.

4. Once your submission is accepted, you need to create thumbnails of the images that will be displayed with your phage's profile.

    1. From the main PhageDB page select the "Data" dropdown menu and click on "Thumbnails."

    2. Type in your phage's name.

    3. Read the instructions for "Making Thumbnails for Plaque and EM Pictures for PhagesDB."

    4. Upload images that fulfill the requirements in the instructions.

5. If you need to modify or add data to your phage's PhagesDB profile, you can do so at any time by clicking on the "Data" dropdown menu and selecting "Modify Phage."

D. Once your phage is sequenced, the SEA-PHAGES team will add data, such as the phage cluster, the genome sequence, and the type of ends. Finally, when your phage has been annotated, submitted to GenBank, and published, the SEA-PHAGES team will add the GenBank accession number and publication data. Phages MUST have GPS coordinates for GenBank submission. Only phages that have been, or will be, archived at the University of Pittsburgh should be entered into the database.

Protocol 7.3: Archiving your Phage Sample

Objective:

To prepare a high-titer lysate for long-term storage

Rationale:

Your phage samples are a valuable resource for current and future in-depth research. There are two objectives to archiving your phage. First, when prepared and stored properly, your phage samples can remain viable for decades. Second, you need to verify the data (including names and titers of your samples) entered at PhagesDB is complete and accurate for easy referencing. You will create three samples for archiving; two will be stored at the University of Pittsburgh, in the lab of Dr. Graham Hatfull, and another at your institution.

Important: This protocol is provided as a classroom supplement to the complete SEA-PHAGES Lysate Archiving Protocol on phagesdb.org. This protocol is updated periodically so please check that you are using the most up-to-date version of this protocol.

Supplies:

- Barcoded tubes provided by the Hatfull lab
- Sample tubes provided by your institution
- DMSO mixture
- Lysate (at least 5 x 10^9 pfu/ml)

Procedure:

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

B. Certify phage samples on Phagesdb.org.

1. Make certain that the details of your phage samples have been recorded on the phages database at phagesdb.org according to the protocol Entering Phages into the Actinobacteriophage Database (7.2).

2. Double-check the spelling of the phage name and the recorded titer of the lysate. Alert your instructor if you find any discrepancies.

C. Prepare archiving tubes.

1. Retrieve two barcoded tubes for storage at the University of Pittsburgh and a third tube for storage at your institution. This third tube does not need to be barcoded.

2. Properly label each tube with your phage's name according to Figure 7.3-1C, making sure it is legible.

D. Prepare DMSO/lysate mixture.
Important: Turn off your flame, as DMSO and its vapors are flammable!

1. Dispense 4.0 ml of your high-titer lysate into a sterile 15 ml conical tube.

2. Add 280 µl DMSO to the tube, cap the tube, and then vortex the mixture.

E. **Important:** Dimethyl sulfoxide (DMSO) is a cryoprotectant that prevents ice crystals from forming. (Ice crystals can cause damage to phage particles during long-term storage at -80 °C.)Aliquot the mixture into the storage tubes

1. Pipette the lysate/DMSO mixture into each of the labeled bar-coded tubes until the tube is approximately three-quarters full. The remainder can go into the archive tube (not bar-coded) stored at your institution.

2. Avoid overfilling and underfilling the tubes.

3. Take care to close the tubes properly.

F. Prepare the tubes for storage

1. Place the barcoded tubes in the storage box provided in the same order as shown on the archive list. (Your instructor will request a complete archive list once all information is entered in PhagesDB.)

2. Store samples for shipment to University of Pittsburgh at 4 °C.

3. Samples stored at your institution can be frozen immediately.

Helpful Tips:

- A video of this protocol can be viewed at: https://www.youtube.com/watch?v=5GjAjky81D4 . (Again, ignore the beads.)

Chapter:7 Tips & Hints

Making Webbed Plates

- When making webbed plates, you will want to standardize as many variables between preparing the initial lysate and the webbed plates. Using the same batch of cells (culture) and plates (so that they have the same water concentration), as well as adsorption and incubation time will ensure consistent results.

- The moisture content of the plates has a huge impact on the quality of the resulting webbed plates. It is best to use fresh plates that have been cured (left out on the bench, lids on and facing down, to dry) for approximately 24 hours.

- Once a webbed plate has been observed after at least 24 hours of incubation, you can allow this plate to incubate for an additional 24 hours to allow for further phage replication. These additional rounds of phage replication would ideally fully clear the remaining bacteria on the plate, which will increase the titer of the resulting lysate.

- Once the desired amount of lysate is created, it must be titered according to the spot titer protocol or by doing a full plate titer.

- When pooling lysates take care not to dilute a high titer lysate with a lysate made from a marginal plate with a low titer. It is recommended not to pool lysates from different rounds of infection.

Microscopy

Chapter 8: Electron Microscopy

Chapter 8: An Overview

Phages, like most microorganisms, are too small to be seen with the naked eye. Traditional compound light microscopes, like those found in most biology labs, use a series of lenses to magnify a specimen and a light source to illuminate it. A simple light microscope can magnify an object 40x to 1,000x. Even the most sophisticated light microscopes cannot magnify an object beyond ~ 1,500x due to the properties of light and its ability to resolve, or distinguish between, two objects that are less than 0.2 µm apart. Although light microscopy allows us to see individual microorganisms such as bacteria, it is not sufficient for viewing phages. To overcome this limitation, scientists employ microscopes that use a beam of electrons instead of light, which allows for magnification up to 10,000,000x.

Transmission electron microscopy (TEM) is based on the same principles as light microscopy, but it uses a beam of electrons rather than light to “illuminate” the specimen. Electron microscopes are usually very large (Figure 8.0-1) and use a hot wire, called a filament, to produce a stream of electrons that are focused into a beam by magnets. Much the same way as light-microscopes rely on glass slides that are transparent to light, you must use slides that are transparent to electrons. Electron microscope slides, which are called grids, are typically made of thin copper or nickel mesh and can be coated with carbon and heat-resistant plastics such as formvar, depending on the sample to be examined. Biological samples and the carbon/plastic coating are transparent to electrons, yet they are extremely fragile, so the metal mesh provides stability and support. For liquid phage samples, such as lysates, 400-mesh copper grids coated with carbon and formvar are appropriate (“400-mesh” means that there are 400 holes in the copper foil of the grid).

Figure 8.0-1. Transmission electron microscopy (TEM).

An ideal TEM sample has a concentration of 10^{10} pfu/ml and should be relatively free of cellular membranes and debris. You can concentrate your sample and remove debris by pelleting your phage in a microcentrifuge and resuspending the pellet in fresh phage buffer. When pelleting a phage sample the densest biomolecule is DNA, followed by protein, with

lipid membranes being the lightest (even lighter than water). Because phage particles are composed of both DNA and protein, they sediment to the bottom of a centrifuge tube faster than either lipid membranes or host proteins. By pelleting only the phage particles in your lysate and then resuspending the pellet in clean phage buffer, you will have a clean, concentrated sample for electron microscopy.

When a phage sample is applied to a grid, the phage particles adhere to the carbon coating by means of electrostatic interactions. The longer the lysate sits on the grid, the more phage particles stick to the coating. Using distilled water, you can rinse away extraneous salts and cell debris from the grid, and then stain the grid with a heavy metal salt, such as uranyl acetate (UA). The best images are obtained when the stain is carefully dried into a thin film across the entire grid, pooling around the phage and barely coating their surfaces. When the grid is placed in the electron microscope, electrons within the beam penetrate biological components, such as phage and the carbon/plastic grid coating, but are scattered by the heavy metal stain surrounding the phage and by the copper support mesh. The electrons that pass through the sample strike a phosphorescent screen and create an image called a micrograph. A good micrograph should provide a clear outline and some appreciable detail of the phage.

A lot can be learned about a phage based on its morphology. For example, you can tell if it is a siphoviridae with a long flexible tail (Figure 8.0-2A), a myoviridae with a contractile tail (Figure 8.0-2B), or a podoviridae (Figure 8.0-2C), with a stumpy noncontractile tail. Two phages with similar plaque morphologies may be distinguishable by calculating their respective head and tail sizes from a micrograph. The morphology may also provide information about a phage's genome. For instance, phages that have large capsids typically contain more DNA (that is, a longer genome) than phage with smaller capsids. Phages with long tails have a long tape-measure gene, which codes for a protein that determines the length of its tail.

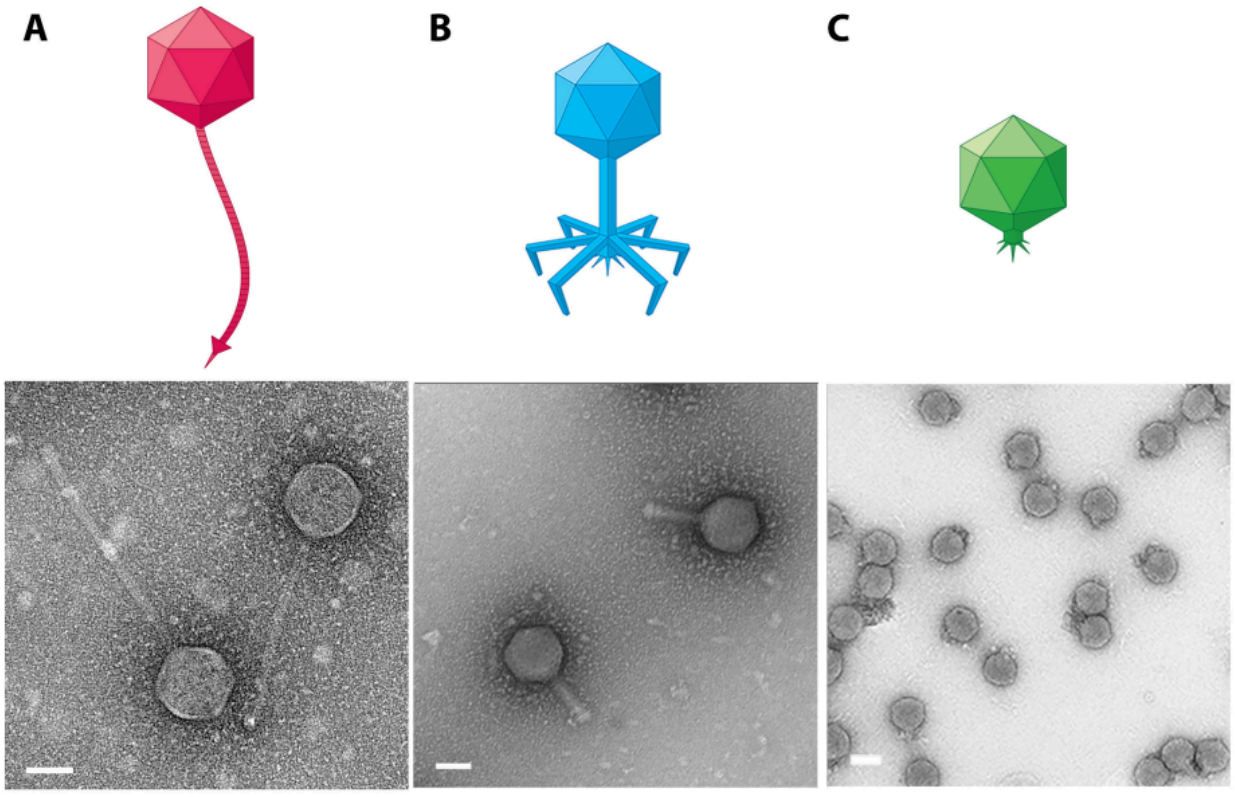


Figure 8.0-2. Phage morphology. Cartoons and transmission electron micrographs show three different phage morphologies. (A) Mycobacteriophage Scarlett is a siphoviridae with a long flexible tail. (B) Mycobacteriophage Gabriel is a myoviridae with a short contractile tail. (C) Cyanobacteriophage Syn5 is a podoviridae with a stumpy noncontractile tail. Size bars = 50 nm.

Protocol 8.1a: Mounting Phage Samples for TEM and Staining with Uranyl Acetate

Objective:

To prepare your phage sample for viewing with a transmission electron microscope

Rationale:

Individual phage particles are too small to be seen with light microscopes, necessitating the use of transmission electron microscopy (TEM). To observe your phage using TEM, a sample of your lysate should first be concentrated and cleared of protein and bacterial membrane debris. This is accomplished by pelleting your phage in a microcentrifuge and resuspending the phage pellet in clean phage buffer.

The concentrated sample is then placed on an electron microscopy grid. Once on the grid, salts and remaining bacterial debris are rinsed away and the sample is stained with uranyl acetate. When the stained grid is placed in the electron microscope, the beam of electrons penetrates the phage particles, but not the stain surrounding your phage, making the phage visible on the dark background.

Important: You will use uranyl acetate (UA) as your stain. UA is a hazardous substance requiring special safely precautions. Please follow your instructor's directions for handling and disposing of UA-exposed materials.

Supplies:

- High-titer lysate
- Phage buffer
- EM reverse forceps
- 200–400 mesh carbon–formvar-coated copper grids
- Wedges of Whatman filter paper for wicking
- Sterile, *filtered* water
- 1 % uranyl acetate (filtered immediately before use)

Procedure:

A. Prepare your phage samples.

a. Aseptically transfer 100 µl of your high-titer lysate into a sterile microcentrifuge tube. The ideal concentration of your resuspended sample is 10^{10} pfu/ml, so you

should titer your lysate and then choose an appropriate volume, centrifugation time, and resuspension volume according to Table 8.1a-1.

Table 8.1a-1. Centrifuge times to pellet phage samples in a microcentrifuge.

Initial concentration (pfu/ml)	Initial volume	Time	Final resuspension volume
10^{10}	100 µl	22 min	100 µl
$5x10^{9}$	100 µl	22 min	50 µl
$1x10^{9}$	500 µl	30 min	50 µl
$5x10^{8}$	500 µl	30 min	10 µl
$1x10^{8}$	1000 µl	40 min	10 µl

b. Balance the tube(s) and centrifuge according to Table 8.1a-1 at 4 °C at top speed in a microcentrifuge to pellet the phage particles to the bottom of the tube.

c. Using a micropipettor, carefully remove as much of the supernatant as possible without disrupting the pellet. You should remove the majority of the supernatant, leaving as little liquid as possible without dislodging the pellet. (If you can't see the pellet, don't worry, it is still there! You just have a really clean sample.)

d. Resuspend the pellet in the proper volume of phage buffer (Table 8.1a-1) and mix gently using the pipette tip. Note that vigorous pipetting can damage the phage particles.

e. Proceed with the rest of the protocol immediately to avoid damaging the phage heads.

B. Prepare your work area. (This may have been done before class by your instructor.)

a. Put on a fresh pair of gloves.

b. Cover the designated work area with bench paper or a large Kimwipe to create a clean work surface.

c. Using EM forceps, remove a fresh grid from a box of unused grids, touching only the very edge of the grid. If you are not using reverse forceps, slide the silicon clamp down the forceps so that they stay closed around the grid when you let go of the forceps.

d. Place the forceps on the paper so that the grid's dark-and-shiny side is facing UP.

C. Mount and stain your phage.

a. Using a micropipettor, place 5 µl of lysate onto the grid without touching the tip to the grid itself.

b. Allow the lysate to sit on the grid for at least 2 to 10 minutes, but do not let the sample evaporate completely! During this time the phage will settle and adsorb

onto the grid.

c. Rinse the grid once using the following method:

1. Using the forceps, turn the grid so it is at a 45° angle.

2. Carefully pipette 60 µl of sterile water across the dark-and-shiny face of the grid, allowing it to drip off the other side into a Petri dish. It will take about six big drops.

3. Rotate the grid back so that the dark-and-shiny side is facing up again. If necessary, wick away any excess water by placing a fresh wedge of filter paper against one edge of the grid.
Important: Work quickly and carefully! Do NOT allow the grid to dry out or the phage capsids can collapse.

d. Add 5 µl of 1 % uranyl acetate to the grid.
Important: Uranyl acetate is a very toxic compound. You should wear gloves throughout this procedure and when working in any area where this material has been used.

e. Immediately begin to wick off excess stain by using a wedge of filter paper. UA staining occurs by leaving a very thin layer of stain dried across the entire grid. You should continue to wick away the stain until the surface of the grid looks like a rainbow oil slick. Then allow the grid to air dry before putting it safely back into the grid box.

D. Observe your phage.

a. Place your grid in the designated grid box for storage. Be sure to accurately record the location of your grid in the box.

b. Transport your samples to your EM facility for imaging.

E. Calculate the capsid diameter and tail length relative to the size bar.

a. Using a ruler, measure the widest point (edge-to-edge, not vertex-to-vertex) of the capsid and the length of the tail (excluding the capsid and any visible tail fibers). If possible, measure multiple phage heads and tails and average their respective values.

b. Measure the length of the size bar with the ruler.

c. Using the known and relative lengths of the size bar, calculate the length of the capsid and tail.
Example:
The phage in the electron micrograph below has an average capsid diameter of 1.6 cm (16 mm) and a tail length of 4.8 cm (48 mm). The 100 nm size bar is measured to be 2.4 cm long.

To find the scaled (actual) size of the tail set up a simple ratio and solve for the unknown scaled size:
(scaled size bar) / (measured size bar)=(unknown scaled sized) / (measured size)
(100 nm) / (24 mm)=(unknown scaled tail size) / 48 mm
unknown scaled tail size=(100 nm)(48 nm) / (24 mm)
unknown scaled tail size=200 nm

d. Compare your capsid and tail lengths with those of your classmates.

e. Record your findings for entry in Phagesdb according to the protocol **Entering Phage into the Actinobacteriophage Database** (7.2).

F. **Helpful Tips:**

a. The EM forceps are very delicate. Never set them down without first replacing the protective cover.

b. It is extremely important that the grids do not dry out, especially between the last water wash and the addition of the stain. The capsid proteins destabilize in water (because there are no salts available) and the capsid will collapse or burst open if the grid dries out before stain is added. The stain acts as a fixative and will help stabilize the capsids when they dry.

c. Positively stained capsids (the phage heads look black because the stain has permeated the capsid) should not be used when describing the size or the morphology of the phage. Positive staining and capsid collapse can be caused by allowing the grid to dry out.

d. If a white precipitate is visible on the grids after drying, a new grid needs to be made. The precipitates are leftover salts from the lysate that were not wicked off completely and can damage the TEM.

e. Best results are obtained when FRESH lysates are used for the EM staining experiments.

Protocol 8.1b: Mounting Phage Samples for TEM and Staining with Uranyl Acetate (using Pelco Tabs)

Objective:

To prepare your phage sample for viewing with a transmission electron microscope

Rationale:

Individual phage particles are too small to be seen with light microscopes, necessitating the use of transmission electron microscopy (TEM). To observe your phages using TEM, a sample of your lysate should first be concentrated and cleared of protein and bacterial membrane debris. This is accomplished by pelleting your phage in a microcentrifuge and resuspending the phage pellet in clean phage buffer. The concentrated sample is then placed on an electron microscopy grid. Once on the grid, salts and remaining bacterial debris are rinsed away and the sample is stained with uranyl acetate. When the stained grid is placed in the electron microscope, the beam of electrons penetrates the phage particles but not the stain surrounds the phage, making it visible on the dark background.

Important: You will use uranyl acetate (UA) as your stain. UA is a hazardous substance requiring special safely precautions. Please follow your instructor's directions for handling and disposal of UA-exposed materials.

Supplies:

1. High-titer lysate
2. EM forceps
3. Pelco tabs
4. 200–400 mesh carbon–formvar-coated copper grids
5. Wedges of Watman filter paper for wicking
6. Sterile *filtered* water
7. Phage buffer
8. 1 % uranyl acetate

Procedure:

1. Prepare phage samples.
    1. Aseptically transfer 1 ml of your high-titer lysate into a sterile microcentrifuge tube.

2. Balance the tube(s) and centrifuge for 1 hour at 4 °C at top speed to concentrate the phage particles at the bottom of the tube.

3. Using a micropipettor, carefully remove as much supernatant as possible without disrupting the concentrated phage at the bottom of the tube.

4. Add 100 µl of phage buffer and let resuspend at 4 °C for 30 minutes to one hour.

5. Proceed with the rest of the protocol immediately to avoid damaging the phage heads.

2. Prepare your work area. (This may have been done before class by your instructor.)

    1. Put on a fresh pair of gloves.

    2. Cover the designated work area with bench paper or a Kimwipe to create a clean work surface.

    3. Remove the cover from a 5 x 5 cm piece of parafilm, and place the parafilm into the lid of Petri dish.

    4. Place a PELCO Tab or small piece of double-sided tape onto the parafilm in the lid of the Petri dish. Expose the adhesive or the tab.

    5. Using EM forceps, remove a fresh grid from a box of unused grids, touching only the very edge of the grid.

    6. Place the grid dark-and-shiny side UP, on the edge of the tab or double-sided tape so that only the very edge of the grid (no more than 0.5 mm) is touching the adhesive.

3. Mount and stain your phage.

    1. Using a micropipettor, place 10 µl of lysate onto the grid without touching the tip to the grid itself.

    2. Allow the phage settle and attach onto the grid for at least 2 minutes, or according to the times in Table 8.1b-1.

Table 8.1b-1. Approximate times that it should take phage to settle onto the EM grid based on titer.

Lysate titer	Approximate time
10^6 -10^7	5-7 minutes
10^8	5 minutes
≥ 10^9	2 minutes

3. Using a small wedge of filter paper, wick off the excess fluid.

4. Rinse the grid two times by the following method:

    1. Carefully pipette 10 µl of sterile water onto the grid. Allow it to sit for 2 minutes.

    2. Wick off the water using a fresh wedge of filter paper.
    Important: Work quickly and carefully! Do NOT allow the grid to dry out!

5. Add 10 µl of 1 % uranyl acetate to the grid.
    Important: Uranyl acetate is a very toxic compound. You should wear gloves throughout this procedure and when working in any area where this material has been used.

6. Let it sit for 2 minutes.

7. Wick off excess stain by using a wedge of filter paper. UA staining occurs by leaving a very thin layer of stain dried across the entire grid. You should continue to wick away the stain until the surface of the grid looks like a rainbow oil slick. Then allow the grid to air dry before putting it safely back into the grid box.

4. Observe your phage.

    1. Place your grid in the designated grid box for storage. Be sure to accurately record the location of your grid in the box.

    2. Transport your samples to your EM facility for imaging.

5. Calculate the capsid diameter and tail length relative to the size bar.

    1. Using a ruler, measure the widest point (edge-to-edge, not vertex-to-vertex) of the capsid and the length of the tail (excluding the capsid and the tail tip). If possible, measure multiple phage heads and tails and average their respective values.

    2. Measure the length of the size bar with the ruler.

    3. Using the known and relative lengths of the size bar, calculate the length of the capsid and tail.

    4. Example: The phage in the electron micrograph below has an average capsid diameter of 1.6 cm (16 mm) and a tail length of 4.8 cm (48 mm). The 100 nm size bar is measured to be 2.4 cm long.

To find the scaled (actual) size of the tail set up a simple ratio and solve for the unknown scaled size:(scaled size bar) / (measured size bar)=(unknown scaled sized) / (measured size)(100 nm) / (24 mm)=(unknown scaled tail size) / 48 mmunknown scaled tail size=(100 nm)(48 nm) / (24 mm)unknown scaled tail size=200 nm

6. Compare your capsid and tail lengths with those of your classmates.

7. Record your findings for entry in Phagesdb according to the protocol **Entering Phage into the Actinobacteriophage Database** (7.2).

Helpful Tips:

- The EM forceps are very delicate. Never set them down without first replacing the protective cover.

- It is extremely important that the grids do not dry out, especially between the last water wash and the addition of the stain. The capsid proteins destabilize in water (because there are no salts available) and the capsid will collapse or burst open if the grid dries out before stain is added. The stain acts as a fixative and will help stabilize the capsids when they dry.

- Positively stained capsids (the phage heads look black because the stain has permeated the capsid) should not be used when describing the size of the morphology of the phage. Positive staining and capsid collapse can be caused by allowing the grid to dry out.

- If a white precipitate is visible on the grids after drying a new grid needs to be made. The precipitates are leftover salts from the lysate that were not wicked off completely and can damage the TEM.

- Best results are obtained when FRESH lysates are used for the EM staining experiments.

Protocol 8.1c: Mounting Phage Samples for TEM and Staining using the Parafilm Drop Method

Objective:

To prepare your phage sample for viewing with a transmission electron microscope

Rationale:

Individual phage particles are too small to be seen with light microscopes, necessitating the use of transmission electron microscopy (TEM). To observe your phages using TEM, a sample of your lysate should first be concentrated and cleared of protein and bacterial membrane debris. This is accomplished by pelleting your phage in a microcentrifuge and resuspending the phage pellet in clean phage buffer. The concentrated sample is then placed on an electron microscopy grid. Once on the grid, salts and remaining bacterial debris are rinsed away and the sample is stained with uranyl acetate. When the stained grid is placed in the electron microscope, the beam of electrons penetrates the phage particles but not the stain surrounds the phage, making it visible on the dark background.

Important: You will use lanthanide salts as your stain. Please follow your instructor's directions for handling and disposing of these materials.

Supplies:

- High-titer lysate
- Phage buffer
- EM reverse forceps
- 200–400 mesh carbon–formvar-coated copper grids
- Wedges of Whatman filter paper for wicking
- Sterile, filtered water
- Appropriate stain (e.g. 2 % uranyl acetate, ammonium molybdate or lanthanide salts) Important: Heavy metals should be handled with caution. You should wear gloves throughout this procedure and when working in any area where this material has been used.

Procedure:

A. Prepare your phage samples.

  - Aseptically transfer 100 µl of your high-titer lysate into a sterile microcentrifuge tube. The ideal concentration of your resuspended sample is 1010 pfu/ml, so

you should titer your lysate and then choose an appropriate volume, centrifugation time, and resuspension volume according to Table 8.1a-1.

trial|Initial concentration (pfu/ml)	Initial volume	Time	Final resuspension volume
10^{10}	100 µl	trial|22 min	100 µl
$5x10^{9}$	100 µl	22 min	50 µl
$1x10^{9}$	500 µl	30 min	50 µl
$5x10^{8}$	500 µl	30 min	10 µl
$1x10^{8}$	1000 µl	40 min	10 µl

- Balance the tube(s) and centrifuge according to Table 8.1a-1 at 4 °C at top speed in a microcentrifuge to pellet the phage particles to the bottom of the tube.
- Using a micropipettor, carefully remove as much of the supernatant as possible without disrupting the pellet. You should remove the majority of the supernatant, leaving as little liquid as possible without dislodging the pellet. (If you can't see the pellet, don't worry, it is still there! You just have a really clean sample.)
- Resuspend the pellet in the proper volume of phage buffer (Table 8.1a-1) and mix gently using the pipette tip. Note that vigorous pipetting can damage the phage particles.
- Proceed with the rest of the protocol immediately to avoid damaging the phage heads.

B. Prepare your work area. (This may have been done before class by your instructor.)

- Put on a fresh pair of gloves.
- Cover the designated work area with bench paper or a large Kimwipe to create a clean work surface.
- Cut a 5x5 cm piece of parafilm and place into the lid of a Petri dish with the waxy side facing up.

C. Prepare lysate, water wash, and stain.

- Using a micropipettor, transfer 10-15 µl of high titer lysate to the surface of the parafilm. It will make a droplet.
- In a separate area of the parafilm, transfer 15 µl of sterile, filtered water to make a separate droplet.
- In a third area of the parafilm, transfer 15 µl of stain to make a separate droplet.

D. Mount and stain your phage.

- Using EM forceps, remove a fresh grid from a box of unused grids, touching only the very edge of the grid.
- Place the forceps on the high titer lysate droplet so that the grid's dark-and-shiny side is facing down toward the liquid. Carefully release the grid. The grid will float on the droplet.
- Allow the grid to sit on the lysate for at least 2 to 10 minutes (use longer times if lysate titer is not higher than 10^9 pfu/ml). During this time the phage will adsorb onto the grid.
- Rinse the grid once using the following method:
    a. Carefully grasp the edge of the grid with the forceps. While holding, use a small (2-to-3-cm) wedge of Whatman filter paper to wick off the excess fluid.
    b. Place the grid, dark-and-shiny side down, onto the droplet of water. Leave for 30 seconds.
    c. Remove the grid from the drop with forceps. Wick excess liquid immediately using an unused corner of the filter paper wedge.
    Important: Work quickly and carefully! Do NOT allow the grid to dry out or the phage capsids can collapse.
- Place grid on droplet of staining liquid with dark-and-shiny side down. Allow to stain for 2 minutes.
- Carefully grasp edge of grid with the forceps. Immediately begin to wick off excess stain by using a wedge of filter paper. Lanthanide staining occurs by leaving a very thin layer of stain dried across the entire grid. You should continue to wick away the stain until the liquid no longer enters the filter paper. Then allow the grid to air dry before putting it safely back into the grid box.

E. Observe your phage.

- Place your grid in the designated grid box for storage. Be sure to accurately record the location of your grid in the box.
- Transport your samples to your EM facility for imaging.

F. Calculate the capsid diameter and tail length relative to the size bar.

- Using a ruler, measure the widest point (edge-to-edge, not vertex-to-vertex) of the capsid and the length of the tail (excluding the capsid and any visible tail fibers). If possible, measure multiple phage heads and tails and average their respective values.
- Measure the length of the size bar with the ruler.

- Using the known and relative lengths of the size bar, calculate the length of the capsid and tail.
  Example:
  The phage in the electron micrograph below has an average capsid diameter of 1.6 cm (16 mm) and a tail length of 4.8 cm (48 mm). The 100 nm size bar is measured to be 2.4 cm long.

To find the scaled (actual) size of the tail set up a simple ratio and solve for the unknown scaled size:
(scaled size bar) / (measured size bar)=(unknown scaled sized) / (measured size)
(100 nm) / (24 mm)=(unknown scaled tail size) / 48 mm
unknown scaled tail size=(100 nm)(48 nm) / (24 mm)
unknown scaled tail size=200 nm

- Compare your capsid and tail lengths with those of your classmates.
- Record your findings for entry in Phagesdb according to the protocol **Entering Phage into the Actinobacteriophage Database** (7.2).

G. **Helpful Tips:**

- The EM forceps are very delicate. Never set them down without first replacing the protective cover.
- It is extremely important that the grids do not dry out, especially between the last water wash and the addition of the stain. The capsid proteins destabilize in water (because there are no salts available) and the capsid will collapse or burst open if the grid dries out before stain is added. The stain acts as a fixative and will help stabilize the capsids when they dry.

- Positively stained capsids (the phage heads look black because the stain has permeated the capsid) should not be used when describing the size or the morphology of the phage. Positive staining and capsid collapse can be caused by allowing the grid to dry out.

- If a white precipitate is visible on the grids after drying, a new grid needs to be made. The precipitates are leftover salts from the lysate that were not wicked off completely and can damage the TEM.

- Best results are obtained when FRESH lysates are used for the EM staining experiments.

Chapter:8 Tips & Hints

Working with the delicate EM grids.

- For transmission electron microscopy, phage samples are deposited on 200-400 mesh carbon-stabilized, formvar coated copper TEM grids. It is best if these grids are “fresh” (<6 month)=" and=" not=" saved=" from=" year=" to=" year=" since=" the=" formvar=" gets=" brittle=" with=" age.=" when=" storing=" unused=" grids,=" place=" in=" a=" dry=">

- TEM forceps come in two varieties. Traditional forceps are held closed with your thumb and index finger, while reverse forceps remain closed at rest, and are opened by exerting pressure with your thumb and index finger. Which protocol you use to stain your grids will depend on which forceps you want to use. For protocol 8.1a you need reverse forceps, but protocol 8.1b is better suited to regular forceps. Either forcep will work well for protocol 8.1c. A summary of the protocols is provided below:

    - **Protocol 8.1a** uses reverse forceps to hold the grid while the sample and stain are added to the grid surface. The grid is rinsed by holding the forceps and the grid at an angle and dropping sterile filtered water across the grid surface. Using the reverse forceps reduces damage to the delicate grid since it is only manipulated when it is removed from the grid box, and again when it is returned to the grid box. The rest of the time it is safely held in the forceps.

    - **Protocol 8.1b** uses sticky Pelco tabs in the bottom of a Petri dish to hold the grid in place while the sample, rinses, and stain are deposited on the grid and wicked off using filter paper. It can be difficult to move the grids from the grid box onto the Pelco tab, and off again, without damaging the grid. A video protocol from Gonzaga University is available here.

    - **Protocol 8.1c** is called the parafilm drop method, in which a droplet of phage sample, water, and stain are placed on a piece of parafilm. The grid is then “floated” on these liquid droplets, sequentially.

- When removed from the grid box you will see that the girds have a “dark and shiny” side and a “light and matte” side. You will want to put the sample on the “dark and shiny” side. This dark side is the side that formvar has been layered on and covered with the carbon. The light side actually has some depth to it, and the sample and stain can actually pool in the boxes made by the mesh rather than spreading evenly across the carbon surface. Such pooling will negatively impact your ability to visualize your phage by TEM.

- These grids are very delicate and should not be crumpled or bent prior to viewing on the TEM. To avoid damaging the grids, handle them carefully and try to touch only the outer edge with the forceps when moving them between grid boxes and the preparation area. However, even if the grids do get crumpled or bent, it is usually possible to unfold them and use them to visualize phage anyway.

- Most commercially purchased formvar coated grids have been glow-discharged for you. If you are preparing your own grids you will need to treat them in a glow-discharge unit. Carbon and plastic are hydrophobic, while phage lysates are hydrophilic. Imagine

trying to evenly spread a thin film of water across a piece of saran wrap! The water beads and pools. The same thing would happen to phage samples if the grids were not glow-discharged prior to application of the sample. In a glow-discharge unit, grids are placed under vacuum on a metal surface, and high-voltage electricity is added to the system at a slight distance. In a glass glow discharge unit, it is possible to see a purple glow evenly surrounding the high voltage source through the vacuum and the metal plate that hold the grids. By glow-discharging grids for up to a minute, it is possible to artificially charge the surface of the carbon/plastic, thus allowing hydrophilic samples to evenly spread across it.

Staining

- The method presented for staining your EM grids utilizes uranyl acetate, a toxic compound for which you should read the MSDS information carefully. When dealing with uranyl acetate, you should wear gloves and exposure should be minimized. You should set up a designated staining station where all work using uranyl acetate will be done and waste collected. The uranyl acetate waste will need to be saved separately and disposed of according to your institution's hazardous waste policies.

- Set up a "staining area" on a piece of absorbent bench paper. This will help you keep the uranyl acetate in one area as well as provide a clean white surface in case a grid is accidentally dropped.

- It is important that the 1-2 % uranyl acetate be filtered after it is made, before use. The 1- 2 % uranyl acetate can be made ahead of time and stored for long periods of time (years!) if it is protected from light with aluminum foil.

- A staining alternative to uranyl acetate is non-toxic lanthanide salts (e.g. UranyLess). You can substitute lanthanide salts for uranyl acetate and simply follow the rest of the protocol. The contrast is not usually as good in the TEM images, and so we recommend uranyl acetate.

Other Tips

- It is important to have a scale bar on each micrograph so that the size of the capsid and tail can be measured. Comparing the measurements of phage particles can be important for determining if a phage is unique or may be the same as another phage. For example, multiple phages can produce a common plaque morphology but may have very different capsid and tail dimensions. The scale bar is also required if the micrograph is to be included in research presentations and publications. Please remind your microscopist to add a scale bar to each image.

DNA Extraction

Chapter 9: Extracting Phage DNA

Chapter 9: An Overview

Once you have obtained a high-titer lysate of your clonal phage population, you can further characterize your phage by analyzing its genome. A genome is defined as an organism's complete set of genetic material, comprising all of its genes and regulatory sequences. While virus genomes are unique in that they can be made of RNA or DNA, most phages isolated in this course use DNA as their genetic material. To characterize your phage's DNA, you first need to extract it from your phage lysate. Once isolated, you can study the phage DNA by using experiments like restriction enzyme digestion and DNA sequencing.

Your lysate contains your clonal phage population as well as the remnants of lysed bacterial cells, including bacterial nucleic acids (DNA and RNA), proteins, and lipids. Because the presence of bacterial DNA and RNA will interfere with future experiments designed to study your phage genome, the initial step in DNA extraction is the destruction of bacterial nucleic acids by using enzymes called nucleases. Your phage genomic DNA is surrounded by the protein shell of the phage capsid and therefore protected from the nucleases. After a brief incubation period, the nucleases are inactivated with the addition of denaturants like guanidine hydrochloride. Denaturants unfold all proteins, including the nucleases and phage capsids, thereby simultaneously inactivating the nucleases and releasing phage genomic DNA into your lysate.

Once the phage genomic DNA has been released, you can purify it from the rest of the lysate. First, you add a resin containing microscopic polymer beads that bind to the long DNA molecules of your phage genome. This resin/DNA mix is then loaded onto a column that contains a very fine filter that prevents the beads and bound DNA from escaping the column. This allows you to "wash" away unwanted contaminating molecules such as proteins, lipids, nucleotides, denaturant, and salts with isopropanol. The DNA is then released, or eluted, from the resin by adding hot water to the column and collecting the liquid that contains your phage genomic DNA. The concentration of DNA in your sample is determined by using spectrophotometry, fluorimetry, or by viewing it on an agarose gel.

The protocol you will use takes advantage of a commercially available kit. Kits like these are available from many different vendors and are designed to make common molecular biology protocols, like DNA extractions, easier, faster, and generally more accessible. Not too long ago, kits were not readily available and scientists spent much of their time mixing solutions and spending hours performing protocols that now only take a few minutes. These kits are not magic, but instead are based on the inherent chemical properties of the molecules involved, like proteins and DNA. Understanding what each step accomplishes, and the chemistry behind it, is especially important when it comes to troubleshooting and understanding why an experiment didn't go as planned. It is also important to keep in mind that not all protocols or kits will work with all organisms, so understanding the principles behind what you are doing can be crucial to your success.

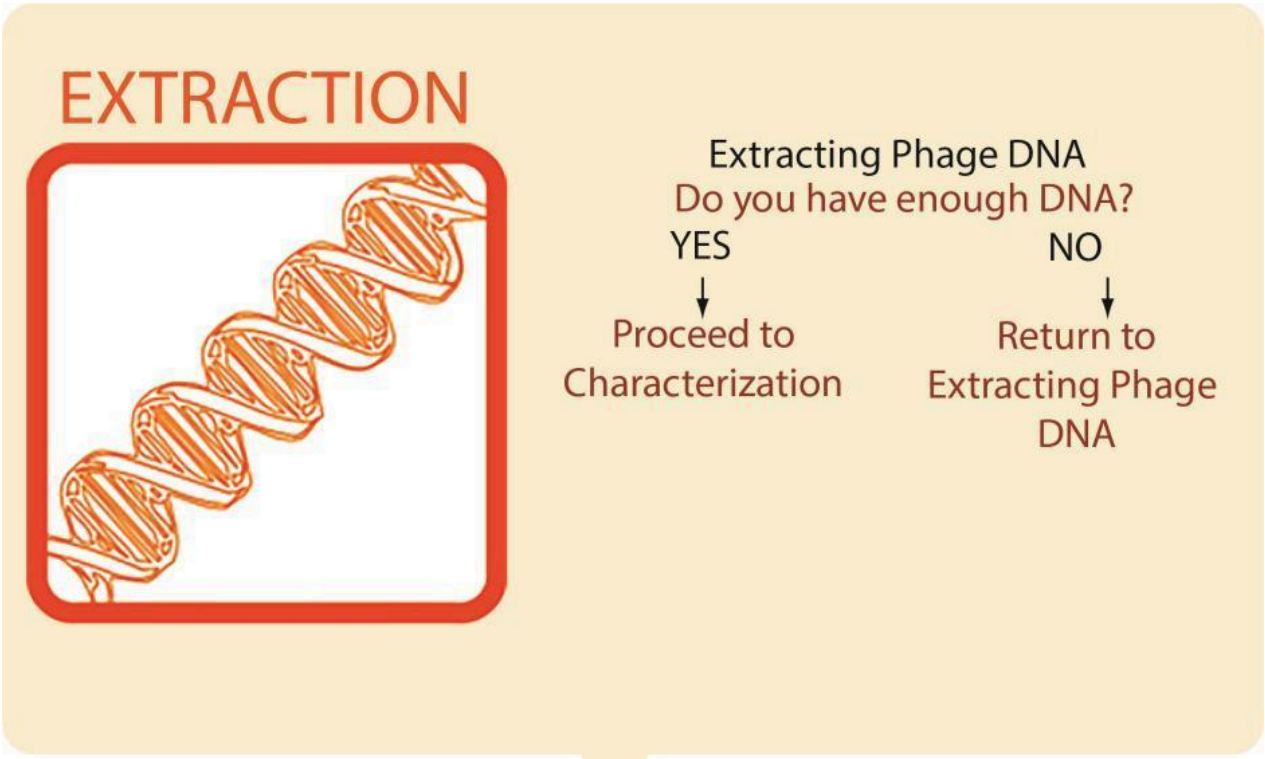


Figure 9.0-1. Flow chart of phage DNA extraction experiments..

Protocol 9.1: Phage DNA Extraction

Objective:

To isolate genomic DNA from phage

Rationale:

Once you have a prepared a phage lysate, your phage DNA can be isolated and studied. The first step in extracting phage DNA is to remove bacterial DNA and RNA from your lysate using nuclease enzymes. Next, the phage DNA is released from the phage capsid, bound to a solid matrix according to its inherent chemical properties, rinsed, and eluted, yielding a sample of pure phage DNA.

Supplies:

- 1 ml phage lysate (titer ≥ 5 x 10^9 pfu/ml)
- Nuclease mix
- EDTA (0.5 M) (optional)
- Proteinase K (20 mg/ml) and SDS (10 %) (optional)
- 2 ml DNA clean-up resin (Promega Wizard DNA Clean-Up Kit)
- 2 DNA clean-up columns (Promega Wizard DNA Clean-Up Kit)
- 3 ml syringes
- 6 ml 80 % isopropanol, freshly prepared
- ddH2O pre-warmed (95 °C)

Procedure:

A. Prepare your bench and assemble your supplies.

B. Degrade bacterial DNA/RNA in high-titer phage lysate.

1. Aseptically transfer 1 ml of phage lysate into a microcentrifuge tube.

2. Wearing gloves and working in the designated area, add 5 µl nuclease mix to the lysate.
   Important: The enzymes (RNase in particular) are very stable and can persist and contaminate equipment and supplies throughout the laboratory. Take precautions to keep and use them in the designated area.

3. Mix gently but thoroughly by repeated inversions—do not vortex!

4. Incubate at 37 °C for 10 minutes or room temperature for 30 minutes.

5. Remove and discard your gloves before returning to your bench.

6. Optional: Add 15 µl EDTA to the nuclease-treated lysate and mix gently.

    a. EDTA will inactivate the nucleases by chelating, or binding, divalent cations required by the nucleases for activity.

7. Optional: Add 0.5 µl Proteinase K and 50 µl SDS to the nuclease-treated lysate and mix gently. Incubate at 37 °C for 10 minutes.

    a. Proteinase K is added to degrade the nucleases added in Step 2. SDS stimulates the activity of Proteinase K.

C. Denature the protein capsid to release phage DNA.

1. Put on a fresh pair of gloves.

2. Add 2 ml of DNA clean-up resin to a 15 ml conical tube.

    a. The DNA resin is a slurry solution containing microscopic polymer beads. Make sure that the bottle of resin is well mixed, the precipitate dissolved by heating to 37 °C, and the beads resuspended before aliquoting your 2 ml. (Your instructor may have done this for you.)
    Important: The resin contains guanidinium thiocyanate, a chemical that denatures proteins. Do not get it on your skin!

3. Transfer your nuclease-treated phage lysate from the microcentrifuge tube to the 15 ml conical tube containing resin.

4. Mix the solution by gently inverting the tube repeatedly for 2 minutes.

D. Isolate the phage genomic DNA.

1. Label two Wizard Kit columns with your initials.

2. Remove the plungers from two 3 ml syringes and attach a column to each syringe barrel.

3. Follow the steps below for each column at the same time:

    a. Set the column and syringe barrel on a new microcentrifuge tube.

    b. Transfer 1.5 ml of phage DNA/resin solution to the column using a pipette.

    c. Do not discard the empty 15 ml conical tube.

d. Insert a plunger into the syringe and carefully push all the liquid through, collecting the flow-through in the used 15 ml conical tube from above.
Important: The DNA is bound to the polymer beads that pack into the column as the liquid is pushed through. It is VERY important to maintain a firm, gentle, unrelenting, and even pressure on the syringe. Do not let the plunger pop out of the syringe barrel because releasing the vacuum will ruin the column.

e. Once the liquid is expelled, maintain pressure on the plunger as you dry residual liquid by touching the tip of the column to a paper towel.

f. Unscrew the column from the syringe barrel before releasing the plunger and set the column into a clean microcentrifuge tube.

g. Remove the plunger from the syringe barrel, and then reattach the syringe barrel to the column.

4. Wash the salts from the DNA (now in the column) with the following steps for each column:

    a. Add 2 ml 80 % isopropanol to each syringe barrel/column and push the liquid through the column, repeating steps 3(d)–3(f).

    b. Repeat twice, for a total of three isopropanol washes.

5. Remove residual isopropanol.

    a. With each column in a fresh 1.5 ml microcentrifuge tube, spin at 10,000 × g for 5 minutes.

        i. The column will prevent the microfuge tube lids from closing. Arrange the open tubes in the centrifuge so that the lids point toward the center of the rotor.

    b. Transfer columns to new 1.5 ml microcentrifuge tubes. Spin at 10,000 × g for 1 additional minute to remove any residual isopropanol.

    c. Evaporate the last traces of isopropanol by removing your columns from the microcentrifuge tubes and placing them directly in a 90 °C heating block for 60 seconds.
    Important: Leaving the columns in the heat block for more than 1 minute can lead to DNA damage.

6. Elute the phage DNA from the columns.

    a. Place each column in a clean microcentrifuge tube and apply 50 µl of 90 °C sterile ddH_2O directly to each column.
    Important: Keep the ddH_2O in the heating block so that it remains at 90 °C.

    b. Incubate columns for 1 minute at room temperature.

c. Spin at 10,000 × g for 1 minute in a microcentrifuge.

d. Combine the products from both microcentrifuge tubes into one tube; this is your eluted phage DNA.

E. Determine the concentration of your phage DNA.

1. Using a spectrophotometer (fluorimeter, or Nanodrop) and a protocol from your instructor, quantify your phage DNA.

2. Place at 4 °C for short-term storage (1–2 weeks) or at -20 °C for long-term storage.

Helpful Tips:

- Make sure you have a titer of 5 x 10^9 PFU/ml or higher before proceeding with the protocol, or you may not isolate enough DNA.
- If your titer is less than 5 x 10^9 PFU/ml you can increase the concentration by pelleting your phage.
- Spinning above 10,000 x g can lead to the column getting stuck in the microcentrifuge tube, the microcentrifuge tube breaking, and both getting stuck in the microcentrifuge.
- Insufficient rinsing with isopropanol will lead to residual guanidinium that will affect your ability to estimate DNA concentration via spectrophotometry.
- Failure to remove all of the isopropanol can cause problems when loading agarose gels later.
- A brief video demonstrating this protocol is available here.

Protocol 9.2a: Phage DNA Extraction Following Precipitation with PEG

Objective :

To concentrate liquid phage samples prior to extracting DNA

Rationale:

Once you have a prepared a phage lysate, your phage DNA can be isolated and studied. If you have a lysate with a titer lower than 5 x 10^9 PFU/ml, the phage can be concentrated prior to DNA isolation to increase yield. The first step in extracting phage DNA is to remove bacterial DNA and RNA from your lysate using nuclease enzymes. After precipitating you phage using phage precipitant solution containing polyethylene glycol (PEG), and pelleting in a high speed centrifuge, the phage DNA is released from the phage capsid, bound to a solid matrix according to its inherent chemical properties, rinsed, and eluted, yielding a sample of pure phage DNA.

Supplies:

- 4 - 10 ml phage lysate
- Phage precipitant solution (0.4 ml per 1 ml phage lysate)
- Nuclease mix
- 15 ml Oak Ridge Tube (optional)
- 15 ml conical tubes
- Sterile water (0.5 ml)
- 2 ml DNA clean-up resin (Promega Wizard DNA Clean-Up Kit)
- 2 DNA clean-up columns (Promega Wizard DNA Clean-Up Kit)
- 3 ml syringes
- 6 ml 80 % isopropanol, freshly prepared
- ddH2O pre-warmed (95 °C)

Procedure:

A. Prepare your bench and assemble your supplies.

B. Degrade bacterial DNA/RNA in phage lysate.

1. Aseptically transfer 4 - 10 ml of phage lysate into a conical tube.
   Note: This step can be done in an Oak Ridge tube if an appropriate centrifuge for these tubes are available.

2. Wearing gloves and working in the designated area, add 5 µl nuclease mix (per 1 ml phage lysate) to the lysate.
   Important: The enzymes (RNase in particular) are very stable and can persist and contaminate equipment and supplies throughout the laboratory. Take precautions to keep and use them in the designated area.

3. Mix gently but thoroughly by repeated inversions, and incubate at 37 °C for 10 minutes or at room temperature for 30 minutes.

4. Optional: Add 15 µl EDTA (per 1 ml phage lysate) to the nuclease-treated lysate and mix gently.

   a. EDTA will inactivate the nucleases by chelating, or binding, divalent cations required by the nucleases for activity.

5. Optional: Add 0.5 µl Proteinase K and 50 µl SDS (per 1 ml phage lysate) to the nuclease-treated lysate and mix gently. Incubate at 37 °C for 10 minutes.

   a. Proteinase K is added to degrade the nucleases added in Step 2. SDS stimulates the activity of Proteinase K.

C. Precipitate phage particles.

1. Add 0.4 ml of phage precipitant solution to every 1.0 ml of the nuclease-treated lysate, and cap the tube.

2. Mix gently but thoroughly by inversion.

3. Prepare 1 ml aliquots of the mixture in microcentrifuge tubes.

4. Incubate 30 minutes on ice or, for maximum yield, overnight at 4 °C.

5. Place the tube in a high-speed centrifuge, balance the tubes, and spin at 10,000 × g for 20 minutes. Centrifugation should be done at 4 °C.

6. Decant the supernatant being careful not to disturb the pellet. Drain excess liquid from the pellet by inverting for 2 to 3 minutes on a paper towel. Discard the paper towel and your gloves.
   Important: Remove as much phage precipitant solution as possible with out disturbing the phage pellet. Failure to do so will interfere with or clog the column and significantly reduce your DNA yield.

D. Re-suspend the phage pellet.

1. Add 0.5 ml of sterile dd$H_2$0 to the pellet in one microcentrifuge tube.

2. Gently re-suspend the pellet by pipetting up and down.

3. Transfer the resuspension to the pellet in the next centrifuge tube, and gently re-suspend the pellet by pipetting up and down.
   Note: Skip step 3 and 4 if using one Oak Ridge tube per lysate.

4. Continue to do so until all the phage pellets have been resuspended in the 0.5 ml of ddH_20.

A. Denature the protein capsid to release phage DNA.

1. Put on a fresh pair of gloves.

2. Add 2 ml of DNA clean-up resin.

   a. The DNA resin is a slurry solution containing microscopic polymer beads. Make sure that the bottle of resin is well mixed, the precipitate dissolved by heating to 37 °C, and the beads resuspended before aliquoting your 2 ml. (Your instructor may have done this for you.)
   Important: The resin contains guanidinium thiocyanate, a chemical that denatures proteins. Do not get it on your skin!

3. Mix the solution by gently inverting the tube repeatedly for 2 minutes.

B. Isolate the phage genomic DNA.

1. Label two Wizard Kit columns with your initials.

2. Remove the plungers from two 3 ml syringes and attach a column to each syringe barrel.

3. Follow the steps below for each column at the same time:

   a. Set the column and syringe barrel on a new microcentrifuge tube.

   b. Transfer 1.25 ml of phage DNA/resin solution to the column using a pipette.

   c. Do not discard the empty 15 ml conical tube.

   d. Insert a plunger into the syringe and carefully push all the liquid through, collecting the flow-through in the used 15 ml conical tube from above.
   Important: The DNA is bound to the polymer beads that pack into the column as the liquid is pushed through. It is VERY important to maintain a firm, gentle, unrelenting, and even pressure on the syringe. Do not let the plunger pop out of the syringe barrel because releasing the vacuum will ruin the column.

   e. Once the liquid is expelled, maintain pressure on the plunger as you dry residual liquid by touching the tip of the column to a paper towel.

   f. Unscrew the column from the syringe barrel before releasing the plunger and set the column into a clean microcentrifuge tube.

g. Remove the plunger from the syringe barrel, and then reattach the syringe barrel to the column.

4. Wash the salts from the DNA (now in the column) with the following steps for each column:

    a. Add 2 ml 80 % isopropanol to each syringe barrel/column and push the liquid through the column, repeating steps 3(d)–3(f).

    b. Repeat twice, for a total of three isopropanol washes.

5. Remove residual isopropanol.

    a. With each column in a fresh 1.5 ml microcentrifuge tube, spin at 10,000 × g for 5 minutes.

        i. The column will prevent the microfuge tube lids from closing. Arrange the open tubes in the centrifuge so that the lids point toward the center of the rotor.

    b. Transfer columns to new 1.5 ml microcentrifuge tubes. Spin at 10,000 × g for 1 additional minute to remove any residual isopropanol.

    c. Evaporate the last traces of isopropanol by removing your columns from the microcentrifuge tubes and placing them directly in a 90 °C heating block for 60 seconds.
    Important: Leaving the columns in the heat block for more than 1 minute can lead to DNA damage.

6. Elute the phage DNA from the columns.

    a. Place each column in a clean microcentrifuge tube and apply 50 µl of 90 °C sterile ddH_2O directly to each column.
    Important: Keep the ddH_2O in the heating block so that it remains at 90 °C.

    b. Incubate columns for 1 minute at room temperature.

    c. Spin at 10,000 × g for 1 minute in a microcentrifuge.

    d. Combine the products from both microcentrifuge tubes into one tube; this is your eluted phage DNA.

C. Determine the concentration of your phage DNA.

1. Using a spectrophotometer (fluorimeter, or Nanodrop) and a protocol from your instructor, quantify your phage DNA.

2. Place at 4 °C for short-term storage (1–2 weeks) or at -20 °C for long-term storage.

Helpful Tips:

- Spinning above 10,000 x g in the microcentrifuge can lead to the column getting stuck in the microcentrifuge tube, the microcentrifuge tube breaking, and both getting stuck in the microcentrifuge.

- Insufficient rinsing with isopropanol will lead to residual guanidinium that will affect your ability to estimate DNA concentration via spectrophotometry.

- Failure to remove all of the isopropanol can cause problems when loading agarose gels later.

Protocol 9.2b: Phage DNA Extraction Following Precipitation with Zinc Chloride

Objective :

To concentrate liquid phage samples prior to extracting DNA

Rationale:

Once you have a prepared a phage lysate, your phage DNA can be isolated and studied. If you have a lysate with a titer lower than 5 x 10^9 PFU/ml, the phage can be concentrated prior to DNA isolation to increase yield. The first step in extracting phage DNA is to remove bacterial DNA and RNA from your lysate using nuclease enzymes. After precipitating you phage using zinc chloride, and pelleting in a high speed centrifuge, the phage DNA is released from the phage capsid, bound to a solid matrix according to its inherent chemical properties, rinsed, and eluted, yielding a sample of pure phage DNA.

Supplies:

- phage lysate (4 - 10 ml)
- 15 ml conical tube
- 2 M $ZnCl_2$ (25 µl per 1 ml of phage lysate), freshly prepared and filter sterilized
- Nuclease mix
- 0.1 M EDTA (0.5 ml)
- Proteinase K (20 mg/ml) and SDS (10 %) (optional)
- 2 ml DNA clean-up resin (Promega Wizard DNA Clean-Up Kit)
- 2 DNA clean-up columns (Promega Wizard DNA Clean-Up Kit)
- 3 or 5 ml syringes
- 6 ml 80 % isopropanol, freshly prepared
- ddH_2O pre-warmed (95 °C)

Procedure:

A. Prepare your bench and assemble your supplies.

B. Degrade bacterial DNA/RNA in ml phage lysate.

1. Aseptically transfer 4 - 10 ml of phage lysate into a 10 ml conical tube.

2. Wearing gloves and working in the designated area, add 5 µl nuclease mix (per 1 ml phage lysate) to the lysate.
   Important: The enzymes (RNase in particular) are very stable and can persist and contaminate equipment and supplies throughout the laboratory. Take precautions to keep and use them in the designated area.

3. Mix gently but thoroughly by repeated inversions, and incubate at 37 °C for 10 minutes or at room temperature for 30 minutes.

C. Precipitate phage particles.

1. Using a 5 ml pipette, portion out the nuclease-treated lysate as 1 ml aliquots in clean microcentrifuge tubes.

2. To each 1 ml of nucelase-treated lysate, add 25 µl $ZnCl_2$.

3. Mix gently but thoroughly by inversion.

4. Incubate at 37 °C for 10 minutes.

5. Place the tube in a high-speed centrifuge, balance the tubes, and spin at 10,000 × rpm for 1 minute.

6. Remove and discard the supernatant from each microcentrifuge tube using a micropipettor, being careful not to disturb the pellets. This pellet contains your phage particles. Proceed immediately to Step D.

D. Re-suspend the phage pellet in 1.0 ml of EDTA.

1. Distribute a total of 1.0 ml of 0.1M EDTA equally between all the microcentrifuge tubes containing a pellet from Step C6. For example, if you have 5 microcentrifuge tubes from Step C6, add 200 µl of EDTA to each microcentrifuge tube.

2. Working quickly but gently, re-suspend each pellet by pipetting up and down.

3. Once all the pellets have been resuspended, combine all the resuspensions into one microcentrifuge tube.

4. Optional: Add 0.5 µl Proteinase K and 50 µl SDS to the nuclease-treated lysate and mix gently. Incubate at 37 °C for 10 minutes.

   a. Proteinase K is added to degrade the nucleases added in Step 2. SDS stimulates the activity of Proteinase K.

A. Denature the protein capsid to release phage DNA.

1. Put on a fresh pair of gloves.

2. Transfer the 1.0 ml of phage pellet resuspension to a clean 15 ml conical tube.

3. Add 2 ml of DNA clean-up resin to the phage pellet resuspension.

    a. The DNA resin is a slurry solution containing microscopic polymer beads. Make sure that the bottle of resin is well mixed, the precipitate dissolved by heating to 37 °C, and the beads resuspended before aliquoting your 2 ml. (Your instructor may have done this for you.)
    Important: The resin contains guanidinium thiocyanate, a chemical that denatures proteins. Do not get it on your skin!

4. Mix the solution by gently inverting the tube repeatedly for 2 minutes.

A. Isolate the phage genomic DNA.

1. Label two Wizard Kit columns with your initials.

2. Remove the plungers from two syringes and attach a column to each syringe barrel.

3. Follow the steps below for each column at the same time:

    a. Set the column and syringe barrel on a new microcentrifuge tube.

    b. Transfer 1.5 ml of phage DNA/resin solution to the column using a pipette.

    c. Do not discard the empty 15 ml conical tube.

    d. Insert a plunger into the syringe and carefully push all the liquid through, collecting the flow-through in the used 15 ml conical tube from above.
    Important: The DNA is bound to the polymer beads that pack into the column as the liquid is pushed through. It is VERY important to maintain a firm, gentle, unrelenting, and even pressure on the syringe. Do not let the plunger pop out of the syringe barrel because releasing the vacuum will ruin the column.

    e. Once the liquid is expelled, maintain pressure on the plunger as you dry residual liquid by touching the tip of the column to a paper towel.

    f. Unscrew the column from the syringe barrel before releasing the plunger and set the column into a clean microcentrifuge tube.

    g. Remove the plunger from the syringe barrel, and then reattach the syringe barrel to the column.

4. Wash the salts from the DNA (now in the column) with the following steps for each column:

    a. Add 2 ml 80 % isopropanol to each syringe barrel/column and push the liquid through the column, repeating steps 3(d)–3(f).

b. Repeat twice, for a total of three isopropanol washes.

5. Remove residual isopropanol.

    a. With each column in a fresh 1.5 ml microcentrifuge tube, spin at 10,000 × g for 5 minutes.

        i. The column will prevent the microfuge tube lids from closing. Arrange the open tubes in the centrifuge so that the lids point toward the center of the rotor.

    b. Transfer columns to new 1.5 ml microcentrifuge tubes. Spin at 10,000 × g for 1 additional minute to remove any residual isopropanol.

    c. Evaporate the last traces of isopropanol by removing your columns from the microcentrifuge tubes and placing them directly in a 90 °C heating block for 60 seconds.
    Important: Leaving the columns in the heat block for more than 1 minute can lead to DNA damage.

6. Elute the phage DNA from the columns.

    a. Place each column in a clean microcentrifuge tube and apply 50 µl of 90 °C sterile ddH_2O directly to each column.
    Important: Keep the ddH_2O in the heating block so that it remains at 90 °C.

    b. Incubate columns for 1 minute at room temperature.

    c. Spin at 10,000 × g for 1 minute in a microcentrifuge.

    d. Combine the products from both microcentrifuge tubes into one tube; this is your eluted phage DNA.

B. Determine the concentration of your phage DNA.

1. Using a spectrophotometer (fluorimeter, or Nanodrop) and a protocol from your instructor, quantify your phage DNA.

2. Place at 4 °C for short-term storage (1–2 weeks) or at -20 °C for long-term storage.

Helpful Tips:

- Spinning above 10,000 x g in the microcentrifuge can lead to the column getting stuck in the microcentrifuge tube, the microcentrifuge tube breaking, and both getting stuck in the microcentrifuge.
- Insufficient rinsing with isopropanol will lead to residual guanidinium that will affect your ability to estimate DNA concentration via spectrophotometry.

- Failure to remove all of the isopropanol can cause problems when loading agarose gels later.

Chapter:9 Tips & Hints

Enough DNA?

- DNA yield per phage lysate processed should ideally be >8 µg of purified phage DNA (at a concentration >100 ng/µl), for restriction enzyme digests and DNA sequencing.
  Important: The minimum amount of DNA required for sequencing is 4 µg, at 40 ng/µl.

- There are many little things that can help with the success of the DNA extraction protocol but the most important factor is starting with a phage titer of 10^9 pfu/ml or greater. In our experience, titers below this value do not yield adequate amounts of DNA. However, if a titer greater than 10^9 pfu/ml cannot be obtained, you can try concentrating the sample using several strategies presented below. Regardless of the method you choose to concentrate a phage lysate, it is important to use freshly prepared lysates. This is because phage particles in older lysates are often less stable than in fresh lysates, and the process of concentrating phage can physically damage some fraction of phage particles, often resulting in the release of DNA from the phage particles. Presented here are 3 strategies for concentrating phage:

  1. **Protocol 9.2a: DNA Extraction following Phage Precipitation with PEG.** This protocol involves spinning the lysate at 10,000 x g after precipitation using ZnCl2. It uses ZnCl2 to precipitate phage prior to DNA extraction, and is as reliable for concentrating phage as using PEG. This protocol is faster and does not require centrifugation at 4 °C.

  2. **Protocol 9.2b: DNA Extraction following Phage Precipitation with ZnCl2** uses sticky Pelco tabs in the bottom of a Petri dish to hold the grid in place while the sample, rinses, and stain are deposited on the grid and wicked off using filter paper. It can be difficult to move the grids from the grid box onto the Pelco tab, and off again, without damaging the grid. A video protocol from Gonzaga University is available here.

Treatment with nucleases:

- Digestion time of host genomic DNA with RNase and DNase can be shortened or lengthened by increasing/decreasing the temperature. The enzymes are most active at 37 °C. In a pinch, this time can be shortened to 5 minutes at 37 °C, as the host DNA does not generally interfere with assembly of the phage genome sequences. Likewise, the phage genomic DNA is protected from the enzymes, and a lengthier incubation will not hurt the phage DNA.
  Important: If your phage is particularly unstable, it may be worth incubating the sample at 30 °C or room temperature, instead of 37 °C.

- For some phages, the may be nucleases that are co-purified with your DNA. We have observed this for phages isolated using Microbacterium foliorum. In these instances, the addition of cations during the restriction enzyme digest reaction leads to rapid degradation of phage DNA by those nucleases. To avoid co-purification of nucleases, ProteinaseK can be added during the DNA extraction process to degrade the

nucleases. The addition of ProteinaseK in the DNA Extraction protocol is presented as an optional step.

- It is a good idea to set up a separate area where to work with the nucleases, and use a dedicated set of pipettes, to avoid the dispersal of DNase and RNase around the lab.

Other important considerations when extracting phage DNA:

- It is important to use **freshly made 80 % isopropanol** since the alcohol will evaporate and change in concentration overtime. Too low of an isopropanol concentration in the wash will cause the DNA to elute off the column during the wash step.

- Prior to elution, the column is placed directly in the 90 °C heat block (not inside the 1.5 ml tube, but directly into the block). This step removes any residual isopropanol remaining after the two spins. Again, this is a critical step to assure accurate DNA concentrations, as well as preventing the restriction enzyme reactions from floating out of the wells prior to gel electrophoresis. If RE enzyme samples float when the gels are loaded, you can use DNA loading dye at a final concentration of 2x instead of 1x. Alternatively, you can supplement your loading dye with glycerol to 50 % (v/w). Commercially purchased 6 x loading dye typically has glycerol at 30 % (v/w).
  Important: The DNA is eluted using water heated to 90 °C in the heat block. The kit instructs that TE (Tris EDTA) be used for elution, but the sequencing protocol at the University of Pittsburgh requires the samples be in water.

- Using high quality microcentrifuge tubes with a reinforced lip will prevent the tube from collapsing during centrifugation with the DNA isolation columns. These tubes are available from Eppendorf as Polypropylene, Snap-cap, Flex-tubes (Fisher Scientific Catalog Number 05 402).
  Important: Spinning DNA isolation columns in non-reinforced microcentrifuge tubes beyond 10,000 rpm should be avoided, as the columns can break into the centrofuge tubes and become stuck in the centrifuge rotor.

- All wash steps in the DNA extraction protocol (9.1) are performed on the DNA clean-up columns by forcing isopropanol through the column with a syringe. An alternative protocol (9.1b) has been included in this Instructor's Guide, in which all but the last wash step are performed in a microcentrifuge tube and by centrifugation. The advantage of this alternative protocol is that the tedious task of forcing isopropanol over the column is minimized. Though DNA yields reported using this protocol are similar to that when using protocol 9.1, it should be noted that this protocol has not been extensively tested.
  Important: If using the alternate protocol (9.1b), it will be important to ensure that the resin is not remove along with the supernatant during washes.

Characterization

Chapter 10: Characterization

Chapter 10: An Overview

Another step in characterizing your phage is to analyze its genome. Ideally, we would sequence the genome of every new bacteriophage isolated during the SEA-PHAGES course. Unfortunately, sequencing technology has not yet progressed to the point where this is feasible in the available time frame. Luckily, you can still get a sense of the character of your phage's genome by comparing the genetic "fingerprints" generated by restriction endonuclease enzymes.

Restriction Enzymes

A restriction enzyme recognizes and cuts a specific 4–6 base pair sequence of DNA, called a restriction site. The incubation of a piece of DNA with a restriction enzyme is called a restriction digestion. An example of a restriction site is GGATCC, recognized by the restriction enzyme BamHI. The number of times a particular enzyme like BamHI cuts your phage DNA depends on the number of times the restriction site occurs in the genome. For example, if GGATCC occurs once in your phage genome and the genome is mixed with BamHI in a restriction digestion, the genome will be cut into two fragments of DNA. Whereas if GGATCC occurs twice in the phage genome of a classmate and the genome is mixed with BamHI in a restriction digestion, your classmate's phage genome will be cut twice yielding three fragments of DNA. The number and sizes of fragments that are generated are part of the genetic fingerprint of your phage. By performing multiple restriction digestions with different enzymes, you can create a robust genetic fingerprint of your phage, which can then be compared to those of other phages.

Gel Electrophoresis

You will use gel electrophoresis to separate the DNA fragments in your restriction digestions and visualize the genetic fingerprint of your phage. Gel electrophoresis is a common way to separate and visualize macromolecules like DNA, RNA, and proteins. Even though the exact protocols differ for different macromolecules, the rationale remains the same: charged macromolecules migrate through a molecular sieve (a gel) when an electric current is applied. DNA is negatively charged, and therefore, when placed in a gel and exposed to current, it migrates toward the positive electrode. As the charged macromolecules move through the porous matrix of the gel, the smaller molecules encounter less resistance and move faster than the larger molecules. As a result, the DNA fragments become separated by size.

To perform DNA gel electrophoresis, first the gel is cast by melting agarose (a substance extracted from seaweed) in buffer and then it is poured into the mold of an electrophoresis apparatus where it solidifies after cooling. The gel mold includes a comb that creates depressions called "wells" in the gel into which you pipette your phage DNA sample. Since DNA is invisible to the naked eye, a fluorescent DNA-binding dye is added to the gel prior to pouring. When bound to DNA, the dye fluoresces (glows) when exposed to ultra violet light. As a result, you are able to visualize the location and abundance of the DNA in the gel. It is important to note that these dyes work by binding to DNA—meaning they can also bind to your DNA! Therefore, always be cautious and wear gloves when working with these mutagenic dyes. Traditionally, ethidium bromide (EtBr) has been the most popular dye, but now less toxic alternatives are available and should be considered. After the gel has cooled and set, it is placed in the electrophoresis apparatus, submerged in buffer, and then it is

ready to be loaded with your phage DNA sample. Before loading your restriction digest samples into the gel, you must mix them with a loading buffer. The loading buffer typically contains glycerol—which helps your sample sink to the bottom of the well and not diffuse into the buffer—and one or more dyes such as bromophenol blue. Bromophenol blue is a dye that migrates through the gel at a similar rate as a 300 bp DNA fragment when exposed to electricity. Its use allows you to see your sample as you pipette it into the wells of the gel and to track the movement of the DNA through the gel during electrophoresis.

Once your samples are loaded into the wells on the gel, the electrophoresis apparatus is connected to a power source and an electric current flows through the gel, causing the DNA fragments to begin migrating. Because the different-sized DNA fragments migrate at different speeds, each fragment will end up as a band on the gel that is visible when the gel is exposed to the proper wavelength of light. The number and sizes of these bands are the genetic fingerprint of your phage!

A photograph of your phage's genetic fingerprint (Figure 10.0-1) contains valuable information about your phage that can be used for characterization and comparison purposes. To determine the size of each fragment, or band, as it appears on the gel, a ladder containing DNA fragments of known size is also run on the gel. This ladder can be used to estimate the size of each band from your sample, or, when used to generate a standard curve, to determine the exact size of each band. Once you have determined the number and sizes of the bands from your restriction-digested phage sample, you can interpret them to see how they compare with other characterized phages.

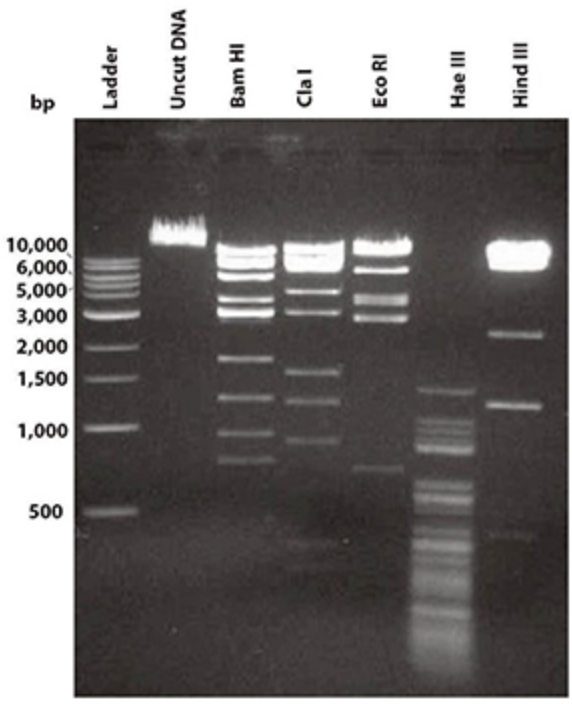


Figure 10.0-1. Restriction enzyme gel. A 0.8 % agarose gel loaded with a 10 kb ladder and phage DNA. Each lane is labeled according to its contents.

Chapter 10: Tips & Hints

- If uncut DNA from Protocol 10.1 appears to be degraded when run on an agarose gel (i.e. a smear without the discrete band typical of uncut phage genomic DNA), there may be nucleases persisting in your DNA prep. Nuclease in your DNA prep, if present, will be inactive because of the lack of cofactors required for activity. However, upon the addition of restriction enzyme buffer (i.e. the uncut control in Protocol 10.1), the necessary cofactors for nuclease activity are introduced. To test for the presence of nucleases in your DNA prep, include an additional control in Protocol 10.1 in which neither restriction enzyme nor buffer are included.

- The percentage of agarose can be adjusted to resolve different sized bands: 0.8 % will resolve larger bands (greater than 10 kb), 2.5 % may help resolve the size of very small bands (less than 500 bp).

- The voltage of the gel can be adjusted to run the gels faster or slower. If you run a gel above 100 Volts, the bands may smear.

- Stains other than EtBr can be used for visualization of DNA bands and may be less toxic, as well as eliminate the use of a UV light box. These dyes are generally more expensive but the benefits may outweigh the cost.

Protocol 10.1: Setting Up Restriction Enzyme Digests

Objective:

To cut your phage genome into multiple fragments based on its DNA sequence

Rationale:

Restriction endonuclease enzymes recognize and cleave DNA at specific 4–6 bp sequences called restriction sites. Digestion of a phage genome with a given restriction enzyme results in the genome being cut into fragments of different sizes, depending on the number and locations of the restriction sites. Since the genome sequences of different phages are highly variable, the frequency and location(s) of a particular restriction site are not constant across all phages' genomes. As a result, digestion of different genomes with the same enzyme may yield different numbers and sizes of DNA fragments. These fragments constitute a genetic "fingerprint" that is unique to each phage.

Supplies:

- Phage DNA
- 37 °C water bath
- 65 °C heat block
- Microcentrifuge tubes
- Restriction enzymes* with buffers

* Specific sets of restriction enzymes are best suited for studying phages isolated on a given bacterial host. Refers to the specific host information page for the recomemnded enzymes to use.

Procedure:

A. Prepare genomic DNA.

1. Gently mix your DNA sample by either flicking the closed tube with your finger or vortexing it on low.
2. Incubate the tube at 65 °C for 10 minutes, and then quickly place it on ice. Quick spin the tube in a microcentrifuge for less than 1 minute to move all of the liquid to the bottom of the tube.
3. Using the concentration of your DNA sample, calculate the volume of DNA sample needed to obtain 0.5 µg of DNA.

   Example: You want to digest 0.5 µg of your phage DNA. If your DNA sample has

a concentration of 125 µg/ml, calculate how many µl of DNA are needed to obtain 0.5 µg:
µl DNA=0.5 µg (ml/125 µg)(1000 µl/1 ml)=4 µl

B. Set up restriction enzyme digest reactions.

1. Set up a reaction in a microcentrifuge tube for each enzyme according to Table 10.1-1. Include a negative control in which no restriction enzyme is added.

Solution	Volume
Sterile ddH_2O	To final volume of 25 µl
10X Reaction Buffer	2.5 µl
Restriction Enzyme	0.5 µl
trial|Phage Genomic DNA	Equivalent to 0.5 µg

2. **Important**: Add your phage DNA last to prevent contamination of the enzyme stocks!

3. Mix the contents of each tube gently and quick spin the tube in a microcentrifuge for less than 1 minute to move all of the liquid to the bottom of the tube.

4. Incubate at 37 °C for up to 1 hour. (Note: Some enzymes may require only 15 minutes, so follow your instructor's directions.)

C. Quick spin the tube in a microcentrifuge for less than 1 minute to move all of the liquid to the bottom of the tube. Store at -20 °C until ready to use.

D. To visualize your digested phage DNA, follow the protocols for **Casting Agarose Gels** (10.2) and **Analyzing Restriction Enzyme Gels** (10.4).

Helpful Tips:

- Do not vortex genomic DNA on high because it can cause it to shear.
- Restriction enzymes must be kept cold at all times. Keep them in an insulated ice block or ice bucket unless they are being used. When handling the tubes, hold them by the top to avoid heating the enzyme with your fingers.
- Be attentive when pipetting small volumes. It is best to pipette small volumes into liquid already in the tube because they can adhere to the pipette tip via cohesion.
- Highly concentrated phage genomic DNA tends to aggregate in solution. Heating for 15 minutes at 55 °C before pipetting can help ensure consistent concentration.
- Remember that 1 ng/µl=1 µg/ml.

Protocol 10.2: Casting Agarose Gels

Objective:

To prepare an agarose gel for electrophoresis

Rationale:

Gel electrophoresis is a common method of separating DNA fragments according to size. The gel is prepared using the polymer agarose that is purchased as a powder, suspended in liquid buffer, and melted before use. The concentration of agarose needed in your gel depends on the size of DNA fragments you want to separate. Typically, agarose gels are prepared at concentrations of 0.7 %–2 %.

Supplies:

- Agarose
- 1X TBE running buffer (TBE = 0.089 M Tris-Boric Acid, 0.002 M-EDTA)
- DNA dye (ethidium bromide or alternative)
- Erlenmeyer flask
- Electrophoresis apparatus and power supply

Procedure:

A. Pour a 0.8 % (w/v) agarose gel.

1. Set up gel apparatus according to your instructor's directions.
2. Prepare enough 0.8 % agarose gel to cover the tips of the gel combs by ~2–3 mm.

   Example: You want to prepare 30 ml of 0.9 % agarose. How much powdered agarose will you add to 30 ml of buffer?

   concentration of solute (w/v, %) = [(mass agarose, *g*) / (volume agarose, *ml*)] x 100
   0.9 % = [(X, *g*) / (30 *ml*)] x 100
   [(0.9 %) (30 *ml*)] / 100 = (X, *g*)
   X = 0.27 *g*

   a. Weigh out the appropriate mass of agarose powder, and then transfer the powder to an Erlenmeyer flask.

b. Add the appropriate volume of 1X TBE buffer to the agarose powder. Swirl gently to mix. Take note of the volume of liquid in the flask.

c. Heat the mixture in the microwave just until it boils (1–2 minutes). As soon as it boils, stop heating.

d. Using a heat-resistant glove or mitt, *carefully* remove the flask from the microwave, remembering to face the open mouth of the flask away from yourself and others.

e. Very gently, swirl to mix and then examine the solution for small transparent clumps. If clumps remain, return the flask to the microwave and continue heating until the mixture boils again. Repeat this process until no clumps are visible.
Important: Take care not to splash the hot liquid or touch the hot flask.

f. Check the volume of the solution. If it has decreased, bring it back to the original volume using ddH_2O. Swirl to mix.

g. Allow the solution to cool to between 50 °C and 60 °C (very warm to the touch but not hot enough to burn). This typically takes 10–15 minutes.

3. Once the solution is sufficiently cool, put on latex gloves and add the gel dye of your choice.

   a. If using ethidium bromide (EtBr) as a DNA dye, add enough to achieve a final concentration of 0.5 µg/ml in the agarose solution. Swirl to mix.
   Important: Use extreme caution when handling EtBr and other DNA dyes. They are mutagens that intercalate (insert) between nucleotide bases.

   Example: You want 100-ml agarose with 0.5 µg/ml EtBr and therefore need to use an unknown volume of 10 mg/ml EtBr stock. (Remember that 10 mg/ml = 10 µg/µl). Using the $C_1V_1 = C_2V_2$ equation:

   (100 ml) (0.5 µg/ml EtBr) = (X) (10 mg/ml)
   (100)(0.5 µg) = X (10 µg/µl)
   X = 5 µl

4. Pour the agarose/EtBr mixture into the prepared gel apparatus, being careful not to introduce any bubbles. Insert the comb to cast the wells.

5. Allow the gel to cool for 20–30 minutes.

6. Once the gel has solidified, very carefully remove the comb by pulling it slowly straight up. Once the comb is removed, gently lift the gel platform out of the casting tray.

7. Place the platform with the solidified gel into the gel box. The wells should be at the (-) cathode end of the box, where the black lead is connected.

8. Pour 1X TBE buffer into the gel apparatus until your gel is submerged by ~ 1/4 inch of buffer.

B. Load your samples and run the gel according to your instructor's directions and the **Gel Electrophoresis** (10.3) protocol.

Helpful Tips:

- If you want to achieve better resolution and separation of smaller fragments you can run up to a 2% agarose gel.
- TAE running buffer can be substituted for TBE.
- When microwaving agarose, use an Erlenmeyer flask or bottle that can accommodate at least 10 times the volume being heated to prevent it from boiling over.

Protocol 10.3: Gel Electrophoresis of Restriction Enzyme Digests

Objective:

To separate DNA fragments via agarose gel electrophoresis

Rationale:

DNA fragments can be separated by size using an agarose gel. When exposed to an electric current, the DNA molecules migrate through the sieve-like agarose gel because of their inherent negative charge. Smaller DNA fragments move more easily, and therefore more quickly, than larger DNA molecules through the meshwork of agarose polymers. After electrophoresis, the gel is illuminated using specific wavelengths of light to visualize the stained DNA and photographed. The sizes of DNA fragments, called bands, are calculated by comparing them to a DNA ladder containing DNA fragments of known size.

Supplies:

- Pre-poured agarose gel
- DNA loading dye
- Electrophoresis apparatus and power supply
- DNA cut with restriction enzymes
- DNA ladder

Procedure:

A. Set up the gel electrophoresis equipment with a gel prepared according to the Casting Agarose Gels(10.2) protocol.

    1. Wearing gloves, orient the gel in such a way that the wells are closest to the cathode (black electrode).

B. Prepare your restriction enzyme digest samples for electrophoresis.

    1. Add 5 µl of concentrated 6x loading dye to each 25 µl restriction enzyme sample.

    2. Place samples at 65 °C, either in a heat block or a hot water bath, for 5 minutes. Immediately place the samples on ice to cool, and then spin them in a microcentrifuge for ~ 15 seconds at 10,000 rpm.

        1. This step prevents annealing of the cohesive ends of phage DNA.

C. Load the gel in the following order:
Ladder Uncut DNA Enzyme-1 Enzyme-2 Enzyme-3 Enzyme-n

1. Carefully load the gel with the proper volume of DNA ladder. Each manufacturer is different, so follow the manufacturer's instructions.

2. Using a fresh tip on your micropipettor for each sample, pipette 20 µl (or as much will fit in the well) of each RE reaction into the wells in the order suggested above.

    1. Holding the pipette in both hands, place your elbows on either side of the gel apparatus.

    2. Situate your eyes directly above the wells to make the wells easier to see.

    3. Place the pipette tip directly above the well, just below the surface of the buffer. Do not try to get the pipette tip into the well, or you might puncture the bottom of the gel.

    4. Slowly depress the pipette plunger, allowing the solution to slowly sink into the well.

    5. Remove the pipette from the gel before releasing the plunger.

3. Draw a picture of the gel in your lab notebook, making note of where your samples are relative to your classmates' samples. (Once the gels are loaded, everyone's samples look alike!)

4. Plug the electrodes into the appropriate locations on the power supply. Turn on the power supply and set the voltage to 100 V.
Important: Remember, DNA runs toward the RED electrode!

5. Run the gel until the blue dye front has migrated at least 3.5 inches from the well. This will take approximately 1 hour.

6. Turn off the power supply.

D. Photograph the gel.

1. Using gloves, carefully remove the gel from the electrophoresis chamber.

2. Photograph your gel and obtain a copy of the gel photograph, following your instructor's directions.

3. Label your copy of the gel photograph to include the names of each enzyme used above its corresponding lane. Below is an example of a properly labelled gel photograph (Figure 10.3-1).

4. Include this labelled copy of the gel photograph in your laboratory notebook and upload a copy to phagesDB.

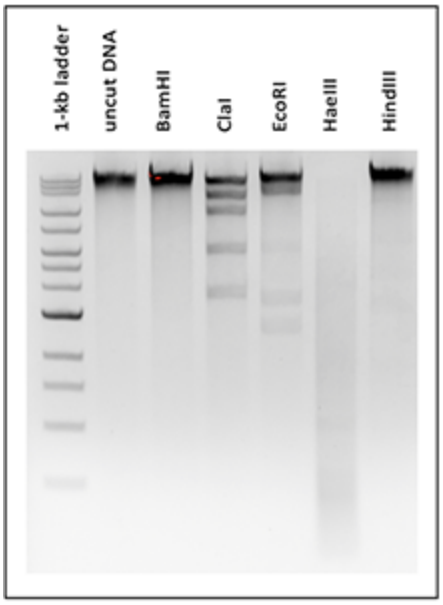


Figure 10.3-1. Example of a well-labelled restriction enzyme gel photograph, which includes information about the various enzymes used.

E. Clean up your work area.

1. If another gel isn't going to be run, empty the buffer into the sink, rinse out the gel apparatus, and set it aside to dry. (The buffer can be reused, so check to see if anyone else will need to run a gel in the next few days.)

2. Dispose of your gel as directed by your instructor.

Helpful Tips:

- Loading dyes are prepared at a higher concentration than used in the gel. If your loading dye is labeled as 6X, this means it is six times as concentrated as its working concentration (1X).

- If your samples float when you try to load the gel, they contain residual isopropanol. Consult with your instructor, who may suggest you add additional DNA loading dye. The excess glycerol should cause the samples to sink to the bottom of the well.

Protocol 10.4: Analyzing Restriction Enzyme Gels

Objective:

To examine and interpret the results of your restriction enzyme digest

Rationale:

DNA fragments of a phage genome cleaved by restriction enzymes can be separated by size using gel electrophoresis. The result is a series of DNA bands on the gel that reflect the sizes of each DNA fragment. The number of DNA fragments and the size of each fragment is a genetic “fingerprint” for your phage and can be used as a diagnostic tool for comparing phages.

Supplies:

- Photo of gel
- Ruler
- DNA ladder with band sizes

Procedure:

A. Perform a qualitative gel assessment.

1. Obtain a printout of your gel photo or access a digital image.

a. Label each lane according to its contents.

b. Using the “map” of the DNA ladder, label each ladder band with its size in base pairs.

c. Looking at each lane, and the gel as a whole, answer these questions:

i. Is there DNA in each lane? How do you know?

ii. Did each restriction enzyme cut the phage DNA? How do you know?

iii. Which restriction enzyme cut the most? Which cut the least?

iv. If there is a problem with your gel, can you identify the cause?

d. Upload a labeled digital image of your gel to the profile for your phage that you created on phagesDB.

B. Perform a quantitative gel assessment—calculate band size.

1. Using a ruler and a pen, draw a line at the top of the gel photo, above the wells or below the wells. The line will act as a reference for measuring the distance migrated by the DNA (Figure 10.4-1).

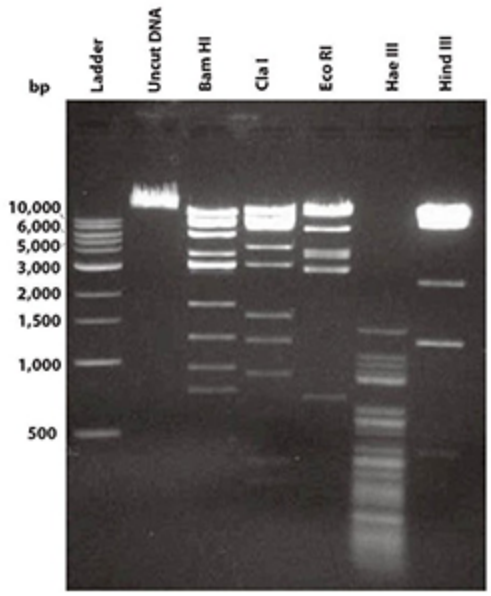


Figure 10.4-1. Sample restriction enzyme gel. Agarose gel with lanes and ladder labeled.

2. Make a standard curve of the DNA ladder.

    a. Using Table 10.4-1 as a guide, make a table in your notebook or an Excel spreadsheet.

    b. Fill in the values for known DNA fragments sizes of the ladder.

        i. DNA ladders (also known as standards or markers) contain DNA fragments of known sizes. The fragment sizes are manufacturer-specific; therefore, it is important to use the proper map when assigning band sizes to your ladder.

    c. Measure the distance migrated, in centimeters, of each band in the ladder. Start at the line you drew at the top of the gel and stop at the top of each band.

    d. Record these data in the table or spreadsheet.

    e. Create a semi-log plot of these data points to create a standard curve. The graph can be made by hand or by using Excel.
    Table 10.4-1. Sample data table for creating DNA standard curve for agarose gel shown in Figure.

Distance Traveled (mm)	DNA Ladder Fragment Size (bp)
15	10,000

17	6,000
20	3,000
23	2,000
27	1,500
31	1,000
50	500

.

To create a graph by hand on semi-log graph paper:

i. Label the axes according to the example in Figure 10.4-2

- The y-axis is divided into three “cycles,” each with 10 sections representing logarithmic distances. Using a semi-log plot lets you visualize exponential data without calculating the logarithm.

ii. Plot the data accordingly and create a “best fit” line.

To create a graph using a spreadsheet in Excel:

iii. Select the data entered in your spreadsheet in the format shown in Table 10.4-1.

iv. Insert a marked scatter plot.

v. Format the y-axis to use a logarithmic scale, add major and minor gridlines, and label the axes.

vi. Right-click a data point to add a trend line, making sure it is exponential. Have the line equation displayed on the chart.

3. Estimate restriction fragment lengths by using the standard curve. For each restriction enzyme:

   a. Measure the distance (cm) migrated, from the line drawn through the wells to the top of each band.

   b. Find this distance on the x-axis of your graph and move straight up until you intersect your standard curve. From that point, move left until you intersect the y-axis. This value is the size (bp) of your restriction fragment.

   c. Make a table of these data in your notebook.

      i. You can double-check your estimates by substituting the distance migrated (x) into the equation of the best-fit line and calculating the size in bp (y).

4. Compare the results from your gel with those for other bacteriophage

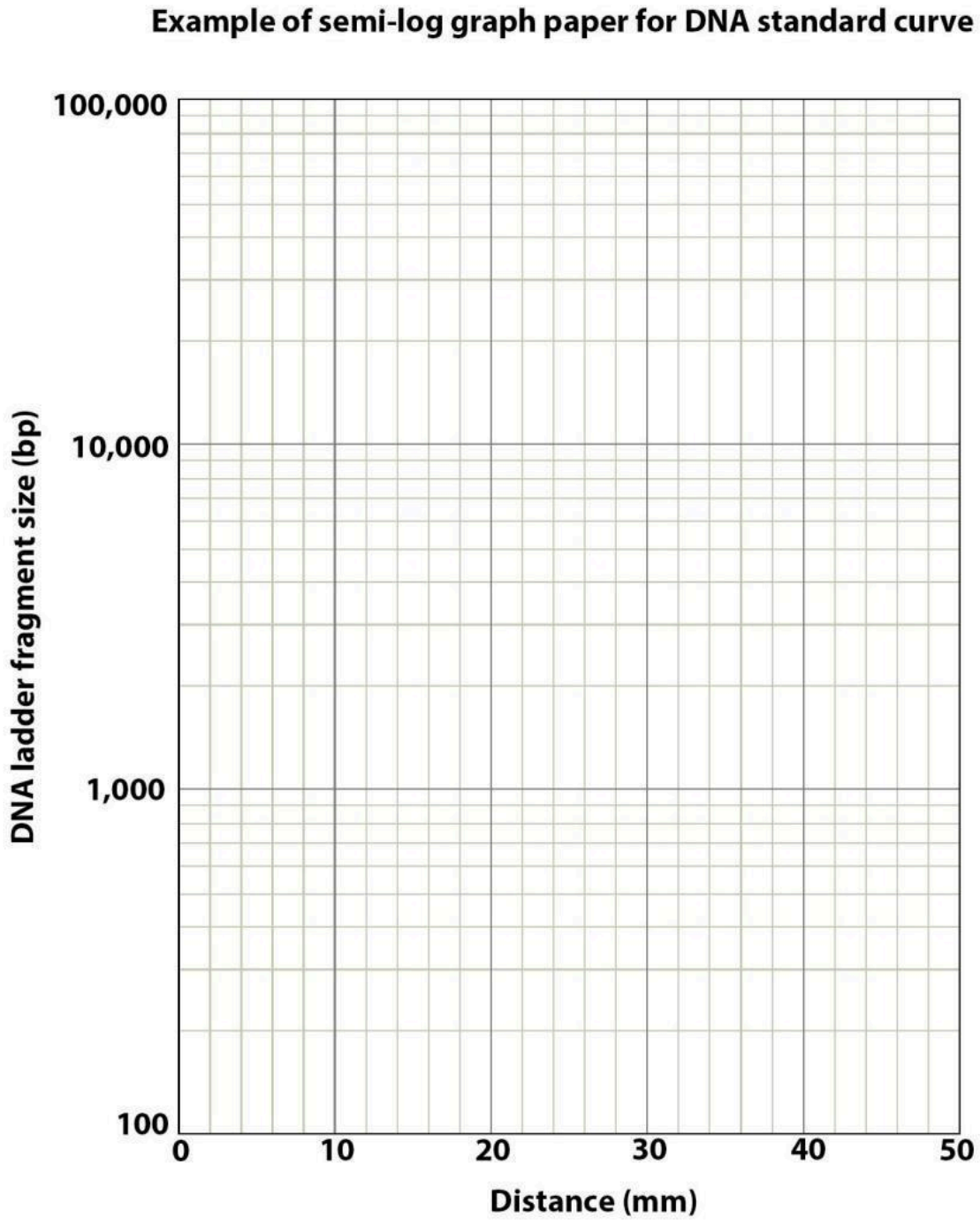


Figure 10.4-2. Example of semi-log graph paper for DNA standard curve. The x-axis is logarithmic, allowing exponential data to fit on the graph and be linearized without taking the logarithm. The y-axis is the distance migrated by the bands in the DNA ladder.

Furthur Discovery

Chapter 11: Further Discovery

Chapter 11: An Overview

The microbial world is a dynamic place, constantly changing and evolving. Bacteria must compete for resources, such as food, oxygen, and light, and aggressively defend themselves against other microbes to be successful. Bacteria have many ways of rendering themselves insensitive to phage infection; consequently, bacteriophages must constantly evolve to remain competitive. This continual warfare drives the gene flow between bacteria and bacteriophages. Whereas some of the mechanisms by which bacteria can become sensitive or insensitive to phage infection are well understood (and are described below), many more remain to be discovered.

Mechanisms of Bacterial Insensitivity

Bacterial insensitivity to phage infection can be categorized, according to mechanism, as immunity, exclusion, or resistance. Bacteria can acquire insensitivity to infection if phage proteins from a previous phage infection are expressed. This insensitivity to infection is due to either **immunity** or **exclusion**, depending on the phage proteins expressed and how they act to prevent further infection.

Bacteria can also acquire insensitivity to phage infection because of DNA mutations that cause changes in the bacterial receptor that is recognized by a phage for infection. In addition, bacteria may possess phage defense systems such as restriction endonucleases or CRISPR arrays (discussed in Chapter 1) that protect them from phage infection. Bacterial insensitivity resulting from these kinds of defense systems, including mutation, is called resistance. Immunity, exclusion, and resistance are described in more detail below.

Lysogeny

A basic characteristic of a phage is its life cycle. The phages isolated in the SEA-PHAGES program are all capable of forming a plaque, which means they can undergo the lytic cycle. However, some phages that form plaques have an alternate life cycle option; that is, after infection this type of phage represses the lytic functions of its genes and has its genome stably maintained within the bacterial cell throughout multiple rounds of cell division. This latent form of phage is called a prophage. **Lysogeny** occurs when the phage genome is integrated into the bacterial genome through homologous recombination or through the circularization of the phage genome and its maintenance as a large plasmid in the bacterial host. Bacterial cells that carry the phage genome are called **lysogens** and phages that form them are said to be **temperate**.

Repressors

During lysogen growth the expression of genes involved in the lytic cycle is repressed. In many actinobacteriophages this occurs through the constant expression of a protein known as an immunity repressor. This repressor protein binds to specific sequences on the prophage genome at sites called **operators** to block transcription initiation or at sites called **stoperators** to prevent protein elongation. This means that the lysogen contains excess immunity repressor proteins that diffuse freely through the bacterium during its normal life cycle. Should another phage infect the lysogen (a process called **superinfection**), the immunity repressor proteins can bind to the new invading phage DNA if that genome

contains the same specific operator and stoperator sequences. In this way, immunity repressor proteins offer the **lysogen** immunity to superinfection from phages identical or closely related to the prophage.

Immunity repressors are highly specific for their operator and stoperator sequences. Even a single base change in the operator/stoperator sequence can alter or prevent binding to the target DNA. Therefore, phages that cannot infect a lysogen, because of actions of the immunity repressor, share at least some genomic similarity to the prophage's operator/stoperator sequences. Frequently, the inability of two different actinobacteriophages to infect each other's lysogens, called **homoimmunity**, is a property shared by members of the same subcluster. It is important to note that lysogens are only considered "immune" to superinfection if the immunity repressor is the mechanism by which infection is prevented.

Exclusion

In addition to the immunity repressor protein, some phages express proteins that can alter the surface of the bacterial cell. When these proteins or carbohydrates appear on the bacterial cell surface, the prophage can prevent other phages from binding to the cell and injecting their DNA. This ability is called surface **exclusion**. Unlike immunity, which is generally restricted to genomically related phages, exclusion depends on interactions between the tail fiber(s) of the infecting phage, which is the primary means of attachment to the cell, and the receptor on the surface of the lysogen. Tail fibers are highly mosaic and modular, and they may or may not be shared among phages that are otherwise very similar at the genomic level.

Other Factors

Finally, it is important to note that not that every phage infection of a bacterial population will yield intrinsically insensitive host cells. There have been cases of transient insensitivity owing to the stage of a bacterial cell cycle as well as permanent resistance caused by mutation. A liquid culture of bacteria in stationary phase likely has 10^8–10^9 cells per milliliter! A mutant present in the culture at only a few tenths of a percent will rapidly become the dominant strain when the bulk of the cells in the culture are destroyed by phage infection.

So how do you tell the difference between a true lysogen and a naturally resistant bacterial cell in the lab? Lysogens will be insensitive to superinfection from all subsequent attempts at infection with the initial phage—no matter how many times the lysogen is streak-purified or regrown from a liquid stock. Also, liquid cultures of lysogens will release phage into the supernatant of the culture. This is because some of the cells in the population are undergoing spontaneous induction of the lytic cycle at all times. And finally, the gold standard for lysogen confirmation is to demonstrate that the prophage is in the cell. This can be done using a technique called polymerase chain reaction (PCR) and amplifying the new junction between the bacterial and phage genomes. Or confirmation can be done by carefully repeating rounds of purification and then sequencing of all of the DNA in the cell to look for the presence of the prophage genome.

It is possible to isolate lysogens by carefully purifying them away from exogenous infecting phage. Once isolated, lysogens can be used to create bacterial lawns to test the ability of other phages to infect the new lysogen. This immunity data can be helpful in characterizing

a whole collection of sequenced or unsequenced phages. It is likely that temperate phages carry a variety of genes that are expressed in lysogens and influence phage sensitivity. Therefore, exploring these patterns can provide valuable insights into phage mechanisms of bacterial sensitivity and insensitivity to bacteriophage.

Protocol 11.1: Creating Lysogens by Streaking from a High Titer Spot

Objective:

To create a lysogen with your phage

Rationale:

The phages isolated in the SEA-PHAGES program are all capable of forming plaques, which means they can undergo the lytic cycle. However, some phages that can form plaques are temperate phage; that is, after infecting a host bacterium this type of phage can repress the lytic functions of its genes, with the result that its genome is stably maintained within the host cell throughout multiple rounds of cell division. The host bacterium that contains a stable copy of a phage genome is called a lysogen. Lysogens are isolated by carefully purifying them away from exogenous infecting phage. Once isolated, lysogens can verified through a patch test, a phage-release test, and a sensitivity assay.

Supplies

- Host bacterial culture (250 µl)
- Agar plate
- Top agar
- Phage buffer
- Microcentrifuge tubes

Procedure:

Day 1

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

B. Create a spot plate of your lysate.

1. Serially dilute your lysate to 10^{-8} according to the Serial Dilutions (6.2) protocol.
2. Make a spot plate according the Spot Titer (6.4) protocol.

Day 2

A. Check plates each day for a “mesa.”

1. A mesa is an overgrowth of bacterial cells surrounded by the zone of clearing caused by phage infection and lysis. It will look like a cloudy center in of one of your spots. A mesa is caused by bacterial cell growth in the presence of phage and therefore is an excellent source of lysogens.

    a. A mesa will not likely appear on day one. A true mesa is generally as well grown as the unspotted lawn by day two or three (Figure 11.1-1).

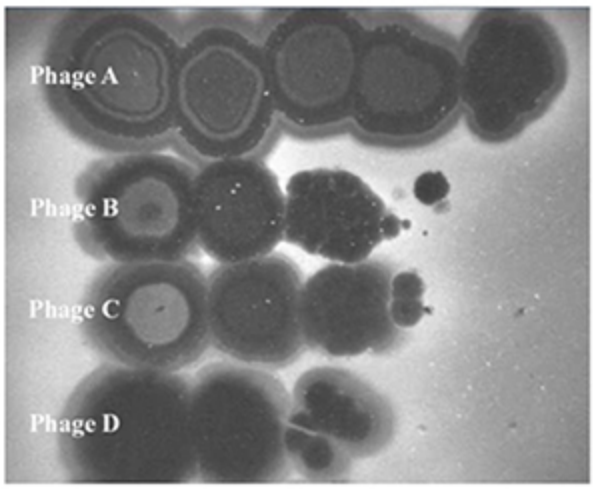


Figure 11.1-1. This picture depicts four phages spotted at 10-fold dilutions (100 - 10-4). A mesa is recognizable for phages A, B, and C. However, phage D may also have produced lysogens. Continue with the protocol regardless of whether there is visible bacterial growth in the spot. Note that phage A has mesas to at least the 10-3 spot, while phages B and C have visible mesas to 10-2. Warning: do not plate multiple phages for lysogen isolation on a single plate as depicted in the photo; you could select double-lysogens in the process!

B. Streak cells from the mesa onto a new plate.

1. Label the bottom of an agar plate.

2. Using a sterile stick, pipette tip, or sterile loop, touch the mesa growing in the spot.

    a. If you are using a wire loop, sterilize it by heating it in a flame until red hot and then allowing it to cool before picking up the bacteria.

3. Touch the stick or loop to the agar and streak back and forth across the top one-third of the agar. Discard the stick or reflame the loop.

    a. The objective of this step is to spread the bacteria out as much as possible.

4. With a new wooden stick (or sterile loop), drag through the first streak once and continue to streak back and forth in an adjacent one-third of the agar, making sure not to overlap the first streak again.

a. This second streak allows you to spread just a fraction of the bacteria from the first streak, thereby diluting it.

5. Repeat step 4 to create a third streak on the remaining one-third portion of the plate.

    a. This third streak further dilutes the bacteria sample. Hopefully, you will have individual bacterial cells in this third streak that will grow into isolated colonies.

6. Incubate at the appropriate temperature for 2–4 days.

Day 3

A. Test and confirm putative lysogens.

1. Choose 6–10 colonies to test for lysogeny. Not all of them will be lysogens. Verify the lysogens via Patch Assay (Protocol 11.3) and Liquid Phage Release Assay (11.4), or PCR amplify the attachment sites in the lysogen.

Helpful Tips:

- When making a lysogen, you may need three rounds of streak purification to remove exogenous phage from your initial pick from the mesa. Each round requires approximately 4 days of incubation.
- Remember, you will be streaking cells, not phage, as is discussed in the purification protocol. A plate streaked with phage and nothing else will look completely blank no matter how long you incubate it for.
- Never plate for mesas with more than one phage per plate during isolation. You run the risk of creating double lysogens if phage spots are side by side.
- Try to pick up cells from the top of the colony. Do your best to avoid scraping the agar surface around your colony to avoid picking up additional exogenous phage.

Protocol 11.2: Creating Lysogens & Determining Efficiency of Lysogeny using Phage-Seeded Plates

Objective:

To create a lysogen with your phage

Rationale:

The phages isolated in the SEA-PHAGES program are all capable of forming plaques, which means they can undergo the lytic cycle. However, some phages that can form plaques are temperate phage; that is, after infecting a host bacterium this type of phage can repress the lytic functions of its genes, with the result that its genome is stably maintained within the host cell throughout multiple rounds of cell division. The host bacterium that contains a stable copy of a phage genome is called a lysogen. Lysogens are isolated by carefully purifying them away from exogenous infecting phage. Once isolated, lysogens can verified through a patch test, a phage-release test, and a sensitivity assay.

Supplies

- Phage lysate, with a known titer that is >10^{9} PFU/ml
- Host bacteria (250 µl/phage being tested)
- Agar plates (5 plates per phage being tested, and 5 additional plates for the control)
- Phage buffer
- Microcentrifuge tubes
- 5 ml serological pipettes
- Sterile glass beads or spreader

Procedure:

Day 1

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

B. Dilute the phage lysate to 1 x 10^{9} PFU/ml, in a final volume of 500 µl of phage buffer.

C. Seed plates with phage.

1. Obtain 5 agar plates and label them with the name of the phage, the name of the host bacterium, and one of the following five dilutions: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

2. To each of these 5 plates, add 100 µl of your phage sample that has been diluted to 1 x 10^9 PFU/ml.

3. Spread the lysate evenly across the plate, either using sterile glass beads or a spreader.
   Note: If using glass beads, leave the beads in the plate for a subsequent step. If using a spreader, the spreader needs to be sterilized only once and after spreading lysates across all five plates.

D. Prepare a dilution series of host bacteria.

1. Arrange 5 microcentrifuge tubes in a rack and label them 10^{-1}, 10^{-2}, 10^{-3},….10^{-6}.

2. Fill each microcentrifuge tube with 450 µl of phage buffer.

3. Add 50 µl of your undiluted host bacteria culture to the tube labelled "10^{-1}" and mix by pipetting up and down.

4. Using a new clean pipette tip, transfer 50 µl of the "10^{-1}" sample to the "10^{-2}" tube and mix well.

5. Continue each successive dilution until you get to your last tube.

E. Spread host bacteria on control plates.

1. Obtain 5 new agar plates and label them with the name of the host bacterium and one of the following five dilutions: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.
   Note: these are your control plates.

2. To each of these 5 plates, add 100 µl of host bacteria of at corresponding dilution. For example, add 100 µl of host bacteria at the 10^{-2} dilution onto the plate labelled with that dilution.

3. Spread the culture evenly across the plate, either using sterile glass beads or a spreader.
   Note: If using a spreader, the spreader needs to be sterilized only once and after spreading culture across all five plates.

F. Spread host bacteria on phage-seeded plates.

1. Obtain the 5 agar plates that have already been seeded with phage.

2. To each of these 5 plates, add 100 µl of host bacteria of at corresponding dilution. For example, add 100 µl of host bacteria at the 10^{-2} dilution onto the plate labelled with that dilution.

3. Spread the culture evenly across the plate, either using sterile glass beads or a spreader.
   IMPORTANT: If using a spreader, and unlike before, the spreader must be sterilized after each plate.

G. Incubate the plates at the appropriate temperature, and for the duration is typically takes for this host bacterium to form colonies, typically 2 – 5 days (see Host Basics).

Day 2

H. Check plates each day for colony formation.

1. Colonies that form on the phage-seeded plates are mostly lysogens, though a small fraction of host bacteria may contain a mutation that renders them insensitive to infection by your phage and can also form colonies on the phage-seeded plates.

I. Once colonies and clearly visible, photograph all plates.

J. Calculate the rate of lysogeny, also known as the Efficiency of Lysogeny (EOL).

1. EOL = [(CFU on a phage-seeded plate) / (CFU on the corresponding control plate)] x 100.

   Note: CFU = colony forming units (i.e. colonies that form on a plate). The best plates to use for counting colonies contain 20 – 500 colonies.

   For example, if there are 250 colonies on the phage-seeded plate with the 10^{-5} dilution of host bacteria, and there are 500 colonies on the corresponding control plate with the 10^{-5} dilution of host bacteria, then
   EOL = [(250)/(500)]*100 = 50 %

2. The efficiency of lysogeny for a temperate phage can range from 0.1% - 100%.

K. Purify 5 – 10 candidate lysogens for verification.

1. Obtain as many agar plates as candidate lysogens that will be verified.

2. Label the bottom of the agar plate.

3. Using a sterile stick, pipette tip, or sterile loop, pick a candidate lysogen from a phage-seeded plate.
   Note: Pick a representative and well-isolated colony.If you are using a wire loop, sterilize it by heating it in a flame until red hot and then allowing it to cool before picking up the bacteria.

4. Touch the stick or loop to the new agar plate and streak back and forth across the top one-third of the agar. Discard the stick or reflame the loop.

   i. The objective of this step is to spread the bacteria out as much as possible.

5. With a new wooden stick (or sterile loop), drag through the first streak once and continue to streak back and forth in an adjacent one-third of the agar, making sure not to overlap the first streak again.

i. This second streak allows you to spread just a fraction of the bacteria from the first streak, thereby diluting it.

6. With a new wooden stick (or sterile loop), drag through the second streak once and continue to streak back and forth in an adjacent one-third of the agar, making sure not to overlap the first streak again.

    i. This third streak further dilutes the bacteria sample. Hopefully, you will have individual bacterial cells in this third streak that will grow into isolated colonies.

7. Repeat Step S3 – S6 for each candidate lysogen to be verified.

8. Incubate the plates at the appropriate temperature to allow for colonies to form.

Day 3

L. Check streak plates each day for colony formation.

M. Purify the candidate lysogens further through one more round of streak for colonies.

1. Select an isolated and representative colony for each candidate lysogen, and repeat Steps S1 - S8.

Day 4

N. Verify the lysogens via Patch Assay (Protocol 11.3) and Liquid Phage Release Assay (11.4)

Helpful Tips:

- Try to pick up cells from the top of the colony. Do your best to avoid scraping the agar surface around your colony to avoid picking up additional exogenous phage.

Protocol 11.3: Verification of Potential Lysogens via Patch Assay

Objective:

To identify lysogens by patching putative lysogens onto a layer of host cells and incubating to observe clearing in the lawn caused by lysis

Rationale:

All lysogen cultures release phages into the environment as the individual cells spontaneously undergo the lytic cycle. Thus, it is possible to differentiate true lysogens from insensitive bacterial cells by testing for the presence of phage in the cells. This method tests for phage release by patching putative lysogens onto a host-seeded plate, incubating them until cell growth is visible, and observing signs of cell lysis.

Supplies

- Plates with bacterial colonies that are potential lysogens
- Agar plates
- Top agar
- Host bacterial culture (250 ml)
- Sterile sticks or wire loops for bacterial streaking

Procedure:

Day 1

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

B. Prepare two plates for this procedure.

1. Draw identical grids on the bottom of two agar plates and number the squares 1–10 (or however many putative lysogen colonies you are testing).

2. Label both plates. One plate is your experimental plate on which you will observe lysis around lysogens on a host cell lawn after incubation; the other plate is your lysogen-only plate, which will contain your patched lysogens.

    a. You will pick confirmed lysogens from the lysogen-only plate for future experiments.

3. Obtain a 250 µl culture of host bacteria.

4. For this part of the experiment you will need 3 ml of molten top agar. Your instructor may provide this for you or you may need to make it according to protocol found in the Toolbox .

5. Using a sterile 5 ml pipette, transfer 3 ml of molten top agar to a culture tube containing host bacteria and then immediately draw the solution back into the same pipette.

    a. Dispense the top agar-bacteria mixture onto the experimental plate ONLY.

    b. Gently, but quickly, tilt the plate in multiple directions until the top agar mixture evenly coats the agar plate.

    c. Do not prepare a lawn on the lysogen-only plate.

6. Let the top agar set and dry completely. This should take approximately 15 minutes.

C. Make your patch plates.

1. Pick a colony from the lysogen streak plate, made according to the protocol Creating and Testing Lysogens , Streak Method (11.1), by lightly touching a sterile loop or sterile toothpick to the center of an isolated colony.

    a. If you are using a wire loop, sterilize it by heating it in a flame until red hot and then allowing it to cool before picking up the bacteria.

2. Streak a small quantity of candidate lysogen cells to each plate by dragging the loop or toothpick gently across the surface of the lysogen-only plate first, followed by the experimental plate (the plate with the prepared bacterial lawn), as shown in Figure 11.2-1.

    a. Do not dig into the agar surface of the places. The surface of the lysogen-only plate is far more firm that that of the experimental plate with the prepared host cell lawn.

    b. You may get better results on the prepared lawn by lightly touching the top agar with the loop or toothpick in a series of 6–8 adjacent “taps” instead of dragging it through the top agar.

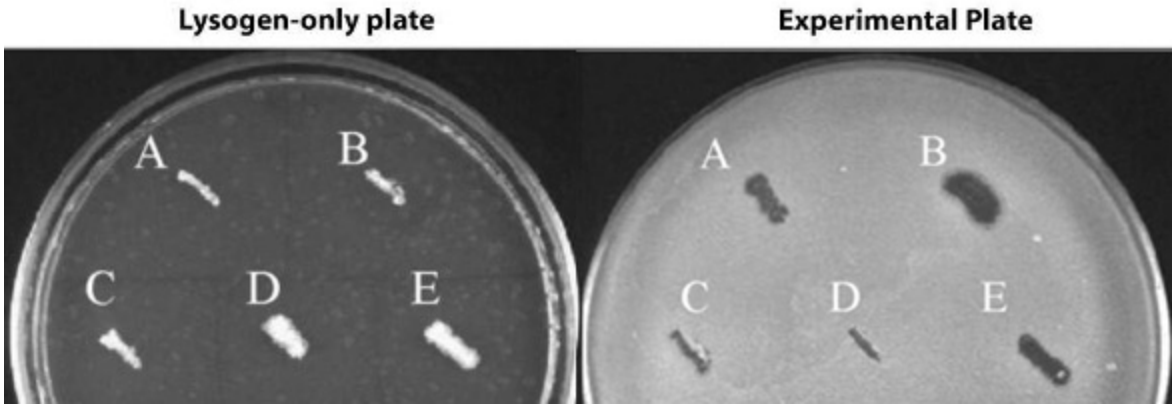


Figure 11.3-1. Patch plates to test putative lysogens. The lysogen-only plate is a plain agar plate with no host bacterial lawn and patched with five putative lysogen colonies. The experimental plate has the same bacterial colonies patched onto a wild-type host bacterial lawn. Patches A, B, and E test positive as lysogens because of clearing from phage release. Patches C and D do not show clearing caused by phage release, but only removal of the lawn from the patching process.

Important: Use the same loop or toothpick for both plates, without getting a new one, because you want the same colony to grow on both plates.

Day 2

A. Identify lysogens.

    a. Look for clearings in the lawn caused by cell lysis around the patches on the experimental plate (Figure 11.3-1). These are candidate lysogens.

B. Streak each positive lysogen candidate from the lysogen-only plate onto an agar plate to isolate single colonies.

    a. Label the bottom of an agar plate.

        a. Indicate if it is your first, second, or third round of lysogen purification.

    b. Using a sterile stick, pipette tip, or sterile loop, touch the center of the lysogen colony you are purifying from the lysogen-only plate.

        a. If you are using a wire loop, sterilize it by heating it in a flame until red hot and then allowing it to cool before picking up the bacteria.

    c. Touch the stick or loop to the agar and streak back and forth across the top one-third of the agar. Discard the stick or reflame the loop.

        a. The objective of this step is to spread the bacteria out as much as possible.

    d. With a new wooden stick (or sterile loop), drag through the first streak once and continue to streak back and forth in an adjacent one-third of the agar, making

sure not to overlap the first streak again.

a. This second streak allows you to spread just a fraction of the bacteria from the first streak, thereby diluting it.

e. Repeat step 4 to create a third streak on the remaining one-third portion of the plate.

a. This third streak further dilutes the bacteria sample. Hopefully, you will have individual bacterial cells in this third streak that will grow into isolated colonies.

f. Incubate at the appropriate temperature for 2–4 days (until single colonies are visible).

C. Repeat the process of picking isolated colonies from the streak plates and streaking to isolation three consecutive times to successfully purify a lysogen.

D. Perform the patch test again on your new lysogen candidates (taken from single colonies from your third round of purification) to confirm that they are indeed lysogens.

a. Unlike the first time you performed the patch test, these cells will have no exogenous phage from the initial high-titer mesa spots associated with the lysogens.

E. You are ready to inoculate a liquid culture for testing using the protocols for Liquid Phage Release Assay (Protocol 11.4) and Sensitivity Assay (11.5).

a. An aliquot(s) of any and all lysogens that are confirmed should be frozen for future use.

Helpful Tips:

- It is very important that potential lysogens are purified away from exogenous phage that may have stuck to the cells during the initial mesa creation. Otherwise, you will never be able to differentiate between a lysogen releasing new phage via spontaneous induction and a mutant resistant cell line that has been coated in a large amount of the initial phage.

- Make sure that the top agar is completely set before you make your patches.

Protocol 11.4: Verification of Potential Lysogens via Liquid Phage Release Assay

Objective:

To determine if a putative lysogen is releasing phage into a liquid culture

Rationale:

In a lysogen culture phages are constantly being released because of spontaneous induction of the lytic cycle. A liquid culture of a lysogen will have a fairly high titer of phage in the supernatant that can be detected via spot titering on a wild-type host lawn.

Supplies:

- Purified streak plate of lysogen candidate
- Liquid bacterial growth media
- Culture tubes
- Top agar
- Agar plate
- Microcentrifuge tubes

Procedure:

Day 1

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

B. Make a liquid culture of a potential lysogen.

1. Using a sterile stick, pipette tip, or sterile loop, pick a single colony of your potential lysogen from a streak purification plate prepared according to the protocol for **Creating Lysogens & Determining Efficiency of Lysogeny: Phage-Seeded Plates (Protocol 11.2)** **or** **Verification of Potential Lysogens: Patch Assay (11.3)**.

2. Inoculate 5 ml of appropriate medium (in a culture tube) with the picked colony by swishing the stick, tip, or loop in the media.

    a. Incubate the tube by shaking it at 220 rpm for 2–4 days.

    b. This culture can also be used to prepare sensitivity assays and to create a freezer stock of your lysogen.

Day 2

A. Prepare a lawn with wild-type host cells and top agar according to the Spot Test (5.6) or Spot Titer (6.4) protocols.

B. Prepare the supernatant of the lysogen liquid culture.

1. Pipette 500 µl of your potential liquid lysogen culture into a 1.5 ml microcentrifuge tube.
2. Spin at top speed in a microcentrifuge for 1 minute to pellet the bacterial cells.
3. Remove 100 µl of the supernatant, and transfer it to a new 1.5 ml microcentrifuge tube.
4. Make 10-fold serial dilutions of the supernatant to 10-8 in phage buffer according to the Serial Dilutions (6.2) protocol.
5. Pipette 3 µl spots of each dilution onto the prepared lawn and let them sit until they are absorbed.
6. Incubate at the appropriate growth temperature for 24–48 hours.

Day 3

A. Observe the spot plates for plaques.

1. If individual plaques appear in the higher dilution spots (i.e., most dilute samples) you may have a stable lysogen in your initial liquid culture.
2. If no plaques appear, this is most likely NOT a lysogen.

B. You can continue to investigate your lysogen by using it to perform a Sensitivity Assay (Protocol 11.5) with other phages.

Helpful Tips:

- Liquid cultures started from single colonies may experience clumping as seen with wild-type *M. smegmatis* $mc^2$155. To avoid this you may want to use the detergent Tween in your starting liquid culture media. If Tween is used, you must passage this first culture into a Tween-free media before testing for phage sensitivity because the presence of Tween in the culture can inhibit phage attachment to the host.
- When you create a freezer stock of your lysogen from a liquid culture, remember to test and save the same liquid culture. Do not assume that different colonies from the same plate are identical.
- Be careful to not confuse “killing from without” with phage infection. Phage infection will yield plaques at low lysate concentrations. “Killing from without” will produce a zone of clearing that dilutes out and will not produce plaques. This is because phages

that can kill a bacterial cell from without are not undergoing a normal lytic cycle. Killing from without occurs when cells are exposed to a high concentration of phage and die without the phage propagating normally.

Protocol 11.5: Sensitivity Assay

Objective:

To test the ability of a phage or panel of phages to infect a confirmed lysogen

Rationale:

Sensitivity assays are spot titers performed on a confirmed lysogen lawn to test those cells' sensitivity to phage infection. Immunity to superinfection is defined as the ability of a lysogen to resist infection from a bacteriophage via its immunity repressor. Although the results will not tell us definitively the mechanism behind cellular insensitivity to phage infection, it is a good first line of inquiry.

Supplies:

- Liquid cultures of potential lysogens
- Liquid culture of wild-type host cells
- Lysates of phages to test on the lysogens
- Agar plates
- Phage buffer
- Top agar

Procedure:

Day 1

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

   1. You will need a liquid lysogen culture prepared according to the Testing and Verification of Potential Lysogens: Liquid Phage Release (11.3) protocol.

   2. You will also need a liquid wild-type bacterial host culture.

B. Prepare bacterial lawns of each bacteria to be tested, including all potential lysogens and wild-type host cells, according to the Spot Test (5.6) or Spot Titer (6.4) protocols.

   1. Label the plates, including the cell type (wild type, lysogen) and name(s) of the phage(s) being tested.

   2. Let plates solidify.

C. Perform the 10-fold Serial Dilutions (6.2) protocol on the phage lysates you wish to assay for their ability to superinfect the lysogen.

1. Lysates should be diluted to 10-8 in phage buffer.

2. Record the titers of the undiluted lysates in your notebook.

D. Spot dilutions and controls on each prepared bacterial lawn.

1. One at a time, aseptically transfer 3 µl of each lysate dilution onto the prepared bacterial lawns.

   a. Dilutions of up to four lysates can be spotted on a single plate if you pipette carefully.

   b. Always use new tips for each spot. You do not want to contaminate the lysate dilutions with the various cells onto which you are plating.

2. Use 3 µl of sterile phage buffer as the negative control.

3. Allow the liquid from the spots to dry completely.

4. Carefully invert the plates and incubate at the proper temperature for 24-48 hours.

   a. To prevent the spots from running, you can postpone inversion until after the first 24 hours have passed.

Day 2

A. Check for evidence of insensitivity.

1. Check for spots and individual plaques after 24 and 48 hours.

   a. Do you have individual plaques in your higher dilutions (i.e., most dilute samples)? See Figure 11.4-1 for an example.

      i. Calculate the titers of phage in the lysate able to infect the lysogen and the wild-type host according to the Spot Titer (6.4) protocol.

   b. If you have spots only on samples of lower dilutions (i.e., the most concentrated samples), you are probably witnessing “killing from without.” This is a phenomenon by which a high-titer lysate can cause cell lysis when it is spotted on a lawn. However, spots of more dilute samples do not produce individual plaques. Killing from without is not indicative of immunity.

2. Photograph your results.

B. Interpret your immunity assay results.

1. Calculate plating efficiency.

    a. Calculate the efficiency according to the formula: Titer of phage on lysogen / titer of phage on wild type host = plating efficiency

    b. The simplest plating efficiencies to interpret are either 0, because the phage did not infect the lysogen, or 1, because the phage infected both strains of bacteria equally.

        i. Some plating efficiencies are more difficult to interpret. For example, some phage lysates will appear to have a substantially lower titer when plated on your lysogen than when plated wild-type cells. This is called reduced plating efficiency. This can be due to a variety of reasons, including surface exclusion or restriction.

2. What can you determine about your phage from your results?

    a. Do you see equivalent infection on your control and experimental plates for your initial phage, or is your phage unable to infect its own lysogen, as in Figure 11.5-1?

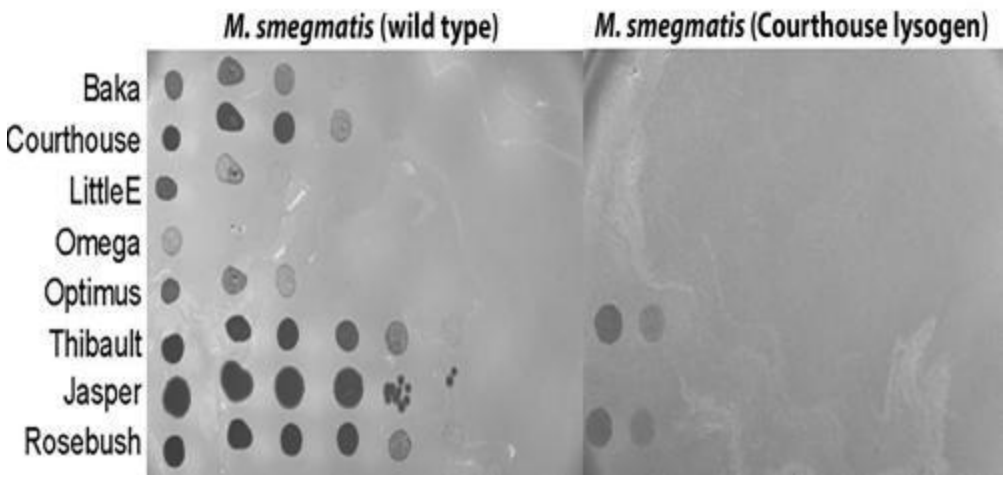


Figure 11.5-1. Sensitivity assay example. Portions of two plates are shown. The left plate has a lawn of wild-type M. smegmatis mc2155. The right plate has a lawn of the M. smegmatis mc2155 lysogen of the Cluster J phage Courthouse. Eight phages were serially diluted and plated on both lawns, and their names are listed on the left. The top six phages are members of Cluster J (Baka through Thibault), Jasper is a member of cluster A1, and Rosebush is a member of Cluster B2. The Courthouse lysogen is insensitive to infection from the Cluster J phages, most likely through immunity and the action of the Courthouse immunity repressor expressed within the lysogen. Some “killing from without” is visible in the Thibault row. Phage Jasper is also unable to infect the lysogen, most likely due to the presence of the A1 immunity repressor gene that Courthouse also carries in its genome (in addition to its regular Cluster J one). Finally, Rosebush is also unable to infect the lysogen, most likely due to exclusion or restriction, as Rosebush does not appear to have any obvious integration machinery or immunity repressor.

3. What can you learn from patterns of infection with other phages?

a. Examining the genomes of sequenced phages may lead you to some interesting genome features. However, do not limit yourself to testing only sequenced phages because you may learn something about the character of unsequenced phages, based on their ability to infect a panel of known lysogens.

Helpful Tips:

- Be careful to not confuse “killing from without” with phage infection. Phage infection will yield plaques at low lysate concentrations. “Killing from without” will produce a zone of clearing that dilutes out and will not produce plaques, as phages that can kill bacteria from without are not undergoing a normal lytic cycle. Killing from without occurs when cells are exposed to a high concentration of phage and die without the phage propagating normally. A good analogy of cell death from phage “killing from without” is dropping a water balloon on a box of needles. You will not make any more needles, but you will definitely break your water balloon.

- A lysogen will not be sensitive to infection by the same phage that created it.

- No immunity assay is valid unless a wild-type control is included in the assay. Why?

- If an attP/attB site is identified, design PCR primers to amplify across the attL or attR sites of the integrated prophage. Design control primers to amplify only across the attB site. The attB primers should work on a non-lysogen, but will be too far apart to work on an intact lysogen.

Protocol 11.6: Host Range Assay

Objective:

To test the ability of a phage to infect an alternative host from which it was isolated

Rationale:

Host range assays are spot titers performed to test the ability and efficiency of a given phage to infect bacteria different from the isolation host. Results are reported as a ratio that relates how well the phage infects the test bacteria compared to the isolation host, called Efficiency of Plating (EOP).

Supplies

- Liquid cultures of test bacteria
- Liquid culture of isolation host
- Phage lysates with a titer of > 5 x 10^9 pfu/ml
- Agar plates
- Phage buffer

Procedure:

Day 1

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

1. You will need a liquid culture for each bacterial species that you plan to test for host range of your phage. You will also need a liquid culture of the bacterial host on which you initially isolated your phage.

2. You will need an aliquot of phage lysate for each phage you wish to test, with a minimum titer of >5 x 10^9 pfu/ml.

B. Prepare a bacterial lawn for each bacteria to be tested, including the original isolation host, according to the Spot Titer (6.4) protocol.

1. Label the bottom of each agar plate your name, the date, and the bacterial species. The configuration of how you will spot the dilutions of you phage(s) should be determined now, labeling accordingly. See Figures 6.4-1 and 11.5-1 for examples.

2. Once your bacterial lawn has been prepared, allow plates dry for at least 20 minutes.

C. Perform serial dilutions on the phage lysates you wish to assay for their ability to infect alternate hosts.

1. Prepare ten-fold dilutions of phage lysate(s) according to **Serial Dilution** (6.2) protocol.

D. Spot dilutions on each prepared bacterial lawn.

1. One at a time, aseptically transfer 3 µl of each sample dilution onto the prepared bacterial lawns.

    a. Always use new tips for each spot to avoid contamination.

2. Use 3 µl of sterile phage buffer as the negative control.

3. Allow the liquid from the spots to dry completely.

4. Carefully invert the plates and incubate at the proper temperature for 24-48 hours.

Day 2

A. Check for evidence of phage infection.

1. After 24 and 48 hours, check each spot on the agar plates.

    a. Do you have individual plaques at some spots? The ability to observe individual plaques will allow you to calculate a titer.

    b. *If you have spots of lysis but no observable individual plaques for any dilutions, this will require further investigation and involve repeating the experiment using full plates (i.e. Plaque Assay (6.1) instead of spot tests).

2. Calculate the titer, in pfu/ml, of phages able to infect each host tested, including the isolation host, according to the Spot Titer (6.4) protocol.

3. Photograph your results. Carefully record your observations.

B. Interpret your host range assay results.

1. Do you see equivalent infection on your isolation host and the test bacteria for your phage? Is it reduced or is your phage unable to infect the experimental host bacteria?

2. Calculate Efficiency of Plating (EOP).

    a. For each host and phage tested, calculate the EOP according to the formula:(Titer of phage on test species) / (Titer of phage on isolation host) = EOP

b. The simplest interpretation of results are that the phage infected both types of bacteria equally, reported as 1, or the phage did not infect the test host, reported as 1/number of phage particles plated**.

c. Record EOPs and interpretations in your notebook.

Helpful Tips:

- *Be careful to not confuse “killing from without” with phage infection. Phage infection will yield plaques at low lysate concentrations. “Killing from without” will produce a zone of clearing at high lysate concentrations with no observable plaques as the lysate is diluted. Killing from without occurs when cells are exposed to a high concentration of phage and die without the phage propagating normally.

- **Do not report EOP as “0”, instead report the EOP as 1/highest number of phage particles plated. If you calculated your phage lysate titer as 5 $x10^{9}$ pfu/ml from plaques observed on the isolation host and you spotted 10 µl of the undiluted sample, the highest number of phage particles you spotted is 5 x 10^{7} particles. You observed less than 1 plaque on the test bacterial species, and should record your results as < 1/(5=" x=" /> ^{7}).

- No host range assay is valid unless the isolation host is included in the assay. It is also not valid if the titer of the phage is low.

- It is not informative to report the results as +/-.

- If you are testing new hosts where no phages have been found, it may be difficult to optimize the conditions for this experiment. Consider phage-hunting on those hosts!

Toolbox

Chapter 12: Toolbox

Chapter 12: An Overview

This chapter contains a collection of protocols that apply to a broad range of experiments. Some, like Plaque Streak Plates are alternative protocols that you may not use, while others, like Making Top Agar, will be used often.

Protocol 12.1: Making Top Agar (1X Middlebrook Top Agar)

Objective:

To make molten agar/media mixture for growing bacterial lawns on agar plates

Rationale:

Mixing a small amount of bacteria with molten top agar and spreading it over the surface of an agar plate results in a smooth lawn perfect for viewing bacteriophage plaques. When working with M. smegmatis, top agar is made by mixing 2X Middlebrook Top Agar with an equal volume of 7H9 liquid media.

Supplies:

- 100mM CaCl2 (sterile) stock (1 ml)
- 7H9 liquid media neat (50 ml aliquot)
- 2X top agar (50 ml aliquot)

Procedure:

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

B. Prepare 7H9 media.

1. Using aseptic technique add 1 ml of CaCl2 to a 50 ml aliquot of 7H9 neat media.
2. Place in 55 °C bath.

C. Melt the 2X top agar in a microwave.

1. Make sure the cap is loose!
2. The 2X top agar should come to a boil but not boil over.
3. The agar must be completely melted, so carefully swirl the bottle and check to make sure there are no clumps.
4. Once the agar is completely melted, place the 2X top agar in a 55 °C bath.

D. Aseptically combine the hot 2X top agar and 7H9/CaCl2 to make 1X top agar.

1. Keep at 55 °C until use.

2. The CaCl2 will precipitate out of solution and form a hazy layer on the bottom of the bottle. It will go back into solution when swirled.

Protocol 12.2: Plaque Streak Plates

Objective:

To generate well-isolated plaques containing only one kind of phage

Rationale:

Before you begin characterizing a phage, you need to ensure that you have a pure phage population. This can be accomplished by repeatedly isolating plaques. Plaque streak plates are a quick and economical method for doing this. The process relies on streaking a phage sample on agar to dilute the sample. Once bacteria are added to the plate, the diluted phage sample should give rise to well-isolated plaques (Figure 12.2-1). By repeating this process of isolating plaques, you will obtain a clonal phage population. Note that for some phages it is more difficult to obtain well-isolated plaques with this method. It is therefore best to also perform this in combination with at least one round of serial dilution/plaque assay to ensure good purification.

Supplies:

- Plate with plaques to purify or liquid sample from picking a plaque
- Agar plate
- Top agar
- Bacterial host culture
- Sterile sticks (If you do not have sterile sticks, sterile pipette tips can be used.)

Procedure:

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

B. Prepare your negative control plate.

1. Label the bottom of the plate with your name, date, streak plate, and negative control.

2. Using aseptic technique, remove a sterile stick from its packaging.

3. Gently streak back and forth across the top one-third of the plate without lifting the stick from the agar. This is your first streak (Figure 12.2-1).
Important: Try to skim across the top of the agar without gouging it with the stick.

4. With a new wooden stick, drag through the area streaked in the previous step, overlapping ONLY on the first two to three strokes. Continue to streak back and

forth in an adjacent one-third of the agar, making sure to not to overlap the first streak again.

5. Repeat Step 4 to create a third streak on the remaining one-third of the agar.

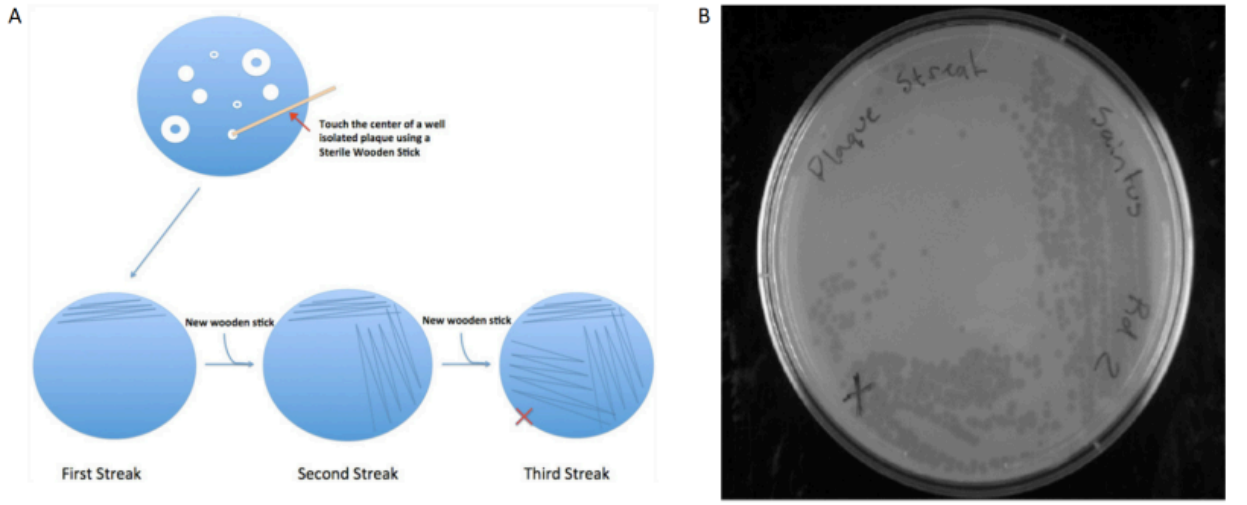


Figure 12.2-1. (A) Streaking for isolation of a phage. The center of a well-isolated plaque is touched with a sterile wooden stick. On the first streak, this stick is used to streak an area of roughly one-third of a fresh agar plate. A new stick is then used to streak an adjacent un-streaked area (one-third) of the plate for the second streak, making sure to overlap the original streaked area with only the first few strokes. Again, a new stick is used for the third streak to make a few overlapping strokes of the second streak. The area where the third streak was made is the most dilute and is designated by the red X. (B) A sample streak plate showing good plaque isolation.

C. Figure 12.2-1. (A) Streaking for isolation of a phage. The center of a well-isolated plaque is touched with a sterile wooden stick. On the first streak, this stick is used to streak an area of roughly one-third of a fresh agar plate. A new stick is then used to streak an adjacent un-streaked area (one-third) of the plate for the second streak, making sure to overlap the original streaked area with only the first few strokes. Again, a new stick is used for the third streak to make a few overlapping strokes of the second streak. The area where the third streak was made is the most dilute and is designated by the red X. (B) A sample streak plate showing good plaque isolation.Gather your phage samples.

1. Your phage sample may be a plaque, either from a plaque assay or another streak plate. It may also be a liquid sample prepared according to the Picking a Plaque (5.4) protocol.

2. If you are picking a plaque directly from a plate, whether from a plaque assay or another streak plate, draw a circle around the plaque on the bottom of the plate and label it. Record the details in your lab notebook.

D. Prepare your agar plates.

1. Obtain as many agar plates as you have phage samples to purify.

2. Label each plate according to the phage sample, as described in step B(1).

E. Make a streak plate.

1. Remove a sterile wooden stick from its packaging.

2. Prepare for your first streak.

    a. If you are working directly from a plaque on a plate, gently touch the center of the plaque once with the edge of the stick.

    b. If you are working from a liquid phage sample, swirl the stick in the sample.

3. Touch the stick to the agar on the appropriately labeled plate and streak back and forth across the top one-third of the agar. Discard the stick.

4. With a new wooden stick, drag through the first streak once and continue to streak back and forth in an adjacent one-third of the agar, making sure to not to overlap the first streak again.

    a. This second streak allows you to spread just a fraction of the phage from the first streak, thereby diluting it.

5. Repeat Step 4 to create a third streak on the remaining one-third portion of the plate.

    a. This third streak further dilutes the phage sample. Hopefully, you will have individual phage particles in this third streak.

6. Mark the area where your third streak ends with an “X” on the bottom of the plate. This is the region that has the least number of phage particles on your streak plate.

7. Repeat Steps 1–6 with a new plate for each phage sample.

F. Add bacteria to your streak plates. For this part of the experiment you will need 3 ml of molten top agar per streak plate. Your instructor may provide this for you or you may need to make it according to the protocol found in the **Toolbox**.

1. For each sample, including your negative control:

    a. Use a sterile 5 ml pipette to aseptically transfer 3 ml of molten top agar to a culture tube containing 250 µl of host bacteria.

    b. Immediately aspirate (suck-up) the mixture back up into the pipette.

        i. Try to avoid generating or withdrawing bubbles, as they can look like plaques on plates.

2. Dispense the top agar/bacteria mixture slowly onto the streak plate, depositing the mixture on the area marked “X.”

a. Do not swirl the plate!

3. Allow the mixture to spread across the plate from the most dilute point to the more concentrated areas. If necessary, tilt the plate very slightly.

4. Repeat this process for each of your samples.

5. Let the plates sit, undisturbed, for ~20 minutes to allow the top agar to solidify.

G. Incubate plates at the proper temperature.

1. After the top agar has solidified (~20 minutes), invert the plates rapidly (but gently). Inverting the plates prevents condensation from dripping onto the top agar in the incubator.

2. Place inverted plates in the proper incubator.

Helpful Tips:

- It is a good idea to streak the phage plaques in duplicate until you have mastered the technique. This will help ensure that you get an isolated single plaque every time.

- Pick your plaques as soon as possible. The longer the phage is on the plate, the farther it diffuses toward and into adjacent plaques.

- It is best to only pick plaques for purification that are well isolated from adjacent plaques. A well-isolated plaque is at least more than 0.5 cm away from adjacent plaques.

Protocol 12.3: Taking Plaque Pictures

Objective:

To keep a visual record of phage plates and plaque morphology

Rationale:

Properly documenting progress and results is the cornerstone of good scientific technique. For this reason, it is imperative that you take pictures documenting plaque morphology throughout the purification process to track your progress and report findings.

Different laboratory equipment exist to take pictures of plaques, but when unavailable, plaque pictures can also be taken with a digital camera or a smart phone. Detailed instructions about how to use a GelDoc or other common imaging instruments should be available at your institution, so only details regarding how to take a good picture with a digital camera are outlined in this protocol.

Supplies:

- Plates to be photographed
- Digital camera

Procedure:

A. Assemble your supplies.

B. The ideal plate for a picture contains about 20–40 plaques, depending on the size of the plaques. At this concentration, individual plaques are clearly visible and features (such as formation of halos) are clear.

C. Remove the lid. The agar side should be facing the lens of the camera.

D. Place the plate on top of a dark surface. Slide a black sheet of paper or black binder underneath the plate if necessary.

E. Make sure the field of view captures the entire surface area of the plate, with little to no space around it.

F. Make sure the lens is exactly parallel to the surface of the plate. Plates photographed at an angle will appear misshapen.

G. Make sure the picture is in focus. Edges of plaques should not be blurry.

H. Do not use a flash. Take the photo under good lighting conditions to avoid glare.

I. Look at the examples in Figure 12.3-1 below. Is your photo acceptable?

J. Include a copy of the photo in the proper location in your laboratory notebook. Include a detailed figure legend.

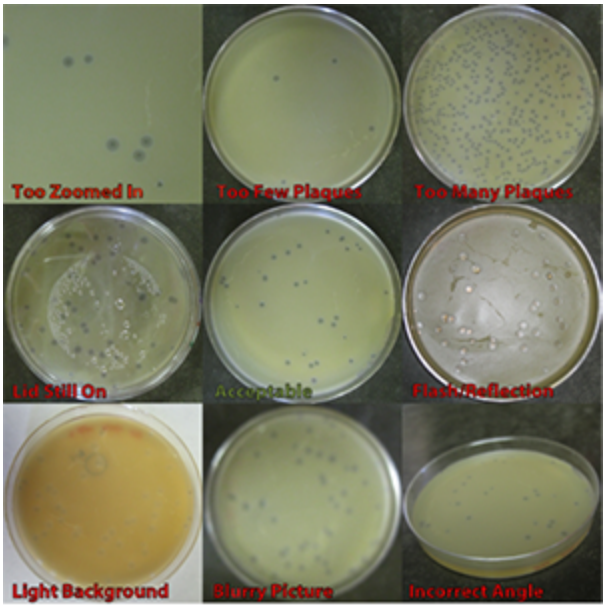


Figure 12.3-1. Examples of poor plate pictures compared with an acceptable plate picture suitable for uploading to the Actinobacteriophage Database.

Preview - Introduction to Bioinformatics

Bioinformatics uses computers to understand biological data. It is a field that is rapidly becoming a critical component in all areas of biology and medicine—from ecologists who study populations and migration patterns to epidemiologists who study emergent diseases and assess threatsto public health. At the most basic level, the field of bioinformatics is aboutrecognizing patterns. We use bioinformatics in the SEA-PHAGES course to find patternsin the sequence of nucleotides in a phage's genome, so we can predict where genes arelocated and better understand phage genetics.

Before we learn how bioinformaticscan help us study phage biology, we need to learn some basic information about genomes.The **genome** of an organism is the sum total of all its genetic information (its DNA). Therefore, we need to understand the molecular structure of DNA, how an organism uses its DNA,and how DNA is organized. Once we have these basics down, we can move on to a discussion of how bioinformatics can help us understand phage genomes.

Components of dsDNA

An organism's genome is organized into discrete units called chromosomes. Unlike our own cells, which have two copies of each chromosome, bacteria and viruses have only one chromosome, or one set of chromosomes. In addition, viral genomes can be made of RNA or DNA and be double-stranded or single-stranded. To date, all phages isolated on hosts of the phylum Actinobacteria have a single chromosome constructed of double-stranded DNA (dsDNA), and the entire genome is contained within a single chromosome.

DNA is made of polymers of the nucleotides adenine (A), thymine (T), guanine (G), and cytosine (C). A nucleotide is a basic unit of DNA, composed of a sugar molecule, a phosphate group, and a base (A, T, G, or C), covalently bonded together (Figure 13.0-1). Individual nucleotides are connected to the nucleotides above them by a covalent phosphodiester bond, forming long polymers called nucleic acids. In dsDNA chromosomes, two of these long nucleic acid strands are connected by hydrogen bonds between bases to form a twisted structure known as a double helix (Figure 13.0-1). Adenines are always paired with thymines and guanines are always paired with cytosines (and vice versa) in a phenomenon known as complementary base pairing.

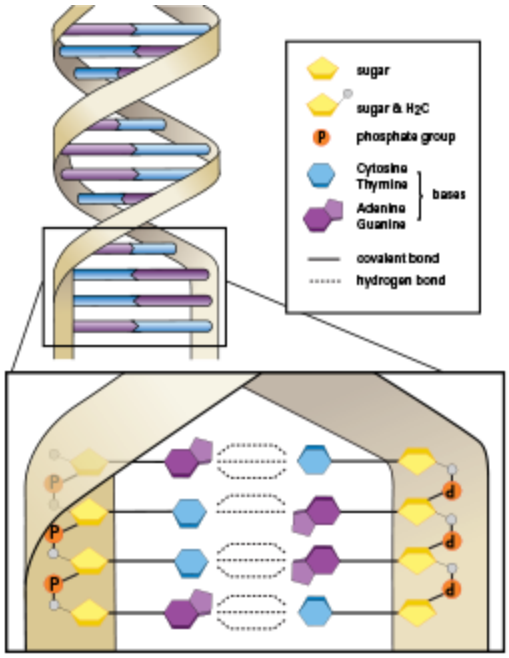


Figure 13.0-1. Structural model of DNA. In dsDNA the sugar and phosphate molecules form the "backbone," while the bases (A, T, G, C) are hydrogen-bonded together down the center. The entire structure twists into a structure known as theDNA double helix.

The Central Dogma

The central dogma of molecular biology describes the flow of biological information through the macromolecules of DNA, RNA, and protein (Figure 13.0-2). It has been a major tenet of molecular biology since the discovery of DNA's structure in 1950s. First outlined by Francis Crick, the central dogma describes the varying ways in which information flows between nucleic acids and from nucleic acids to proteins, and it critically states that information does not flow from proteins to nucleic acids. Thus, information is typically stored as sequences of As, Ts, Gs, and Cs in a DNA genome, transcribed into an intermediary RNA molecule, and translated into a sequence of amino acids in proteins that perform either structural (e.g., phage tail fiber) or enzymatic (e.g., DNA polymerase) activities (Figure 13.0-3).

While the main principles of the dogma—DNA acting as a template for RNA that in turn codes for proteins—remains true, discoveries over the past decades have expanded our understanding of the roles RNA plays in the cell. These include RNA's ability to store information that can be used to generate new DNA during reverse transcription, to catalyze reactions as ribozymes, to regulate gene expression, and to aid in bacterial defense mechanisms against viruses.

Figure 13.0-2. The central dogma of molecular biology.

Transcription is the process by which single-stranded RNA is generated using a DNA template and an enzyme called **RNA polymerase**. The resultant RNA is complementary in sequence to the template DNA, but in RNA the thymine base (T) found in DNA is replaced with the base uracil (U), and the sugar molecules in the backbone are different.

RNA is used during **translation** as a template for making proteins (messenger RNA [mRNA]), as a molecule to match a specific amino acid with a specific nucleotide sequence (transfer RNA [tRNA]), or as a key component of the ribosome (ribosomal RNA [rRNA]). In addition to RNA's informational and structural roles, RNA's chemistry supports a variety of important enzymatic activities. Many small RNA molecules also play key regulatory roles.

Ribosomes are macromolecular structures composed of proteins and rRNAs. They are highly abundant cellular complexes found in all domains of life. **Translation** is the synthesis of proteins by a ribosome using the mRNA sequence as a template. Only specific regions of DNA known as genes are transcribed and translated; other regions of DNA play regulatory roles controlling whether genes are transcribed or not.

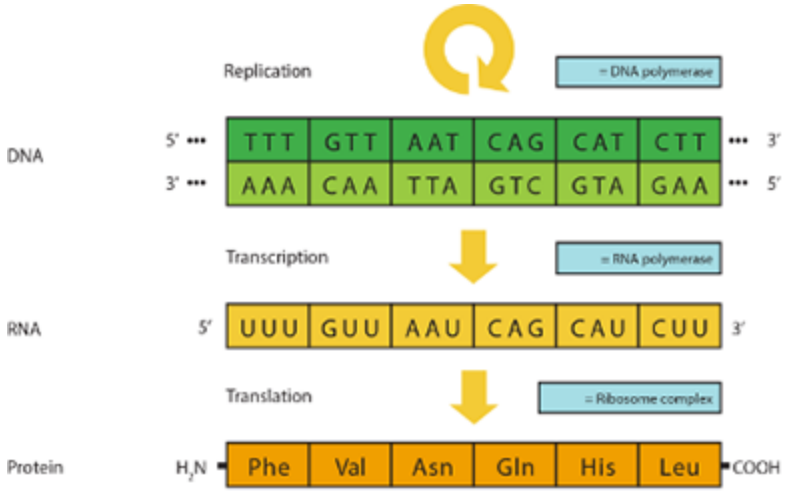


Figure 13.0-3. The central dogma. In biology DNA is transcribed to RNA, which is translated into proteins.

Genes

A **gene** is defined as a unit of DNA on a chromosome that codes for a protein or for RNA. Transcription of a gene results in one of three types of RNA molecules: mRNAs, which will

be translated into proteins; rRNAs, which are incorporated into ribosomes; and tRNAs, which become charged with specific amino acids for use in protein synthesis. Much of the information about genes presented in this chapter is based on what is known about bacterial genes, because they are well understood and share similarities with phage genes. After all, phages must hijack host bacteria transcriptional and translational machinery for the synthesis of their own RNA and proteins.

For a gene to be transcribed and translated, it must contain the following:

1. DNA sequences that signal the start and stop of **transcription**. These regulatory sequences are called promoters and terminators.

2. DNA sequences that signal the start and stop of **translation**. These are known as start (initiation) and stop codons.

3. The DNA sequence that will be translated into a protein. This sequence is called an open reading frame (ORF).

The basic structure of a gene includes the promoter, the terminator, and the ORF (see Figure 13.0-4).

Figure 13.0-4. Basic gene structure.

The Promoter and Terminator

The **promoter** region of a gene is a sequence of nonprotein-coding DNA that is upstream (to the left) of the protein-coding region of the gene. The promoter is where **RNA polymerase**, the enzyme responsible for translating DNA into an RNA transcript, is recruited to the DNA. Bacterial promoters (and some phage promoters) contain two hexameric (six base pair) sequences referred to as the -10 and -35 motifs. These DNA sequences are approximately 10 and 35 base pairs upstream of the **transcription start site (TSS)** (Figure 13.0-5). The -10 and -35 sequences vary among different promoters, but they are all variations of a "consensus" sequence that reflects the most common bases at each of the positions. Typically, agreement of any specific promoter with the consensus sequence is a reflection of promoter strength (the frequency with which mRNA synthesis is initiated); that is, the closer the -10 and -35 sequences are to the consensus, the stronger the promoter.

Bacteria generally have several different types of promoters, which are recognized by different forms of the RNA polymerase containing variations of one of its subunits, called the sigma factor. In Escherichia coli, the predominant promoter types are recognized by sigma 70–associated RNA polymerases and have -10 and -35 consensus sequences of 5'-TATAAT and 5'-TTGACA, respectively. Many other bacteria, including Actinobacteria, share a

similar set of promoters with identical, or very similar, consensus sequences. However, other promoters, including phage promoters, are still not well defined.

The **terminator** is the specific sequence where the RNA polymerase detaches from the template DNA strand, thus stopping transcription and releasing the new RNA.

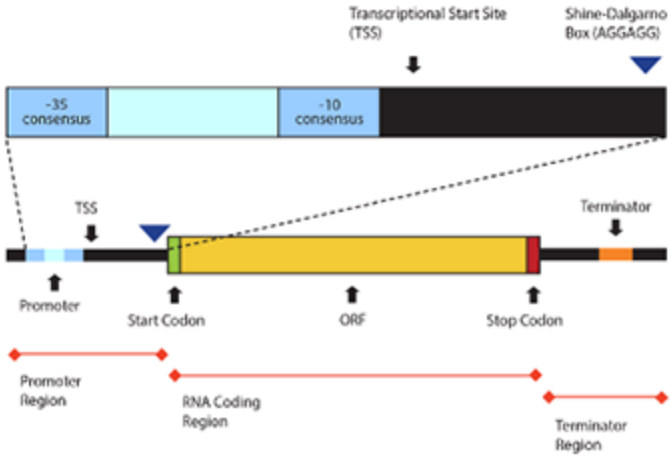


Figure 13.0-5. Detailed gene structure.

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The Open Reading Frame

The **open reading frame (ORF)** is the portion of the gene that encodes the protein. Proteins are composed of long polymers of amino acids. Each of the 20 different amino acids is indicated in DNA and RNA sequences by codons. A **codon** is a triplet of nucleotide bases that, when translated, indicates which specific amino acid should be added to a protein chain. The genetic code (Figure 13.0-6) is universal; that is, the same codons are used for the same amino acids across all organisms (with some exceptions). Each of the 20 common amino acids occurring in proteins is coded for by at least one triplet codon, but in most cases two, four, or six different triplet codons.

Second Letter									
First letter	TTT	Phenylalanine (F)	TCT	Serine (S)	TAT	Tyrosine (Y)	TGT	Cystine (C)	Third Letter
	TTC		TCC		TAC		TGC		
	TTA	Leucine (L)	TCA		TAA	STOP	TGA	STOP	
	TTG		TCG		TAG		TGG	Tryptophan (W)	
	CTT	Leucine (L)	CCT	Proline (P)	CAT	Histidine (H)	CGT	Arginine (R)	
	CTC		CCC		CAC		CGC		
	CTA		CCA		CAA	Glutamine (Q)	CGA		
	CTG		CCG		CAG		CGG		
	ATT	Isoleucine (I)	ACT	Threonine (T)	AAT	Asparagine (N)	AGT	Serine (S)	
	ATC		ACC		AAC		AGC		
	ATA		ACA		AAA	Lysine (K)	AGA	Arginine (R)	
	ATG	Methionine (M)	ACG		AAG		AGG		
	GTT	Valine (V)	GCT	Alanine (A)	GAT	Aspartic acid (D)	GGT	Glycine (G)	
	GTC		GCC		GAC		GGC		
	GTA		GCA		GAA	Glutamic acid (E)	GGA		
	GTG		GCG		GAG		GGG		

Because mRNA is translated using a triplet code and read by the ribosome three nucleotides at a time, the nucleotide sequence in mRNA (which is reflected in the DNA sequence) can be interpreted in three separate ways, or **reading frames**, depending on which nucleotide is used to begin translation. As illustrated in Figure 13.0-7A, if you begin with nucleotide 1 (indicated by the blue arrow), the codons can be grouped as shown in Row 1. If you begin with nucleotide 2 (indicated by the green arrow), the codons would be those in Row 2. And if you begin with nucleotide 3 (indicated by the red arrow),the codons would be those in Row 3

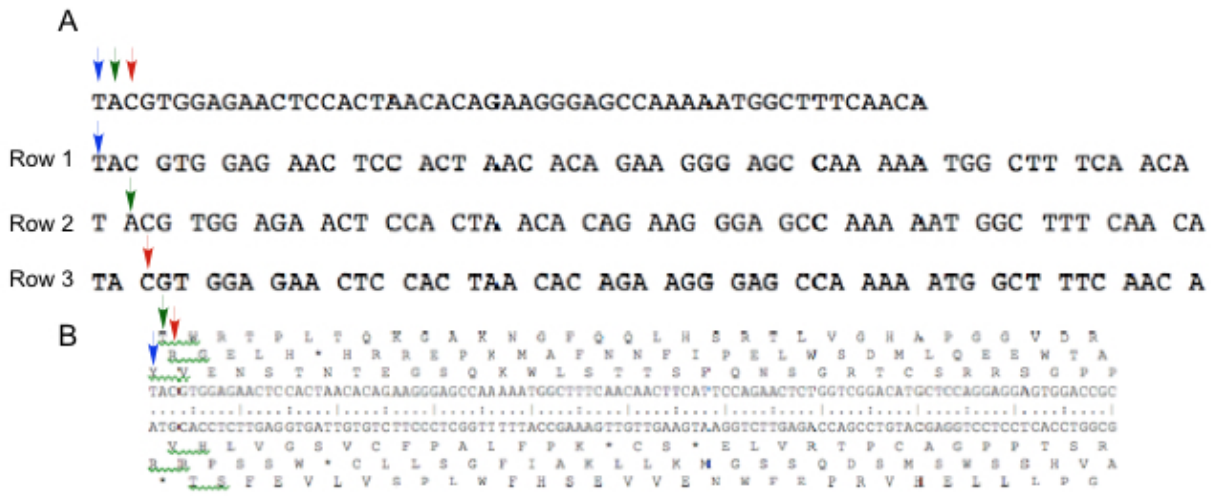

Figure 13.0-7. DNA reading frames. (A) Each strand of DNA has three different reading frames based on which nucleotide is used to start translation. (B) The resulting protein depends on which reading frame is used.

Remember that DNA is double-stranded, so if you have three ways to translate a top strand of DNA in the forward direction (left to right), you also have three ways to translate the complementary strand in the reverse direction (right to left) (Figure 13.0-7B). This means that dsDNA has six possible reading frames!

If each strand of mRNA has three possible reading frames, how does a ribosome know which is the "right" reading frame to make a given protein? Translation begins when a ribosome is recruited to a strand of mRNA and recognizes a start codon. The **start codon** is the site at which the ribosome starts synthesizing the protein by adding amino acids in the order indicated by the mRNA template.

Bacteria and phages share with eukaryotes the use the codon AUG to initiate protein synthesis. But unlike eukaryotes, bacteria and phages sometimes use GUG and UUG (and very occasionally, other codons) to initiate translation. The frequencies with which AUG, GUG, and UUG are used varies enormously; in E. coli, AUG is predominantly used, whereas in mycobacteria, GUG is used almost as frequently as AUG, with UUG used in only about 5 % of genes. All three of these codons are used not only for translation initiation but also as "regular" codons within the gene bodies. However, translation initiation uses a special tRNA (tRNAfMet) that is devoted to initiation. As a consequence, the first amino acid in newly synthesized proteins is always methionine, regardless of whether AUG, GUG, or UUG is used. In some instances the N-terminal methionine is often removed.

How does the protein synthesis machinery distinguish between a codon (such as AUG) used for initiation from one located within a gene body? The answer is that additional sequences are used to promote binding of ribosomes so that initiation can begin. In bacteria and phages there is a specific sequence present just upstream (3–12 nucleotides) of the initiation codon called the **ribosome binding site (RBS)** (also called the **Shine-Dalgarno site**). (See Figure 13.0-5.) It is a short sequence (~8 nucleotides) to which the mRNA can base pair with the 3' end of the rRNA in the small ribosomal subunit (16S rRNA).

Translation stops when the ribosome encounters a **stop codon**, which signals the end of the ORF (Figure 13.0-5). The stop codons UAA, UAG, or UGA (TAA, TAG, or TGA in the DNA sequence) do not code for amino acids, thereby arresting translation at the last codon before the stop codon. The stop codon should not be confused with the transcription terminator. An mRNA molecule is typically longer than just the coding sequence and so extends to the 3' side of the stop codon.

Operons

Unlike eukaryotes, bacteria and bacteriophages organize many (but not all) of their genes into operons. An operon consists of two or more open reading frames (genes) that share a common promoter and a terminator to make up a single transcriptional unit (Figure 13.0-8). This means that one mRNA transcript will include two or more ORFs. These ORFs are then translated as separate proteins by ribosomes. The genes in bacteriophage genomes typically are organized into long operons, with only five or so promoters serving the approximately 100 genes in a 50 kb genome. This allows for the close packing of bacteriophage genes, with little intergenic space allocated for regulatory regions or transcriptional signals.

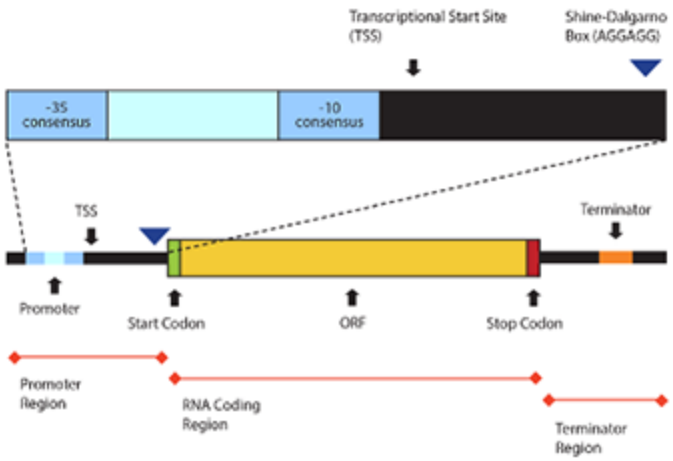

Figure 13.0-8. Operons contain multiple genes under the control of a single promoter, and they have a single TSS and terminator.

Coupled Transcription-Translation

In eukaryotes, transcription occurs in the nucleus and mRNA molecules are transported across the nuclear membrane to the cytoplasm where translation occurs. In bacteria, there is no nuclear membrane, so translation begins as soon as the 5' end of the mRNA is synthesized. Transcription and translation are therefore tightly coupled processes. An important consequence of this is that interrupting the process of translation can result in the premature arrest of transcription. This leads to the phenomenon called **genetic polarity** in which a mutation in one gene appears to inactivate the activity of additional downstream genes. This phenomenon only occurs within operons, usually as a result of a nonsense mutation (a base change converting a codon into a stop codon), an insertion, or a frameshift mutation, that interferes with translation of an upstream gene.

Protiens

Like nucleic acids, proteins are macromolecules. Within a living organism, proteins are involved in nearly every life process. Proteins have many different roles, including catalysis of reactions, structure, transport of other molecules, regulation, and cell signaling. Not all genes in an organism's genome are constantly transcribed and translated, nor are the proteins expressed at the same level. Rather, organisms control which genes are expressed and to what degree, based on the organism's needs at a given time.

Proteins Expressed from Phage Genomes

Bacteriophages vary widely, and like bacteria, they have genes for many different types of proteins, depending on what hosts they infect, what environments they exist in, and what processes they engage in. The phages that infect bacteria of the phylum Actinobacteria carry genes that encode proteins involved in

- Bacterial infection (attachment and insertion)
- Phage DNA synthesis

- Virion head and tail assembly
- DNA packaging into phage particles
- Bacterial lysis
- Establishment and maintenance of lysogens (temperate phages only)

Of course, phages carry many genes in addition to these, including tRNAs, nucleic acid modification enzymes, toxins, and a multitude of genes whose functions we do not yet understand. In fact, the majority of the genes in the genome of every phage (~75 %) encode proteins with no known function.

Phage Genomics

The study of genomics begins with a complete genome sequence. Phage genomes sizes vary from 15 to 250 kilobases (kb), or base pairs, long. When you receive your phage's genome sequence from the DNA sequencing center, it is a long sequence of adenines, thymines, guanines, and cytosines. Although bacteriophage genomes are relatively small compared with bacterial or eukaryotic genomes, even 50,000 bases of sequence are too large and too complicated for a person to analyze easily without the aid of a computer. Computer algorithms make it possible to analyze a genome and predict gene locations and other interesting genome characteristics.

Genome Annotation

Genome **annotation** is the prediction of the genes and other features coded within a phage genome and their potential functions. This is typically the first task in genome analysis. Several guiding principles of annotation are used to predict genome features, and these will be discussed in much greater detail in the bioinformatics portion of the course.

Although it is possible to use computer algorithms to predict the location of ORFs and their possible start and stop codons, each ORF needs to be carefully evaluated. Likewise, predicting the possible functions of the proteins encoded by the proposed genes—and their role in the biology of the phage—requires further study. Functional characterization must be performed in the lab and can involve genetic approaches such as constructing and characterizing mutants, isolating mutants with specific phenotypes, and mapping the mutations. Biochemical strategies can also be performed. These strategies include expressing and purifying proteins to determine their activities and using structural approaches to provide insights into structure-function relationships.

Bioinformatic analyses using computer programs can determine patterns in sequences and similarities to other known proteins. These programs are extremely powerful and many are quick and simple to use. For example, by using sequence alignment tools, it is possible to rapidly screen sequences for similarity to the many thousands of sequences in public databases. These similarities can then be used to predict functions to phage-encoded proteins. Predicted protein functions can provide substantial information about the biology and the life cycles of the phage.

Comparative Genomics

In addition to finding the features of a particular phage genome, we use comparative tools to examine the relationships between genomes, thereby gaining considerable insight into both gene function and genome evolution. Some phages are similar in terms of nucleotide sequence similarity, shared gene content (as determined by similar protein sequences of predicted genes), and genome architecture (genome size, transcriptional organization, and location of genes). These similar phages frequently (but not always) share biological properties such as host range or lifestyle. Careful examination of genome-alignment maps of similar phages can provide clues to genetic novelty that can be explored experimentally. Exploring the relationships between the groups of related genomes yields insights into gene flow, phage evolution, and the global population of bacteriophages.

Bioinformatics Tools

It is helpful to remember that the many computer programs available for genomic analyses are just tools in a large toolbox. You will need to decide which programs to use to address different issues and questions that arise. None of the programs are perfect, so a useful strategy is to use, whenever possible, multiple tools and then test whether they give consistent predictions. Keep in mind that when annotating genomes you are not establishing facts, you are making predictions about genome organization and function. There is no right or wrong answer, but there are ”good,”' well-supported solutions and ”poor” solutions that lack strong support. Also, you should not assume that all the data available in either local or public databases are reliable. Much of the data may be good and well-supported, but some may not be. Information about a sequence may have been supported when the sequence was first characterized, but because databases have grown with new data and sequences, the information about a sequence in the database warrants reinterpretation.

Resources:

See seaphages.org and phagesdb.org for more information on the software used in the bioinformatics portion of the course and instructions on how to download and use it.

Recipes for Solutions

- AD Supplement
- Calcium Chloride (1 M)
- Calcium Nitrate (0.8 M)
- Carbenicllin Stock Solution
- Cycloheximide Stock Solution
- Dextrose (40 %)
- Enrichment Broth
- EDTA
- Glycerol (40 %)
- Isopropanol (80 %)
- Magnesium Chloride (1 M)
- Magnesium Sulphate (1 M)
- Nuclease Mix
- Phage Buffer
- Phage Precipitation Solution
- SDS (10 %)
- Tris Borate EDTA (TBE)
- Tris, pH 7.5 (1 M)
- Tween80 (20 %)
- Uranyl Acetate Stain
- Water
- Zinc Chloride

Recipes for Media

- 7H9 Liquid Medium(Neat)
- 7H9 Liquid Medium(Complete)
- 7H9 Liquid Medium(Complete with Tween80)
- Carbenicllin Stock Solution
- 7H9 Top Agar (1X)
- 7H9 Top Agar (2X)
- Luria Agar (L-Agar) Plates
- Media Supplement for Streptomyces phage
- Nutrient Broth
- Nutrient Broth(with Supplements for S.griseus)
- Nutrient Agar Plates (with Supplements for S. griseus)
- PYCa Agar Plates
- PYCa Liquid Medium
- PYCa Top Agar

Propagating Bacteria and Phage

- Growing bacteria from a frozen stock
- Growing liquid cultures of bacteria from a single colony
- Preparing a culture without Tween80 from one with Tween80
- Preparing frozen stocks of bacteria
- Preparing Additional Phage Lysate & Long-term Storage

Notes

- Notes about Recipes
- Autoclave Setting

- Received Biologicals from the Program? Follow these Next Steps.

AD Supplement

AD Supplement (0.5 L)

For Bacterial Host: *M. smegmatis* mc^2 155 only

Ingredients	Amount	Final Concentration
NaCl	4.25 g	145 mM
Albumin (Fraction V)	25 g	5.0 %
ddH_2O	To 0.5 L	
Dextrose	10 g	2.0 %

To Prepare

1. Add 400 mL of ddH_2O to a 1-L Erlenmeyer flask.
2. Weigh out NaCl and albumin, and slowly, while stirring, add to the ddH_2O in the Erlenmeyer flask.
3. Stir until dissolved.
4. Once dissolved, continue stirring and slowly add the dextrose.
5. Once the dextrose is dissolved, transfer the solution to a 1-L OR 0.5 L graduated cylinder, and bring the volume to 0.5 L with ddH_2O.
6. Sterilize.

To Sterilize

- Filter-sterilize; do not autoclave.

To Store

- At 4 ˚C, indefinitely if not contaminated

Usage

In Recipe:

- 7H9 liquid medium complete

Notes

1. Adding the dextrose last helps keep it from recrystallizing on the bottom of the flask.

2. Make sure the albumin is completely in solution before the filter-sterilization step. To dissolve the albumin, use a large stir bar spinning at a moderate to high speed. Very vigorous stirring will cause the albumin to denature and collect at foam/bubbles at the surface of the solution.

3. 0.5 L of AD supplement is require to prepare 5 L of 7H9 liquid media .

4. Standard medium for the growth of mycobacteria (Middlebrook) generally contains ADC (for albumin, dextrose and catalase) or OADC (ADC plus oleic acid). This guide uses the recipe for ADC but without catalase, hence the term “AD supplement”.

5. Approximately 2.5 L of 7H9 liquid media is used per semester for a class of 20 students.

Calcium Chloride (1 M)

1 M $CaCl_2$ (100 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
CaCl2	14.7 g of $CaCl_2$.2H20 or 11.1 g of $CaCl_2$ (anhydrous)	1 M
ddH_2O	see instructions below	

To Prepare

Note: You will need stock solutions of $CaCl_2$ at 1 M and 100 mM.

For 1 M $CaCl_2$:

1. Add 90 mL of ddH_2O to a large beaker.
2. Weigh out $CaCl_2$ and slowly, while stirring, add to the ddH_2O until the $CaCl_2$ is dissolved.
3. Transfer the solution to a graduated cylinder, and add ddH_2O to 100 mL.
4. Sterilize.

For 100 mM $CaCl_2$:

1. Add 50 mL of 1 M $CaCl_2$ to a large beaker.
2. Add ddH_2O to 500 mL
3. Sterilize*

*if steps 1 & 2 are performed using sterile ddH_2O and sterile 1M calcium chloride, as well as using aseptic technique, there is no need to sterilize the solution.

To Sterilize

- Filter-sterilize; do not autoclave.

To Store

- At room temperature, indefinitely.Do not refrigerate

Usage

In Recipe:

- 7H9 liquid medium complete and top agar
- PYCa liquid medium, agar, and top agar.
- Phage buffer

Notes

1. CaCl2 should not be autoclaved as it can precipitate out of solution at high temperatures.
2. Due to the high concentration of salts in Middlebrook 7H9 media, $CaCl_2$ has a tendency to precipitate out of solution. As such, $CaCl_2$ will ideally be added to 7H9 media immediately before the solution is used.
3. 100 mL of both 1 M $CaCl_2$ and 100 mM $CaCl_2$ should be sufficient for preparing all reagents requiring $CaCl_2$ for ~ 20 students per semester.

Calcium Nitrate (0.8 M)

0.8 M $Ca(NO_3)_2$ (100 mL)

For Bacterial Host: *Streptomyces spp. only*

Ingredients	Amount	Final Concentration
$Ca(NO_3)_2$	18.89 g of $Ca(NO_3)_2.4H_20$	0.8 M
ddH_2O	To 100 mL	

To Prepare

1. Add 80 mL of ddH_2O to a large beaker.
2. Weigh out $Ca(NO_3)_2$ and slowly, while stirring, add to the ddH_2O until the $Ca(NO_3)_2$ is dissolved
3. Transfer the solution to a graduated cylinder, and add ddH_2O to 100 mL.
4. Sterilize.

To Sterilize

- Autoclave.

To Store

- At room temperature, indefinitely. Do not refrigerate

Usage

In Recipe:

- Media Supplement for *Streptomyces* phage

Notes

Carbenicllin Stock Solution

Carbenicillin (CB) at 1000X

For Bacterial Host: *M. smegmatis* mc^2155 only

Ingredients	Amount	Final Concentration
Carbenicillin (powder)	50 mg/ml	stock = 1000X

To Prepare

1. Weigh out 100 to 600 mg of carbenicillin powder and place it in a 10-15 mL tube.
2. Divide the number of milligrams of carbenicillin by 50 to determine the volume (in milliliters) of ddH_2O to add (e.g., for 477 mg of CB, add 9.5 mL ddH_2O).
3. Stir or shake until dissolved.
4. Filter-sterilize (0.22µm pore size) into a fresh sterile tube.
5. Prepare 1 mL aliquots and freeze at -80 °C

To Sterilize

- Filter-sterilize (0.22µm pore size) into a fresh sterile tube.

To Store

- At -80 °C for 3 years.
- At 4 ºC for ≤ 60 days.

Usage

In Recipe:

- 7H9 Liquid Medium

Label Stock Solution

- CB, 1000X, date, initials

To Prepare Final Solution

1. Remove a frozen aliquot, allow to thaw, and use as follows. Do not return thawed aliquot to -80 °C . Instead, store thawed aliquots at 4 °C

2. When using, add stock solution at 1:1000. For example, for 1 L of medium, add 1 mL of stock.

Notes

1. If your host bacteria is resistant to CB, it can be added to prevent growth of other bacteria.

2. Mechanism of action: CB is a semi-synthetic penicillin that interferes with bacterial cell-wall synthesis.

3. 50 mL of carbenicillin should be sufficient per semester for a class of ~ 20 students.

Cycloheximide Stock Solution

Cycloheximide (CHX)

For Bacterial Host: General

Ingredients	Amount	trial|Final Concentration
Cycloheximide (powder)	10 mg/ml	stock = 1000X

To Prepare

1. Weigh out 50 to 100 mg of cycloheximide powder and place it in a 10-15 mL tube.
2. Divide the number of milligrams of cycloheximide by 10 to determine the volume (in milliliters) of ddH_2O to add (e.g., for 51 mg of CHX, add 5.1 mL ddH_2O).
3. Stir or shake until dissolved.
4. Filter-sterilize (0.22µm pore size) into a fresh sterile tube.
5. Prepare 1 mL aliquots and freeze at -80 °C.

To Sterilize

- Filter-sterilize (0.22µm pore size) into a fresh sterile tube.

To Store

- At -80 °C for 1 year.
- At 4 °C for ≤ 60 days.

Usage

In Recipe:

- 7H9 Liquid Medium
- PYCa Liquid Medium
- Luria Agar
- PYCa Agar

Label Stock Solution

- Remove a frozen aliquot, allow to thaw, and use as follows. Do not return thawed aliquot to -80 C. Instead, store thawed aliquots at 4 C.

- When using, add stock solution at 1:1000. For example, for 1 L of medium, add 1 mL of stock.

Notes

1. CHX is added to media to prevent growth of fungi and yeast.

2. Mechanism of action: CHX inhibits protein biosynthesis in eukaryotic organisms. It interferes with the peptidyl transferase activity of the 60S ribosome, thus blocking translational elongation.

3. 50 mL of cycloheximide should be sufficient per semester for a class of ~ 20 students.

Dextrose (40 %)

40 % Dextrose (200 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
Dextrose anhydrous	80 g	40 %
ddH_2O	To 200 mL	

To Prepare

1. Add 100 mL of ddH_2O to a beaker, and then add the dextrose while stirring.
2. Top up with ddH_2O to 200 mL, and continue stirring until dextrose has dissolved. If the dextrose does not fully dissolve, gently warm the solution on the stir plate (or in a microwave).
3. Filter-sterilize the solution. Do not autoclave.

To Sterilize

- Filter-sterilize. Do not autoclave.

To Store

- At room temperature, indefinitely if not contaminated.

Usage

In Recipe:

- PYCa media
- PYCa agar plates
- PYCa top agar
- Media Supplement for *Streptomyces* phage

Notes

1. 100 mL of 40 % dextrose should be sufficient for preparing all reagents and media requiring dextrose, per semester for a class of ~ 20 students.

Enrichment Broth

Enrichment Broth (100 mL)

For Bacterial Host: *M. smegmatis* mc^2155 only

Ingredients	Amount	Final Concentration
7H9 liquid media neat	99 mL	1X
100 mM $CaCl_2$ stock	1 mL	1 mM

To Prepare

1. All ingredients must be added to a sterile bottle or flask using aseptic technique. The final medium can be filter-sterilized.

To Sterilize

- All ingredients are sterile. The final medium can be filter-sterilized.

To Store

- At room temperature, for up to 1 year. Discard if solution is discolored, contaminated or has precipitation.

Usage

- In Protocol: 5.2, 5.5

Notes

1. If antibiotics or antimicrobials are added, the medium must be stored at $4^{o}C$.
2. A good rule of thumb is that $CaCl_2$ should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because $CaCl_2$ will precipitate out when cooling after autoclaving or when refrigerated.
3. 30 ml of Enrichment broth is required per student per soil sample tested.

EDTA

EDTA 0.5 M (100 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
EDTA disodium salt dihydrate	18.61 g	0.5 M
ddH2O	To 100 mL	

To Prepare

1. Weigh out EDTA and place in a large beaker.
2. Add approximately 80 mL of ddH_2O and stir until dissolved.
3. Adjust the pH to 8.0 with NaOH.
4. Transfer the solution to a graduated cylinder and add ddH_2O to 100 mL.

To Sterilize

- Filter-sterilize or autoclave

To Store

- At room temperature, indefinitely if not contaminated.

Usage

- In Protocol: 9.1a, 9.1b, 9.2a, 9.2b

Notes

1. For protocol 9.2b, prepare a 0.1 M EDTA solution by diluting the 0.5 M EDTA solution in water.

Glycerol (40 %)

40 % Glycerol (500 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
ddH_2O	To 500 mL	40 %
Glycerol (also known as glycerin or glycerine)	200 ml	

To Prepare

1. Measure 250 mL ddH_2O and place in a large beaker.
2. Slowly add 200 mL of glycerol while stirring.
3. When the glycerol has completely mixed with the water, transfer the solution to a graduated cylinder.
4. Bring the volume to 500 mL with ddH_2O . Mix.
5. Sterilize

To Sterilize

- Filter-sterilize or autoclave.

To Store

- At room temperature, indefinitely if not contaminated.

Usage

In Recipe:

- I7H9 liquid medium

In Protocol:

- Making glycerol stocks

Notes

1. 100 mL of 40 % glycerol should be sufficient for all reagents and media requiring glycerol per semester.

Isopropanol (80 %)

80 % Isopropanol (50 ml)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
99% Isopropanol	40 ml	80 %
ddH_2O	To 50 ml	

To Prepare

1. Measure 40 ml isopropanol and place in a sterile conical tub
2. Slowly add ddH_2O to 50 ml.
3. Mix using a vortex or by inversion.

To Sterilize

- Not required

To Store

Important: Do Not Store.

- 80 % isopropanol should be prepared and used within the same day.

Usage

- In Protocol: 9.1, 9.2

Notes

- Prepare 80 % isopropanol only as needed. Isopropanol will evaporate with time, resulting in a solution with reduced isopropanol concentration. Isopropanol solutions below 80 % will result in premature elution of DNA during phage DNA extraction.
- 6 mL of isopropanol is used per attempt at DNA extraction from phage lysates.

Magnesium Chloride (1 M)

1 M $MgCl_2$ (100 mL)

For Bacterial Host: *Streptomyces spp.* only

Ingredients	Amount	Final Concentration
$MgCl_2$ hexahydrate*	20.33 g	1 M
ddH_2O	To 100 mL	
***$MgCl_2$** hexahydrate can be substituted with 9.5 g of **$MgCl_2$** anhydrous in this recipe		

To Prepare

1. Weigh out $MgCl_2$ and place in a large beaker containing approximately 75 mL of ddH_2O , and stir until $MgCl_2$ is dissolved.
2. Transfer the solution to a graduated cylinder, and add ddH_2O to 100 mL.
3. Sterilize

To Sterilize

- Autoclave or filter-sterilize

To Store

- At room temperature, for a year.

Usage

In Recipe:

- Media Supplement for *Streptomyces* phage

Notes

Magnesium Sulphate (1 M)

1 M $MgSO_4$ (100 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
trial|$MgSO_4$ (anhydrous)	12.04 g	1 M
ddH_2O	To 100 mL	

To Prepare

1. Weigh out $MgSO_4$ and place in a large beaker containing approximately 95 mL of ddH_2O , and stir until $MgSO_4$ is dissolved.
2. Transfer the solution to a graduated cylinder, and add ddH_2O to 100 mL.
3. Sterilize.

To Sterilize

- Autoclave or filter-sterilize

To Store

- At room temperature, for a year.

Usage

In Recipe:

- Phage buffer

Notes

1. 10 mL of $MgSO_4$ is required to prepare 1 L of phage buffer.
2. 100 mL of $MgSO_4$ is sufficient for 1 – 5 semesters for a class of ~ 20 students.

Nuclease Mix

Nuclease Mix (5 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
NaCl	0.09 g	150 mM
ddH_2O	4.25 mL	
DNase 1	250 µL of 5-mg/mL stock	0.25 mg/mL or 500 KU/ml
RNase A	125 µL of 10-mg/mL stock	0.25 mg/mL or 12.5
Glycerol	2.5 mL	50 %

To Prepare

Note: Begin by preparing the RNase A and DNase 1 stock solutions according to the manufacturer's protocol. RNAse A stock solutions are typically prepared in Tris-Cl (10 mM; pH 7.5) and NaCl (15 mM), and includes a 5-min heating step at 100 ˚C.

1. Dissolve the NaCl in 4.25 mL of ddH_2O in a sterile 15-mL conical tube.
2. Transfer 2.1 mL of the NaCl solution into a new 15-mL conical tube.

   Note: the remaining 2.1 mL can be stored indefinitely and used at a later date to prepare more nuclease mix.
3. Add the glycerol to the NaCl solution, and mix with gentle inversion.
4. Add the RNase and DNase stock solutions, and mix with gentle inversion.
5. Add ddH_2O to a final volume of 5 mL, and mix with gentle inversion until the solution is homogeneous.
6. Aliquot into microcentrifuge tubes.

To Sterilize

- Not required

To Store

- At -20 ºC, for up to 2 years.

Usage

- In Protocol: 9.1

Notes

1. Be extra careful not to contaminate pipettes, benches, or other reagents with this nuclease mixture, because it will degrade any DNA samples.

2. Prepare DNase I and RNase A stock solutions according to the instructions provided by the manufacturer.

3. 5 µL of nuclease mix is used per attempt at DNA extraction from phage lysates.

4. Estimate 1-2 attempts at DNA extraction per student.

Phage Buffer

Phage Buffer (PB; 1 L)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
1 M Tris stock (pH 7.5)	10 mL	10 mM
1 M $MgSO_4$ stock	10 mL	10 mM
NaCl	4 g	68 mM
ddH_2O	up to 1 L	
100 mM $CaCl_2$ stock	10 mL	1 mM
Glycerol (100 %), if using*	100 mL	10 %

To Prepare

1. Add 800 mL of ddH_2O to a large flask or beaker.
2. Add all ingredients to the water, while stirring. Glycerol should be added at this stage, if using (see notes*).
3. Stir until all components have completely dissolved.
4. Add ddH_2O to 1 L.
5. Filter-sterilize.
6. Aliquot into sterile bottles, flasks, or tubes in 50-mL or 100-mL (or other) portions as needed.

To Sterilize

- Filter-sterilize

To Store

- At room temperature, for up to 3 years. Discard if solution is discolored, contaminated or has precipitation.

Usage

In Protocol:

- 5.3, 5.4, 5.6, 6.1, 6.2, 6.3, 6.4, 6.5, 7.1, 8.1a, 8.1b, 11.1, 11.4

Notes

1. *If your phage is particularly unstable in phage buffer, you may consider supplementing your phage buffer with glycerol to 10 %.

2. 1 L of phage buffer is sufficient per semester for a class of ~ 20 students.

Phage Precipitation Solution

Phage Precipitation Solution (100 mL)

[If using, prepare as necessary]

For Bacterial Host: General

trial|Ingredients	Amount	Final Concentration
ddH_2O	To 100 mL	
Polyethylene glycol 8000 PEG 8000)	30 g	30 %
NaCl	19.3 g	3.3 M

To Prepare

1. Add 60 mL of ddH_2O , followed by 19.3 g NaCl, to a glass bottle with a screw cap, and swirl until the NaCl is completely dissolved.
2. Slowly add 30 g of PEG 8000. Add only a few grams at a time, alternately swirling and gently heating in a microwave (e.g. by heating only for 10 sec at a time) until all the PEG is dissolved.
3. Add sterile water to 100 mL.
4. Add a clean magnetic stir bar and stir until homogeneous.
5. Filter-sterilize.
6. Dispense into sterile bottles or tubes.

To Sterilize

- Filter-sterilize.

To Store

- At room temperature, indefinitely. Discard if solution is deteriorating, discolored, contaminated or has precipitation.

Usage

- In Protocol: 9.1 of Instructors Guide

Notes

1. The NaCl and the PEG 8000 are difficult to dissolve. Several iterations of heating and stirring may be required. If there are precipitates in the solution, do not use it! Rather, attempt to dissolve particulates by alternately heating the solution in a microwave and swirling. Allow the solution to cool before using. Use this solution only at room temperature.

2. Up to 4 mL of phage precipitation buffer is used per attempt at DNA extraction using the PEG precipitation protocol.

SDS (10 %)

SDS (10 %)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
SDS powder	1 g	10 %
ddH_2O	up to 10 mL	

To Prepare

Important: SDS may cause respiratory irritation if inhaled. If using loose powder of SDS (instead of micropellets), a mask should be used when weighing out the powder.

1. Weigh out 1g of SDS powder and place it in a 50 mL tube.
2. Add ddH_2O to 10 mL.
3. Invert the tube gently and repeated until the SDS is fully dissolved. Vigorous shaking will lead to excessive foaming.
4. Prepare 1 mL aliquots.

To Sterilize

- Filter-sterilize (0.22µm pore size) into a fresh sterile tube.

To Store

- At room temperature, indefinitely if not contaminated

Usage

- In Protocol: 9.1a, 9.1b, 9.2a, 9.2b

Label Stock Solution

- 10 % SDS

Notes

1. 2 mL of 10 % SDS should be sufficient per semester for a class of ~ 20 students.

Tris Borate EDTA (TBE)

1X TBE

For Bacterial Host: General

Ingredients	Amount	Final Concentration
10X TBE	100 mL	1X (89-mM Tris, 89-mM boric acid, and 2-mM EDTA)
ddH_2O	900 mL	

To Prepare

1. Dilute the 10X TBE to 1X with the volumes above (i.e., 100 and 900 mL). There is no need to check or adjust the pH of this solution, and it does not have to be sterilized before use.

To Sterilize

- Not required

To Store

- At room temperature, indefinitely. Discard if discolored, contaminated or has precipitation.

Usage

- In Protocol: 10.2

Notes

1. Volume of buffer will depend on size of gel and gel tank

Tris, pH 7.5 (1 M)

1 M Tris (base), pH 7.5 (100 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
Trizma base	12.11 g	1 M
ddH_2O	To 100 mL	

To Prepare

1. Add approximately 95 mL of ddH_2O to a large beaker.
2. Add Trizma base to the breaker while stirring.
3. When the Trizma base is dissolved, bring the pH to 7.5 with HCl.
4. Transfer the solution to a graduated cylinder, and add ddH_2O to 100 mL
5. Sterilize

To Sterilize

- Autoclave or filter-sterilize

To Store

- At room temperature, for up to a year.

Usage

In Recipe:

- Phage buffer

Notes

1. 10 mL of 1M Tris is required to prepare 1 L of phage buffer.
2. 100 mL of 1M Tris is sufficient for 1 – 5 semesters for a class of ~ 20 students.

Tween80 (20 %)

20 % Tween80 (100 ml)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
ddH_2O	To 100 ml	20 %
Tween80	20 ml	

To Prepare

1. Measure 60 mL of ddH_2O and place in a medium-size beaker.
2. Slowly add 20 mL of Tween80 while stirring.
3. Once the Tween80 has completely dissolved, transfer the solution to a graduated cylinder and bring to 100 mL with ddH_2O.
4. Sterilize

To Sterilize

- Filter-sterilize. Do not autoclave

To Store

- At room temperature,indefinitely if not contaminated.

Usage

In Recipe:

- 7H9 liquid medium

Notes

1. It may take a while for Tween80 to dissolve. Add Tween 80 slowly and alternate between stirring and warming the solution. For example, alternate the beaker between the stir plate and at 55 ºC water bath to dissolve the Tween80.
2. 100 mL of 20 % Tween80 is sufficient for 1 – 10 semesters for a class of ~ 20 students.

Uranyl Acetate Stain

Uranyl Acetate Stain

For Bacterial Host: General

Ingredients	Amount	Final Concentration
Uranyl acetate	0.1 g	1 % w/v
ddH_2O	10 ml	

To Prepare

1. Weigh out the uranyl acetate and place in a conical containing approximately 9 mL of ddH_2O, and stir
2. When the uranyl acetate is dissolved, bring the final volume up to 10 mL with ddH_2O.

To Sterilize

- Prepare a 0.22-µm filter by passing 3 mL of ddH_2O through the filter. Discard the filtered ddH_2O. Filter-sterilize the uranyl acetate solution and dispense in 1-mL aliquots.

To Store

- Cover each aliquot in foil to protect from light. Store at room temperature, for up to 3 years. Discard appropriately if precipitation occurs.

Usage

In Protocol:8.1a, 8.1b

Notes

1. This is a highly toxic compound. Always wear gloves when preparing or working with it.
2. 5 µL of uranyl acetate stain is used per sample to be imaged.

Water

Water, sterile (200 mL)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
ddH_2O	200 ml	

To Prepare

1. Measure 200 mL ddH_2O (ideally deionized water) and place bottle with a cap.
2. Sterilize

To Sterilize

- Filter-sterilize or autoclave.

To Store

- At room temperature, indefinitely if not contaminated

Usage

In Recipe:

- 80 % Isopropanol

In Protocol:

- DNA Extraction
- Restriction Enzymes Digests

Notes

1. 500 mL of sterilized water should be sufficient for all reagents and experiments per semester.

Zinc Chloride

Zinc Chloride (2 M)

For Bacterial Host: General

Ingredients	Amount	Final Concentration
$ZnCl_2$	10.9 g	2 M
ddH_2O	To 40 ml	

To Prepare

1. Weigh out $ZnCl_2$ and place in a conical tube containing approximately 35 mL of ddH_2O, and vortex until $ZnCl_2$ is dissolved.
2. Add ddH_2O to 40 mL.
3. Sterilize.

To Sterilize

- Filter-sterilize

To Store

- Important: Do Not Store.
  *Prepare immediately before use.

Usage

In Protocol:9.2b

Notes

1. * When prepared at this high concentration, $ZnCl_2$ will precipitate after ~ 1 hour at room temperature.

7H9 Liquid Medium(Neat)

Middlebrook 7H9 Liquid Medium: Neat (900 mL)

For Bacterial Host: *M. smegmatis* mc^{2}155 only

Ingredients	Amount	Final Concentration
7H9 broth base	4.7 g	
40% glycerol stock	5 ml	
ddH_2O	To 900 ml	

To Prepare

1. Add broth base to 850 mL of ddH_2O in an Erlenmeyer flask.
2. While stirring, add the glycerol. Stir until the broth base powder is completely dissolved.
3. Bring up to 900 mL with ddH_2O in a graduated cylinder.
4. Aliquot into 90-mL, 450-mL, or 900-mL portions as needed.
5. Sterilize

To Sterilize

- Autoclave.

To Store

- At room temperature,indefinitely if not contaminated or discolored.

Usage

In Recipe:

- Growing *Mycobacterium smegmatis* mc^{2}155
- 1X 7H9 top agar

Notes

1. This medium will be diluted by the addition of 10 % (v/v) AD supplement (see Middlebrook 7H9 Liquid Medium: Complete). This is why medium is dispensed in 9/10 volumes (e.g., 450 mL).

2. 5 L of 7H9 liquid media should be sufficient per semester for a class of 20 students.

7H9 Liquid Medium(Complete)

Middlebrook 7H9 Liquid Medium: Complete (100 mL)
(For bacterial growth without Tween 80)

For Bacterial Host: *M. smegmatis* mc^{2}155 only

Ingredients	Amount	Final Concentration
7H9 liquid medium: neat	89 ml	1X
AD supplement	10 ml	10 %
CB stock	100 µl	50 µg/ml
CHX stock	100 µl	10 µg/ml
100 mM $CaCl_2$ stock	1 ml	1 mM

To Prepare

1. All ingredients must be added to a sterile bottle or flask using aseptic technique. The final medium cannot be autoclaved or filter-sterilized.

To Sterilize

- All ingredients are already sterile

To Store

- At 4 ºC, for up to 30 days. Discard if media is discolored or contaminated.

Usage

In Recipe:

- Growing *Mycobacterium smegmatis* mc^{2}155

Notes

1. Make complete 7H9 medium as needed; refrigeration may cause the $CaCl_2$ to precipitate. If antimicrobials and AD supplement have been added, the medium must be stored at 4 °C.

2. A good rule of thumb is that $CaCl_2$ should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because $CaCl_2$ will precipitate out when cooling after autoclaving or when refrigerated.

7H9 Liquid Medium(Complete with Tween80)

Middlebrook 7H9 Liquid Medium: Complete with Tween80
(For bacterial growth withTween 80)

For Bacterial Host: M. smegmatis mc^{2}155 only

Ingredients	Amount	Final Concentration
7H9 liquid medium:	90 ml	1X
AD supplement	10 ml	10 %
CB stock	100 µl	50 µg/ml
CHX stock	100 µL	10 µg/ml
100 mM $CaCl_2$ stock	1 ml	1 mM
20 % Tween80 stock	250 µL	0.05 %

To Prepare

1. All ingredients must be added to a sterile bottle or flask using aseptic technique. The final medium cannot be autoclaved or filter-sterilized.

To Sterilize

- All ingredients are already sterile

To Store

- At 4 ºC, for up to 30 days. Discard if media is discolored or contaminated.

Usage

In Recipe:

- Growing *Mycobacterium smegmatis* mc^{2}155

Notes

1. In liquid culture, *Mycobacterium smegmatis* tends to clump. When liquid cultures are grown from bacterial colonies, Tween80 should be added to 0.05% to prevent clumping.

2. This culture is then sub-cultured into medium without Tween80 for use in phage infections.

3. Make complete 7H9 medium as needed; refrigeration will cause the calcium to precipitate. If antimicrobials and AD supplement have been added, the medium must be stored at 4 °C.

4. A good rule of thumb is that $CaCl_2$ should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because $CaCl_2$ will precipitate out when cooling after autoclaving or when refrigerated.

Carbenicllin Stock Solution

Carbenicillin (CB) at 1000X

For Bacterial Host: *M. smegmatis* mc^2155 only

Ingredients	Amount	Final Concentration
Carbenicillin (powder)	50 mg/ml	stock = 1000X

To Prepare

1. Weigh out 100 to 600 mg of carbenicillin powder and place it in a 10-15 mL tube.
2. Divide the number of milligrams of carbenicillin by 50 to determine the volume (in milliliters) of ddH_2O to add (e.g., for 477 mg of CB, add 9.5 mL ddH_2O).
3. Stir or shake until dissolved.
4. Filter-sterilize (0.22µm pore size) into a fresh sterile tube.
5. Prepare 1 mL aliquots and freeze at -80 °C

To Sterilize

- Filter-sterilize (0.22µm pore size) into a fresh sterile tube.

To Store

- At -80 °C for 3 years.
- At 4 ºC for ≤ 60 days.

Usage

In Recipe:

- 7H9 Liquid Medium

Label Stock Solution

- CB, 1000X, date, initials

To Prepare Final Solution

1. Remove a frozen aliquot, allow to thaw, and use as follows. Do not return thawed aliquot to -80 °C . Instead, store thawed aliquots at 4 °C

2. When using, add stock solution at 1:1000. For example, for 1 L of medium, add 1 mL of stock.

Notes

1. If your host bacteria is resistant to CB, it can be added to prevent growth of other bacteria.

2. Mechanism of action: CB is a semi-synthetic penicillin that interferes with bacterial cell-wall synthesis.

3. 50 mL of carbenicillin should be sufficient per semester for a class of ~ 20 students.

7H9 Top Agar (1X)

1X Middlebrook Top Agar (TA; 100mL)

For Bacterial Host: *M. smegmatis* $mc^2$155 only

Ingredients	Amount	Final Concentration
100 mM $CaCl_2$ stock	1 ml	1 mM
7H9 liquid media neat	50 ml	
2X TA	50 ml	1 X

To Prepare

1. Using aseptic technique, add 1 mL of $CaCl_2$ stock to the 50 mL of 7H9 medium.
2. Place into a 55 °C water bath.
3. Melt the 2X TA in a microwave. Make sure the cap is loose! The agar must be completely melted, so carefully swirl the bottle and check to make sure there are no clumps. The 2X TA should come to a boil but not boil over.
4. Once the agar is completely melted, place the 2X TA into a 55 °C water bath. When both solutions are at 55 °C, aseptically add the medium-calcium mixture to the 2X TA and swirl.

To Sterilize

- All ingredients must be added using aseptic technique. The final medium cannot be autoclaved or filter-sterilized.

To Store

- In the 55 °C water bath ≤ 7days. Cooling to room temperature will cause the $CaCl_2$ to precipitate out of solution.
  Discard if media is discolored or contaminated.
  Repeated melting and solidifying of top agar will cause the agar to deteriorate and not solidify properly on plates.

Usage

- In Protocol:
  5.3, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.1,12.2

Notes

1. The final agar concentration will be 0.4%

2. A good rule of thumb is that $CaCl_2$ should be added to all solutions that come in contact with phage, and ideally, this should happen immediately before the solution is used. This is because $CaCl_2$ will precipitate out when cooling after autoclaving or when refrigerated.

3. 3 – 5 mL of top agar is used per plating.

7H9 Top Agar (2X)

2X Middlebrook Top Agar (2X TA; 1 L)

For Bacterial Host: *M. smegmatis* mc^2155 only

Ingredients	Amount	Final Concentration
7H9 broth base	4.7 g	
Agar	8 g	0.8 %
ddH_2O	1 L	

To Prepare

1. Place ddH_2O and a stir bar, then broth base and agar, into an Erlenmeyer flask; put onto a stir plate.
2. Stir until the broth base powder is completely dissolved (the agar will not dissolve until it is heated).
3. Autoclave the flask, and cool to 55 °C in a 55 °C water bath.
4. Gently swirl the solution to evenly distribute the agar.
5. In a hood, using aseptic technique, dispense into sterile bottles using only half the available volume (e.g., for a 100 mL bottle, add 50 mL of 2X TA; this will allow you to add 50 mL of media directly to the molten 2X TA to prepare 1X TA). Keep any lids loose until the agar has solidified.
6. Tighten lids for storage.
7. To melt stored top agar for use, heat in a microwave using 30 second intervals until agar has completely melted.
8. Repeated melting and solidifying of top agar will cause the agar to deteriorate and not solidify properly on plates.

To Sterilize

- Autoclave

To Store

- As a solid, store at room temperature, indefinitely. Discard if media is discolored or contaminated.

Usage

In Recipe:

- 1X Middlebrook Top Agar

In Protocol:

- 12.1

Notes

1. 2.5 L of 2X top agar is sufficient for per semester for ~ 20 students

Luria Agar (L-Agar) Plates

2X Middlebrook Top Agar (2X TA; 1 L)

For Bacterial Host: *M. smegmatis* $mc^2$155 only

Ingredients	Amount	Final Concentration
Luria broth base	15.5 g	
Agar	15 g	
ddH_2O	To 1 L	
CB stock (if using)	1.0 mL	50 µg/mL
CHX stock	1.0 mL	10 µg/mL

To Prepare

1. To 1 L of ddH_2O in a large flask, add 15.5 g of Luria broth base and 15 g agar. Mix well with a magnetic stir bar.
2. Autoclave the flask.
3. Cool to 55 °C in a 55 °C water bath.
4. Once the agar has cooled to 50-55 °C, aseptically add antimicrobials as necessary.
5. Mix well by swirling, and avoid bubbles.
6. Using aseptic technique, pour agar into Petri dishes.
7. Allow the agar to set overnight.*

To Sterilize

- Autoclave (see above)

To Store

- At 4 °C, for up to 3 months. Plates can still be used beyond 3 months, although the antimicrobials may have been partially degraded. Discard if plate is discolored or contaminated.

Usage

- In Protocol:
  5.3, 5.4, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.2

Notes

1. Prior to adding antibiotics or antimicrobials, the cooled medium can be aliquoted into sterile bottles, allowed to solidify, and stored at room temperature. The solidified agar can be melted by autoclaving for 5 mins, or in a microwave. Antimicrobials should only be added once the freshly melted agar has cooled to 55 ˚C.

2. Prior to use, agar plates stored at 4 ˚C should be allowed to warm to room temperature.

3. 1 L of agar is sufficient to prepare 50 agar plates.

4. * Though the agar will set within 1 – 2 hours, allowing the plates to sit at room temperature overnight helps remove some water content within the agar. Drying plates in this manner helps prevent top agar, when used, from slipping on the surface of the agar.

5. Approximately 1,500 agar plates are used per semester per class of 20 students. Consider preparing plates in batches of 500 plates.

Media Supplement for Streptomyces phage

Media Supplement for *Streptomyces* phage (32.5 mL)

For Bacterial Host: *Streptomyces* spp. only

Ingredients	Amount	Final Concentration
1 M $MgCl_2$	10 mL	10 mM (after addition to culture media)
0.8 M $Ca(NO_3)_2$	10 ml	8 mM (after addition to culture media)
40% Dextrose	12.5 mL	0.5% (after addition to culture media)

To Prepare

1. Aseptically add each component to a sterile container to mix or add each directly to cooled, sterile culture media (per 1 L).

2. Mix by gently swirling.

To Sterilize

- Autoclave each component separately.

To Store

- At room temperature, for up to 1 year. Discard if solution is discolored, contaminated or has precipitation.

Usage

- Nutrient Broth with Media Supplement for *Streptomyces* phage, Nutrient Agar with Media Supplement for *Streptomyces* phage

Notes

1. If using a different species of *Streptomyces* for phage isolation, a different base media may be used with the same supplement.

Nutrient Broth

Nutrient Broth (1 L)

For Bacterial Host: *Streptomyces* spp. only

Ingredients	Amount	Final Concentration
Nutrient broth powder	8 g	1X
ddH_2O	To 1 L	

To Prepare

1. Mix 8 g of Nutrient broth powder with 1 L of ddH_2O in a large flask and mix well with a magnetic stir bar.
2. Aliquot as needed.
3. Sterilize

To Sterilize

- Autoclave

To Store

- At room temperature or 4 °C.

Usage

- *Streptomyces* liquid culture growth for starter cultures.

Notes

1. Because *Streptomyces* cultures are prone to clumping when grown in liquid, it is highly recommended that all cultures contain 5% w/v PEG 8000, which serves as a physical dispersant, and that cultures be shaken vigorously in a baffled flask. PEG can be added to mixture prior to autoclaving.
2. 5 L of 7H9 liquid media should be sufficient per semester for a class of 20 students.

Nutrient Broth(with Supplements for S.griseus)

Nutrient Broth with Media Supplement for *Streptomyces* phage (1 L)

For Bacterial Host: *Streptomyces* spp. only

Ingredients	Amount	Final Concentration
Nutrient broth powder	8 g	1X
ddH_2O Media	To 1 L	
Supplement for *Streptomyces* phage	32.5 mL	

To Prepare

1. Mix 8 g of Nutrient broth powder with 1 L of ddH_2O in a large flask and mix well with a magnetic stir bar.
2. Aliquot as needed.
3. Autoclave.
4. Add Media Supplement for *Streptomyces* phage (32.5 mL/1L) after media has cooled.

To Sterilize

- Autoclave

To Store

- At room temperature or 4 °C.

Usage

- *Streptomyces* liquid culture growth in which phage infection will occur (including enrichment cultures)

Notes

1. Because *Streptomyces* cultures are prone to clumping when grown in liquid, it is highly recommended that all cultures contain 5% w/v PEG 8000, which serves as a physical dispersant, and that cultures be shaken vigorously in a baffled flask. PEG can be added to mixture prior to autoclaving.

Nutrient Agar Plates (with Supplements for S. griseus)

Nutrient Agar with *Streptomyces* phage supplement (NA+) Plates (1 L)

For Bacterial Host: *Streptomyces* spp. only

Ingredients	Amount	Final Concentration
Nutrient agar powder	23 g	1X
ddH_2O	To 967.5 L	
Media Supplement for *Streptomyces* phage	32.5 mL	

To Prepare

1. Mix 23 g of Nutrient agar powder with ddH_2O to 967.5 mL total volume in a large flask and mix well with a magnetic stir bar.
2. Autoclave the flask.
3. Cool to 55 °C in a 55 °C water bath.
4. Once the agar has cooled to 50-55 °C, aseptically add 32.5 mL of sterile Media Supplement for *Streptomyces* phage.
5. Mix well by swirling, and avoid bubbles.
6. Using aseptic technique, pour agar into Petri dishes.
7. Allow the agar to set overnight.*

To Sterilize

- Autoclave (see above)

To Store

- At room temperature or 4 °C, for up to 3 months. Discard if plate is discolored or contaminated.

Usage

- In *Streptomyces* plate growth protocols.

Notes

1. Prior to adding media supplements, the cooled medium can be aliquoted into sterile bottles, allowed to solidify, and stored at room temperature. The solidified agar can be

melted by autoclaving for 5 mins, or in a microwave. Supplements should only be added once the freshly melted agar has cooled to 55 ˚C.

2. Prior to use, agar plates stored at 4 ˚C should be allowed to warm to room temperature.

3. * Though the agar will set within 1 – 2 hours, allowing the plates to sit at room temperature overnight helps remove some water content within the agar. Drying plates in this manner helps prevent top agar, when used, from slipping on the surface of the agar.

4. 1 L of agar is sufficient to prepare 50 agar plates.

5. Approximately 1,500 agar plates are used per semester per class of 20 students. Consider preparing plates in batches of 500 plates.

PYCa Agar Plates

PYCa Agar Plates (1 L)

For Bacterial Host: General (excluding M. smegmatis and Streptomyces spp.)

Ingredients	Amount	Final Concentration
Agar	15.0 g	1.5 %
Yeast extract	1 g	1 g/L
Peptone	15 g	15 g/L
1 M Cacl2	4.5 mL	4.5 mM
Dextrose (40 %)	2.5 mL	0.1 %
ddH_2O	990 mL	
CHX stock	1.0 mL	10 µg/mL

To Prepare

Note: A video protocol for this recipe card is available here.

1. Place water and a stir bar, followed agar, peptone and yeast extract, into an Erlenmeyer flask. Put onto a stir plate.
2. Stir until the yeast extract and peptone are completely dissolved (the agar will not dissolve until it is heated).
3. Autoclave the flask, and cool to 60 °C in a 60 °C water bath.
4. In a hood, aseptically add dextrose and CaCl2.
5. Add antibiotics/antimicrobials stock solutions, if using.
6. Mix well by swirling, and avoid bubbles.
7. Using aseptic technique, pour agar into Petri dishes.
8. Allow the agar to set overnight.*

To Sterilize

- Autoclave

To Store

- At 4 °C, for up to 3 months. Plates can still be used beyond 3 months, although the antimicrobials may have been partially degraded. Discard if plate is discolored or contaminated.

Usage

- In Protocol: 5.3, 5.4, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.2

Notes

1. Prior to adding antibiotics or antimicrobials, the cooled medium can be aliquoted into sterile bottles, allowed to solidify, and stored at room temperature. To melt many batches of solidified agar, autoclave for ~ 10 -15 mins. Alternatively, melt the agar in small batches using a microwave. Antimicrobials should only be added once the freshly melted agar has cooled to 55 ˚C.

2. Prior to use, agar plates stored at 4 ˚C should be allowed to warm to room temperature.

3. * Though the agar will set within 1 – 2 hours, allowing the plates to sit at room temperature overnight helps remove some water content within the agar. Drying plates in this manner helps prevent top agar, when used, from slipping on the surface of the agar.

4. 1 L of agar is sufficient to prepare 50 agar plates.

5. Approximately 1,500 agar plates are used per semester per class of 20 students.

   - Consider preparing plates in batches of 500 plates.

   - Often, a limiting factor to preparing plates in large quantities is adequate space at 4 ˚C to store those plates.

PYCa Liquid Medium

PYCa Liquid Medium (1 L)

For Bacterial Host: General (excluding *M. smegmatis* and *Streptomyces* spp.)

Ingredients	Amount	Final Concentration
Yeast extract	1.0 g	1 g/L
Peptone	15.0 g	15 g/L
ddH_2O	990 ml	
1 M $CaCl_2$ stock	4.5 mL	4.5 mM
Dextrose (40 %)	2.5 mL	0.1 %
CHX stock	1.0 mL	10 µg/mL

To Prepare

1. Add 990 mL of ddH_2O into a large flask. Then weigh out and add peptone and yeast extract to the flask.
2. Stir until completely dissolved.
3. Autoclave.
4. Once cool, aseptically add dextrose and $CaCl_2$.
5. Aliquot as 100-mL, 500-mL, or 1000-mL portions, in sterile vials, as needed.*
6. If using CHX, add prior to use.

* It may be more convenient to aliquot the media (lacking dextrose and calcium chloride) into vials prior to sterilization by autoclaving. In this case, the vials do not need to be sterile, but dextrose and calcium chloride stock solutions should still only be added after sterilization. You will also need to adjust the volume of dextrose and calcium chloride stock solutions added to each vial.

To Sterilize

- Autoclave.

To Store

- At room temperature, indefinitely, or at 4 °C for up to 30 days if antimicrobials have been added.

- Discard if media is discolored or contaminated.

Usage

- In Protocol: 5.2, 5.5, & 12.1

Notes

1. If antimicrobials have been added, the medium must be stored at 4 °C.
2. 5 L of PYCa liquid media should be sufficient per semester for a class of 20 students.

PYCa Top Agar

PYCa Top Agar (PYCa TA at 1X concentration; 1 L)

For Bacterial Host: General (excluding *M. smegmatis* and *Streptomyces* spp.)

Ingredients	Amount	Final Concentration
Agar	4.0 g	trial|0.4 %
Yeast extract	1 g	trial|1 g/L
Peptone	15 g	15 g/L
1 M $CaCl_2$	4.5 mL	4.5 mM
Dextrose (40 %)	2.5 mL	0.1 %
ddH_2O	990 mL	

To Prepare

Note: You may choose to prepare a large volume of media and then transferring aliquots into smaller vials after sterilization (as described below), or preparing the media in smaller vials before sterilization. If choosing the latter, adjust the weight and volumes of each media component accordingly.

1. Add water and a stir bar, followed by agar, peptone and yeast extract, into an Erlenmeyer flask; put onto a stir plate.
2. Stir until the yeast extract and peptone is completely dissolved (the agar will not dissolve until it is heated).
3. Autoclave the flask.
4. Once autoclaved and cooled slightly (not below 60 ˚C), add dextrose and $CaCl_2$, using aseptic technique. Gently swirl the flask to mix the solution.
5. In a hood, using aseptic technique, dispense into 50 mL or 100 mL sterile bottles.
6. If using immediately, maintain top agar in the molten form by placing it at 55 ˚C. If not, keep bottle lids slightly loose until the agar has solidified. Once solidified, tighten lids for storage.
7. To melt stored top agar for use, make sure the cap is loose, and heat in microwave using 30 second intervals. The top agar should come to a boil but not boil over. As the agar must be completely melted, **carefully** and gently swirl the bottle with the loose cap facing away from you and others. Check to make sure there are no clumps.

8. Once the agar is completely melted, place the top agar into a 55 °C water bath to cool before use.

9. Repeated melting and solidifying of top agar will cause the agar to deteriorate and not solidify properly on plates.

To Sterilize

- Autoclave

To Store

- As a solid, store at room temperature, indefinitely. Discard if media is discolored or contaminated.

- As molten agar, store for up to 30 days. Agar stored in its molten form deteriorates over time and may not solidify properly on plates.

Usage

In Protocol:

- 5.3, 5.6, 6.1, 6.4, 6.5, 7.1, 11.1, 11.2, 11.3, 11.4, 12.1, 12.2

Notes

1. 5 L of PYCa top agar is sufficient for per semester for ~ 20 students .

Growing bacteria from a frozen stock

Growing bacteria from a frozen stock (Streak Plate)

To Prepare

Note: A video demonstrating this protocol is available at here.

1. Refer to Host Basics in the Phage Discvery Guide for specific guidelines when working with a particular bacterial host. In particular, take note of the appropriate media (agar) and incubation temperature.

2. Label an appropriate agar plate.

3. Once you are ready to streak a plate, obtain your frozen stock from the freezer and complete Step 4 quickly to minimize the amount of thawing of your frozen stock.

4. Using an inoculation loop or sterile wooden stick, scrape the surface of a frozen stock, and immediately return the stock to the freezer.

5. Aseptically spread the bacteria at the end of the loop or wooden stick onto the agar plate by streaking for single colonies.

6. Incubate for 2 – 3 days at the appropriate temperature.

7. Monitor growth over time to ensure bacterial growth parameters are as expected (e.g. time for colony formation and color/texture of colonies.

8. Once single colonies are visible and large enough for picking (see image of plate in “Host Basics” of the Phage Discovery Guide), plates should be stored either at room temperature or at 4 ˚C. To prevent over drying, the plates should be wrapped in parafilm.

To Store

- At room temperature or at 4°C.

Usage

- In Protocol: Streak plate

Notes

1. This is your P0FF (passage 0 from frozen) stock. Depending on the host bacteria, this stock culture can be stored at room temperature or at 4°C for several weeks. We recommend storage for no more than a couple of weeks.

2. We suggest that you start P0FF stock cultures every week or every other week.

Growing liquid cultures of bacteria from a single colony

Growing liquid cultures of bacteria from a single colony

To Prepare

Note: A video demonstrating this protocol is available at here.

1. Refer to Host Basics in the Phage Discovery Guide for specific guidelines when working with a particular bacterial host. In particular, take note of the appropriate media (liquid) and incubation temperature.

2. Add 50 mL of the appropriate liquid medium to a 250-mL baffled flask. If using, add antibiotics or antimicrobials.

3. Using an inoculation loop or sterile wooden stick, pick a small piece from the middle of a single colony of bacteria on an agar plate. Avoid using colonies from plates older than 2 weeks.

4. Aseptically transfer the bacteria from the loop or wooden stick into the medium in the flask.

5. **Tween80 should be added to 0.05% when culturing bacteria that tend to clump (e.g. *M. smegmatis* $mc^2$155).** Cultures prepared with Tween80 must be subcultured to remove Tween80. See instructions for subculturing from cultures prepared with Tween80.

6. Incubate, with shaking (250 rpm), at the recommended temperature.

7. Monitor growth over time to ensure culture growth parameters are as expected (e.g. duration before culture is expected to become saturated)

8. Once the culture is saturated, store at room temperature.

Important: Whenever a liquid culture of bacteria is prepared, the culture should be tested as follows:

- Prepare a streak plate from the culture, and monitor for the expected growth rate and colony morphology of the bacterial strain. If the growth rate or colony morphology is not as expected, the culture may be contaminated.

- Prepare a top agar lawn using an aliquot of the bacterial culture, and test the ability of a host-specific phage to infect the top agar lawn using a spot test (Protocol 5.6). If the host-specific phage is unable to infect the bacterial lawn, the bacterial culture may be contaminated.

To Store

- At room temperature or at 4°C, for 1 – 2 weeks.

Usage

In Protocol:

- Growing liquid cultures for use in phage experiments

Notes

1. This is your P1FF (passage 1 from frozen) stock. This stock culture can be stored at room temperature, the duration for which will depend on the bacterium. Check Host Basics in this Guide for information about a specific bacterium.

2. It can be aseptically aliquoted into smaller volumes for storage (this minimizes the potential for contamination).

3. We recommend that you start P1FF stock cultures weekly, depending on use and the size of a given class. Regardless, because of the possibility of contamination, a backup P1FF culture should be available at all times.

4. Single colonies from an agar plate can be used to inoculate multiple P1FF cultures for as long as the plate remains uncontaminated.

5. If your PIFF does **NOT** contain Tween80, this culture can be used for experiments with phage.

6. Tween80 is added to cultures for some bacteria to minimize clumping during bacterial growth. **The presence of Tween80, however, will likely inhibit phage infection.**
   For this reason:
   To grow liquid cultures for use in phage experiments, sub-inoculate from a P1FF culture into liquid media without Tween80. For instructions, see the recipe card “Growing liquid cultures of bacteria for use in phage experiments.”

Preparing a culture without Tween80 from one with Tween80

Preparing a culture without Tween80 from one with Tween80

To Prepare

Note: This protocol assumes a saturated liquid culture has been prepared, and contains Tween80.

1. Add 50 mL of the appropriate liquid media (i.e., without Tween80) to a 250-mL baffled flask. For larger volumes, adjust accordingly (see notes). If using, add antibiotics or antimicrobials.

2. Aseptically, add a 1:1000 volume of the saturated culture containing Tween80. For example, for a 50-mL culture, add 50-µL of your culture that contains Tween80.

3. Incubate, with shaking (250 rpm), at the recommended temperature.

To Store

- At room temperature or at 4°C.

Usage

- In Protocol: 5.2 - 7.2

Notes

A culture prepared from a single colony is known as a P1FF. A new culture prepared from a PIFF is known as a P2FF.

1. The addition of small (≤1:1000) volumes of the PIFF culture (containing Tween80) when preparing a sub-culture without Tween80 reduces the likelihood of bacterial clumping in subculture. If clumping does occur, continue incubating the subculture up to an additional 48 hours.

2. This culture can be stored at room temperature, the duration for which will depend on the bacterium. Check Host Basics in this guide for information about a specific bacterium.

3. To prepare additional cultures without Tween80, it is best to go back to the initial P1FF stock culture, so long as the P1FF culture is not contaminated, is not clumpy, or has not been stored for beyond the recommended time (see Host Basics in this guide)

4. The volume of a culture should be approximately one-fifth the size of the flask. For example, for 200 mL of bacterial culture, use a 1-L baffled flask. This maximizes aeration when the bacteria are grown on a shaker.

5. Tween80 will inhibit phage infection. It is advisable to double-check that any medium that will be used in phage experiments does not include Tween80.

Preparing frozen stocks of bacteria

Preparing frozen stocks of bacteria

To Prepare

Note: A video demonstrating this protocol is available at here.

1. Label the appropriate number of cryo-tubes with the name of the bacteria, the date of preparation, and initials of the person preparing the stock.
2. Add 300 µL of 40 % glycerol to each tube.
3. Add 700 µL of bacterial culture to each tube.
4. Mix by inversion.
5. Store at -80 °C.

To Store

- at -80 °C, indefinitely.

Notes

1. To use frozen bacterial stocks, refer to the recipe card “Growing bacterial cultures from frozen a stock.”
2. It is important that only one person access any given glycerol stock. When a glycerol stock is first used, it should be labelled with the name of the person who is using it.

Preparing Additional Phage Lysate & Long-term Storage

Preparing Additional Phage Lysate & Long-term Storage

To Prepare

1. First, determine the titer of your phage lysate by performing a full plate titer (Protocol 6.5).

2. Based on the calculated titer, prepare webbed plates (Protocol 7.1).

   Note: You can flood those plates from Step.1 that are webbed or have very high densities of plaques. This lysate should also be titered as part of Step.4.

3. Prepare additional lysate by flooding the webbed plates (Protocol 6.3). Proceed to Step 4, ideally as soon as Protocol 6.3 is complete.

4. Determine the titer of the freshly prepared phage lysate by performing a full plate titer (Protocol 6.5). Ideally, the titer of the lysate will be at 10^9 pfu/ml or greater. If the titer is below 10^9 pfu/ml, return to Step 2 and attempt the following modification: When webbed plates have been identified, incubate those webbed plates for an additional 24 - 48 hrs such that the bacteria remaining within the "webbing" have also been lysed by phage before proceeding to Step 3.

5. Next:

   1. **To prepare freezer stocks,** add 1 ml of the high-titer phage lysate to a labeled cryotube, then add 70 µL of DMSO. Mix by inversion and store at -80 °C. Ideally, 3 - 4 freezer stocks will be prepared.

   2. **To maintain a working stock,** store the lysate at 4 °C.

   Important: The stability of phage particles in lysate stored at 4 °C will vary from phage to phage. Generally, phage stability parallels the titer, with phages more stable at higher titers. Regardless, the titer of phage lysates can drop from one day to another, or remain stable for weeks. It is therefore important to recalculate the titer of your lysate periodically.

   3. **To archive phage lysates,** refer to the archiving protocol (Protocol 7.3).

      Note: You will receive bar-coded cryo-tubes and DMSO from the program in the fall of Year 1. All samples for archiving at the University of Pittsburgh should be prepared as described in the Phage Discovery Guide.

To Store

- Freezer stocks -80 °C, indefinitely. Working stocks at 4 °C, with the titer checked periodically (e.g. biweekly)

Notes

1. To use frozen phage lysates, scrape a few microliters from the surface of the frozen lysate using a sterile micropipette tip, and immediately return the stock to the freezer to prevent thawing of the frozen stock. Repeated freeze-thaw cycles can damage phage particles.

2. Transfer the scraped lysate to a microcentrifuge tube pre-filled with 100 µL of phage buffer.

3. The resuspension can then be used to prepare a working stock of phage lysate.

4. Avoid thawing the frozen stock.

Received Biologicals from the Program? Follow these Next Steps.

Received Biologicals from the Program? Follow these Next Steps.

Bacteria

Bacterial strains are typically shipped as glycerol/freezer stocks to program members, and are intended for use in preparing additional freezer stocks for long-term storage.Upon receiving these glycerol/freezer stocks, store these stocks at -80 °C.

To prepare additional glycerol/freezer stocks:

1. Streak out bacteria to obtain single colonies. Follow the instructions provided in "Growing bacteria from a frozen stock".

2. Within days of obtaining single colonies, prepare a liquid culture of the bacterial strain. Follow the instructions provided in "Growling liquid stock of cultures from a single colony".

3. Once the liquid culture is saturated, prepare additional frozen stocks following the instructions provided in "Preparing frozen stocks of bacteria".

Important: Whenever a liquid culture of bacteria is prepared, the culture should be tested as follows:

- Prepare a streak plate from the culture, and monitor for the expected growth rate and colony morphology of the bacterial strain. If the growth rate or colony morphology is not as expected, the culture may be contaminated.

- Prepare a top agar lawn using an aliquot of the bacterial culture, and test the ability of a host-specific phage to infect the top agar lawn using a spot test (Protocol 5.6). If the host-specific phage is unable to infect the bacterial lawn, the bacterial culture may be contaminated.

Phage

- Phage are typically shipped as working stocks (i.e. the stock lacks a cryo-protectant and should not be placed in a freezer). Upon receiving phage lysate from the program, store the lysate at 4 °C. Within weeks of receiving the lysate, prepare additional lysate for long-term storage and for use in research following instruction provided in 'Preparing Additional Phage Lysate & Long-term Storage'.

Notes about Recipes

Notes About Recipes:

About ddH2O

Water should be double-distilled at a minimum. Filtered water (e.g., Millipore) is ideal. Regardless of water availability, consistency of preparation is essential (i.e., always use the same water).

About Labeling

Clearly label all media and solutions, and date the additions of supplements. Sterile solutions should also be clearly labeled as such, and any autoclave tape (indicating sterility) should be left on the bottles.

About Adjusting the pH of Solutions

Be sure to check each recipe for the proper acid or base solution that should be used to adjust the pH (if needed) of each solution or reagent.

Autoclave Setting

When using an autoclave, selecting the correct settings is crucial.

Cycle Type:

Most autoclaves are equipped with 3 settings: Liquid, Wrapped (or Vacuum), andUnwrapped (or Gravity).

- **Liquid Cycle**: When sterilizing liquids by autoclave, the LiquidCycle should be selected. The Liquid Cycle is programmed to release the chamber pressure slowly at the endof the cycle, which prevents your super-heated liquids from rapidly bubbling over.

- **Wrapped (or Vacuum) Cycle**: Most consumables and glassware used,as well as biohazard waste generated, should be sterilized in an autoclave using the Wrapped (or Vacuum)Cycle. This cycle mechanically replaces the air in the chamber with steam, which allows the steam to accessareas where air may otherwise be trapped (e.g. pipette tip boxes). Note that this cycle is notpre-programmed to release chamber pressure slowly, and should not be used for liquids.

- **Unwrapped (or Gravity) Cycle**:Unwrapped utensils can be sterilized using this cycle. Unlike the Wrapped Cycle, the Unwrapped cycleuses gravity to displace the air in the chamber. As such, air trapped in containers (e.g. pipette tipboxes) may not be replaced with steam, limiting sterilization.

Cycle temperature and duration:

- **Liquids:** Liquids should be autoclaved at 121 ˚C (250 ˚F). The cycle duration varies depending on the amount of liquid being autoclaved. As a rule, 500 ml of liquid should be autoclaved for 40 mins. An extra5 minutes should be added to the overall duration for each additional 500 ml of liquid.

- **Non-liquids:** Most consumables and glassware used, as well as biohazard waste generated, should be sterilized in an autoclave set at 121 ˚C (250 ˚F) for 20 mins.