# Genomics Research Initiative Laboratory

## BILD70

Fall 2021

1-5 PM Wednesday (1-1:30 Zoom lecture; 2-5 in-person)

1-5 PM Friday (1-1:30 Zoom lecture; 2-5 in-person)

York Hall, Room 4406


| INSTRUCTOR: | E-MAIL | OFFICE |
|---|---|---|
| Dr. Joe Pogliano | jpogliano@ucsd.edu | 4111 NSB, 822-4074 |
| **TEACHING ASSISTANT:** | | |
| Emily Armbruster | earmbrus@ucsd.edu | 4109 NSB |
| **PREPARATION ROOM:** | | |
| Dr. Ana Gomez | | 2105 Tata Hall, 858-246-0952 |

| Date | Class Outline | Laboratory reading | Lecture Reading (Veloxsci) |
|---|---|---|---|
| 1. F Sept 24 | Zoom:<br>-Student introductions<br>-Course introduction and outline<br>-Safety<br><br>Demo:<br>-Pipet exercises<br>-Serial dilutions<br>-Swabbing surfaces<br><br>Laboratory:<br>-Laboratory safety<br>-Pipet exercises<br>-Sampling the environment for microbes<br>-Phage collection methodology | -BYS 1-6<br>-Capture 7<br>-Safety in BILD70 Phage Hunters Laboratory<br>-Serial dilutions handout | |
| 2. W Sept 29 | Zoom:<br>-Planet microbiome overview<br>-Sterile technique and phage enrichments<br><br>Demo:<br>-Sterile technique<br>-Set up enrichments<br><br>Laboratory:<br>-Practice sterile technique<br>-Set up enrichment cultures for Bacillus phage | -BYS 1-8<br>-Capture 7-8<br>-Lab Manual p.10 (enrichments) | Chapter 2: Introduction to Microbiomes |
| 3. F Oct 1 | Zoom:<br>-Phage life cycle overview<br>-Filtering enrichments<br><br>Demo:<br>-Spinning & filtering enrichment<br>-Pouring top agar<br>-Streaking phage for purification<br><br>Laboratory:<br>-Test enrichment cultures for *B. subtilis* phage<br>-Practice streaking *B. subtilis phage* | -Capture 8<br>-Capture 17-22<br>-Lab Manual p.11 (filtering enrichments)<br>p.14 (using top agar)<br>p. 17-18 (plaque purification by streaking)<br>Tame 5-8 (purification by streaking) | |
| 4. W Oct 6 | Zoom:<br>-Importance of pure culture<br>-Limitations of culturing techniques<br>-Purifying phage | -Capture 17-22<br>-Tame 1-8<br>-Lab Manual p. 17-18 (purification by streaking) | |

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| | Demo:<br>-Picking new plaques for streak purification<br><br>Laboratory:<br>-Photograph plates<br>-Pick *B. subtilis* phage plaques and streak purify (from your own plate or from a classmate's) | Tame 5-8<br>(purification by streaking) | |
| 5. F Oct 8 | Zoom:<br>**-"My Favorite Microbiome" presentations**<br><br>Laboratory:<br>-Photograph plates, -Continue *B. subtilis* phage plaque purification: pick plaques and streak purify | -Lab Manual p. 17-18<br>(purification by streaking)<br>Tame 5-8<br>(purification by streaking) | |
| 6. W Oct 13 | Zoom:<br>-Breaking and Entering: Details of phage infection<br>-Discussion of Lecture Reading<br><br>Laboratory:<br>-Photograph plates, -Continue *B. subtilis* phage plaque purification: pick plaques and streak purify | -Lab Manual p. 17-18<br>(purification by streaking)<br>Tame 5-8<br>(purification by streaking) | Chapter 3:<br>The Bacterial Outer Membrane; answer assigned homework questions |
| 7. F Oct 15 | Zoom:<br>-Alumni Career day panel (40 min)<br>-Discussion of Lecture Reading (20 min)<br><br>Laboratory:<br>-Photograph plates, -Continue *B. subtilis* phage plaque purification: pick plaques and streak purify | -Lab Manual p. 17-18<br>(purification by streaking)<br>Tame 5-8<br>(purification by streaking) | Chapter 4:<br>Antibiotic Resistance; answer assigned homework questions |
| 8. W Oct 20 | Zoom:<br>-Discussion of phage therapy benefits & limitations based on answers to homework assignment.<br>-How to make a 1 plate lysate<br><br>Demo:<br>-Picking plaques into phage buffer<br><br>Laboratory:<br>-Start 1 plate lysate: Pick single *B. subtilis* phage plaque into phage buffer, make serial dilutions, plate with bacteria | -Tame 9-13 (making a 1 plate lysate) | Chapter 5: The Phage Whisperer **AND** Chapter 6: Mycobacteriophage and Phage Therapy; answer assigned homework question |
| 9. F Oct 22 | Zoom: | Tame 9-13 | Chapter 1: |

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| | -Nucleus-forming Jumbo phage<br>-Spot titer lecture<br><br><u>Demo</u>:<br>-Harvesting & filtering lysate<br>-Spot titer: pour a lawn, make dilutions, spot 5ul<br><br><u>Laboratory:</u><br>-Harvest, filter and spot titer 1 plate lysate<br>-Store *B. subtilis* phage lysate at 4 degrees | Tame 15-18<br>Tame 27-29<br>Lab Manual p 19 -20 (Harvesting lysate and spot titer) | The Phage Nucleus; answer assigned homework questions |
| 10. W Oct 27 | <u>Zoom:</u><br>Justin Meyer guest lecture<br><br><u>Demo:</u><br>Setting up selection plates for phage-resistant bacteria<br><br><u>Laboratory:</u><br>-Select for 201phi2-1 resistant *Pseudomonas chlororaphis* colonies<br>-Submit *B. subtilis* phage for electron microscopy | | Chapter 7: Introduction to the Evolution of Viruses; answer assigned homework questions |
| 11. F Oct 29 | <u>Zoom:</u><br>-Introduce CRISPR mechanism<br>-Phage-resistant mutant selection results<br><br><u>Demo:</u><br>-How to purify phage-resistant mutants<br><u>Laboratory:</u><br>-Pick and purify 201phi2-1 resistant colonies in the presence of phage. -Repeat selection if needed | Handout: Isolating phage resistant mutants | Chapter 8: CRISPR-Cas Systems |
| M Nov 1 | <u>Laboratory:</u><br>-Pick and purify 201phi2-1 resistant *Pseudomonas chlororaphis* colonies with no phage on plates. | | |
| 12. W Nov 3 | <u>Zoom:</u><br>-Microscopy lecture<br>-Foldscope<br><br><u>Laboratory:</u><br>-Purify phage-resistant *P. chlororaphis* mutant colonies with no phage on plates.<br>-Assemble and examine samples with Foldscope | | |

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| 13. F Nov 5 | Zoom:<br>**CRISPR Application presentations (and Foldscope results)**<br><br>Laboratory:<br>-Purify phage resistant mutant without phage on plate | | |
| 14. W Nov 10 | Zoom:<br>Discuss today's experiment: purpose of spot titer and how to perform it.<br><br>Laboratory:<br>-Test P. chlororaphis colonies for phage resistance by spot titer: perform spot titer on wild type strain and one phage-resistant mutant. | Tame 16-18<br>Lab Manual p 20 (Spot titer) | |
| 15. F Nov 12 | Zoom:<br>-Lecture and Discuss Evolutionary tradeoff. Discuss MIC tests Why are they performed, how are they performed<br><br>Demo:<br>How to use MIC test strips<br><br>Laboratory:<br>-Evolutionary trade-off test #1: Test 201phi2-1 resistant *Pseudomonas chlororaphis* strain for antibiotic resistance (evolutionary tradeoff) by performing MIC test.<br>-Set up biofilm assay plates (96 well plate) | Handout MIC testing | |
| 16. W Nov 17 | Zoom:<br>-Discuss Biofilm homework questions<br><br>Demo:<br>-Biofilm assay<br><br>Laboratory:<br>-Evolutionary trade-off test #2: Test 201phi2-1 resistant *P. chlororaphis* strains for changes in biofilm production | Handout: Biofilm assay | Chapter 9 Microbial Biofilms; answer assigned homework questions |
| 17. F Nov 19 | Zoom:<br>-Discuss Fungal homework questions<br><br>Laboratory:<br>-Discuss electron microscopy results | | Chapter 10: Antifungal Drug Discovery; answer assigned homework questions |

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| | -Phage capsid measurement exercise (Use of ImageJ)<br>-Repeat experiments as needed | | |
| 18. W Nov 24 | **Open Lab** | **Open Lab** | **No Lecture** |
| 19. F Nov 26 | **No Class** | **Thanksgiving** | **No Class** |
| 20. W Dec 1 | Powerpoint presentation of phages | | |
| 21. F Dec 3 | **Practicum Final**<br>Lab Clean up | | |
| **Finals Week** | **No Class** | **No Class** | **No Class** |

## Campus Safety Requirements and Expectations

Keeping our campus healthy takes all of us. You are expected to follow the campus safety requirements and pursue personal protection practices to protect yourself and the others around you. These include:

- **Participate in the university's daily screening process.**
  Everyone must complete a Daily Symptom Survey to access a university-controlled facility.
- **Participate in the university's testing program.**
  All students are required to participate in the COVID-19 Testing program as required by their vaccination status:
    - Unvaccinated students with approved exceptions must complete a COVID-19 test twice a week.
    - Students who are fully vaccinated must complete a COVID-19 test once a week, for the first four weeks of the quarter.
- **Wear a well-fitted face covering that covers your nose and mouth at all times.**
  Everyone is required to wear face coverings indoors regardless of vaccination status. If you see someone not wearing a face covering or wearing it incorrectly, then kindly ask them to mask up.
- **Monitor the daily potential exposure report.**
  Every day the university will update the potential exposure report with building and some classroom information and the dates of exposure. Download the CA COVID Notify app to your phone to receive an alert if you have been potentially exposed to COVID-19.
- **Assist in the contact tracing process.**
  If you're contacted by a case investigator, it means you have been identified as close contact, please respond promptly. You must assist with identifying other individuals who

might have some degree of risk due to close contact with individuals who have been diagnosed with COVID-19.

- **Contact the instructional team if you are impacted by COVID-19**

Please note that due to the ongoing COVID-19 Pandemic, changes may be made in response to new developments and information.

**Lab effort and conduct (100 points total, 20 collaborative)**
Students will be evaluated on overall laboratory performance, including mastery of lab methods (including lab safety procedures), professional behavior towards other students, instructors and TA, coming to lab prepared, and contributing to collaborative team efforts. Everyone will start off with full credit, with points deducted at the discretion of the instructors and TA for consistently arriving late or leaving early, lapses in safety procedures, failing to clean up properly, breaking or abusing equipment, unexcused absences, and for failing to work well with classmates.

**Assigned Reading, Presentations and Homework Questions (100 points total)**
The textbook for the course is Veloxsci BILD 70 Phage Hunters Fall 2021. Use this link to purchase the text: https://www.veloxsci.com/texts/LC65DH17-1632265-1-linked. For each of the 10 textbook chapters, except for Chapter 2 and Chapter 8, there will be assigned reading questions posted under the Quizzes tab on the course Canvas. The goal is to assess understanding of the assigned reading and broaden knowledge of the chapter topic. Homework questions will be due at 11:59 pm the day before class. There will be a group presentation assignment for Chapter 2 and Chapter 8 (details regarding the presentation format will be provided in the classroom).

**Practicum (100 points)**
Towards the end of the quarter, we will have a practicum test (rather than a final exam) in which each student will perform a variety of key lab skills (ie: titering a phage stock, interpreting results, troubleshooting) at various stations set up throughout the lab. The practicum is cumulative. Attendance is mandatory. There is no way to make-up the practicum. This year the practicum is on Friday, December 3rd.

**Laboratory notebook (200 points)**
Students are required to keep a laboratory notebook, in which they record how experiments were performed, their results, data interpretation and future experiments. The general goals of lab notebooks are to (1) record your results, (2) allow anyone to repeat the experiment exactly as you did it, (3) provide a resource for trouble shooting experiments, with sufficient detail to later recognize small differences in experimental protocols (such as slight differences in time or mixing method) that can make the difference between a successful experiment and a failure, and (4) provide a legal record of your discoveries for future patenting activities (!). Just as in any scientific lab, we hope to publish our findings, and your lab notebooks must provide information

on exactly where and how each phage was isolated or we cannot include your results or consider it as a candidate for sequencing. Even phage that are not sequenced will be submitted to the Howard Hughes Medical Institute for archiving and possible use in future experiments, but only if your lab notebooks provide sufficient documentation of the phage you isolate.

Notebooks must be legible and neat enough for others to follow, but they do not have to be beautiful. Write in pen and if you make a mistake cross it out and write the correction. Do not erase or add or remove pages! Tape in photos of gels, plaques, etc. Bring the notebook to class everyday, because it will be periodically checked and graded during class time. The first notebook check will be for feedback so that you can modify your note-taking habits. The section "Before You Start", Part B in the lab manual provides a good overview of lab notebooks.

**You need to write down everything you do either when or before you do it, your memory does not suffice!** It is a good habit to write down everything you plan to do *before* you do it (noting any changes to this plan in the margin as you go), and to use this (rather than the lab manual) to guide what you do in the lab. You should read the protocol and write an outline in your lab notebook before you come to class, leaving space to write any modifications to this protocol.

**Powerpoint presentation of your results and online archive reports (100 points)**
At the end of the class, each student will give a 3 to 5 minute presentation of the results of your research. Details regarding the presentation format will be provided in the classroom.

**Summary:**
100 points – Effort and conduct
100 points – Assigned reading (presentations and homework questions)
100 points – Practicum
200 points – Notebook
100 points – Final Presentation
600 points total

**Academic Integrity**
Cheating is not tolerated. Scientific research is completely dependent on the integrity and transparency of the scientists involved. All work should be your own. This can feel less clear-cut for laboratory classes where you do almost all of your work with a lab partner. You will share data (numbers and outcomes) with your lab partner, but the words, interpretations, and notes should be your own words. The UCSD Office of Academic Integrity defines cheating as follows:

"Cheating occurs when a student attempts to get academic credit in a way that is *dishonest, disrespectful, irresponsible, untrustworthy or unfair."*

All incidents of cheating will be reported to the Office of Academic Integrity. If you have any questions about academic integrity or cheating, please ask any of the instructors or your TA.

When in doubt, ask first. We also encourage you to visit the website of the Office of Academic Integrity at UCSD: http://academicintegrity.ucsd.edu

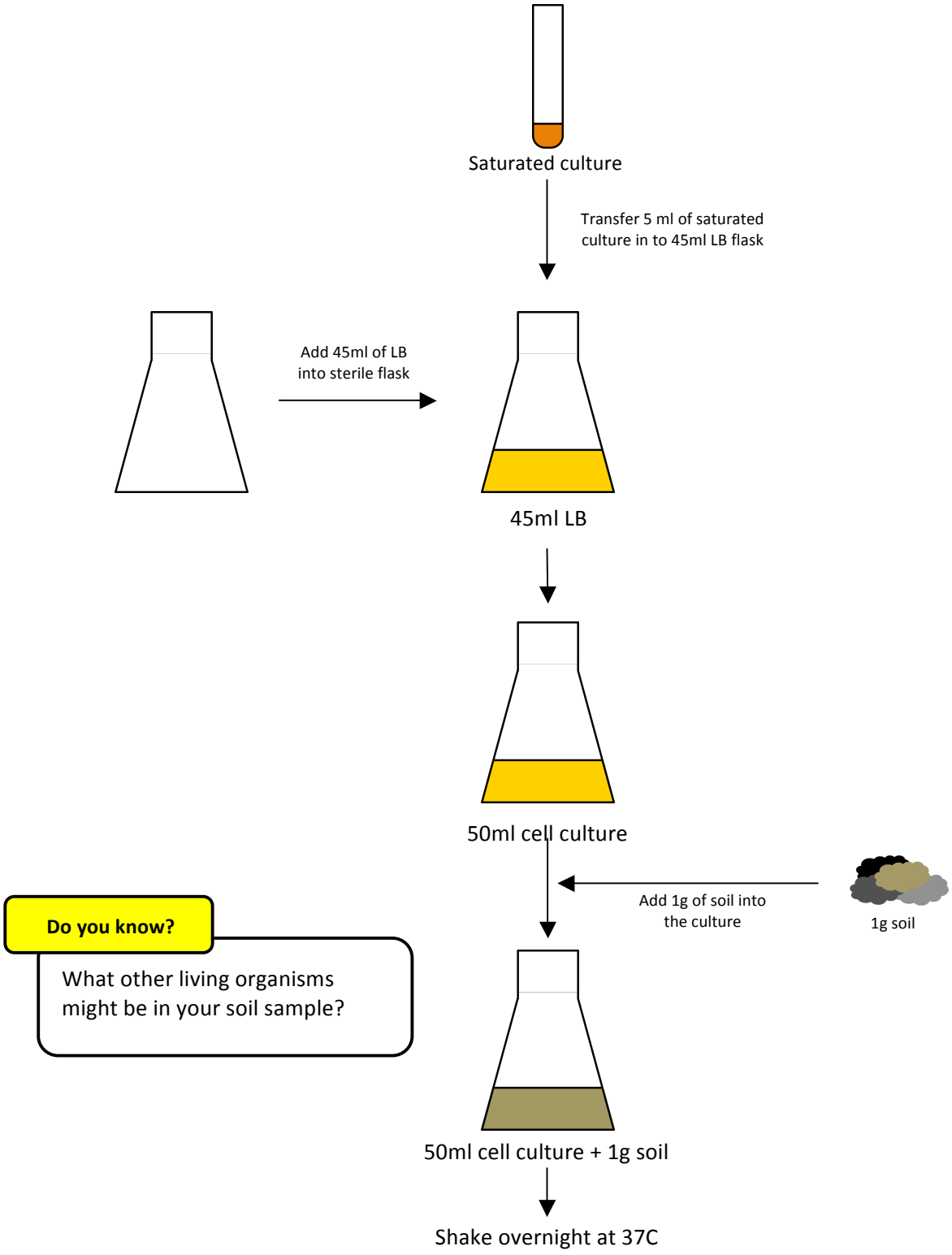
**Additional information:**

LAB SAFETY TRAINING – Enrolled and waitlisted students MUST successfully complete the Biology Lab Safety Training and Assessment before the first lab session: https://biolabclass-safetyquiz.ucsd.edu/introduction. Please note that courses offered by other departments (Chemistry, for example) may have additional safety training requirements.

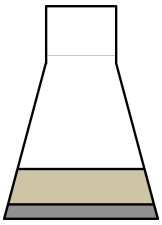
ATTENDANCE – Enrolled and waitlisted students MUST attend the first lab session. Additional details: http://biology.ucsd.edu/go/ug-labs.

ADD/DROP DEADLINES are different for lab courses than lecture courses. Students who drop a Biology lab class after the end of the second class meeting will be assigned a "W". Additional details: http://biology.ucsd.edu/go/ug-labs.

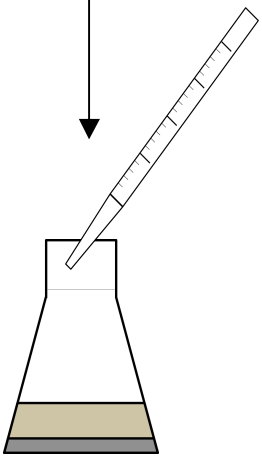
**Preparing enrichment culture (per 1 soil sample)**



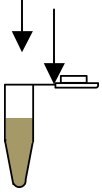
## Phage sample preparation



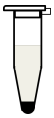
Allow O/N culture to settle

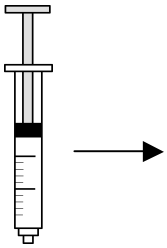


Take 1.5ml of supernate and transfer to microcentrifuge tube

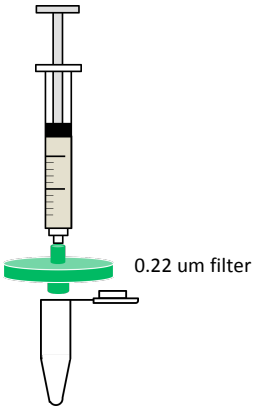


Spin down 1min





Transfer 1ml of supernate to a syringe by micropipette




To filter, dispense the liquid through the filter into the new microcentrifuge tube

Undiluted phage sample ($10^0$ tube)

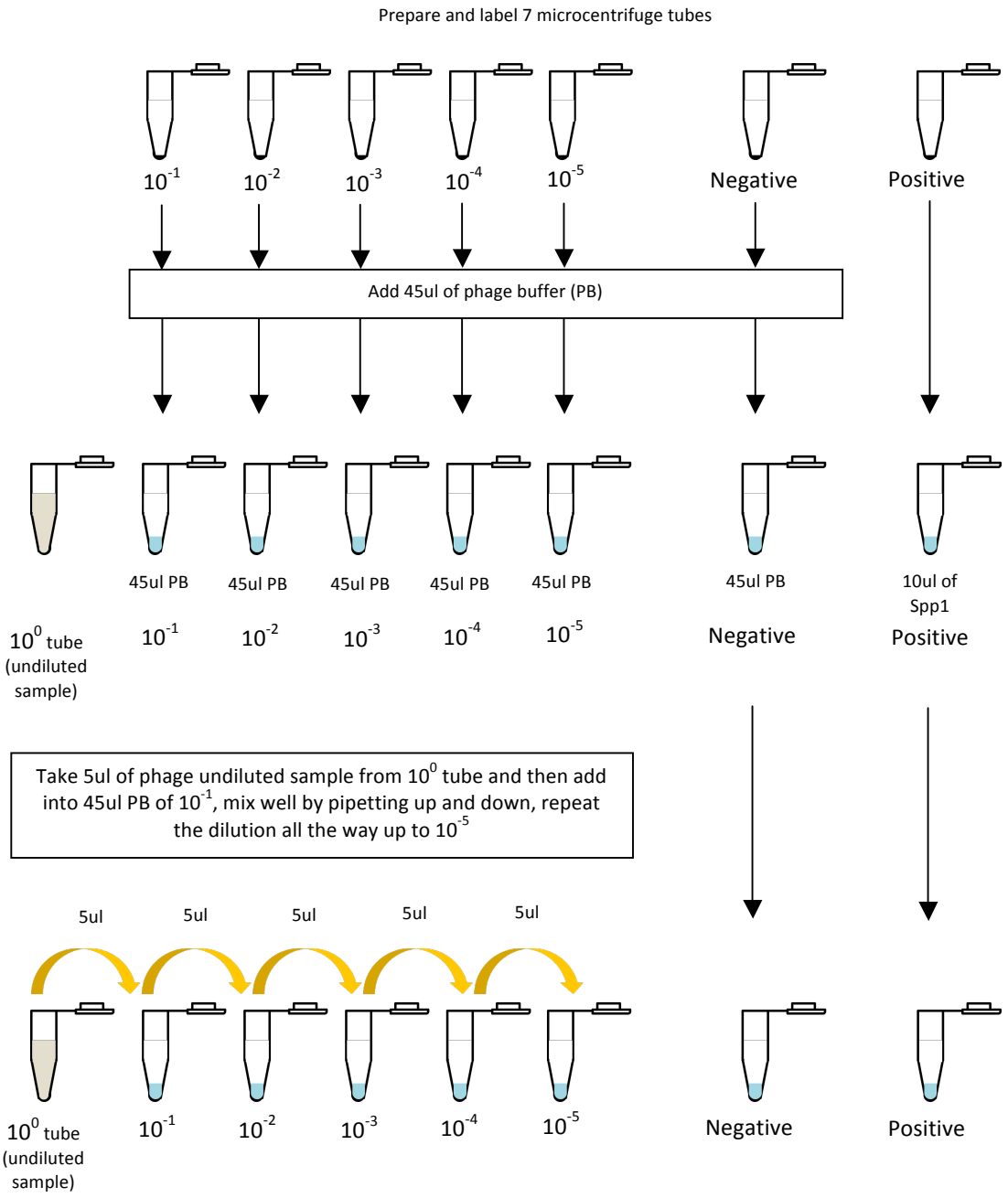
**Do you know?**

What is 0.22um filter used for?

What is in the “undiluted sample”?

Why the “undiluted sample” is also called 10° tube?

## Serial Dilution

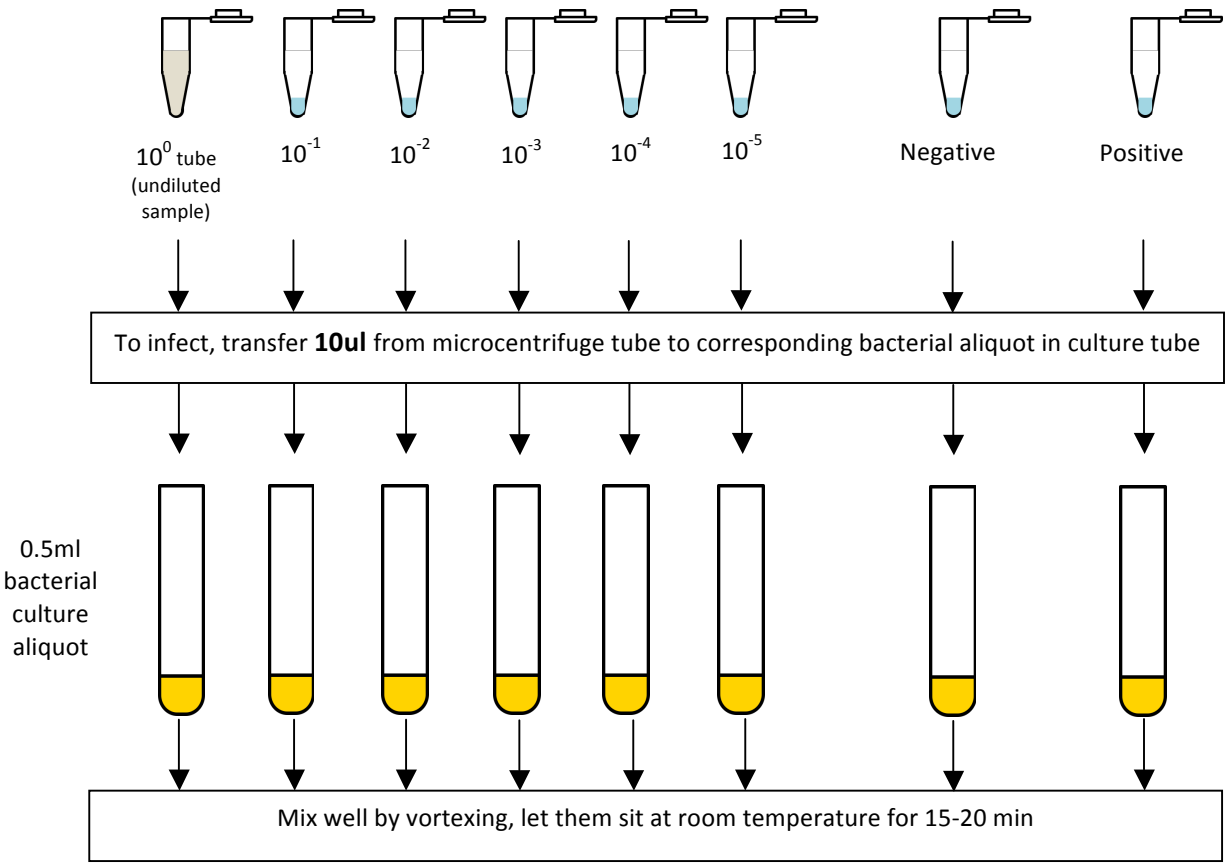



**Do you know?**

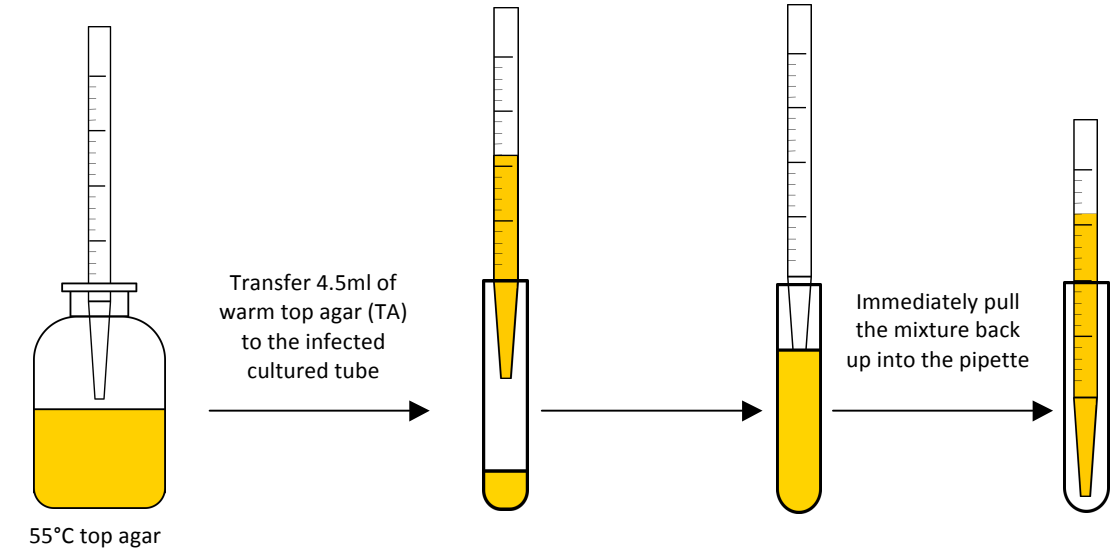
Why do we include "negative" and "positive" controls in the experiment?

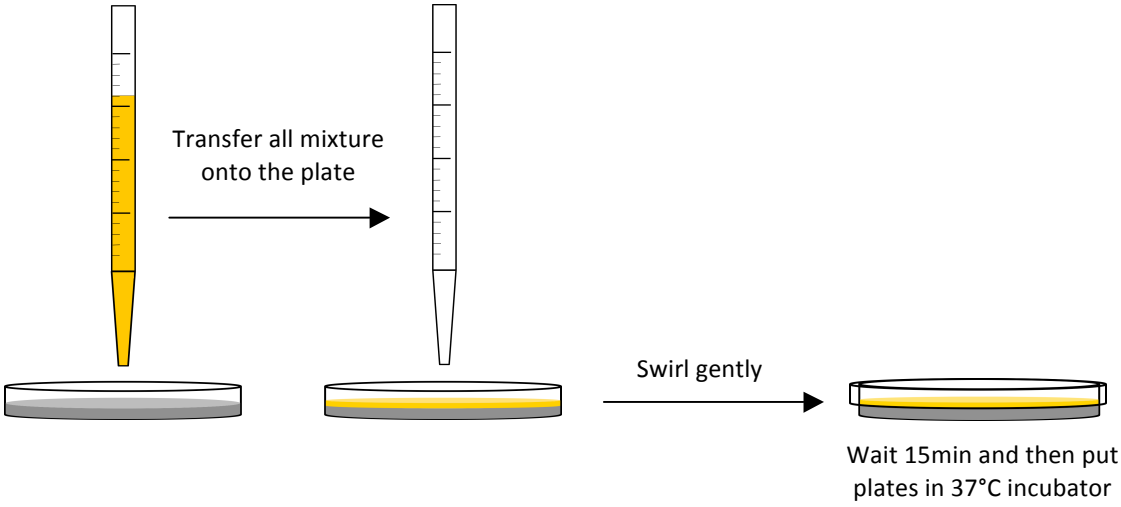
If the $10^{-1}$ tube has 6000 phage/ml, what is the phage density in the $10^{-3}$ tube?

**Infect bacteria with phage sample**

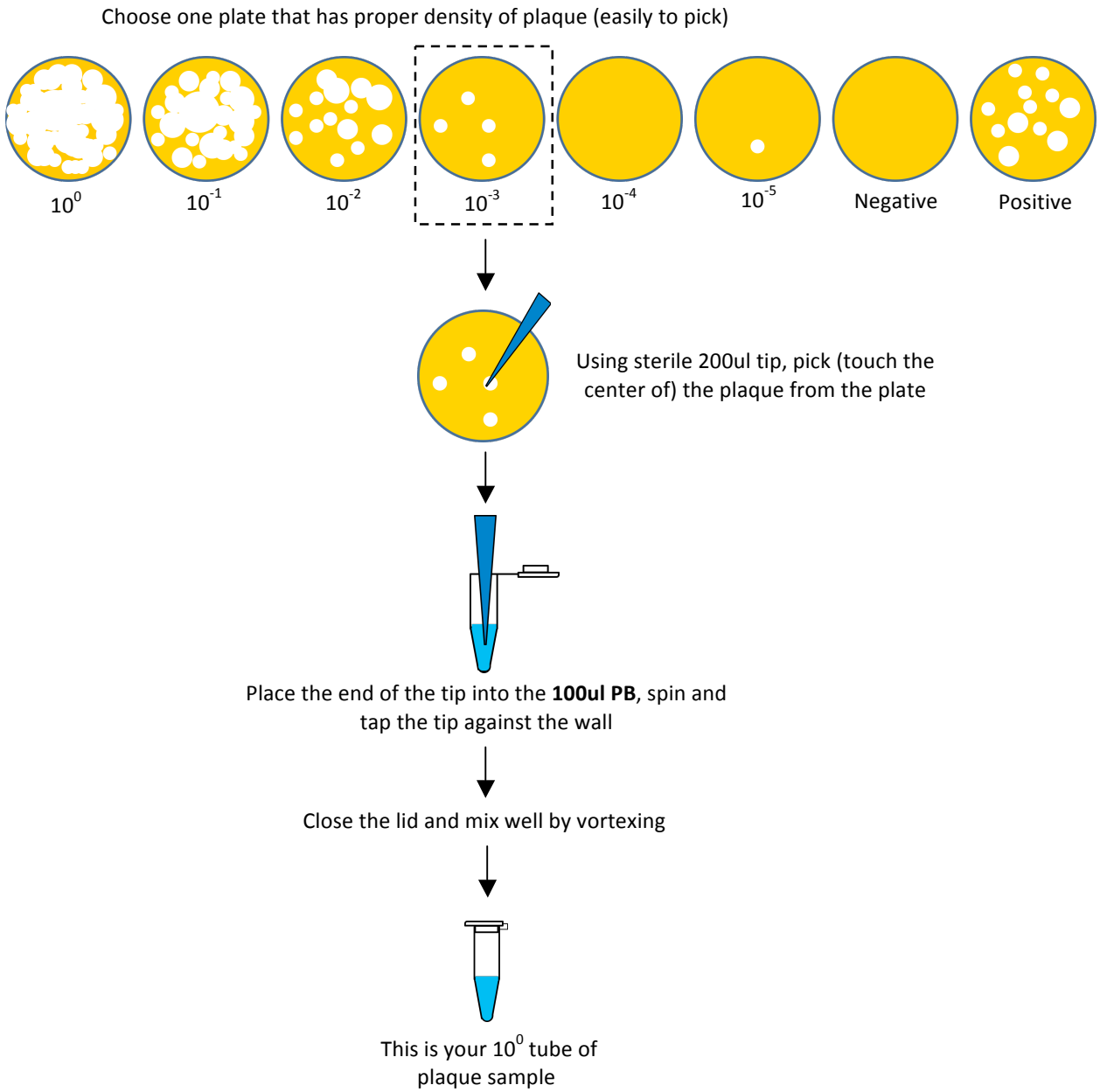


## Plating sample



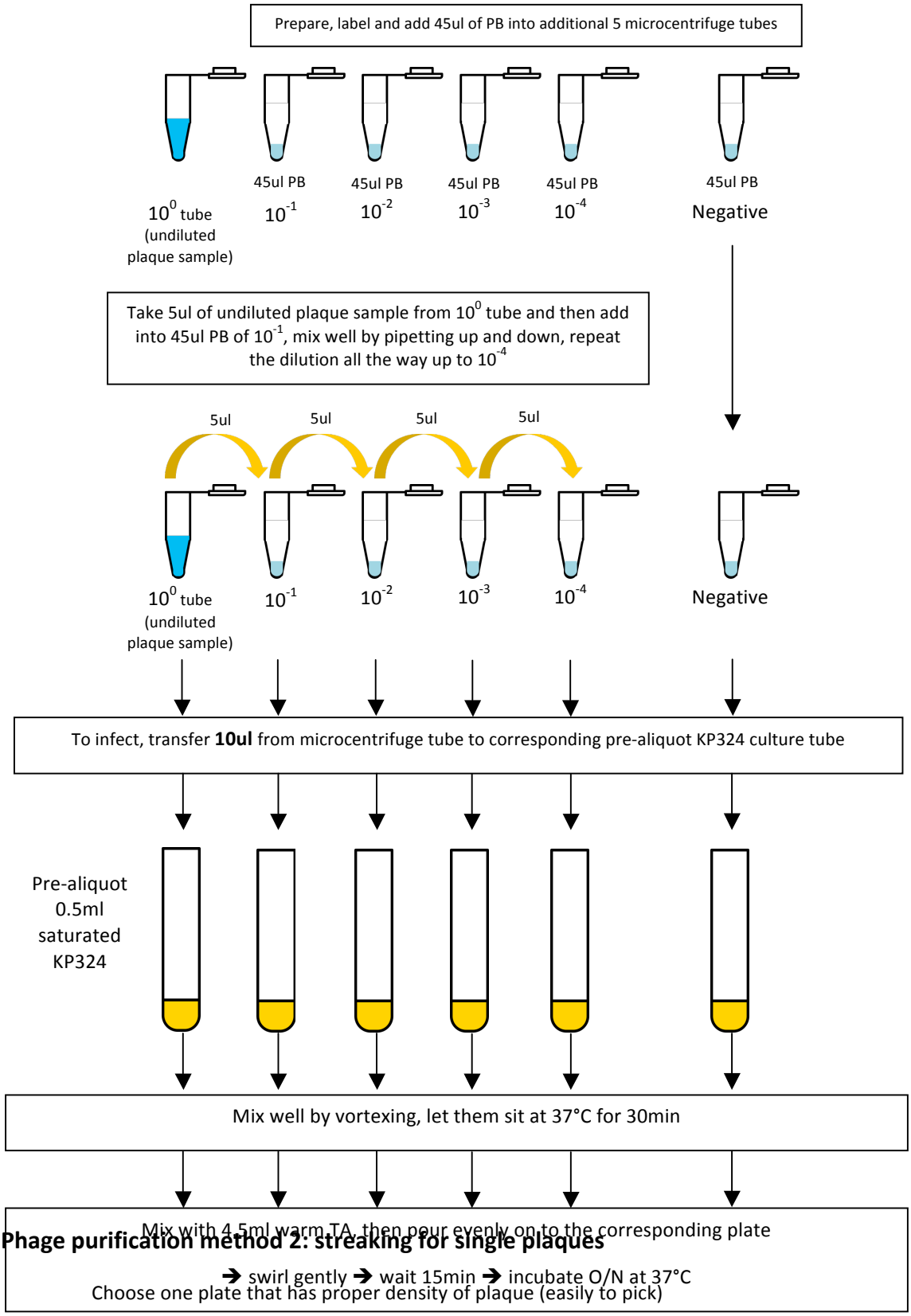
## Phage purification method 1: serial dilution



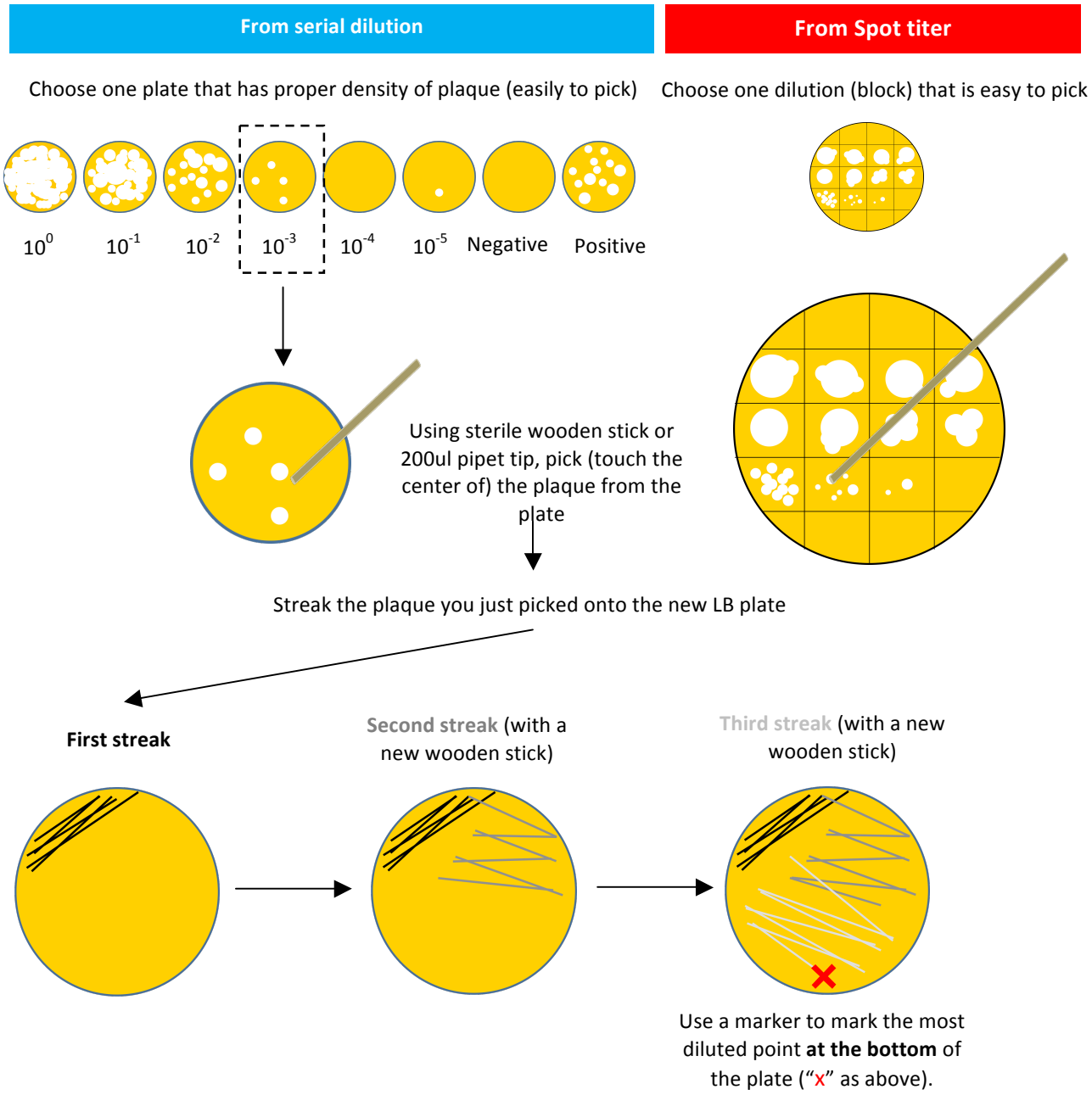
Visual outline and protocol by Poochit Nonejuie adapted from Marcy Erb and the HHMI

## Phage purification method 1: serial dilution

Choose one plate that has proper density of plaque (easily to pick)



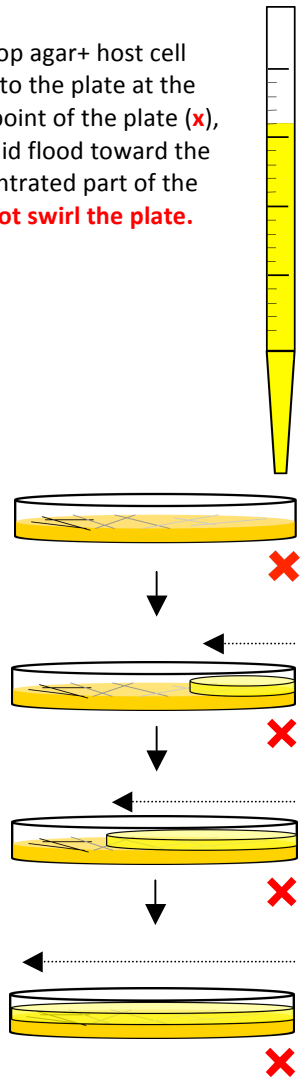

## Phage purification method 2: streaking for single plaques




**Phage purification method 2: streaking for single plaques continued**

**Pouring top agar plus host cell mix onto streaked phage**

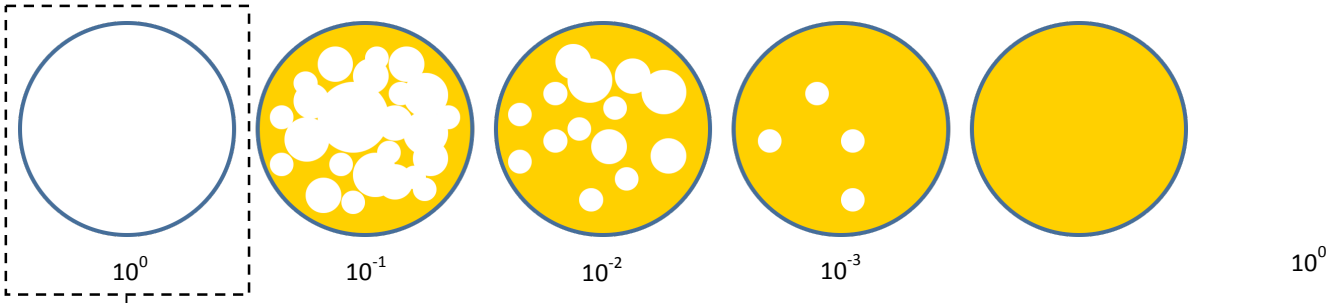
Transfer “top agar+ host cell mixture” onto the plate at the most diluted point of the plate (**x**), allow the liquid flood toward the more concentrated part of the plate. **Do not swirl the plate.**

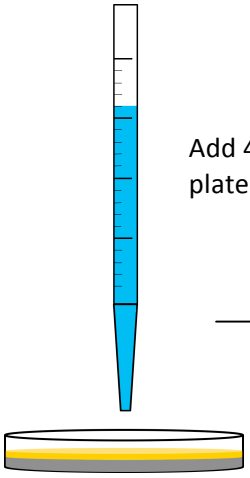


If top agar does not completely cover the plate, you can **tap** the plate a bit or **gently tilt** the plate toward the most concentrated part until top agar covers all over the plate

## Harvest lysate

Choose one plate that the bacteria lawn is nearly clear (all cells are lysed)

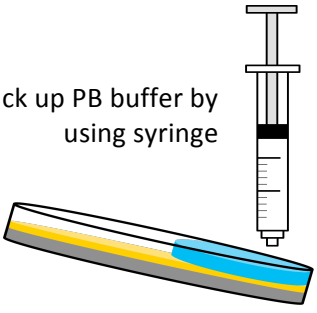


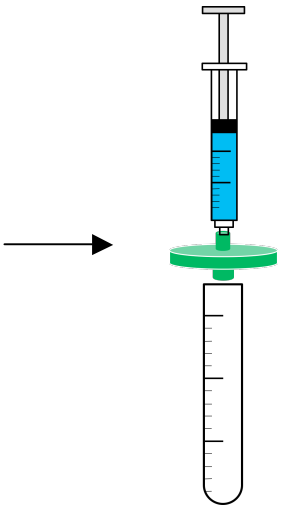



Add 4.5 ml of PB onto the plate and swirl gently

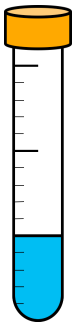
Wait 2-4 hr at room temperature

Suck up PB buffer by using syringe





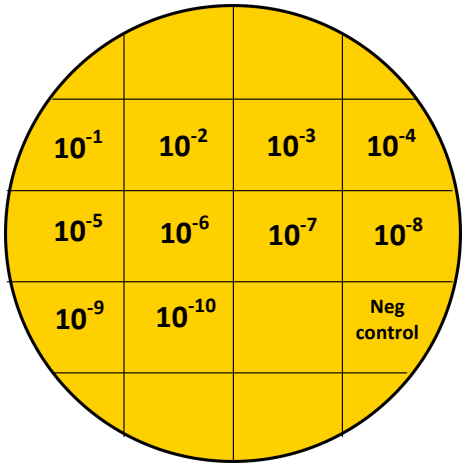
Filter phage lysate through 0.22um filter into new 15ml sterile plastic tube



Cap the tube and label properly.

**This is your purified phage lysate!!!!!**

**Spot titer**

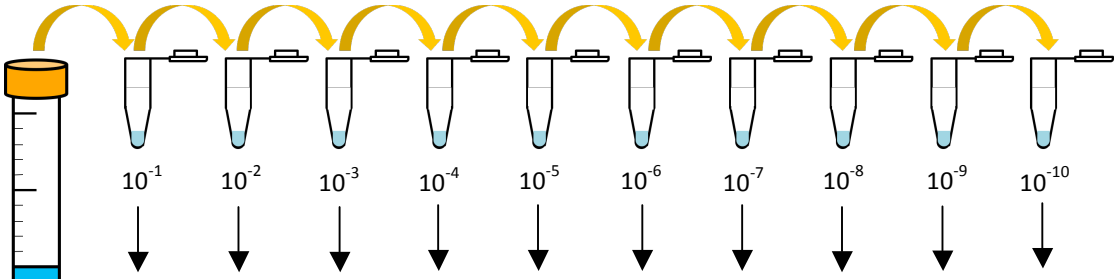



**Label the bottom of the plate**

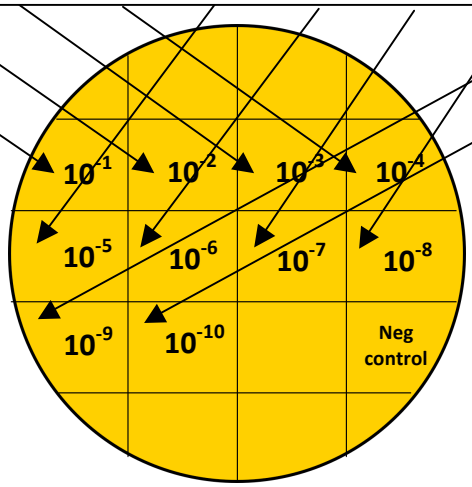
**Make a lawn of bacteria (*B. subtilis* KP324) by mixing 0.5ml of saturated bacteria culture with 4.5ml warm top agar and then overlay the plate. Just like we did as usual but no infection required since we need bacterial lawn for this step**

Prepare, label and add 45ul of PB into additional 10 microcentrifuge tubes

Dilute your purified phage lysate all the way up to $10^{-10}$




Spot **5ul** of all phage dilution samples onto the corresponding blocks on the grid




**5ul** of PB for negative control

# SEA-PHAGES

## Laboratory Manual

**Science Education Alliance**
Howard Hughes Medical Institute
Chevy Chase, Maryland

**Science Education Alliance**

**David J. Asai, Ph.D.**
Director, Precollege and Undergraduate Science Education Program

**Cheryl Bailey, Ph.D.**
Senior Program Officer

**Lucia P. Barker, Ph.D.**
Program Officer

**Kevin W. Bradley**
Research Technician

**Razi Khaja**
Bioinformatics Specialist

**Melvina F. Lewis**
Program Assistant

**Credits**

Design: VSA Partners, New York

Illustrations: Marjorie C. Leggitt

**In Situ**

*Capture* fig. 5, *Capture* fig. 6, *Capture* fig. 7, *Tame* fig. 4, *Tame* fig. 6: Annie O'Neill/Tom Little

*Capture* fig. 4: Adapted from an illustration at www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/phage/lysis.html, courtesy of Stanley Maloy, San Diego State University

*Capture* fig. 9: Adapted from fig. 6 in M. Feiss and W. Widner, Bacteriophage λ DNA packaging: scanning for the terminal cohesive end site during packaging, *Proc Natl Acad Sci USA* **79** (1982): 3498–3502, courtesy of Michael Feiss. ©1982 National Academy of Sciences, USA.

*Capture* fig. 11: Adapted from fig. 9-15 in B. Alberts et al., *Essential Cell Biology: An Introduction to the Molecular Biology of the Cell* (New York: Garland Publishing, 1998), 288.

*Dissect* fig. 1: Lucia P. Barker

Appendix 1 photos: Annie O'Neill/Tom Little; DNA gels courtesy of FOTODYNE Incorporated

Appendix 5 photos: Courtesy of Fisher Scientific; capillary tweezers courtesy of Ted Pella, Inc.; DNA grids courtesy of Jim Burke, CIDDE/ University of Pittsburgh; Melvina Lewis

**In Silico**

*Analyze* figs. 24-27, 29, and 31: Lucia P. Barker

HHMI
HOWARD HUGHES MEDICAL INSTITUTE

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# Preface

The Science Education Alliance (SEA) and its first offering, Phage Hunters Advancing Genomics and Evolutionary Science (PHAGES), *formerly the National Genomics Research Initiative in Phage*, are new ventures for HHMI. We believe that beginning undergraduate students should experience real, inquiry-based science in person as soon and as often as possible. We recognize that science is a collaborative and open process. A very real and necessary part of science is sharing what does and does not work and building new knowledge on a foundation of commonly held information. Through the Alliance, we are creating a structure in which students and faculty share ideas, problems, and findings in order to align the learning experience with the practice of science.

Much of modern life science research is developing on the borders of biological and physical science. Taking this into account, our course starts in vivo, in the field, with the isolation of new viruses from nature. It then progresses to the laboratory bench for an in vitro molecular analysis. Finally, it ends in silico, with a bioinformatics analysis of the isolated genomes.

In short, answering questions by discovery and constructing new knowledge by sharing carefully crafted information are all hallmarks of science. It is our hope that the Alliance, the new course, and this guide should all help bring the excitement and fulfillment of science to beginning students across the nation.

**Peter J. Bruns, Ph.D.**
Vice President, Emeritus
Howard Hughes Medical Institute

# Welcome

You are about to embark on a unique experience in which novice scientists across the country will work together to address a single question of scientific interest. An *authentic research* experience. An experience that will expose you, the new investigator, to the ***process*** of doing science. This adventure will be about making discoveries—discoveries about mycobacteriophages, and discoveries about yourself. This means that an observation has been made and a problem identified or a question posed, and scientific tools will be available for you to use to address the problem or question. It means that no one knows with absolute certainty whether those tools will work to address the situation at hand. It means that you will have the opportunity to formulate testable hypotheses, make predictions, design experiments to address the hypotheses, analyze data, and decide what the next best step is. It means that there will be times of uncertainty. It means that there will be successes, and there will be some challenges. You will learn that no one person ever has all the answers. You will not be alone in that experience. Science is a collaborative and collegial endeavor. You are part of a national network of experienced and novice scientists engaged in a single effort. You have at your disposal a vast array of resources available from the Howard Hughes Medical Institute, a national genome sequencing center, and colleges and universities across the country. We are happy you are part of this alliance. We look forward to learning of your discoveries.

**The Staff of the Howard Hughes Medical Institute's**
**Science Education Alliance**

# Overview

This manual provides a framework for exposing you, the novice scientist, to the *process of doing science* while you explore mycobacteriophages, a focus of the Phage Hunters Advancing Genomics and Evolutionary Science. The SEA-PHAGES has two major components: a wet lab and genomics. The manual breaks these components down into steps that are correlated with the major steps of the research experience: **capture** (get the phage from the environment), **tame** (isolate and purify a single phage), **dissect** (analyze aspects of the phage's physical and molecular structure), **analyze** (elucidate the genomic properties), **discover** (identify novel genomic traits), and **share** (disseminate data). The first three sections take place in the wet lab, and the last three involve the science of genomics.

The wet lab experience focuses on using techniques in microbiology, molecular biology, and electron microscopy to characterize phages isolated from the environment. The genomics portion focuses on using bioinformatics tools to facilitate genome finishing, annotation, and comparison as ways to help gain insight into the diversity of mycobacteriophages in the environment and the characteristics of mycobacteriophages that render them unique and distinct from other phages. This type of information could provide a framework for SEA scientists and other researchers to delve into the possible utility of these organisms in a variety of biomedical, health, environmental, and ecological applications.

The Laboratory Manual starts out with very detailed instructions for setting up work areas and doing experiments. The level of detail drops off as you gain confidence and develop proficiency in designing experiments. The manual has three main sections:

1. **In Situ (Part 1)**
   This section acquaints you with important aspects of an authentic research experience and includes the protocols required to isolate, purify, and characterize the mycobacteriophage and background information about phage biology and nomenclature. It contains instructions for maintaining a laboratory notebook and why that is important, the elements required to accurately label reagents and data, and basic microbiological techniques and proficiencies.

   Each procedure includes an **objective**, to provide the procedure's rationale; required **supplies** and **equipment**; and **questions**, to enhance your critical-thinking skills. In addition, the following are included throughout the experimental protocols:

   - **The Big Picture**: Explains where the particular experiment or exercise fits in with everything else
   - **Alerts**: Signal important information related to lab safety and special precautions

- **Notes and Reminders**: Suggest things that are important to consider or remember
- **Food for Thought**: Gets you to think beyond the procedure

At key points throughout the procedure sections, **Decision Trees** guide you through the process of determining the next logical step.

2. **DNA Master (Part 2)**
   This section includes the protocols and training you need to use bioinformatics tools to analyze your phages and compare your data with those posted by other schools in the SEA.

3. **Glossaries and Appendices**
   The Glossaries are available on the SEA wiki at http://www.hhmi.org/seawiki/display/StuEduRes/Laboratory+Manual. Appendices for Part 1 provide additional resources and information, such as a troubleshooting guide, readings, and research paper format.

To enhance the research and learning experience, we encourage you to visit our website at www.hhmi.org/sea.

As you work through the protocols and materials provided in this Laboratory Manual and on our website, please note the places where the information is unclear, incomplete, or too extensive. We welcome any and all suggested improvements. Please enter your comments in the appropriate place on our wiki, accessible through our website.

# Acknowledgments

This Resource Guide and the student Laboratory Manual are the result of hours of discussions with a vast array of enthusiastically supportive individuals dedicated to research and education. We would like to acknowledge and thank Elizabeth Fischer and her staff at Rocky Mountain Laboratories/NIAID/NIH for their expert advice about refining the protocols used for electron microscopy; Elizabeth Summer (Texas A&M University) for hours of discussion concerning the joys and challenges associated with phage biology and research-based laboratory courses and for providing us with her refined DNA-isolation protocol, teaching resources, and editorial comments; the Genomics Education Partnership (GEP) 2006 Cohort for lengthy discussions of appropriate ancillary materials to engage faculty and undergraduates in consortial research projects; Erin Sanders-Lorenz (UCLA) for enthusiastic support of and editorial comments on the Resource Guide; Deborah Jacobs-Sera (University of Pittsburgh) for mycobacteriophage protocols, materials, and enthusiastic support for the project; Graham Hatfull (University of Pittsburgh) for his willingness to impart his expert knowledge to the SEA community; the Department of Energy's Joint Genome Institute for their efforts to accommodate the Alliance and for providing the protocol to assess genomic DNA quality; Annie O'Neill and Tom Little for their expertise during the Troubleshooting Guide photo shoot; Amy Vogelsberger (University of Pittsburgh) for her solution recipes and tips; the students in the pilot course at the University of Pittsburgh for their pioneer spirit and for their extreme patience in enduring the constant questions and comments from the SEA staff as we observed what was happening in the course; Ed Lee (Lawrence Berkeley National Laboratory) for his enthusiasm for all things bioinformatic and for building and refining the workflow; Steve Cresawn for refining the Phamerator to meet the needs of the Alliance and for providing the draft background and protocols; HHMI IT staff for working tirelessly to provide the SEA with the tools and mechanisms necessary for creating, sustaining, and assessing the Alliance; the HHMI Communications staff for providing the appropriate staff and mechanisms to support these unique publications and the growing SEA community; and finally, to the faculty and students of Cohort 2008 for their undeterred support, insight, and constructive criticism of the materials and resources described herein.

# Table of Contents

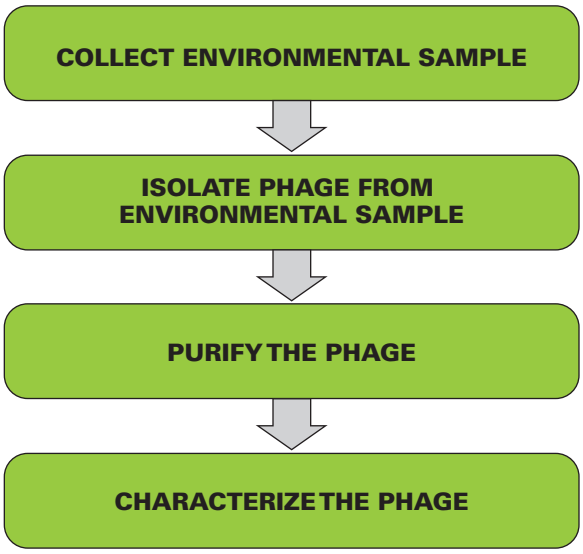
## Part 1 In Situ Section



*continued* ▸

## Contents (*continued*)

## In Situ Overview



# Before You Start

## Contents



**NOTE**

We use live microorganisms in this course. These microorganisms are generally considered safe to use for those with a normal, noncompromised immune response.

**If you have any health or other concerns associated with exposure, please alert your instructor or the laboratory director immediately, or contact your institution's safety office.**

# Part A. Aseptic Technique, or "Keeping It Clean"

## Objective

Each student will learn to minimize the chance of contamination.

## Supplies

- □ 250-mL bottle or flask of water (at least 50 mL water; 1 per 1 to 2 students)
- □ Squeeze bottles containing disinfectant
- □ 15-mL conical tube (1)
- □ 10-mL culture tube (1)
- □ 10-mL serological pipettes (at least 2)
- □ 1-mL serological pipette (at least 1)
- □ Microcentrifuge tubes (2)
- □ Micropipettor tips (100-$\mu$L) (at least 3)

## Equipment

- □ Bunsen burners (1 per student)
- □ Pipettors (e.g., Pipette-Aid; at least 1 per 2 students)
- □ Micropipettor (100- or 200-$\mu$L) (1 per student)
- □ Bottle of "medium" (water)

## Procedure: Getting Started with Aseptic Technique

### A. Prepare your work area.

1. Tidy up your work bench by removing clutter, papers, bottles, etc.
2. Using a squeeze bottle containing disinfectant, such as 70% ethanol (EtOH), dispense enough disinfectant to dampen the entire work surface.
3. Using a paper towel or gauze pad, wipe the entire area, back to front, to evenly spread the EtOH and moisten the entire work surface.
4. If you need more EtOH, add more, and repeat the process from back to front.
5. Allow the EtOH (or other disinfectant) to evaporate—do not wipe dry.

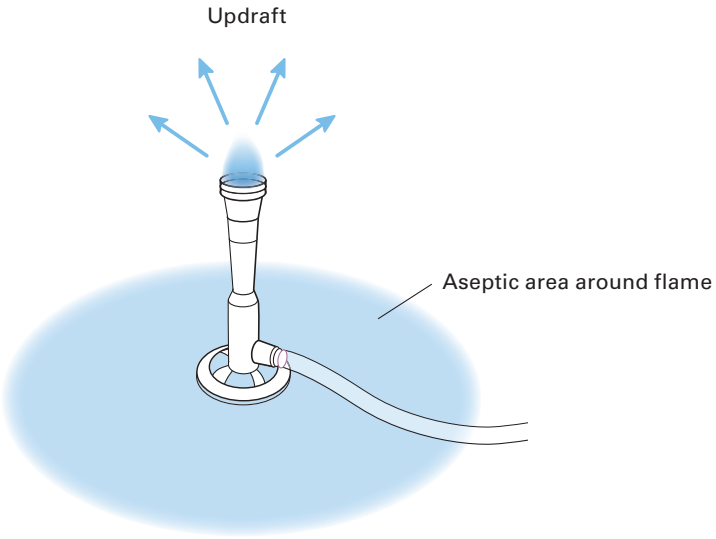
At a minimum, your bench should be wiped down with 70% ethanol at the beginning and end of *every* lab exercise. The bench should also be wiped down whenever there is *any chance* of contamination or spill.

### B. Ignite the Bunsen burner.

1. Light the Bunsen burner, but only ***after*** the disinfectant has completely dried.
2. 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).

**Until one is familiar with using a Bunsen burner, it is important to be constantly mindful of the location of the flame. The flame, even with an apparent blue cone, is not always easy to see!**

**Before fig. 1**
How a Bunsen burner works to keep the work area aseptic.




Aseptic versus sterile: These terms are often used interchangeably, but they should not be. "Sterile" means free of *all* living microorganisms, including bacteria, fungi, and viruses. "Aseptic" refers to an area whose contamination has been minimized.

In your procedures, reference is often made to supplies that are *sterile*, and the expectation is that you will use *aseptic* technique to minimize contamination by undesired microorganisms.

The flame is now providing an updraft (see ***Before*** fig. 1 and "The Big Picture," below). Because heat rises, microorganisms and dust particles will be forced upward and away from the immediate work area. It is important to work within this area, or "zone," careful not to disturb the updraft by rapid movements that dramatically change the air currents around your bench. Work slowly, carefully, and deliberately.

## Procedure: Transferring Sterile Solutions Using Aseptic Technique

### A. Arrange needed supplies on your bench so you have easy access.

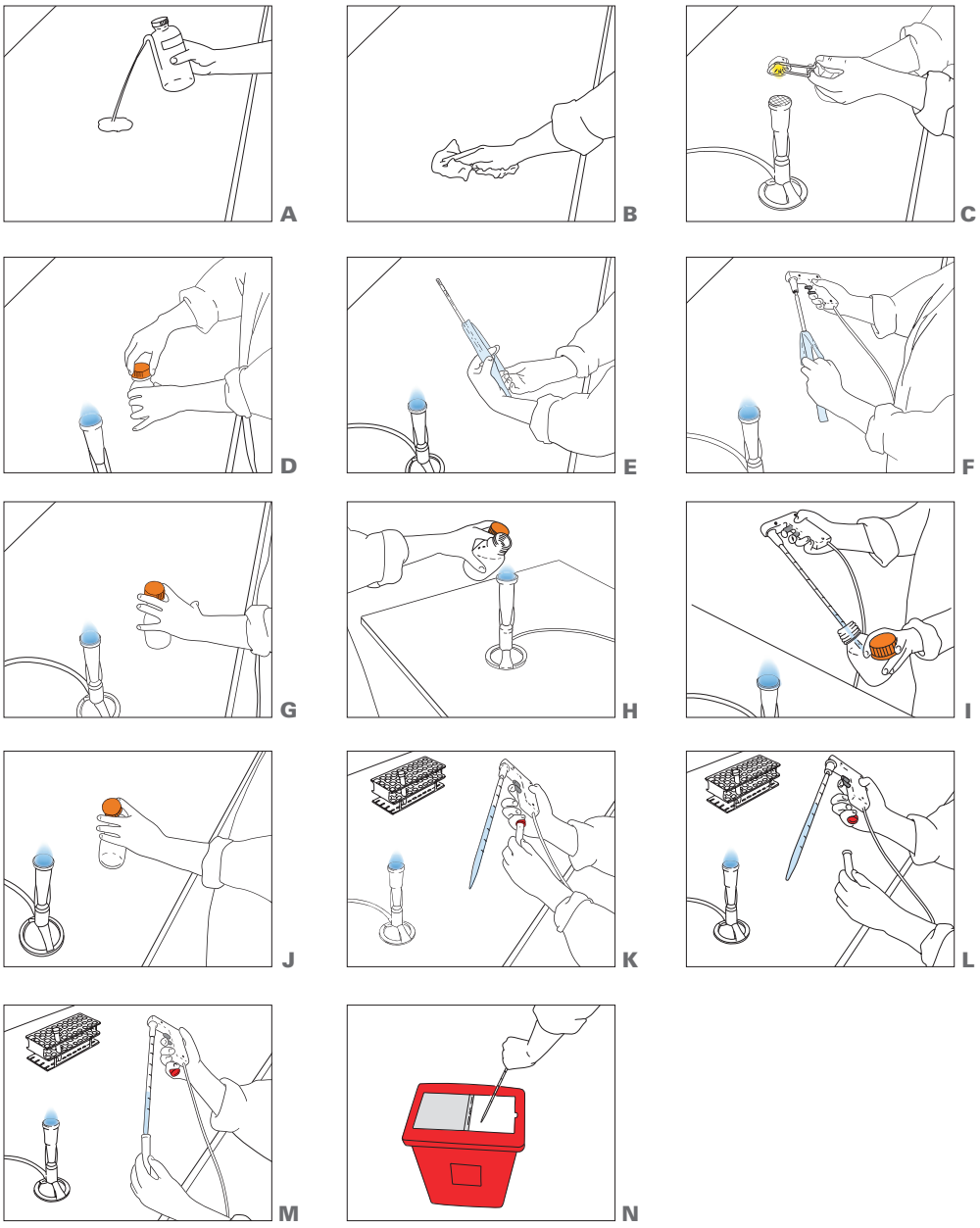
- ☐ A bottle of "medium" (i.e., water in this case)
- ☐ A culture tube and a 15-mL conical tube in a rack
- ☐ A 10-mL pipette
- ☐ A pipettor
- ☐ A Bunsen burner

### B. Transfer 10 mL of medium into a 15-mL conical test tube.

1. Loosen (but do ***not*** remove) the tube and bottle caps.
2. Peel down the wrapper of the pipette (like 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's your dominant hand).
3. Using your other (dominant) hand, place the pipette on the pipettor and remove the wrapper. Do ***not*** touch the tip of the pipette—which is sterile—to any surface!

4. Using your nondominant hand, unscrew the top of the media bottle and leave it between your forefinger and middle finger (see *Before* fig. 2).
5. Use your nondominant hand to pick up the bottle and pass the opening two to three times through the flame of the Bunsen burner. Do ***not*** linger over the flame, and do ***not*** heat the top of the bottle or lip. This technique is called "flaming."
6. Immediately place the serological pipette into the bottle, and, using the pipettor, draw up 10 mL.
7. "Flame" the top of the bottle again and replace the cover.

Do *not* turn the cap upside-down and do *not* set it on the bench. Any area inside the cap of a sterile bottle or sterile tube is considered a "sterile field," not to be compromised!




**Before fig. 2**
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 all your materials, and loosen all bottle and tube caps **(D).** Peel down both sides of the pipette package **(E),** and hold both flaps while attaching the pipettor **(F).** Using your first and second fingers, remove the bottle top **(G),** and pick up the bottle with the same hand and "flame" **(H).** Remove your sample **(I),** and reflame 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.

The goal of "flaming" is to gently heat the air at the bottle opening. This creates a mini-microbiological force field because the air is more likely to come *out* of the bottle than to fall *into* the bottle when the air at the opening is heated (the updraft concept).

8. Using the smallest finger on the hand holding the pipettor, remove the top of the test tube.
9. Dispense the contents of the pipette into the tube.
10. Cover the tube.
11. Discard the used pipette.

**C. Repeat the entire process by transferring 5 mL of water into the 10-mL culture tube.**

1. Transfer 5 mL of water from the medium bottle into the glass culture tube using aseptic technique, this time flaming the top of the glass tube.
2. Pour out the contents of the tube and practice this two to three times until you are comfortable with the process.

### The Big Picture

**Bacteria and fungi (as well as pollen, spores, and other particles) are found in surprisingly high concentrations suspended in the air we breathe. One estimate is that we each breathe in approximately *10,000* organisms per day. Creating an updraft with the Bunsen burner minimizes the possibility of organisms falling onto the bench or into open bottles, tubes, plates, or flasks.**

Remember to avoid passing any of your fingers over the top of sterile bottles, tubes, or flasks, especially when removing or replacing a cap or lid.

3. Choose a partner and critique each other's technique.

Things to watch:

- ☐ Hand and finger placements
- ☐ Movement of pipette and pipette tips
- ☐ Sterile field location(s)
- ☐ Placement and handling of tube and bottle caps

**D. Transfer 100 μL of medium into a microcentrifuge tube.**

1. Arrange on the bench so that you have easy access to each of these items:
   - ☐ A bottle of medium (water)
   - ☐ A 1.0-mL pipette
   - ☐ A 100- or 200-μL micropipettor
   - ☐ A box of pipette tips
   - ☐ Microcentrifuge tubes (2) in a tube rack
2. Transfer 1.0 mL of medium from the bottle to a microcentrifuge tube using the aseptic technique described above, except do not attempt to flame any plastic or microcentrifuge tubes!

3. Wipe down the micropipettor with disinfectant (such as 70% ethanol), and allow it to air-dry.
4. Set the micropipettor to 100 $\mu$L.
5. Using the micropipettor, remove a sterile tip from the box.
6. Open the tube containing the 1.0 mL of medium and remove 100 $\mu$L using the micropipettor.
7. Dispense the 100 $\mu$L of liquid into the second microcentrifuge tube.
8. Pour out the contents of the tube, and practice this two to three times until comfortable with the process.
9. Choose a partner and critique each other's technique.

Things to watch:

- ☐ Hand and finger placements
- ☐ Movement of pipette and micropipettor, and pipette and micropipettor tips
- ☐ Sterile field location(s)
- ☐ Placement and handling of tubes

The micropipettor has a two-stop plunger system. The first stop draws in the desired volume of liquid; the second dispenses the liquid.

### E. Clean up your workbench.

- ☐ Turn off the Bunsen burner.
- ☐ Put away all supplies and solutions.
- ☐ Put any potentially contaminated glassware into a decontaminating solution.
- ☐ Put any other potentially contaminated items into infectious-waste containers.
- ☐ Make sure the bench is clear and free from clutter.
- ☐ Wipe down the bench with disinfectant (such as 70% EtOH), and allow it to air-dry.

## Notes

To keep an area aseptic, remember these important points:

- ☐ Always know where your hands are.
- ☐ Never pass your hands or fingers over the top of a sterile field (such as open bottles or flasks, the inside of tube and bottle caps, and agar plates).
- ☐ Plastic culture tubes and microcentrifuge tubes cannot be flamed, so it is doubly important to work with an open flame and to take care not to pass fingers or hands over any tube openings.
- ☐ The microcentrifuge tube should be kept closed until liquid is transferred and should be closed immediately when 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.

**Food for Thought**

Why is there an "extra" step? In other words, why not transfer 100 µL of media directly into the microcentrifuge tube and save some time and supplies?

- ☐ Always work with an open flame when opening sterile tubes or bottles.
- ☐ Never have more than one tube, bottle, or flask open on the bench at a time.
- ☐ Even if someone else has recently used the bench and the bench top was wiped down with disinfectant, *always* begin your laboratory time by wiping down the laboratory bench. Always *end* laboratory time by wiping down the bench. One bit of dust can contain hundreds of organisms!

## Questions

1. **The food and beverage industries, though held to high standards, are not required to perform this level of aseptic technique during the handling of materials (except, of course, in the microbiological laboratories!). Why isn't there more illness associated with our food supply?**
2. **Why do researchers concern themselves with contamination?**
3. **What do you expect to observe when something is contaminated? Why?**

# Part B. Labels, Laboratory Notebooks, and Laundry Lists

## The Principle of Autonomous Replication

The goal of writing in a laboratory notebook is to create a record that anyone can use to perform the same procedures and obtain the same results—using only your laboratory notebook. This is known as "the principle of autonomous replication."

Indications that you have fulfilled the requirements for the principle of autonomous replication include:

- ☐ The level of detail should be high enough that you can go back at any time and troubleshoot your procedures if, for some reason, a procedure does not work or yields questionable results.
- ☐ Jargon, personal shorthand, and personal abbreviations should be kept to a minimum (unless you have a list of abbreviations in the notebook).
- ☐ Drawings, figures, and tables are encouraged. They must contain enough information that another scientist can interpret them without extensive reference to the text.

## Writing in a Laboratory Notebook

This is an outline of the minimum information required in a laboratory notebook. *Italicized portions are suggestions*. You may, however, want to arrange and organize your notebook in your own way.

**You must write in your lab notebook as you perform the procedure. Your memory is not reliable. Experiments without sufficient documentation must be discarded. The results cannot be disseminated!**

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

**B. 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. *State (or restate) any hypothesis or expected results here. This acts as a framework from which you can build your analysis (below).*

## The Big Picture

**When scientific data are published, the source of the data (namely, the laboratory notebook) belongs to, and must be retained by, the laboratory for *at least 7 years.* The notebook should, therefore, be a document that can be read and understood for years to come even if, and especially when, science moves on from the point of discovery outlined in the notebook.**

**C. Materials.** All materials should be listed. This helps you, and anyone reading the notebook, repeat procedures with the same supplies.

**D. Procedures and protocols.** This should be a comprehensive, accurate, and detailed step-by-step accounting of your procedure. Even if you are repeating a protocol without any changes, write out the exact steps.

It is a good idea to number and initial each page as you complete your write-up.

**E. Results.**

- ☐ The description of any results must be comprehensive and accurate.
- ☐ Primary data (numerical values, photographs, printouts, and all observations) should be listed first.
- ☐ Anything taped into the notebook must be dated and initialed.
- ☐ Primary data can later be sorted or organized into tables, graphs, charts, or diagrams.
- ☐ Any reader should be able to read the results of an experiment and know exactly what happened.

**F. Analysis and interpretation.**

- ☐ An objective and balanced analysis must be documented in the laboratory notebook. Even if the results indicate a simple "yes" or "no" answer, it is important to state that in this section.
- ☐ 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 changing the path that your research has been following.
- ☐ Interpretation of data can sometimes be subjective, but your reasons for a particular interpretation *must* be stated here.

### G. 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 step or steps. *The rationale for this should be clear in your "analysis" section and does not necessarily have to be restated*. This will lead you and the reader into the next procedure with a clear idea of the short- and long-term goals of your work.

*This section can also include a to-do list for the next day of work ("Get more 5-mL pipettes," "Don't forget to prewarm plates," etc.).*

## Labeling Hints: Minimum Labeling Requirements

### A. Each set of tubes, plates, and bottles should be labeled with

- ☐ Your name or initials
- ☐ The date
- ☐ The designation (this can be numbers, letters, or more detailed descriptors)

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

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

### C. For computer files or images

- ☐ Use a consistent designator (e.g., your initials and the date, ML100208) *plus* an image or file number.
- ☐ Make sure that the label on or title of your file(s) matches the information in your laboratory notebook.

## Notes

Every laboratory notebook belongs to the laboratory of the lead researcher (faculty member) on your team. The original notebook cannot leave the laboratory, though laboratory personnel often make copies for their records.

Do *not* remove your notebook from the laboratory.

You may want to leave a few pages at the beginning of the notebook for a table of contents (page numbers for each experiment and protocol) and a list of abbreviations, both standard and personal.

At the beginning, you may want to limit notebook entries to the right side of the open pages. The left side of the notebook can be used for notes, comments, and checklists.

Remember that everything that is labeled should be designated in your laboratory notebook with as much detail as possible. For example, if 10 samples

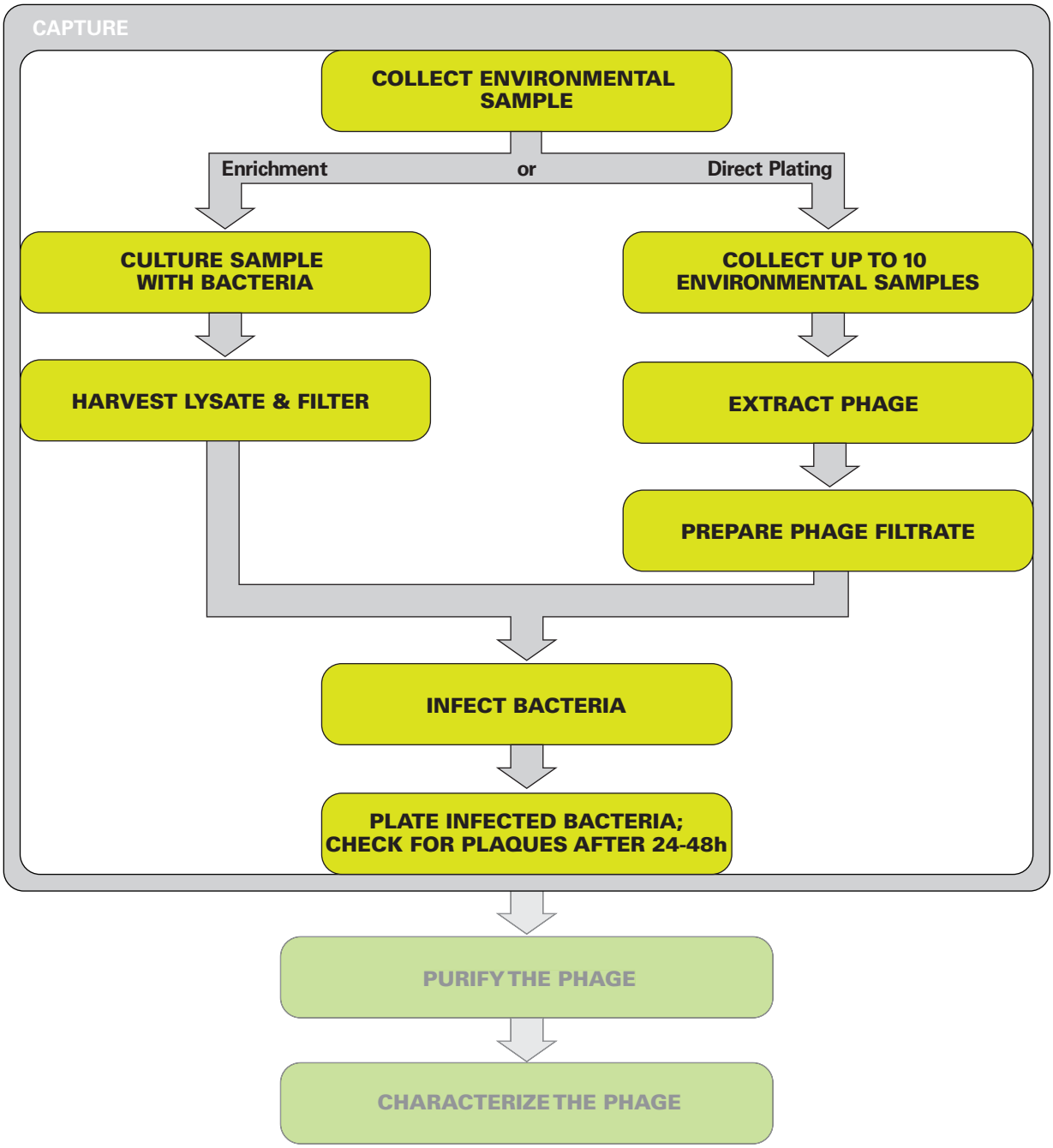
from the environment are labeled A to J on the bench (with, of course, the date and the initials of the scientist), the laboratory notebook should list each designation with the descriptors for each sample site.

## Questions

1. **In commercial and clinical laboratories, a supervisor is often required to sign off daily on the entries of the laboratory scientist. In these settings, all notebooks are usually locked in a safe, or otherwise secured, until the next day of work. Why do you suppose these precautions are taken?**

2. **What are the possible repercussions for a given laboratory or lead researcher if, during a routine audit (i.e., an inspection of laboratory procedures and practices, including documentation), the primary data used in a published work cannot be found or cannot be interpreted? These audits do, in fact, occur, especially if another laboratory obtains conflicting results!**

## You Are Here


CAPTURE
COLLECT ENVIRONMENTAL SAMPLE
Enrichment
or
Direct Plating
CULTURE SAMPLE WITH BACTERIA
COLLECT UP TO 10 ENVIRONMENTAL SAMPLES
HARVEST LYSATE & FILTER
EXTRACT PHAGE
PREPARE PHAGE FILTRATE
INFECT BACTERIA
PLATE INFECTED BACTERIA; CHECK FOR PLAQUES AFTER 24-48h
PURIFY THE PHAGE
CHARACTERIZE THE PHAGE

# Capture

## Contents

# Part A. FYI #1: Introduction to Bacteriophages

## What Are Bacteria?

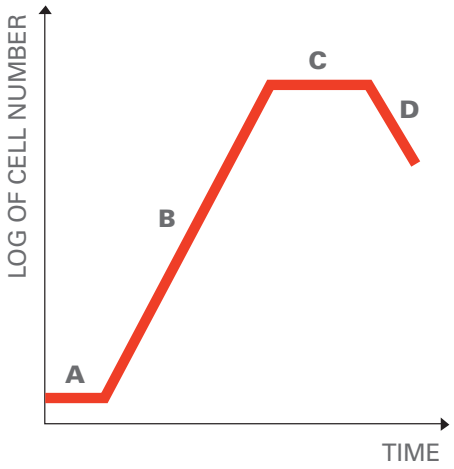
Bacteria are prokaryotes, the simplest free-living life-form.

They are quite small, usually in the range of 1 to 5 microns ($\mu$m) (a human red blood cell is 8 $\mu$m across; a white cell is 12 to 20 $\mu$m). See Cells Alive, "How big is a…" at

www.cellsalive.com/howbig.htm

Different bacteria can cause a variety of infections in humans, from food poisoning to meningitis to boils.

Bacteria grow by binary fission (in other words, they split in half: 1 to 2 to 4 to 8 to 16, etc.). See *Capture* fig. 1.




**Capture fig. 1**
Bacterial growth over time. When inoculated into fresh media, bacteria will take some time to 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 **(C,** the stationary phase**)** and eventually decline **(D,** the decline, or death, phase**)**.

Bacteria can grow very rapidly.

They are generally susceptible to antibiotics (unlike viruses, the "other" germs).

Bacteria are ubiquitous.

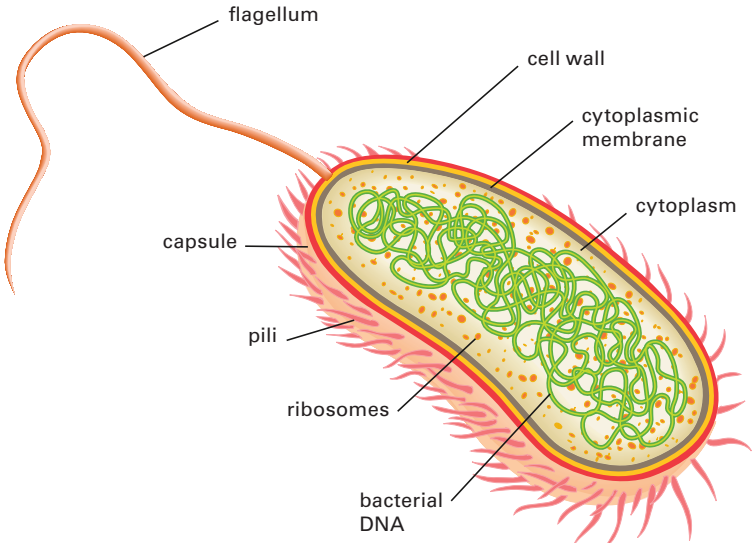
The morphology (form and structure) of different bacteria varies (see *Capture* fig. 2), but the basic components are these:

**Outer structures.** All bacteria have a plasma membrane made of lipids (or "fats") that surrounds the inner workings of the bacterial cell. The cell wall is a collection of molecules that surround the plasma membrane and give the bacteria structural strength. Bacteria can have an additional lipid membrane and/or a slimy capsule that protects them from harsh environments or performs other functions. Some bacteria have appendages called "pili" and "flagella" (singular is "pilus" and "flagellum," respectively). These are hairlike structures that protrude from the bacteria and perform specialized functions.

**Inner structures.** All bacteria contain genetic material, or DNA. Since bacteria do not have a nucleus, the free DNA, or chromosome, is called a "nucleoid." All bacteria also contain ribosomes, which translate the DNA sequence into proteins.

For more information about bacterial cell components, see Cells Alive at

www.cellsalive.com/cells/bactcell.htm




**Capture fig. 2**
Bacterial structure.

## What Are Bacteriophages?

Bacteriophages (often shortened to "phages") are a class of viruses (i.e., all phages are viruses, but not all viruses are phages).

Phages are parasites specific to bacteria. The Ancient Greek word "phagein" means to eat. A phage is an eater (or devourer) of bacteria.

Phage size ranges from 100 to 200 nm. See Cells Alive, "How big is a…," at

www.cellsalive.com/howbig.htm

**Food for Thought**

How is it possible to isolate individual phages if they can only grow inside bacteria?

Phages cannot replicate or propagate outside their host bacteria.

Phages are not susceptible to antibiotics.

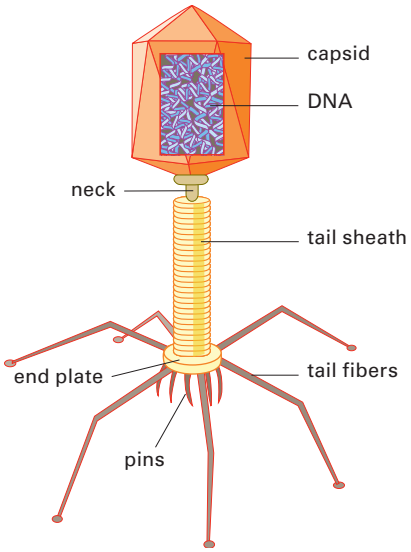
Phages are ubiquitous.

Phages are the most abundant life-form on earth (there are $\approx 10^{31}$ phages worldwide).

Phages can survive in almost any environment, and they can be found both inside and outside bacterial cells.

The morphology of different phages varies (see ***Capture*** fig. 3), but there are three basic components:

- ☐ the **capsid**, also known as the "head," which contains the genetic material;
- ☐ the **genetic material**, which in phages is mostly double-stranded DNA (dsDNA); and
- ☐ the **tail**, a hollow structure or tube. The tail serves two purposes. First, it allows the phage to attach to bacteria. This is a very ***specific*** interaction. The "host range" of the phage is defined by the kinds of bacteria to which the phage can attach. Second, the DNA passes through the tail and into the bacterium. These are the first two steps of infection of the bacterium by the phage.




**Capture fig. 3**
Phage structure.

"Phage" can be singular or used in reference to a collection of the same type of phage particle. For example:

"My favorite phage is the Bronx Bomber."
"I have 750 billion Bronx Bomber phage in this test tube."

"Phages" is the plural referring to a collection of different types of phage particles. For example:

"We study Bronx Bomber and T3 phages in our laboratory."

"Mycobacteriophages" are phages that specifically infect species of bacteria in the genus *Mycobacterium*.

## Who Discovered Phages?

In 1910, Felix d'Herelle saw "clear spots" on a lawn of bacteria. We call them "plaques." He filed this away in the back of his mind until, in 1915, he added

some material from a plaque to a growing (and cloudy, or "turbid") batch of bacteria. This is how d'Herelle described what happened:

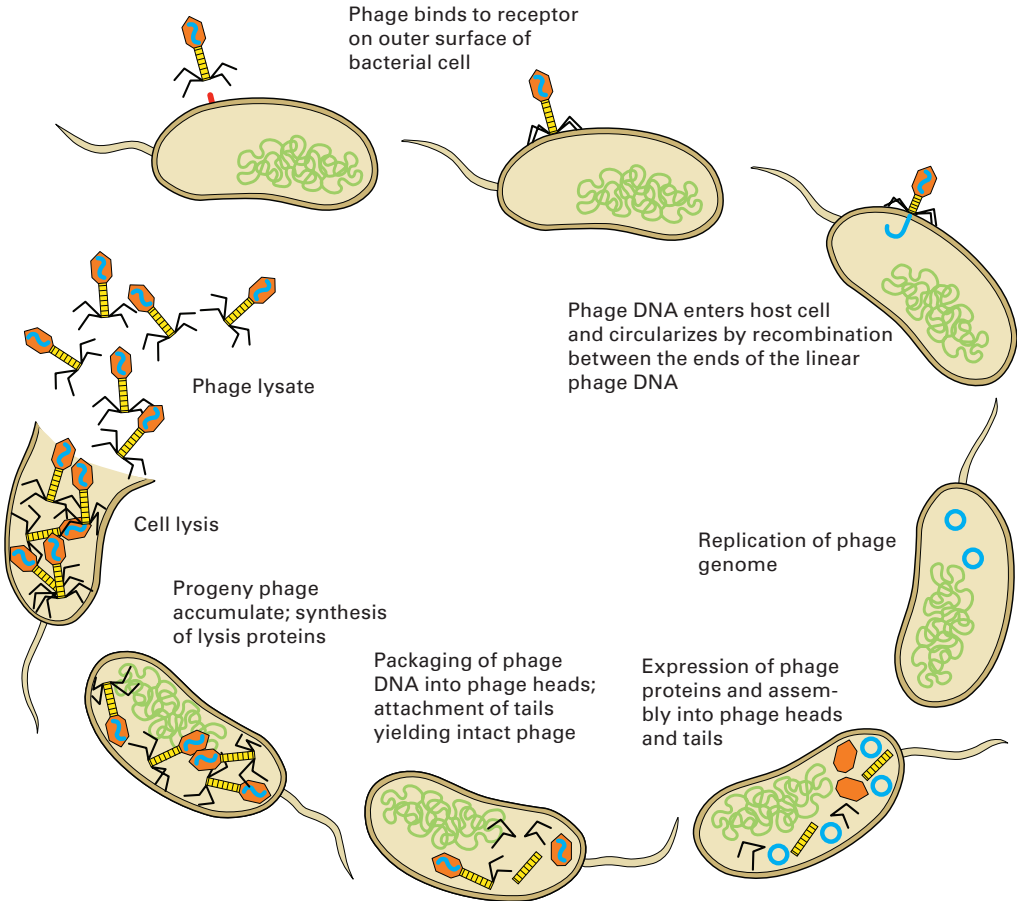
*The next morning, on opening the incubator, I experienced one of those moments of intense emotion which reward the research worker for all his pains: at the first glance I saw that the culture which the night before had been very turbid, was perfectly clear: all the bacteria had vanished, they had dissolved away like sugar in water. As for the agar spread, it was devoid of all growth and what caused my emotion was that in a flash I had understood: what caused my clear spots was in fact an invisible microbe, a filtrable virus, but a virus which is parasitic on bacteria.* (From "The Bacteriophage," by Felix d'Herelle, ***Science News*** 14:44–59 (1949). Translation by JL Crammer.)

Another scientist, Edward Twort, was credited for the independent discovery of phages at the same time. d'Herelle, however, coined the term "bacteriophages."

## What Happens When a Phage Infects a Bacterium?

The phage attaches to the bacterium and injects its DNA into the bacterial cell. The phage structure, now empty and attached to the bacterium, is no longer infectious and is called a "ghost." Using the bacterium's replication

**Capture fig. 4**
Phage life cycle, simple (lytic only).



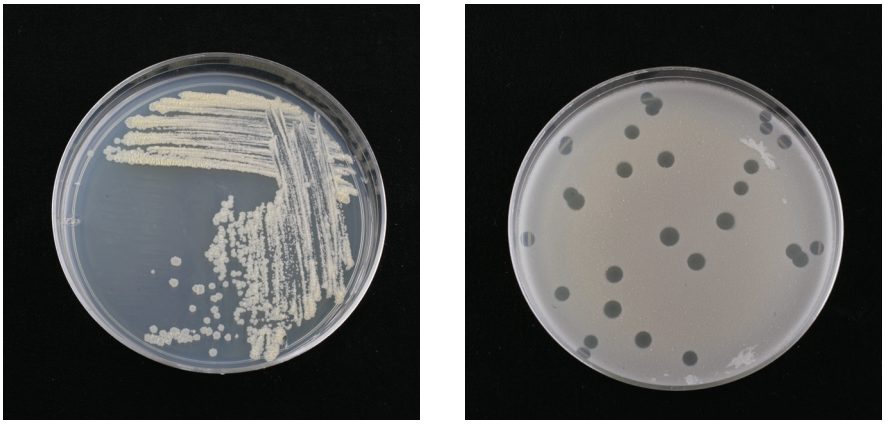
"machinery," the phage genomic DNA makes and assembles copies of itself (including the capsid and tail) until the host bacterium lyses (*Capture* fig. 4). See Cells Alive, "Oh my goodness, my ***E. coli*** has a virus," at

www.cellsalive.com/phage.htm

## How Do You See (and Count) Bacteria and Phages?

**Use a microscope.** You can see individual bacteria with a standard light microscope, but to see phages, you generally need an electron microscope.

**Grow a ton of them.**
**Bacterial colonies**. These are visible collections of bacteria that arose from one bacterium (see *Capture* fig. 5, left plate). When a bacterium is inoculated onto an agar plate containing all the nutrients required for growth, it will



**Capture fig. 5**
Bacterial single colonies (left) and lawn of bacteria with clearing zones (right).

replicate and produce many bacteria. One bacterium becomes two; these two become four, etc. Depending on the bacterial species and doubling time (the time it takes a bacterial population to double), one can see a colony—literally a pile of bacteria—after 16 to 24 hours. These bacteria are identical to the original bacterium from which the colony was formed (i.e., they are "clonal").

**Phage plaques.** If growing bacteria are inoculated into a solid (but soft) medium (called "top agar," or TA) and poured onto the top of an agar plate, a cloudy suspension of bacteria will soon be visible throughout the medium. If even one phage is present, it will infect one of the bacterial cells, replicate within the cell, and lyse the bacterium (i.e., the bacterium will burst open). This lysis will release many (up to 100) individual new phage. The number of new phage released is called the "burst size."

The new phage will travel via diffusion through the TA and infect other bacteria. One infection-lysis cycle yields 100 × 100, or 10,000, new phage particles. This cycle continues, and, because of the bacterial lysis, there is no

cloudy suspension of bacteria radiating outward from the area that contained the original phage. This is called a "clearing zone," or plaque, indicating the presence of an infectious phage particle (see ***Capture*** fig. 5, right plate). The plaques contain billions of infectious phage particles, all identical to the original phage (i.e., they are clonal).

## Why Study Phages Now?

**Genetics.** Phages can be used as tools to move DNA around for cloning, mutation, and other laboratory techniques. Genetic information about different phages allows scientists to compare the phages (e.g., through genome analysis), study biodiversity (e.g., through environmental patterns of evolution), and identify new genes that may be useful for scientific or therapeutic applications.

**Epidemiology.** Some phages can make their host bacterium more deadly to humans. For example, a bacterium called *Escherichia coli* 0H157 causes food-borne illness that is sometimes fatal. The toxin that causes the symptoms was delivered to the bacterium by a phage!

**Therapeutics.** Scientists would like to use phages to kill specific antibiotic-resistant bacteria that cause disease.

**Food for Thought**

Name at least two challenges that researchers might face when trying to develop the use of phages as treatments for bacterial infections.

## The Big Picture

**You are now a part of the Science Education Alliance, or SEA program. In this program, you will discover, manipulate, and sequence a new phage. You will label (or annotate) the entire genome and, in the process, characterize a new life-form. This phage will likely contain new genes and new gene arrangements. The data you and your classmates generate will be used by other scientists to explore some of the research avenues listed under "Why Study Phages Now?" (above). Newly discovered phages and new genes can lead to new strategies for molecular biology and comparative evolutionary biology research, new therapeutics, and the discovery of how different organisms and genes can cause disease. You will be adding to the body of knowledge in a discipline known as "phage genomics."**

# Part B. Isolate a Novel Phage from the Environment: Enrichment of Environmental Samples

## Overview

The enrichment culture technique creates conditions that favor replication of specific bacterial phages. Since phages are obligate intracellular parasites, large numbers of a desired phage can be obtained by adding host bacteria and bacterial media to an environmental sample. By seeding the sample with host bacteria and using nutritional conditions optimized for bacterial growth, the phages that are specific to that bacterial species will infect the bacterial cells and replicate to higher concentrations. These phages can then be isolated at much higher frequency than with direct plating. This protocol is for the isolation of phages specific to *Mycobacterium smegmatis.*

## Objective

To increase the likelihood of each student obtaining a novel phage from the environment.

## Supplies

- ☐ Sterile 15-mL conical tube with cap
- ☐ A ruler
- ☐ A laboratory notebook
- ☐ A labeling pen
- ☐ 0.22-$\mu$m sterilization filters (2)
- ☐ 1.0-mL tuberculin syringes (2)
- ☐ A sample site marker
- ☐ A 250-mL baffled Erlenmeyer flask
- ☐ A GPS unit or Google Earth™
- ☐ 10 mL of phage buffer (PB) supplemented with 1 mM $CaCl_2$
- ☐ 5 mL of an ***M. smegmatis*** culture (48 hours)
- ☐ 0.5-mL aliquots of ***M. smegmatis*** culture (7, in 10 mL glass culture tubes with lids on a tube rack)
- ☐ 40 mL of top agar (TA) supplemented with 1 mM $CaCl_2$
- ☐ 5 mL of 10X 7H9/glycerol broth
- ☐ 5 mL of AD supplement
- ☐ 0.5 mL of 100mM $CaCl_2$
- ☐ Agar plates, 100 mm (7)
- ☐ Sterile 50-mL conical tube with cap
- ☐ Microcentrifuge tubes (7) and tube rack
- ☐ 25-mL sterile disposable pipettes (2)
- ☐ 5-mL sterile disposable pipettes (14)
- ☐ A 1000-$\mu$L and a 100-$\mu$L or 200-$\mu$L micropipettor and tips
- ☐ An automated pipettor for larger volumes
- ☐ A vortexer

## Equipment

- ☐ A 37°C shaking incubator (or a shaker in a large incubator)
- ☐ A centrifuge with a rotor (or adaptors) for spinning 50-mL conical tubes at 3000rpm
- ☐ A microwave oven
- ☐ 37°C incubator with room for 7 100-mm plates per student

## Procedure: Sample Collection and Preparation

### A. Collect a soil sample

1. Label a 15-mL conical tube
2. Place soil into the tube
3. Mark your collection site and note site characteristics in your laboratory notebook (see Capture, page B.4)

### B. Back in the lab, prepare the enrichment culture.

1. Using a clean spatula, add approximately 1 gram (~1/2 teaspoon) of your sample[1] to a 250-mL baffled Erlenmeyer flask.
2. To this flask, aseptically add:
   a. 40 mL of sterile $H_2O$ (using 2 x 25-mL pipettes).
   b. 5 mL of sterile 10X 7H9/glycerol broth.
   c. 5 mL of AD supplement.
   d. 0.5 mL of 100 mM $CaCl_2$.
3. Add 5 ml of late log/early stationary phase *M. smegmatis* culture (48 hour culture) to the flask.

### C. Incubate the flask at 37°C, shaking at 220rpm, for 24 hours.

## Procedure: Harvesting and Preparing the Enriched Sample

### A. Centrifuge the culture.

1. The next day, transfer the contents of the Erlenmeyer flask to a 50-mL conical tube.
2. Balance the tube and spin at 3,000rpm (2,000x*g*) for 10 minutes to pellet (i.e., force to the bottom of the tube) particulate matter, including most of the bacterial cells.

### B. Prepare a phage filtrate.

Assemble the following at your bench:

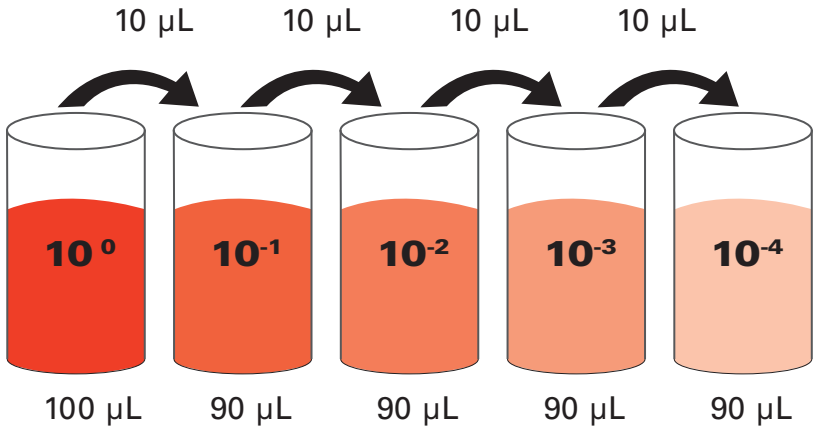
- ☐ Microcentrifuge tubes (7)
- ☐ A microcentrifuge tube rack
- ☐ A permanent-ink marker
- ☐ A 50 mL conical tube
- ☐ A sterilization filter unit

1. Pour the supernatant (liquid) from the centrifuged sample into a fresh 50 mL conical tube.
2. Follow your instructor's directions and filter-sterlize your enrichment sample.
3. Immediately and aspetically cap the tube (be sure it is labeled properly).

This is your $10^0$ (undiluted) enrichment sample. This sample can be stored at 4° C.

### C. Dilute the enrichment sample $10^0$–$10^{-4}$ by using serial 10-fold dilutions.

1. Arrange four microcentrifuge tubes in a tube rack and label them -1, -2, -3, and -4.
2. Add 90 µL of PB to each of these four tubes.
3. Label one microcentrifuge tube "0" and aseptically transfer 100 µL of your $10^0$ (undiluted) enrichment sample into this tube.
4. Add 10 µL of your $10^0$ sample to the "-1" tube and vortex well (see ***Capture*** fig. 6).




**Capture fig. 6**
The serial dilution of phage preparations for a titer. A volume of 10 µL of the undiluted phage sample ($10^0$) in 100 µL of phage buffer is transferred into 90 µL of PB 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.

5. The "-1" tube is your $10^{-1}$ (or 1:10) dilution.
6. Add 10 µL of the "-1" sample to the "-2" tube and vortex well.
7. Continue each successive dilution until you get to the "-4" tube.

### D. For the positive control, obtain an aliquot of phage from your instructor.

### E. For the negative control, filter-sterilize 1.0 mL of PB into an appropriately labeled microcentrifuge tube.

You may want to label your diluted enrichment samples E0, E-1, E-2, etc.

## Procedure: Plaque Screening

### A. Add 50 µL of each sample (including controls) to 0.5 mL of *M. smegmatis*.

Assemble the following:

- ☐ Culture tubes containing 0.5 mL of an ***M. smegmatis*** culture (7).
- ☐ A 100-µL or 200-µL micropipettor.
- ☐ Micropipettor tips.
- ☐ Positive and negative control tubes.
- ☐ Phage enrichment tubes ($10^0$-$10^{-4}$).

For each sample, including the controls:

1. Label the culture tubes containing 0.5 mL of *M. smegmatis* the same way you labeled the microcentrifuge tubes.
2. Using a micropipettor:
   a. Dispense 50 μL of each sample into the appropriate culture tube.
   b. For the negative control, add 50 μL of filter-sterilized PB to the phage-negative control tube.
   c. For the positive control, add 50 μL of the prepared phage solution to the phage-positive control tube.
3. Mix each tube well by vortexing.
4. Allow the tubes to sit at room temperature for 15-30 minutes. This allows the phage(s) to infect the bacteria.

### B. Add 4.5 mL of TA and plate out each sample and each control.

1. Label each of 7 agar plates with sample or control information plus the date and your initials.
2. Remove a bottle of TA from the 55°C water bath.
3. For each sample, including the two control tubes:
   a. Use a sterile 5-mL pipette to aseptically transfer 4.5 mL of TA to the culture tube.
   b. Immediately pull the mixture back up into the pipette.
   c. Transfer the mixture to the appropriate plate and discard the pipette.
   d. Swirl the plate gently to spread the TA evenly over the agar plate.
   e. Let the plates sit undisturbed for at least 20 minutes until the TA has completely solidified.

### C. Incubate the plates at 37°C.

1. After the TA has solidified, invert the plates rapidly but gently.
2. Put all inverted plates into the 37°C incubator.

### D. Check for plaques after 24 hours.

## Notes

Google Earth can be downloaded at http://earth.google.com.

If no plaques are apparent, check again at 48 hr.

If you observe more than one plaque morphology on your enrichment plates, be careful! Though you may well isolate more than one phage, the same phage can produce different plaques depending upon the top agar, the density of bacterial cells present, the age and thickness of the agar plate, etc. Careful notes and tracking of samples is paramount.

# Part B. Isolate a Novel Phage from the Environment: Direct Plating of Environmental Samples

*If you haven't practiced aseptic technique yet, go back to the* Before You Start *section*.

## Overview

You will go to different sites around your college or university, obtain soil samples, and record details about the sites. The samples will be processed in the lab and used for the infection of ***Mycobacterium smegmatis*** mc$^2$155 for a plaque assay and the eventual isolation of a new phage. You will use a screening process to discover a new phage that you can name, characterize phenotypically and genotypically, and make available to other scientists. You will be able to compare and contrast the locations and conditions of the sites from which new phages have been obtained and formulate a hypothesis about the likelihood that different sites will yield phages specific to ***M. smegmatis***. You will also become familiar with the basic skills required to perform a plaque assay.

## Objective

The objective of this procedure is for each student to discover a new phage.

## Supplies

- ☐ 15-mL conical tubes (10)
- ☐ A trowel, spatula, or spoon for obtaining soil samples
- ☐ A ruler
- ☐ A laboratory notebook
- ☐ A labeling pen (permanent-ink marker)
- ☐ 0.22-$\mu$m sterilization filters (10)
- ☐ 1.0-mL tuberculin syringes (10)
- ☐ Microcentrifuge tubes (12) and a tube rack
- ☐ Site markers for sampling sites (10)
- ☐ A GPS unit or Google Earth™
- ☐ 100 mL of phage buffer (supplemented with 1 mM (final) $CaCl_2$)
- ☐ 0.5-mL aliquots of ***M. smegmatis*** culture (12, in 10-mL culture tubes with lids on a tube rack)
- ☐ 60 mL of top agar (TA, supplemented with 1 mM (final) $CaCl_2$)
- ☐ Agar plates, 100-mm (12)
- ☐ 10-mL sterile disposable pipettes (at least 10)
- ☐ 5-mL sterile disposable pipettes (at least 12)
- ☐ An aliquot of a phage preparation for use as a positive control
- ☐ A 1000-$\mu$L micropipettor and a 100- or 200-$\mu$L micropipettor and tips
- ☐ An automated pipettor for larger volumes (e.g., Pipette-Aid)
- ☐ A vortexer

## Equipment

- ☐ 37°C incubator with room for 12 100-mm plates per student
- ☐ A microwave oven
- ☐ A shaking 37°C incubator (or a shaker in a large incubator)

## Procedure: Sample Collection and Preparation

### A. Collect up to 10 soil samples in 15-mL screw-cap conical tubes from a variety of locations around your campus, home, and/or city or town.

1. Label each tube (with your name or initials, the date, and a designator).
2. Using a trowel, spatula, or spoon, transfer a sample to a clean tube. Soil samples should fill the tube approximately one-third to one-half full.
3. For ***each*** sample, record the following in your laboratory notebook:
   - ☐ Date and time of sampling
   - ☐ Approximate air temperature, as indicated in a local weather report, for example
   - ☐ Location (by GPS or Google Earth™)
   - ☐ Depth from which sample was obtained, to the nearest centimeter
   - ☐ Approximate moisture content (i.e., dry, moist, or saturated)
   - ☐ Defining features of the site, including
     - ☐ whether the area is urban or rural
     - ☐ proximity to buildings, foot traffic, major roads, cement, water, and trees
     - ☐ if sloped, the site's compass direction (e.g., north-facing)
     - ☐ other: specify

## B. Back in the lab, extract phage from the soil.

1. Using a 10-mL pipette and pipettor, add enough phage buffer (PB) to flood each sample. The volume added should result (after settling) in a liquid layer distinct from the solid layer.
2. Mix well using a vortexer.
3. Allow the sample to settle for at least 20 minutes.

## C. Prepare a phage filtrate.

Assemble the following at your bench:

- ☐ Microcentrifuge tubes (12)
- ☐ A microcentrifuge tube rack
- ☐ A permanent-ink marker
- ☐ 0.22-$\mu$m filters (12)
- ☐ 1.0-mL syringes (12)

1. For each collected sample:
   a. Use aseptic technique as you label the microcentrifuge tubes (10 sample tubes plus 1 positive and 1 negative control[5]). Place each labeled tube in the rack.
   b. Open the packaging of the 0.22-$\mu$m filter but do ***not*** remove the filter from the packaging.

**The bottom of the 0.22-µm filter is a *sterile field* and should *never* be touched. Use the plastic wrapper (*not* your bare fingers) to manipulate the filter and attach it to the syringe.**

c. Using the syringe, remove 1.0 mL of liquid from the top of the sample tube.

d. Place the syringe into the top of the filter, and remove the filter from the package.

e. Pushing the plunger, dispense the liquid through the filter into the appropriately labeled sterile microcentrifuge tube. Immediately cap the tube.

f. Discard the syringe and filter.

2. For the positive control, obtain an aliquot (sample) of phage from the instructor.

3. For the negative control, filter-sterilize 1.0 mL PB into an appropriately labeled microcentrifuge tube.

The filtrate and tube are sterile and should be manipulated using aseptic technique.

## Procedure: The First Round of Infection—Plaque Screening

### A. Add 50 μL of each sample (or control) to 0.5 mL *M. smegmatis*.

Assemble the following:

- ☐ Culture tubes containing 0.5 mL of an *M. smegmatis* culture (12)
- ☐ Micropipettor, 100-μL or 200-μL
- ☐ Micropipettor tips
- ☐ Positive control (i.e., phage-positive)
- ☐ Phage buffer (PB)

For each sample, including the controls:

1. Label the 12 culture tubes containing 0.5 mL of an *M. smegmatis* culture the same way you labeled the microcentrifuge tubes.

2. Using a micropipettor:

   a. Dispense 50 μL of each sample filtrate into the appropriate culture tube.

   b. For the negative control, add 50 μL of filter-sterilized PB to the phage-negative control tube.

   c. For the positive control, add 50 μL of the prepared phage solution to the phage-positive control tube.

3. Mix each tube well by vortexing.

4. Allow the tubes to sit at room temperature for 15 to 30 minutes. This allows the phage(s) to infect the bacteria.

### B. Add 4.5 mL of top agar (TA, a solid but soft medium) and plate each sample and the controls.

1. Label each of 12 agar plates with sample or control information plus the date and your initials.

2. Remove a 50-mL bottle of TA from the 55°C water bath.

Agar plates should be at room temperature.

3. For each sample, **including the two control tubes:**

   a. Use a sterile 5-mL pipette attached to a pipettor to aseptically transfer 4.5 mL of TA to the culture tube (which already contains the bacteria).

   b. *Immediately* pull the mixture back up into the pipette. Try to avoid bubbles; they can appear to be plaques on a plate.

**The TA should not sit in the pipette more than a few moments, because the agar will begin to solidify.**

   c. Transfer the entire mixture to the appropriately labeled plate, and discard the pipette.

   d. Swirl the plate gently to spread the TA evenly on top of the agar plate.

   e. Let the plates sit, undisturbed, for at least 20 minutes.

### C. Incubate plates at 37°C.

1. After the TA has solidified, invert the plates rapidly (but gently). This prevents condensation from dripping onto the TA.
2. Put all inverted plates in a 37°C incubator.

### D. Check plates for plaques (clear zones) after 24 hours.

Be sure to ascertain that

☐ there are no plaques on the negative-control plate and
☐ plaques are present on the positive-control plate.

**If there are no plaques on your plates after 24 hours, do *not* discard! Check them again at 48 hours.**

## Notes

If you see plaques at 24 or 48 hours, continue on to the Tame section.

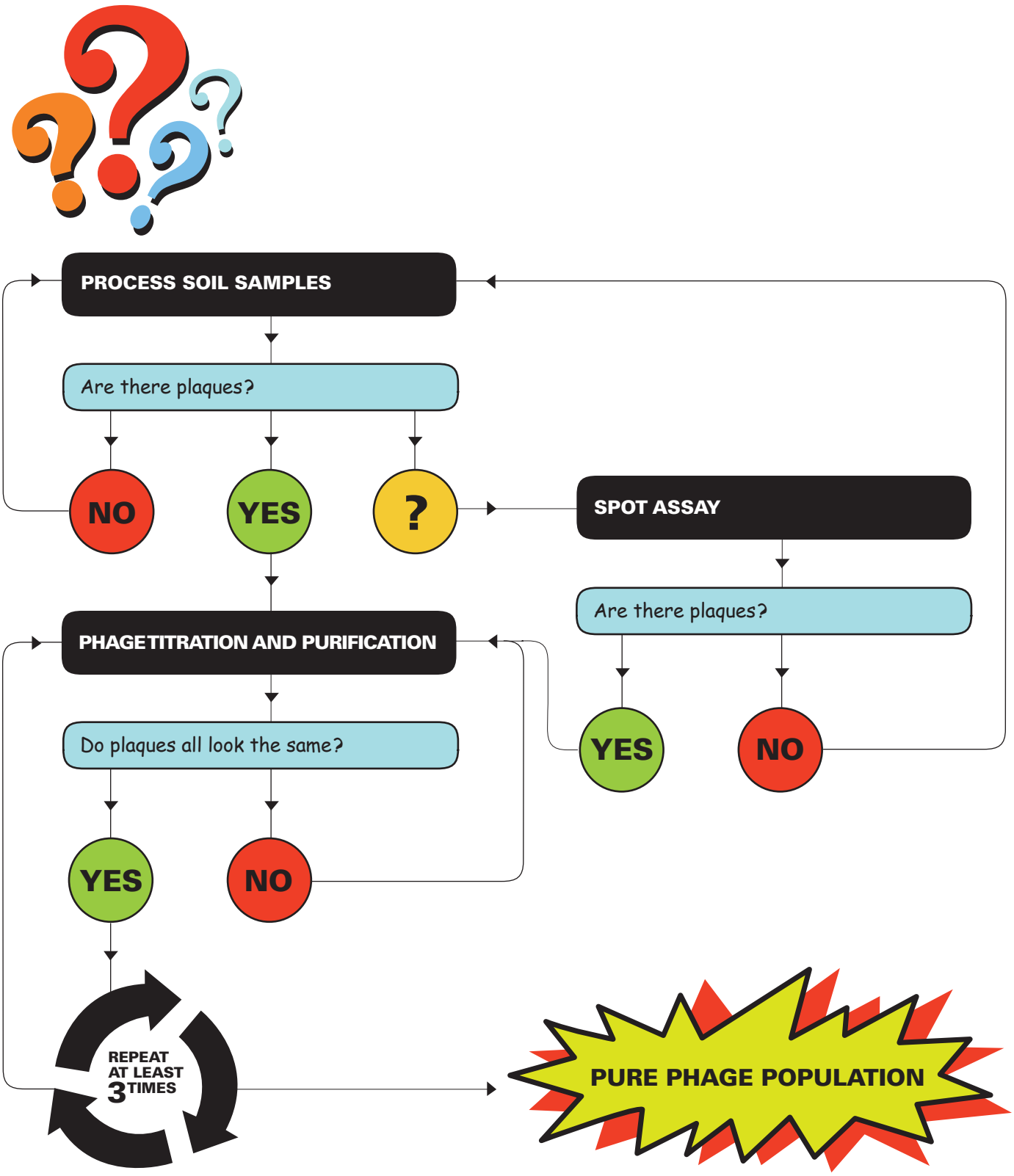
Test anything that could possibly be a plaque by continuing with the plaque assay or spot test (see Decision Tree #1).

Plaques may be difficult to see because they can be very small and the medium can be turbid, so look carefully—and remember, an air bubble in the agar can appear to be a plaque.

If there are no possible plaques, don't be discouraged! Just go out and collect new samples, and repeat the procedures until you discover your new phage.

If you are not sure what a plaque might look like on the agar plate, consult the Troubleshooting Guide (Appendix 1).

## Decision Tree #1
From Soil Sample to Pure Phage



## Questions

1. If students isolate and sequence the DNA of phages from different sites nationwide, what kinds of research questions can be answered with the data they collect, assuming they record all the requested sampling information?

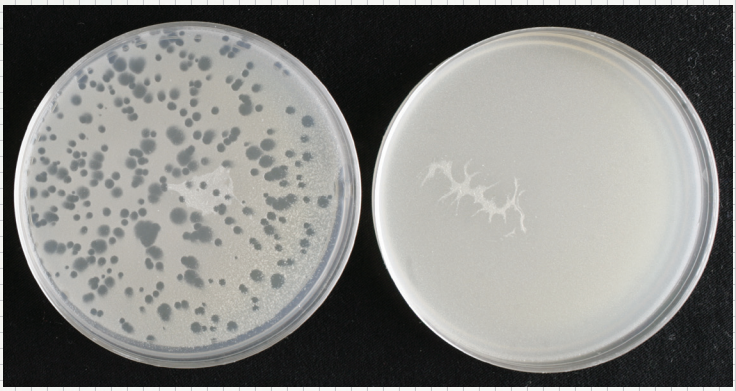
2. Why are both controls needed?

3. Where are phages most likely to be found?

4. If there are thousands of phages in every square inch of soil, why are 10 sites being sampled?

5. The filters used to process the supernatants are designated "0.22 µm." What's the significance of "0.22 µm" relative to the things that might be present in the supernatants?

6. Terry obtained the following results after 24 hours. Which plate in *Capture* fig. 7 represents a negative result with respect to the presence of phage? Explain.



**Capture fig. 7**
Two plates after 24 hours.

# Part C. FYI #2: Bacteriophage Lifestyles

## Lytic vs. Temperate Phages

There are two categories of phages, defined by their life cycle: lytic and temperate. **Lytic phages** have a straightforward life cycle, and they lyse *all* the bacteria they infect. **Temperate phages** can use two different strategies for replication and survival:

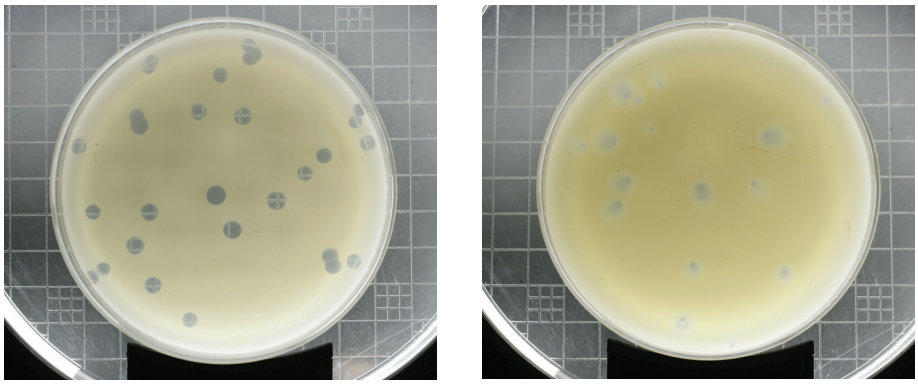
1. replicate and lyse the host bacteria they have infected, just like lytic phages, or
2. enter a quiescent (dormant) state by incorporating their genetic material, or DNA, into the DNA of the host bacterium.

The majority of bacteriophages are temperate.

Within a temperate phage population infecting a bacterial culture, it is likely that some percentage of the phage will enter the lytic life cycle and some will enter the quiescent state.

## Plaque Morphology and Phage Lifestyle

Looking at *Capture* fig. 8, can you make a guess about which phage (left or right plate) is likely to be lytic and which is likely to be temperate?



**Capture fig. 8**
Two phages, likely one temperate and the other lytic.

You may notice a variety of plaque morphologies (appearances) in the plates obtained from processing the soil samples. It is likely that clear plaques (plaques in which very few or no bacteria remain intact) are the result of a **lytic phage**. It is also likely that cloudy, or "turbid," plaques are the result infection with a **temperate phage** (some bacteria remain intact).

A **lytic phage** will likely produce clear plaques.

A **temperate phage** will likely produce turbid plaques.

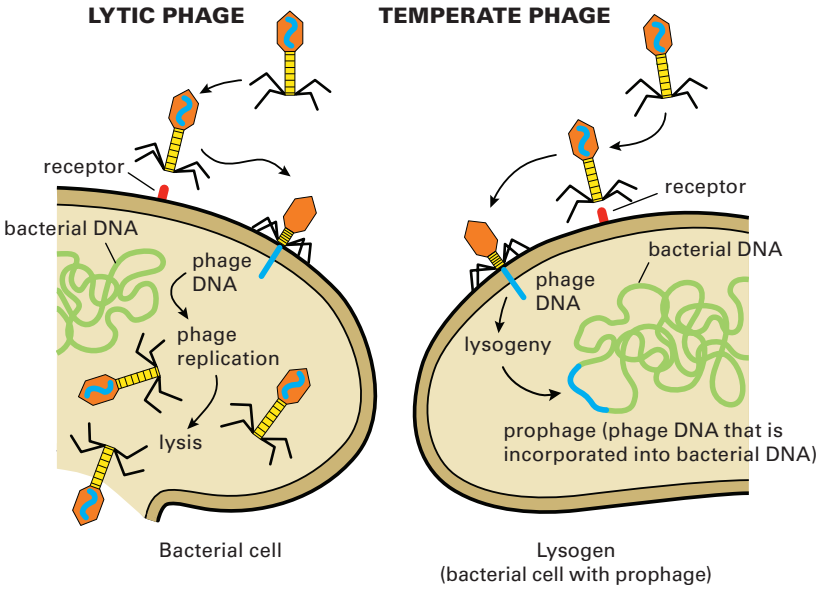
A temperate phage can either enter the lytic cycle or incorporate its DNA into the DNA of the host bacterium. When the latter happens,

- ☐ the phage genome incorporated into the bacterial DNA is called a **prophage**,
- ☐ the mechanism by which a naïve bacterial cell becomes infected with a prophage is called **lysogeny**, and

To learn more about phages isolated by other student researchers, see: http://phagesdb.org/.

☐ the bacterium that contains the prophage is called a **lysogen**. (See *Capture* fig. 9.)

**Capture fig. 9**
Lytic and temperate phage life cycle nomenclature.




## The Infection Process

Regardless of lifestyle, the first requirement for phage replication is infection of a host bacterium. This happens in four discrete steps:

1. **Adsorption** of the phage to the surface of the bacterium. The tip of the tail has specialized structures that adhere specifically to "matching" receptors on the bacterial cell. This specificity defines the host range of the phage. The phage tail will adhere to receptors on some bacteria but not others. Interestingly, the receptors to which the phages adhere serve a specific purpose for the bacterium, as structural proteins or pili, for example. The phages have evolved to use the bacterial receptors to their own advantage.
2. **Irreversible attachment** of the phage to the bacterial cell. Adsorption, though very specific, is weak and reversible. The second phase of attachment is when the phage becomes tightly and irreversibly bound to the bacterial cell.
3. **Penetration** of the bacterial cell wall. If a bacteriophage has a contractile sheath (which resembles a vacuum cleaner hose), irreversible binding of the phage to the bacterial cell leads to the contraction of the sheath. The sheath contracts (the "ribs" on the hose move closer together), and a hollow tail fiber pierces the bacterial cell envelope. Phages without contractile sheaths have other mechanisms for penetrating the bacterial cell wall. For example, some phages produce enzymes that "eat away" at the wall.

4. **Nucleic acid injection** from the phage head. When the phage has gotten through the bacterial envelope, the DNA from the head passes through the hollow tail and enters the bacterial cell. The capsid generally stays attached to the outside of the bacterium as a "ghost."

(Adapted from EP Mayer, "Bacteriology: Bacteriophage [ch. 7]," in *Microbiology and Immunology On-line*, ed. RC Hunt [Columbia, SC: University of South Carolina School of Medicine, 2007], http://pathmicro.med.sc.edu/mayer/phage.htm.)

## The Lytic Cycle

The lytic cycle is very simple. It involves infection, viral assembly, and viral release. See ***Capture*** fig. 4 for more details.

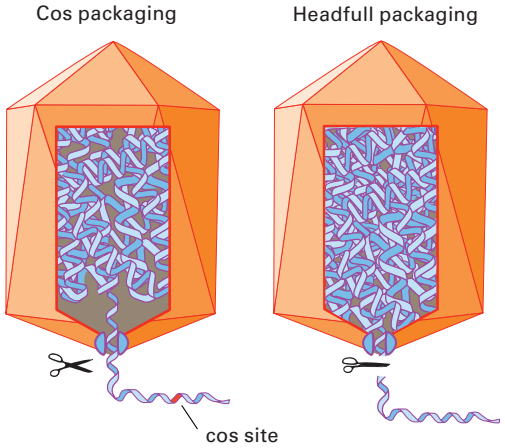
**Injection of DNA.** DNA is linear within the phage head, and it is injected as a linear molecule. When the phage DNA gets inside the bacterium, it immediately ***circularizes***.

**Packaging of DNA.** Newly synthesized DNA is loaded into the assembled capsid of the phage in this way:

1. Head and tail components are synthesized and assembled.
2. The DNA is packaged (or loaded) into the capsid.
3. The tail and the capsid (containing the DNA) are attached to form complete and infectious particles.

There are two mechanisms by which the DNA can be packaged (see ***Capture*** fig. 10):

- ☐ Cos-end packaging: Linear DNA packaged by this mechanism has "cos," or cohesive, ends. The DNA being packaged into the capsid is cut at the cos site, loading specific and precisely cut DNA molecules into the phage head. Later, when the new phage particle infects a bacterium, the DNA is injected as before. The cos ends, which are complementary, will stick together to form a circle of DNA.
- ☐ Headfull packaging: Compared with cos-end packaging, this is much less specific. Linear DNA, which is synthesized as a long chain of repeat genomes, is packaged, or "stuffed," into the phage head until no more can fit.




**Capture fig. 10**
Two kinds of packaging, cos and headfull. During cos packaging, a nuclease, or nucleic acid "cutter" (represented by scissors in this figure), recognizes the sequence at the cos site and cuts the DNA into discrete genomes. During headfull packaging, a nuclease cuts the DNA when the capsid can hold no more DNA. Nuclease activity is likely triggered by shear forces acting on the DNA molecule.

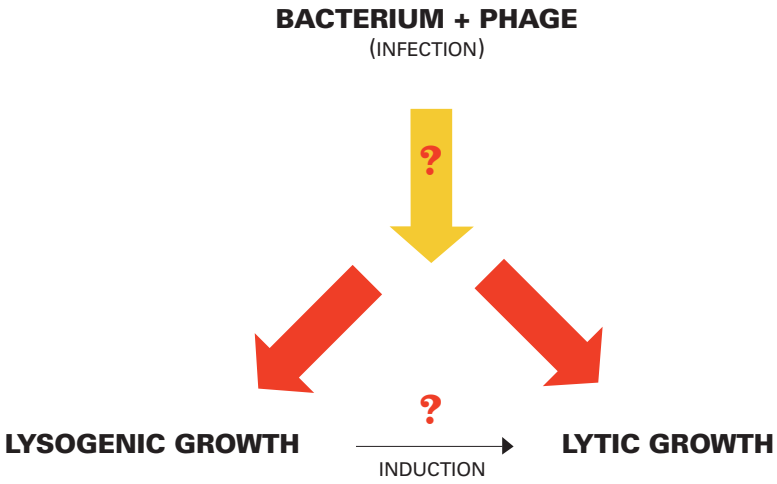
**Food for Thought**

What is the advantage (to the phage) of integrating into the host genome?

## The Temperate Phage Lifestyle

Temperate bacteriophages will not lyse their host bacterium *until* they switch to the lytic life cycle.

A genetic switch, called "induction," can occur in a phage during lysogenic growth that allows the phage to switch from lysogenic to lytic growth (see *Capture* fig. 11).




**Capture fig. 11**
Temperate phage "decisions."

Different genetic signals from the host bacterium will be produced depending on many factors, including the "health" of the host bacterium, the availability of nutrients, and the growth temperature. Certain signals will lead to phage induction. The phage will then remove itself (or "excise") from the host genome and begin the lytic cycle.

The outcome of an infection of a bacterium by a temperate phage is entirely dependent on genetic signals the phage receives from the bacterium:

1. the phage enters immediately into the lytic cycle *or*
2. the phage integrates (or inserts) into the bacterial genome:
   - ☐ The bacterium is now a lysogen.
   - ☐ The phage is now a prophage.
   - ☐ The whole process is called lysogeny.
   - ☐ The phage undergoes lysogenic growth.

Phages are not "lysogenic"--they are temperate!

A temperate phage does three key things during lysogeny:

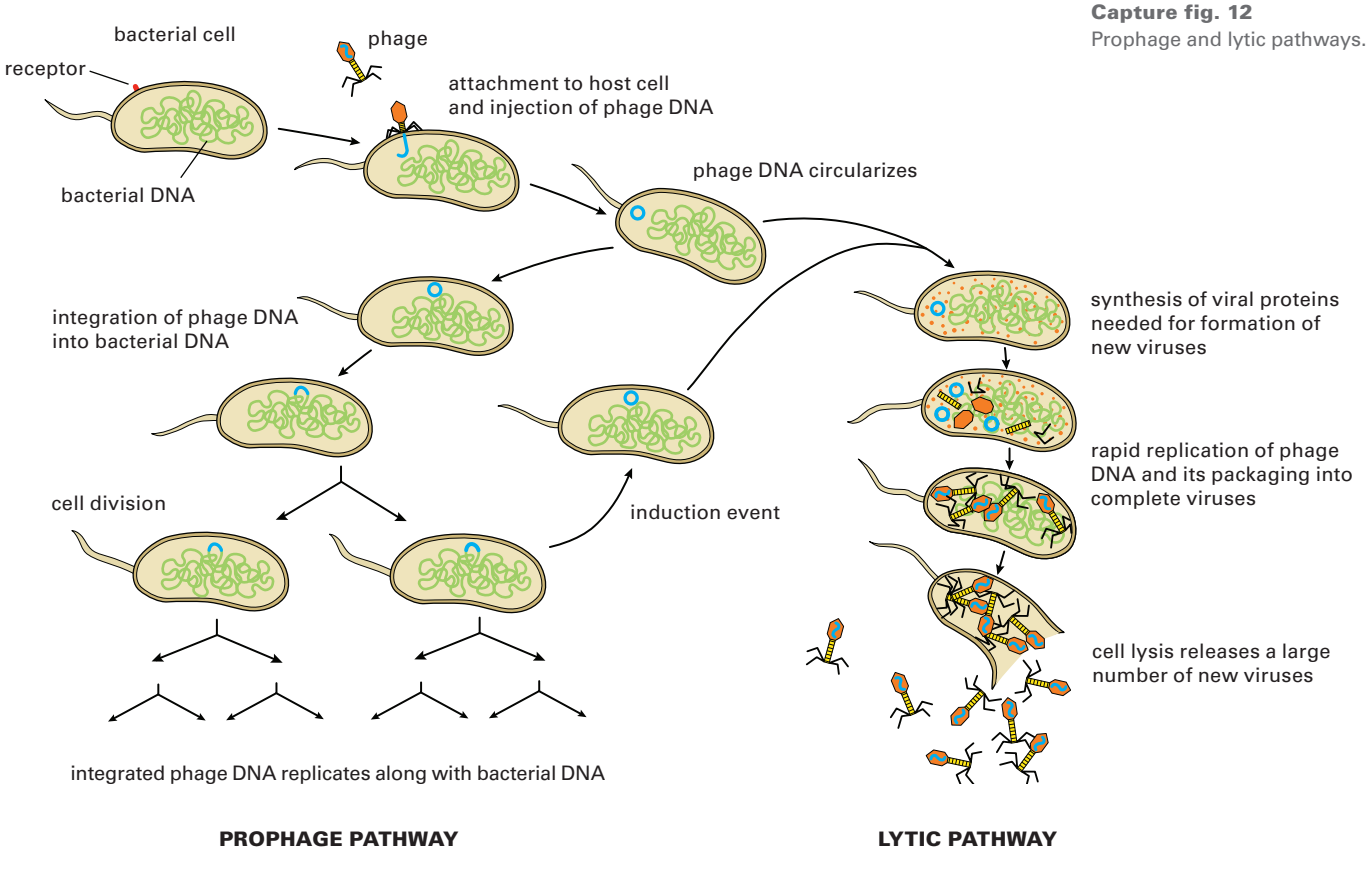
1. Lets the host bacterium live. The temperate phage synthesizes a **repressor** that stops the expression of its lytic genes, thereby inhibiting lytic growth (and thus the host is not lysed). Synthesis of this repressor protein also serves to stabilize the phage within the host bacterial genome.

2. Ensures its own survival. The phage **integrates** its DNA into the host genome, which keeps its own DNA safe. It does this via a mechanism called site-specific recombination, in which the phage genomic DNA inserts into a specific site in the bacterial genome. The site is selected by the phage based on the DNA sequence that defines the site.
3. Ensures that its DNA gets passed along. Each time a bacterium replicates, the prophage is replicated. Each bacterial chromosome, therefore, carries a copy of the prophage, so all bacterial progeny have a copy of the prophage.

The viral DNA integrated in the host genome is called a prophage because it is not truly a phage, but it has the capability of producing more phage. Almost all bacteria that have been sequenced have **at least one prophage** in their chromosome.

Induction occurs when genetic signals from the host bacterium decrease the amount of phage repressor present. The prophage will excise during induction, circularize, and enter the lytic life cycle. When new phage particles are assembled and the host bacterium is lysed, the prophage no longer exists.

To recap, see ***Capture*** fig. 12.



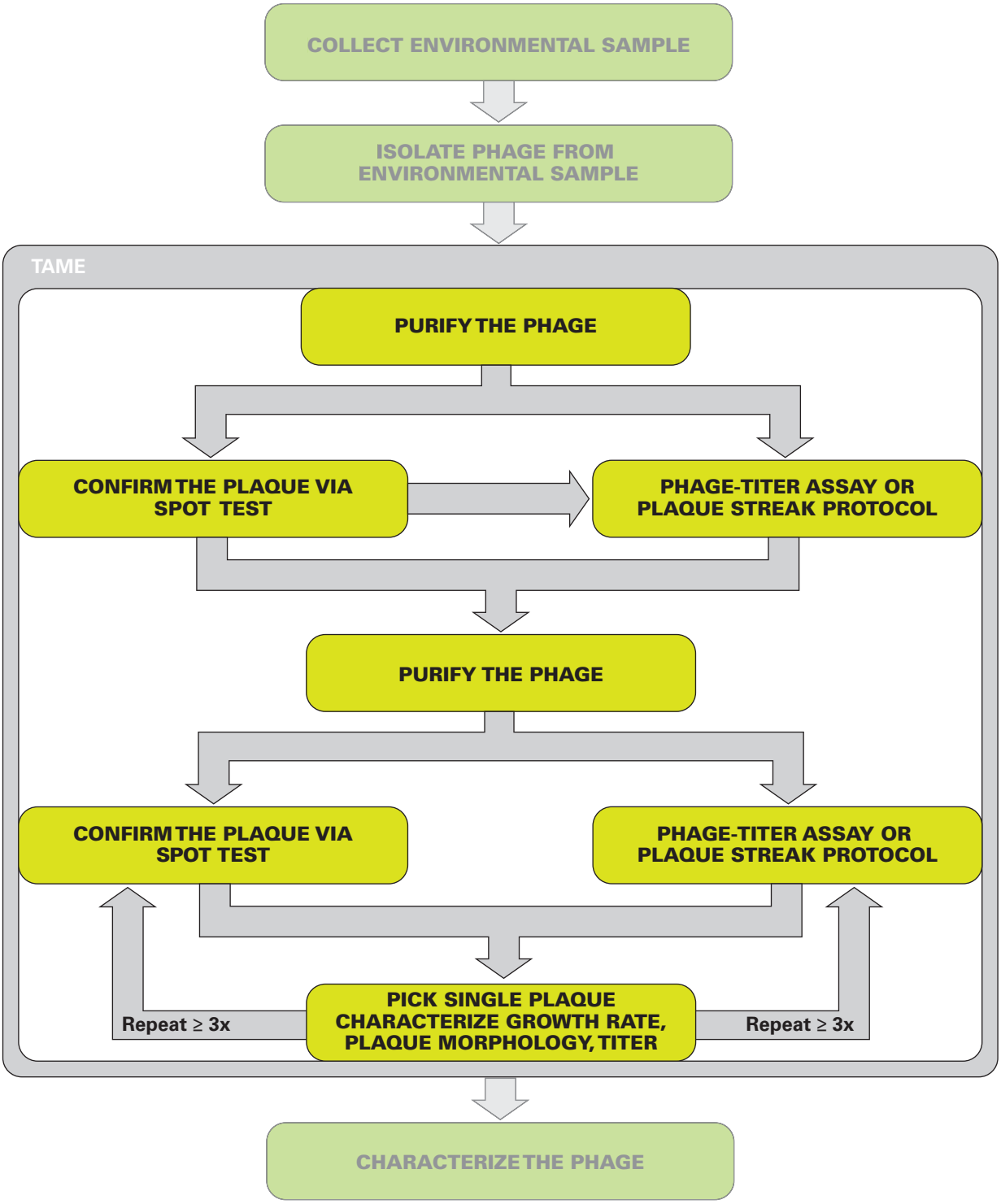

**Capture fig. 12**
Prophage and lytic pathways.

## The Big Picture

**Making good decisions about your approach to studying the phage you isolate depends on being familiar with the basics of phage biology. The investigative process that you are beginning is more than an exercise in laboratory steps and procedures. Though these steps are important, you are embarking on a collaboration with your instructor, your fellow students, and SEA participants across the country to further phage biology and genomics. When you consider the basic science behind your experiments, you will find that you are more productive in the laboratory and that you are in a position to contribute more to the process and to your team of scientists. You should, therefore, ask yourself "why?" as often as you ask "what next?"**

**If you have other questions or want to learn more about phage biology, see Appendix 2 in this manual for websites, references, and other materials.**

## You Are Here


COLLECT ENVIRONMENTAL SAMPLE
ISOLATE PHAGE FROM ENVIRONMENTAL SAMPLE
TAME
PURIFY THE PHAGE
CONFIRM THE PLAQUE VIA SPOT TEST
PHAGE-TITER ASSAY OR PLAQUE STREAK PROTOCOL
PURIFY THE PHAGE
CONFIRM THE PLAQUE VIA SPOT TEST
PHAGE-TITER ASSAY OR PLAQUE STREAK PROTOCOL
Repeat ≥ 3x
PICK SINGLE PLAQUE CHARACTERIZE GROWTH RATE, PLAQUE MORPHOLOGY, TITER
Repeat ≥ 3x
CHARACTERIZE THE PHAGE

# Tame

## Contents

# Part A. Purify the Phage: The Spot Test

## Overview

This is a relatively quick assay used to determine whether spots on a bacterial lawn are actually plaques. Because plaques can vary in size, morphology, and effects on the host bacterial cell (see pictures in Appendix I. Troubleshooting Guide), it can be easy to mistake an air bubble or other anomaly in the top agar for a plaque. If the plaque or plaques are suspect, a spot assay can be performed to determine whether or not phages are present. If the original sample is a true plaque, you will see plaques or clearing within the spot area(s).

## Objective

The objective of this procedure is to determine whether putative plaques contain phage(s).

## Supplies

- ☐ A laboratory notebook
- ☐ A labeling pen
- ☐ Sterile microcentrifuge tubes (2 to 10) and a tube rack
- ☐ A 5-mL sterile disposable pipette and pipettor
- ☐ 50 mL of phage buffer (PB)
- ☐ 0.5 mL of ***M. smegmatis*** culture (in a 10-mL sterile culture tube)
- ☐ 50 mL of top agar (TA) melted and stored at 55°C
- ☐ An agar plate
- ☐ A 100- or 200-$\mu$L and a 20-$\mu$L micropipettor and tips
- ☐ An automated pipettor for larger volumes
- ☐ A vortexer

## Equipment

- ☐ 37°C incubator
- ☐ A microwave oven
- ☐ 55°C water bath

## Procedure: Using a Pipette Tip to Pick a Single Putative Plaque (or Plaques) into 100 $\mu$L of Phage Buffer (PB)

### A. Prepare your work area.

- ☐ Remove clutter.
- ☐ Wipe the bench with disinfectant (allow to air-dry).
- ☐ Start your flame.
- ☐ Arrange your supplies.

### B. Label possible plaques; label and prepare tubes.

1. Aseptically aliquot 100 $\mu$L of PB into microcentrifuge tubes (one tube for each potential phage).
2. Label plaques on agar plate(s) by drawing a small circle (on the ***bottom*** of the Petri dish!) around the area with a labeling pen. Designate all plaques (numbers, letters, or other) with your labeling pen.

Read *all the way through* each procedure carefully before setting up your bench.

All tubes should remain closed except when transfers are being made!

**It is possible that you will obtain more than one plate with putative plaques. If this happens, be sure to label all your putative plaques with a plate designation that makes it easy for you to determine from which sample each plaque was obtained.**

3. Label each microcentrifuge tube with the same designation(s) as that of the plaque(s).

**C. List any putative plaques in your laboratory notebook and note the characteristics (morphology, size, "cloudy" versus "clear," etc.) of each.**

**D. "Pick" putative plaques.**

1. Place a sterile 100-μL tip onto a 100- or 200-μL micropipettor.
2. Open the lid of the Petri dish and keep it in your hand (do *not* invert—remember the sterile field!).
3. Touch the center of the putative plaque with the end of the tip.
4. Return the cover to the Petri dish.
5. Place the end of the tip into the PB in the corresponding microcentrifuge tube and gently tap the tip on the wall of the tube. Discard the tip.
6. Mix the tube well by vortexing.
7. Repeat steps 1 through 6 for each plaque.

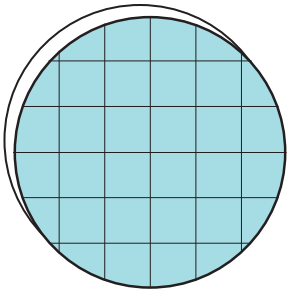
These samples should be stored at 4°C (refrigerated) until the presence of phage is verified.

## Procedure: Preparing a Bacterial Lawn in Top Agar

**A. On your bench, you should have**

- ☐ A 0.5-mL aliquot of *M. smegmatis* in a 10-mL culture tube
- ☐ An agar plate
- ☐ Top agar (TA) from the 55°C water bath[2]
- ☐ A 5-mL sterile pipette and pipettor

**B. Draw a grid onto the bottom of the agar plate (see *Tame* fig. 1).**



**Tame fig. 1**
A grid drawn on the bottom of a Petri dish.

**C. Label the grid with each phage designation, and save a section for the negative (buffer-only) control.**

### D. Prepare a TA-bacteria plate.

1. Using aseptic technique, aspirate (suck up) 4.5 mL of TA into a 5-mL sterile pipette.
2. Transfer the TA to 0.5 mL of bacteria and *immediately* draw the TA back into the same pipette.
3. Add the TA-bacteria mixture to the labeled agar plate.
4. Swirl gently to spread the mixture.
5. Allow to solidify *completely*. This will take at least 10 minutes.

## Procedure: Spotting Putative Plaques and Buffer Control onto a Labeled Plate

### A. On your bench, you should have

- ☐ The labeled TA-bacteria agar plate
- ☐ All possible plaque samples in 100 μL of PB in labeled microcentrifuge tubes
- ☐ A tube with 100 μL of PB only (the negative control)
- ☐ A 20-μL micropipettor
- ☐ A box of sterile 20-μL micropipettor tips

### B. Spot control and samples onto plate.

1. Set the micropipettor to 5 μL.
2. Aseptically transfer 5 μL of PB to the negative-control block on the grid.
3. Transfer 5 μL of all putative phage samples onto the corresponding blocks on the grid. Allow the droplets to soak into the agar for a few seconds, until no apparent liquid remains on the agar.

- **Do not touch the pipette tip to the agar! Hold the tip slightly above the agar, and push the droplet out slowly to avoid splattering.**
- **Avoid making bubbles because they can burst and scatter any phages that may be present across the plate.**
- **Labels on the bottom of the Petri dish are mirror images (i.e., they will appear backwards) of your labeling scheme once you turn the plate over. Be sure to spot your sample in the right place!**

4. Invert the plates and place into the 37°C incubator overnight.

## Procedure: Checking Spot Plate for Plaques

The next day, check spot plate for plaques. If you see clearing zones, or spots, in any of your grid areas, you have picked a genuine plaque![3]

**Before deciding whether you have any plaques, you must make certain your negative control has neither clear zones nor spots!**

The TA must be completely solidified before any sample is spotted onto the plate!

Do not discard the microcentrifuge tubes containing your samples. These should be stored at 4°C (refrigerated) after the spot test until the presence of phage is verified.

**Food for Thought**

Is there a correlation between negative or positive spot test results and the original notes you made on plaque morphology, size, etc.?

**A. By referring to the grid, identify the origin of the phage sample.**

Use the refrigerated sample for the phage-titer assay (see page *Tame* 9).

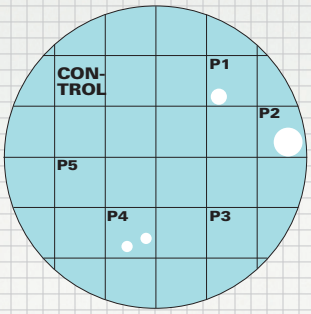
**B. In your laboratory notebook, for each putative plaque tested, be sure to record the following:**

- ☐ whether the spot test was positive or negative
- ☐ plaque size and morphology on the spot plate
- ☐ the number of plaques in the spot

## Questions

1 **What are the advantages and disadvantages of performing the spot test rather than the phage-titer assay (next section)?**

2. **Lee's spot test produced the results shown in *Tame* fig. 2. What conclusions can be drawn from these results?**




**Tame fig. 2**
Results of Lee's spot test.

3. **What are some possible explanations of different plaque morphologies from the *same* original sample? In other words, if you spotted a putative sample that originally appeared to be a clear plaque and, on the spot plate, observe cloudy plaques or a variety of plaque sizes, what could be the reason(s) for this?**

# Part A. Purify the Phage: Plaque Streak Protocol

## Objective

The objective of this procedure is to purify a single phage population from samples with potentially mixed phage populations.

## Supplies

- □ A laboratory notebook
- □ A labeling pen
- □ 5-mL sterile disposable pipettes (3 per phage tested)
- □ 0.5 mL of ***M. smegmatis*** culture in a 10-mL culture tube (3 per phage tested)
- □ Top agar (TA) melted and stored at 55°C (15 mL per phage tested)
- □ Agar plates (3 per phage tested)
- □ Sterile wooden sticks
- □ An automated pipettor for larger volumes

## Equipment

- □ 37°C incubator
- □ A microwave oven
- □ 55°C water bath

## Procedure

### A. Prepare your work area.

- ☐ Remove clutter.
- ☐ Wipe the bench with disinfectant (allow to air-dry).
- ☐ Start your flame.
- ☐ Arrange your supplies.

### B. Label plaques and prepare plates.

1. Label the plaque you intend to pick from a plate containing plaques (or putative plaques) by drawing a small circle around the area with a labeling pen. Designate all picked plaques with your labeling pen.
2. Label each plate with the plaque designation, including a plate for your negative control.

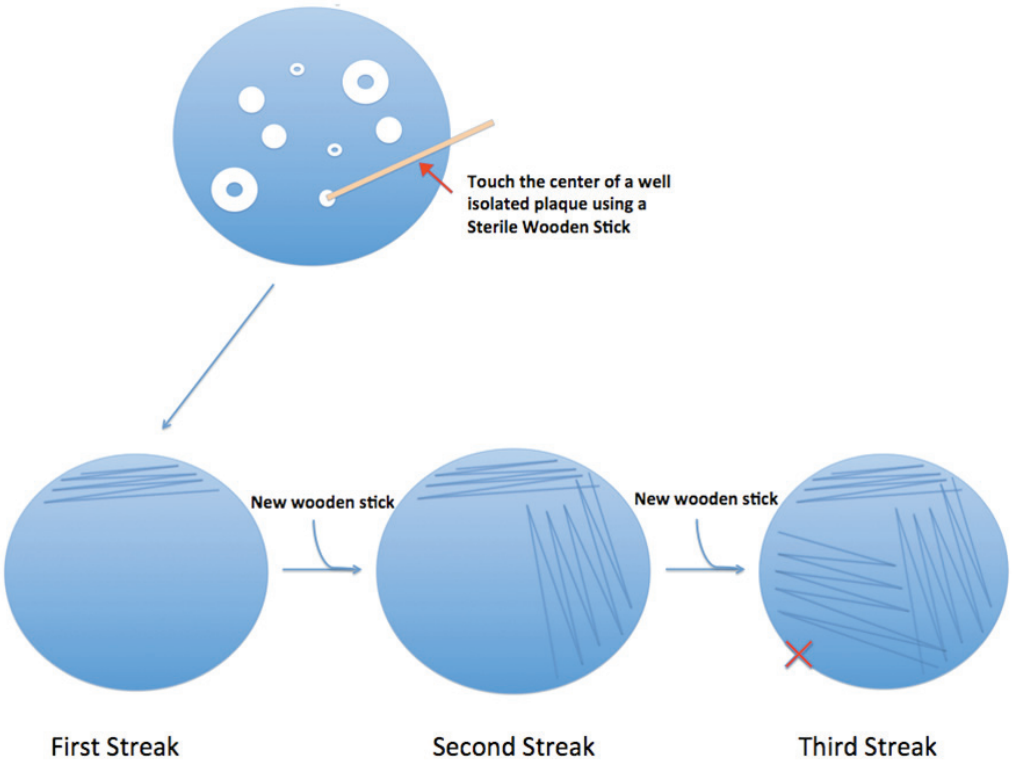
### C. For each plaque being purified, note the morphology and size in your laboratory notebook.

### D. Prepare your negative control plate.

1. Using aseptic technique, remove a sterile wooden stick from its packaging.
2. Open the Petri dish labeled "Negative Control".
3. Gently streak back and forth across the top third of the agar plate (see *Tame* fig. 3), without lifting the stick from the agar.

4. Return the cover to the Petri dish and discard the wooden stick.
5. Carefully remove a new wooden stick.
6. Open the lid of the Petri dish. Beginning in the area streaked in the previous step, streak the adjacent un-streaked portion (~1/3) of the agar making sure to overlap only on the first few strokes.
7. Repeat steps 4-6 for the remaining un-streaked portion of the plate.

**Tame fig. 3**
Streaking for isolation of a phage from a mixed population. 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 on a fresh agar plate. A new stick is then used to streak an adjacent un-streaked area (1/3) of the plate on 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."




It is a good idea to streak the phage plaques in duplicate until you have mastered the technique. This will help to ensure that you get an isolated single plaque every time.

## E. "Pick" plaques and streak on a fresh agar plate.

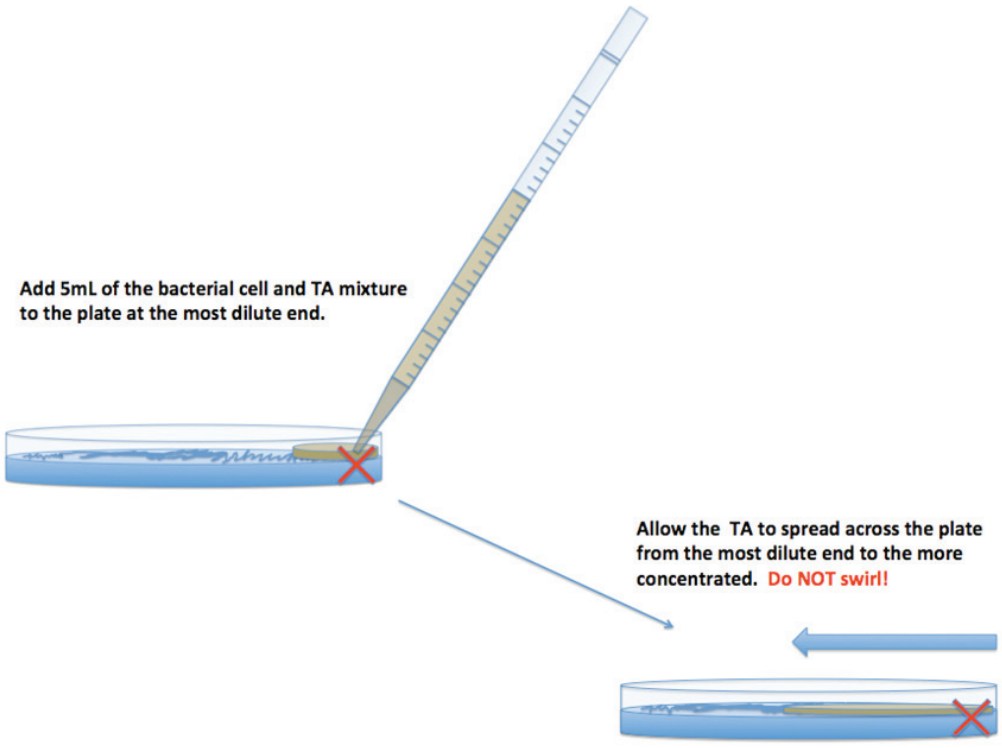
1. Using aseptic technique, remove a sterile wooden stick from its packaging.
2. Open the Petri dish with the (putative) plaque and keep it in your hand (do not invert-remember the sterile field!).
3. Touch the center of a plaque with the end of the stick.
4. Return the cover to the Petri dish.
5. Open the lid of the Petri dish (to be streaked) with the corresponding label.
6. Perform steps 3-7 in section D above.
7. Indicate the most dilute area of the plate with a marker.

## F. Add top agar and bacteria.

1. Add 4.5 mL of TA to 0.5mL aliquot of *M. smegmatis.*
2. Immediately, bring the mixture back up into the serological pipet.
3. Carefully, dispense the TA/bacteria mixture slowly onto the most dilute (marked) area of the streaked plate (see *Tame* fig. 4).

**Do not swirl the plate.**

4. Allow the mixture to spread across the plate from the most dilute point to the more concentrated areas by gently tilting or tapping the plate.
5. Allow the TA to harden.




**Tame fig. 4**
Plating bacterial cells and top agar on a plate streaked with phage. The mixture of cells and top agar is added to the plate at the most dilute point and allowed to spread to the more concentrated regions of the plate without swirling.

## G. Incubate plates at 37°C.

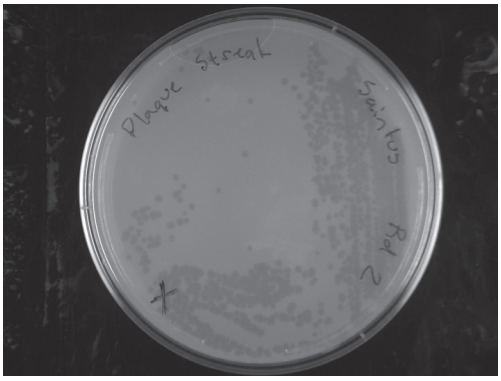
1. After the TA has solidified, invert the plates rapidly (but gently).
2. Put all inverted plates in a 37°C incubator.

## H. Check plates for plaques.

1. Check plates for plaques after 24 hours (see *Tame* fig. 5).
2. Be sure to ascertain that there are no plaques on the negative-control plate.
3. If you do not have plaques, allow the plate(s) to incubate an additional 24-48 hours.
4. Repeat this procedure *at least* two times (more if necessary)until you are relatively certain you have a pure phage population.

**Food for Thought**

Why is "three" the minimum number of times you would streak out individual plaques for isolation? Why would you do additional rounds of purification?




**Tame fig. 5**
Results of a plate streaked with phage and topped with bacteria/top agar after incubation. The most concentrated area of the plate can be completely cleared by the phage while the more dilute parts of the plate yield individual plaques. The "X" indicates where the TA/bacteria was added to the plate.

## Notes

Some samples may include multiple phages that are difficult to isolate and purify. It is extremely important that the final plaque purification contain only a single phage. Continue to pick *well isolated* plaques as you purify your phage. For phages that are particularly difficult to isolate, The Phage-Titer Assay (*Tame* page 9) can also be used.

# Part A. Purify the Phage: The Phage-Titer Assay

## Overview

This assay is the basis for determining the concentration of phage particles in a given solution and conducting successive rounds of purification of an individual phage. This assay is performed once you are certain you are observing an actual plaque on the bacterial lawn. After a series of dilutions and platings, you will count the number of plaques on each plate and calculate the number of plaque-forming units per milliliter of original phage sample. This is called the titer. Once a titer has been established, and you are sure that you have a pure phage population, you can move on to "Final Plaque Purification" (*Tame*, page 15).

## Objective

The objective of this procedure is to determine the concentration of plaque-forming units (pfu) in a given solution and to become comfortable with titer calculations.

## Supplies

- □ A laboratory notebook
- □ A labeling pen
- □ Sterile microcentrifuge tubes (5 per phage tested) and tube rack
- □ 5-mL sterile disposable pipettes (5 per phage tested)
- □ 50 mL of phage buffer (PB)
- □ 0.5 mL of *M. smegmatis* culture in a 10-mL culture tube (5 per phage tested)
- □ 50 mL of top agar (TA) melted and stored at 55°C
- □ Agar plates (5 per phage tested)
- □ A 100- or 200-μL and a 20-μL micropipettor and tips
- □ An automated pipettor for larger volumes
- □ A vortexer

## Equipment

- □ 37°C incubator
- □ A microwave oven
- □ 55°C water bath

## Procedure

### A. Prepare your work area.

- ☐ Remove clutter.
- ☐ Wipe the bench with disinfectant (allow to air-dry).
- ☐ Start your flame.
- ☐ Arrange your supplies.

### B. Label plaques; label and prepare tubes.

1. Aseptically aliquot 100 μL of phage buffer (PB) into microcentrifuge tubes (one per plaque to be tested).

2. Label plaques on agar plate(s) by drawing a small circle (on the *bottom* of the Petri dish!) around the area with a labeling pen. Designate all plaques (numbers, letters, or other) with your labeling pen.
3. Label each microcentrifuge tube with the plaque's designation.

**C. For any plaques, note the morphology, size, and whether "clear" or "cloudy" in your laboratory notebook.**

**D. "Pick" plaques.**

1. Place a sterile tip onto a micropipettor.
2. Open the lid of the Petri dish and keep it in your hand (do *not* invert—remember the sterile field!).
3. Touch the center of the putative plaque with the end of the tip.
4. Return the cover to the Petri dish.
5. Place the end of the tip into the PB in the corresponding microcentrifuge tube and gently tap the tip on the wall of the tube.
6. Discard the tip.
7. Mix the tube well by vortexing. ***This is your "neat," or undiluted, phage sample, also referred to as the $10^0$ plaque sample.***
8. Add "0" or "$10^0$" to the label of this tube.
9. Repeat steps 1 through 8 for each plaque.

Avoid transfer errors. Use two racks and move tubes to a different rack after you add the sample.

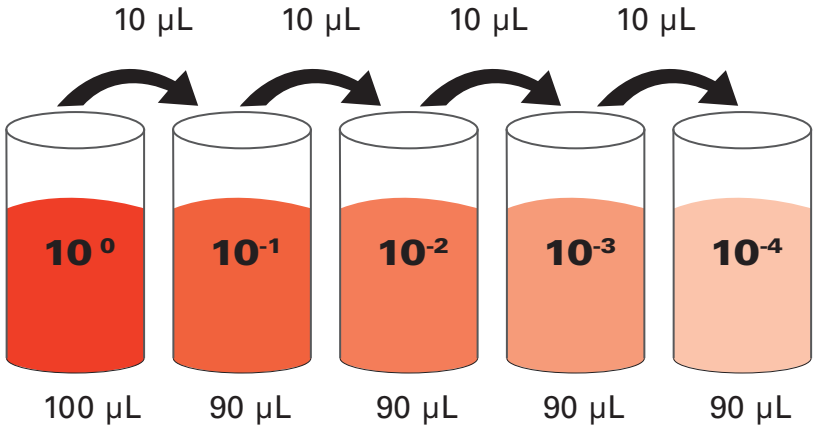
**E. Perform serial 10-fold dilutions.**

For each putative phage, using aseptic technique in every step:

1. Arrange four microcentrifuge tubes in a tube rack and label them –1, –2, –3, and –4.
2. Add 90 $\mu$L of PB to each tube.
3. Add 10 $\mu$L of your $10^0$ (undiluted) phage sample to the "–1" tube and vortex well. (See *Tame* fig. 6.)

**To prevent the sample from spraying out of the tube, gently tap the capped tube on the counter. This forces the fluid to the bottom of the tube, rather than onto the sides or top.**

4. The "–1" tube is your $10^{-1}$ (or 1:10) dilution.
5. Add 10 $\mu$L of the "–1" tube to the "–2" tube and vortex well.
6. Continue each successive dilution until you get to the "–4" tube.




**Tame fig. 6**
The serial dilution of phage preparations for a titer. A volume of 10 µL of the undiluted phage sample ($10^0$) in 100 µL of phage buffer is transferred into 90 µL of PB 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.

## F. Infect *M. smegmatis* cultures with diluted phage solutions.

For each putative phage, using aseptic technique in every step:

1. Label culture tubes (containing 0.5 mL of ***M. smegmatis***) with the phage designation (e.g., "A1") and each dilution ($10^{-1}$). For a set of serial dilutions from $10^{-1}$ to $10^{-4}$ plus a negative control, you will need five tubes.
2. Infect 0.5 mL of ***M. smegmatis*** in the appropriately labeled culture tubes with 10 $\mu$L of the $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions.
3. Add 10 $\mu$L of PB to the negative-control tube.
4. Allow the phage to infect the bacteria for 15 to 30 minutes. Be sure to ***record*** the length of time allowed for infection in your laboratory notebook.

**Food for Thought**

Why is the undiluted sample designated $10^0$?

## G. Add 4.5 mL of top agar (TA) and plate each sample (including the negative control).

1. Label each of the five agar plates (at room temperature) with sample or control information ***plus*** the date and your initials.
2. Remove a 50-mL bottle of top agar (TA) from the 55°C water bath.
3. For each sample, including the negative-control tube:
   a. Use a sterile 5-mL pipette attached to a pipettor to aseptically transfer 4.5 mL of TA to the culture tube (containing the bacteria).
   b. ***Immediately*** pull the mixture back up into the pipette. Try to avoid bubbles.
   c. The TA should not sit in the pipette for more than a few moments, because the agar will begin to solidify.
   d. Transfer the entire mixture to the appropriately labeled plate and discard the pipette.

The "neat," or undiluted, phage sample ($10^0$) and the dilutions should be stored at 4°C (refrigerated) until the phage titer is calculated. All tubes must be well labeled and easily identified.

e. Swirl the plate gently to spread the mixture evenly on top of the agar plate.

f. Let the plates sit, undisturbed, for at least 20 minutes.

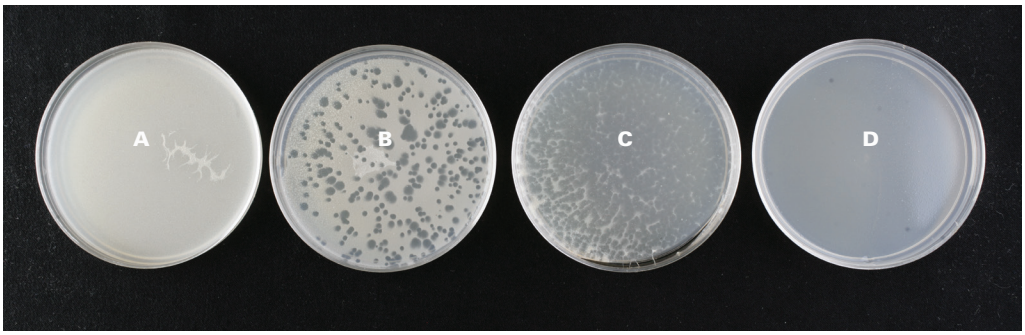
**Use a fresh, sterile pipette for each sample!**

### H. Incubate plates at 37°C.

1. After the TA has solidified, invert the plates rapidly (but gently).
2. Put all inverted plates in a 37°C incubator.

### I. Check plates for plaques.

1. Check for plaques after 24 hours (see *Tame* fig. 7).
2. Be sure to ascertain that there are no plaques on the negative-control plate.




**Tame fig. 7**
Representative appearances of plates from serial dilutions of a phage lysate. **A.** No lysis. **B.** Discrete plaques, pfu can be counted. **C.** A "web pattern" plate. Notice the wispy *M. smegmatis* growth on this plate, in which most of the *M. smegmatis* has been lysed. **D.** Complete lysis. With total bacterial lysis, it is impossible to determine the point (number of pfu) at which this occurred.

3. If you have plaques, proceed to step J. If there are no plaques on your sample plates, or if plaques are small or difficult to see, check them again at 48 hours. If you have plaques at 48 hours, proceed to step J.

### J. For each plaque tested, count the number of plaques on the plate and calculate the titer.

1. Count the number of plaques on a plate with a range of 20 to 200 plaques.
2. Record in your laboratory notebook the name of the putative phage, the dilution plate, the volume tested (in this case, 10 $\mu$L), and the number of plaques on the plate. A suggested template is shown in *Tame* table 1.

| Date: | | | |
|---|---|---|---|
| **Phage designation** | **Number of pfu** | **Dilution** | **Sample volume (µL)** |
| A1 | 78 | –3 | 10 |
| A2 | 62 | –3 | 10 |
| B4 | 106 | –2 | 10 |
| E7 | 93 | –3 | 10 |

**Tame table 1**
Recording titer data for calculating pfu/mL.

3. Calculate your pfu/mL (plaque-forming units per milliliter) according to the formula:

   Titer (pfu/mL) = (pfu/#µL) × (1000 µL/mL) × dilution factor*

   **For a $10^{-3}$ dilution, the dilution is 1000× (1000-fold); the dilution factor is 1000 (or $10^3$).*

   For example: Using Phage A1 in *Tame* table 1,

   pfu/mL = 78 pfu/10 µL × 1000 µL/mL × 1000

   = (7.8 × 1000 × 1000) pfu/mL

   = 7.8 × 1,000,000 pfu/mL

   = 7.8 × $10^6$ pfu/mL

   Do this calculation for each sample listed in *Tame* table 1.

## Notes

Some plates may include multiple phages that are difficult to isolate and purify. It is extremely important that the final plaque purification contain only a single phage. Continue to pick isolated plaques, make dilutions, infect *M. smegmatis*, plate samples, and incubate overnight until plaque morphologies and other characteristics remain consistent. ***Three to six* repeats of this protocol may be required to isolate an individual phage.**

Once you are sure that your phage is pure, you should name it using these phage naming guidelines:

- The name should be 3 to 10 characters long.
- It must contain only letters and numbers (A–Z, a–z, 0–9); other spaces and symbols ($, #, @, !, etc.) are not permitted.
- The first character of the name must be a letter and not a number.
- Short, nonsensical names are discouraged (e.g., "r7B").
- The name should be polite and not offensive in any way.

After naming your phage, proceed to the next section, "Final Plaque Purification."

## The Big Picture

In laboratories where serial-dilution assays are routinely performed, scientists count plates with 20 to 200 pfu (plaque-forming units), as shown in *Tame* fig. 4, plate B. Counting more than 200 plaques takes a long time, and counting fewer than 20 plaques is less accurate (and less replicable) than counting higher numbers. This is because the counting error is proportional to the square root of the number counted, so the greater the number that you count, the smaller the error. For example, the uncertainty in a count of 16 is ±4 plaques with an error of ±25%. The uncertainty of 169 plaques is ±13, or ±7.7%.

## Questions

1. Why do you think that the plaques are called "plaque-forming units," or "pfu," rather than "phage particles"?

2. What are other applications of serial dilution outside the phage world?

# Part A. Purify the Phage: Final Plaque Purification

## Overview

Once you have gone through at least three iterations of the plaque assay and are reasonably sure that each plaque on a given lawn is representative of only one phage, you are ready to accomplish two things. The first is to use PB to flood a lawn containing phage-infected bacteria on TA and then use the resulting solution to purify higher numbers of filter-sterilized phage. The second is to titer that phage-containing solution for use in determining the approximate range needed for an empirical determination of the number of pfu required for lysis of a bacterial lawn on a standard agar plate.

## Objective

The objective of this procedure is to isolate enough pure phage stock that phage titers can be empirically tested as outlined in ***Tame*** B. Empirical Testing of Phage Lysates. This stock lysate, sometimes called the "MTL" for Medium-Titer-Lysate , will be used to make a large batch of pure phage (called the "HTL" for High Titer Lysate"). The HTL will then be used for the remainder of your experiments.

## Supplies

- □ A laboratory notebook
- □ A labeling pen
- □ Sterile microcentrifuge tubes (11) and a tube rack
- □ 5-mL sterile disposable pipettes (1 to 10)
- □ 5-mL syringe
- □ 0.22-$\mu$m syringe filter
- □ A sterile 15-mL tube
- □ A ruler
- □ 10 mL of phage buffer (PB)
- □ 0.5 mL of ***M. smegmatis*** culture in a 10-mL culture tube (1 to 10)
- □ 5 to 50 mL of top agar (TA) melted and stored at 55°C
- □ Agar plates (1 to 10)
- □ A 200-$\mu$L and a 20-$\mu$L micropipettor and tips
- □ An automated pipettor for larger volumes
- □ A vortexer

## Equipment

- □ 37°C incubator
- □ A microwave oven
- □ 55°C water bath

Remember the sterile field, and remember to keep the microcentrifuge tube capped unless transferring sample! The filtrate and tube are sterile and should be manipulated using aseptic technique.

This harvested lysate is sometimes called the "MTL" for Medium-Titer-Lysate.

## Procedure: Harvesting a Plate Lysate

### A. Prepare your work area.

### B. Choose a plate to harvest and add PB.

1. Choose the plate from your most recent titer in which you are sure you have a single phage population and in which the bacterial lawn is nearly cleared (see *Tame* fig. 7, plate C).
2. Add 8 mL of PB and swirl gently.
3. Let the plate sit for 2 to 4 hours at room temperature. Alternatively, the plate can be stored overnight at 4°C.

### C. Filter-sterilize the plate lysate.

1. Invert the flooded plate (lid side down) and allow the lysate to pool in the lid.
2. Label the sterile microcentrifuge tubes you will be using.
3. Retrieve a 0.22-$\mu$m filter.
4. Open the packaging but do *not* remove the filter from the packaging.
5. Using a tuberculin (5-mL) syringe, aspirate the PB from the lid.
6. Place the syringe into the top of the filter and remove the filter from the package.
7. Pushing the plunger, dispense the liquid through the filter into the appropriately labeled sterile 15-mL tube.
8. Immediately cap the tube.
9. Discard the syringe and the filter.

## Procedure: Preparing a Bacterial Lawn in TA

### A. On your bench, you should have

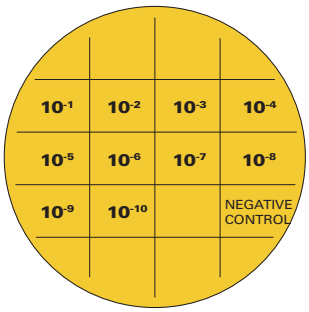
- ☐ A 0.5-mL aliquot of *M. smegmatis* in a 10-mL culture tube
- ☐ An agar plate
- ☐ Top agar (TA) from the 55°C water bath
- ☐ A 5.0-mL pipette and pipettor

### B. Draw a grid onto the bottom of the agar plate.

Label the grid with designations for phage dilutions from $10^{-1}$ to $10^{-10}$ and a negative (buffer-only) control (see *Tame* fig. 8).

### C. Prepare the TA-bacteria plate.

1. Using aseptic technique, aspirate 4.5 mL of TA into a 5-mL sterile pipette.
2. Transfer the TA to 0.5 mL of bacteria.




**Tame fig. 8**
Plate labeled for a high-dilution spot test.

3. *Immediately* draw the TA back into the same pipette.
4. Add the TA-bacteria mixture to the labeled agar plate.
5. Swirl gently to spread the mixture.
6. Allow to solidify *completely*. This will take at least 10 minutes.

## Procedure: Performing a Spot Test on the Phage Lysate

### A. Perform 10-fold serial dilutions from $10^0$ to $10^{-10}$.

1. Arrange 10 microcentrifuge tubes in a tube rack and label them −1 through −10.
2. Add 90 μL of PB to each tube.
3. Add 10 μL of your $10^0$ (undiluted) phage sample to the "−1" tube and vortex well.
4. The "−1" tube is your $10^{-1}$ (or 1:10) dilution.
5. Using a fresh sterile tip, add 10 μL of the "−1" tube to the "−2" tube and vortex well.
6. Using another sterile tip, add 10 μL of the "−2" tube to the "−3" tube and vortex well.
7. Continue to do successive dilutions, each with a fresh sterile pipette tip, until you get to the "−10" tube.

**This is where you have a big decision to make. If you are unsure of any aspect of phage purification or plaque morphology, you may want to perform a plate titer (as in the previous section) on all of your dilutions from $10^{-1}$ to $10^{-10}$.**

### B. Spot control and samples onto plate.

1. Set a micropipettor to 5 μL.
2. Aseptically transfer 5 μL of PB to the negative-control block on the grid.
3. Transfer 5 μL of all phage dilution samples onto the corresponding blocks on the grid.

4. Allow the droplets to soak into the agar until no apparent liquid remains on the agar.
5. Invert the plates and place into the 37°C incubator.
6. Incubate overnight.

### C. Count the number of plaques and calculate the titer.

1. After the overnight incubation, count the number of plaques on each plate.
   a. For the 5-$\mu$L spot test, find the sector on the grid that has 5 to 50 plaques and count those plaques.
   b. For the phage-titer assay (one plate per 10 $\mu$L of each dilution), find the dilution that yields 20 to 200 plaques per plate and count those plaques.
2. Record in your laboratory notebook the count, original volume, and dilution factor.
3. Calculate your pfu/mL (plaque-forming units per milliliter) according to the following formula and example.

   Titer (pfu/mL) = (pfu/#$\mu$L) × (1000 $\mu$L/mL) × dilution factor*

   ***For a $10^{-3}$ dilution, the dilution is 1000× (1000-fold); the dilution factor is 1000 (or $10^3$).***

   For example: 16 plaques were identified on a plate that received a 5-$\mu$L spot of a $10^{-4}$ dilution.

   pfu/mL = 16 pfu/5 $\mu$L × 1000 $\mu$L/mL × $10^4$

   = ($3.2 \times 10^3 \times 10^4$) pfu/mL

   = $3.2 \times 10^7$ pfu/mL
4. Record in your laboratory notebook the following:
   a. the titer of your phage lysate,
   b. the appearance of your plaques (you can also insert a picture), and
   c. the approximate size of your plaques (diameter in millimeters). This will help you estimate the number of individual pfu that you will need for complete lysis of bacteria on an agar plate.

When exponential numbers are multiplied, the exponents are added together. For example, $10^5 \times 10^4 = 10^9$.

## Notes

Now that you have a pure phage at relatively high titer, you will be able to use this lysate for the empirical assays in the next section. You will infect larger numbers of bacteria to obtain a pure and highly concentrated solution of phage particles. This high-titer phage stock will be used for all subsequent procedures, including DNA isolation and electron microscopy (See Decision Tree #2, page *Tame* 19).

## Decision Tree #2

From Pure Phage to High-Titer Phage Stocks

PERFORM EMPIRICAL TEST WITH PHAGE LYSATE

Is there a distinct web pattern?

YES

NO

RECALCULATE DILUTIONS

PERFORM 10-PLATE INFECTION

Is there a distinct web pattern on all the plates?

YES

NO

- FLOOD PLATES WITH PB
- HARVEST PHAGE
- TITER

Do you have at least 10 mL of lysate with a titer of $10^7$ pfu/mL?

YES

NO

PROCEED TO DNA-ISOLATION STEP

## Questions

1. What are some of the properties of individual phages that would lead to higher or lower titers in a given phage preparation?

2. Think of an experiment to perform if you continue to obtain more than one morphology in your phage preparations (e.g., some plagues are large and clear, and some are much smaller and cloudy). How would you determine that an individual phage yielded two different morphologies? What would be a possible mechanism by which one phage could yield two different morphologies?

3. In one to two sentences, describe how each of the following might look:
   a. Doing a titer procedure and forgetting to add the bacteria
   b. Contaminated bacteria
   c. PB contaminated with phage
   d. PB contaminated with bacteria or fungus
   e. Doing a spot test and forgetting to add the top agar
   f. Using top agar that is not completely melted

# Part B. Make Phage Stocks: Empirical Testing of Phage Lysates

## Overview

You will use your phage-lysate titer numbers and the size of your phage to logically determine a range of dilutions to test for almost complete lysis of a bacterial lawn grown in TA on 10 agar plates.

## Objective

The objective of this procedure is to determine the dilutions and volumes required to form "web" patterns of lysis on bacterial lawns grown in top agar (TA). This will lead to a 10-plate infection for the purpose of large-scale phage purification.

## Supplies

- ☐ A laboratory notebook
- ☐ A labeling pen
- ☐ Sterile microcentrifuge tubes (6 to 10) and tube rack
- ☐ 10-mL sterile disposable pipettes (6)
- ☐ A ruler
- ☐ 10 mL of phage buffer (PB)
- ☐ 1.0 mL of *M. smegmatis* culture in a 10-mL culture tube (6)
- ☐ At least 10 mL of the same culture held in reserve
- ☐ 30 mL of top agar (TA) melted and stored at 55°C
- ☐ Agar plates (6)
- ☐ A 200-μL and a 20-μL micropipettor and tips
- ☐ An automated pipettor for larger volumes
- ☐ A vortexer

## Equipment

- ☐ 37°C incubator
- ☐ A microwave oven
- ☐ 55°C water bath

## Procedure: Preparing Your Dilutions

### A. Calculate the approximate number of pfu that will be required for complete bacterial lysis on an agar plate.

There are a couple of ways to do this:

1. On the basis of plaque size:
   a. Use a ruler to estimate the diameter (mm) of both the average plaque on your plate and the bottom of your plate.
   b. Calculate the **radii** of the plaque and of the plate using this relationship: **radius (mm) = 0.5 × diameter.**
   c. Calculate the **areas** of the plaque and the plate using this: Area ($mm^2$) = $\pi r^2$, where $r$ = radius.

d. Estimate the approximate number of pfu needed to cover the agar plate:

$$\text{pfu}_{\text{max web}} = \frac{\text{Area}_{\text{agar plate}}\ (\text{mm}^2)}{\text{Area}_{\text{plaque}}\ (\text{mm}^2)}$$

2. On the basis of lysis of a previous titer plate: If you performed the ***plate titer*** on your phage lysate, you may have seen a plate in which the bacteria were completely, or almost completely, lysed (see plate C in ***Tame*** fig. 7). If this is the case, you'll need to add the same number of pfu to get a similar pattern of bacterial lysis on multiple plates.

**B. Calculate the dilutions you will need to make for an infection that will yield an optimal webbing pattern on an agar plate.**

For example:

1. Your lysate titer is $5 \times 10^6$ pfu/mL and you estimate that you will need 3000 pfu for full lysis of your plate.
2. Use this formula: $\text{Volume (mL)}_{\text{phage stock}} = (\text{pfu}_{\text{desired}})/(\text{pfu/mL}_{\text{phage stock}})$

For this example, (3000 pfu/5,000,000 pfu/mL) = 0.0006 mL, or 0.6 $\mu$L.

3. You therefore would perform serial dilutions (using PB) to $10^{-2}$. This solution now has $5 \times 10^4$ (50,000) pfu/mL.
4. You would then recalculate the volume needed as:

(3000/50,000 mL) = 0.06 mL (60 $\mu$L)

5. To attempt complete lysis of bacteria on an agar plate, you would, therefore, add 60 $\mu$L of a $10^{-2}$ dilution.

These calculations are estimates. Volumes, the age of bacterial cultures, changes in growth conditions, and a variety of other factors will affect the lysis patterns of a given phage. Make a guesstimate about the best concentration to use. Test a range of plaque-forming units to determine the best way to obtain the desired bacterial lysis pattern.

**C. Determine the range of pfu you would like to test in the empirical assay.**

Continuing with the example above, you would test either

- ☐ two 2-fold dilutions above and below your calculated pfu (750, 1500, 3000, 6000, and 12,000 pfu) or
- ☐ two 5-fold dilutions above and below your calculated pfu (120, 600, 3000, 15,000, and 75,000 pfu).

**D. Determine the concentrations and volumes needed for each of the desired lysate additions.**

You can do these calculations using one of the formulas above.

## Procedure: Performing the Empirical Assay

**A. Prepare your work area.**

Remove clutter and wipe down your workbench. You should have

- ☐ Sterile 10-mL culture tubes containing 0.5 mL of *M. smegmatis* (6)
- ☐ Agar plates (6), labeled and prewarmed to 37°C

- ☐ Sterile microcentrifuge tubes (for dilutions)
- ☐ PB
- ☐ A 20-$\mu$L micropipettor
- ☐ A 200-$\mu$L micropipettor
- ☐ Sterile micropipettor tips
- ☐ 5-mL serological pipettes (6)
- ☐ 30 mL of 55°C TA (remove from 55°C water bath right before use)

### B. Perform serial 10-fold (or other) dilutions as determined by your calculations.

1. Arrange microcentrifuge tubes in a tube rack and label them with the appropriate dilution designations (e.g., –1 through –7).
2. Perform serial dilutions using a fresh sterile tip for each volume transfer.
3. Carefully outline in your laboratory notebook the way you performed the dilutions and the volume you will use for each infection.

### C. Infect *M. smegmatis* cultures with diluted phage solutions.

1. Label the six culture tubes (each containing 0.5 mL of *M. smegmatis*) with designations corresponding to your dilution tubes.
2. Label one tube as the negative control. This tube will contain a lawn of bacteria with PB only (no phage). It will be a control for *no* lysis. It should also contain 0.5 mL of *M. smegmatis* culture.
3. Infect each sample tube with the dilution and volume specified by your calculations.
4. Allow the phage to infect the bacteria for 20 to 30 minutes. Be sure to record the length of time allowed for infection in your laboratory notebook. Take careful note of all of the conditions of the infection (time allowed to infect, the culture of *M. smegmatis* used, volumes and dilutions used, etc.) so that you can replicate these parameters when you do the 10-plate infection.

### D. Add 4.5 mL of top agar (TA) and plate each sample (including the negative control).

1. Label each of the six agar plates (prewarmed to 37°C). Return these plates to the 37°C incubator. Remove the plates immediately before you add the top agar to the samples.
2. Remove a 30-mL bottle of TA from the 55°C water bath.
3. For each sample, including the negative-control tube, add the top agar and plate as before.
4. Let the plates sit, undisturbed, for at least 30 to 40 minutes or until the top agar has solidified.

### E. Incubate the plates at 37°C.

## Procedure: Analyzing the Data

The next day, determine which volume-dilution combination yielded the best web pattern of phage-infected *M. smegmatis* growth (see *Tame* fig. 7).

The combination of lysate volume and lysate dilution that yields the best web pattern along with a minimal presence of *M. smegmatis* will be used for the subsequent 10-plate infection.

If you do not have a clear web pattern, repeat the empirical test using different lysate volume and dilution combinations. Increase or decrease your phage concentration depending on your results.

## Notes

Before starting the 10-plate infection, you must be confident with the volume-dilution combination of lysate that yields a web pattern on an agar plate. To standardize the conditions, *the same subculture of* M. smegmatis *must be used throughout the empirical test and 10-plate infection*.

The 10-plate infection (see next section) should also be done very soon (within 2 to 3 days) because the age of the bacterial culture can also affect infection efficiency and plaque size.

## Questions

1. **What are the reasons for performing the empirical test?**
2. **Why not calculate the area of the plaque and infect the 10 plates immediately?**
3. **In addition to human error, list as many different parameters that can vary from one infection to the next that may influence a typical titer procedure.**
4. **Why do you think that a "web pattern" is better than *complete* lysis for isolating high numbers of phage particles?**

# Part B. Make Phage Stocks: The 10-Plate Phage Infection and Harvest

## Overview

You will use your phage-lysate dilution and volume that yielded the best "web pattern" to perform a 10-fold (volume) infection to spread on 10 agar plates. The purpose of this infection and harvest is to obtain large absolute numbers of phage ($>10^{10}$) for later DNA-isolation protocols. If the 10 plates have good web patterns, they will be flooded with phage buffer and allowed to sit for several hours (or overnight) so that phage can diffuse into the PB. The PB-containing phage will be aspirated from the plates, spun down to pellet cell debris (the phages remain in solution), and filter-sterilized. A phage titer will be performed, and the harvest will be considered successful if there are $>10^9$ pfu/mL.

The information recorded for a successful "web formation" in the previous empirical test should be followed ***to the letter*** in the 10-plate infection protocol. Further, the ***same bacterial culture*** should be used, and this protocol should be performed within 2 to 3 days of the empirical test.

Web formation is important for maximum yield. At the same time that the phage are infecting bacterial cells, other (uninfected) bacteria are replicating. A web pattern ensures that there are enough phage to almost completely lyse a growing bacterial lawn. Complete lysis indicates only that enough phage were present to infect and lyse all the bacteria on the plate. If fewer phage are present, each generation of phage released by lysis can go on to infect new bacterial progeny. A good web pattern increases the yield of phage significantly.

## Objective

The objective of this procedure is to obtain a high-titer phage lysate with high enough phage concentrations that students can progress to the DNA-isolation protocol.

## Supplies

- □ A laboratory notebook
- □ A labeling pen
- □ Sterile microcentrifuge tubes (11)
- □ 5-mL sterile disposable pipettes (11)
- □ 25-mL sterile disposable pipettes (16)
- □ Sterile 50-mL conical tubes (2)
- □ Filter-sterilization unit, 0.22-$\mu$m pore size and 100-mL volume
- □ 12.0 mL of ***M. smegmatis*** culture (the ***same*** as used in the previous empirical tests)
- □ 60-mL bottles of top agar (TA) melted and stored at 55°C (2)
- □ Agar plates (11)
- □ 100 mL of phage buffer (PB)
- □ A 200-$\mu$L and a 20-$\mu$L micropipettor and tips
- □ An automated pipettor for large volumes
- □ A vortexer

## Equipment

- ☐ 37°C incubator
- ☐ A microwave oven
- ☐ 55°C water bath
- ☐ High-speed (e.g., Sorvall) centrifuge

## Procedure: Setting Up Your Work Area

### A. Prepare your workbench.

Remove clutter and wipe down your workbench. You should have

- ☐ A flask containing 5.0 mL of *M. smegmatis*
- ☐ A culture tube containing 0.5 mL of the same *M. smegmatis* culture
- ☐ A micropipettor and sterile tips (to add your phage)
- ☐ 25-mL serological pipettes (2) and an automated pipettor
- ☐ 60 mL of 55°C TA (remove from 55°C water bath right before use)
- ☐ Agar plates (11), prewarmed to 37°C and labeled (remove right before use)

### B. Using the volume-dilution combination that yielded the best web pattern in the empirical test, calculate the volume and dilution of lysate necessary for a 10-plate infection.

Multiply the volume of phage used to create a web on one plate by 10 to get the volume needed for all 10 plates. For example, if 60 $\mu$L of a $10^{-5}$ dilution of lysate formed an acceptable web pattern on the test plate, then you will need 60 × 10 = 600 $\mu$L for all 10 plates. Instead of using 600 $\mu$L of a $10^{-5}$ dilution, you could use 60 $\mu$L of a $10^{-4}$ dilution for the infection.

## Procedure: Performing the Large-Plate Infection

**Once the plates are infected, you *must* return the following day to flood the plates and the day after that to harvest and prepare the lysates. This is not a protocol to start on a Friday if you are not planning to work in the lab over the weekend!**

### A. Infect the *M. smegmatis*.

1. In a 100-mL flask, infect 5.0 mL of *M. smegmatis* with the appropriate volume and dilution of lysate.
2. Swirl gently to mix the bacteria and lysate well.
3. Allow phage to infect for the *same* amount of time used to infect for the empirical test (usually 20 to 30 minutes).

### B. Add TA.

1. TA must be between 55°C and 60°C (use directly from water bath; it should be very warm to the touch but not hot enough to burn).
2. Add 45 mL of TA to the flask that contains the infected *M. smegmatis*.
3. Swirl gently to mix, avoiding bubbles.

### C. Immediately plate 5-mL aliquots onto each of the 10 prewarmed agar plates.

1. Using an automated pipettor and a 25-mL pipette, pull up 25 mL of the mixture at a time.
2. Dispense 5 mL per agar plate (five plates per 25-mL pipette load). Swirl plates gently to cover the agar plate completely with the TA mixture. To reduce contamination, change the pipette after dispensing the 25 mL.

### D. Prepare a control plate with 0.5 mL of bacteria plus 4.5 mL of TA.

### E. Allow plates to harden and then invert and incubate at 37°C overnight.

**This harvesting step must be done within 24 hours of the initial infection.**

## Procedure: The Next Day, Harvesting Your Phage

### A. Prepare your work area.

Remove clutter and wipe down your workbench. You should have

- ☐ 10 infected plates with web-pattern lysis
- ☐ 100 mL of PB
- ☐ 25-mL serological pipettes (14) and an automated pipettor
- ☐ 50-mL conical tubes (2)
- ☐ A filter-sterilization unit, 0.22-$\mu$m pore size and 100-mL volume
- ☐ Sterile microcentrifuge tubes (for titer dilutions)
- ☐ A 20-$\mu$L and a 200-$\mu$L micropipettor and tips
- ☐ 5-mL serological pipettes (10)
- ☐ Aliquots of 0.5 mL of saturated *M. smegmatis* culture in 10-mL culture tubes (10)
- ☐ 60 mL of 55°C TA (remove from 55°C water bath right before use)

Be sure that bubbles do not come up the pipette past the cotton plug. This will clog the filter in the automated pipettor, which will lose suction and stop working.

## B. Flood the 10 plates with PB.

1. Using the 25-mL serological pipettes and automated pipettor, add 8 mL of PB to each plate (use one pipette per three plates).
2. Make sure the buffer covers the entire surface of the agar.
3. Let the plates sit for 2 to 4 hours at room temperature. Alternatively, the plates can be stored overnight at 4°C.

## C. Harvest the phage lysate.

1. Using the 25-mL serological pipettes and automated pipettor, remove the PB (now containing phage) from the agar plates. Keep in mind that some of the PB may have seeped *under* the agar. Remove the PB as outlined in *Tame* fig. 9.

**Tame fig. 9**
Removing PB-containing phage from an agar plate. Go through the agar with the pipette and, while tilting the plate up at a slight angle, aspirate the PB from the bottom of the plate. Be very careful not to spill lysate on the bench top, which could contaminate other samples with your phage.

This harvested lysate is sometimes called the "HTL" for High-Titer-Lysate. The HTL will be used for DNA purification..

2. Pool the PB-phage into a 50-mL conical tube.
3. Balance and centrifuge the tubes at 2500×*g* for 20 minutes to pellet cell debris.
4. Transfer the supernatant into a 100-mL filter-sterilization unit.
5. Using suction, filter-sterilize your lysate.
6. Turn off the suction.
7. Using aseptic technique, place the lid on the bottom reservoir of the filter-sterilization unit.
8. Label the bottle containing your lysate for storage at 4°C. You will use this lysate for the titer determination below.

- **Be careful not to spill your lysate onto countertops or equipment. High-titer lysate spills can contaminate the work of others!**
- **Save your lysate. If the titer is high enough, you will use this lysate for the preparation of electron microscopy grids and visualization of your phage. You will also use this lysate for the preparation of phage genomic DNA.**

## Procedure: Titering Your Phage

**A Perform serial dilutions on a sample from the pooled and sterilized lysate.**

**B Use 10 μL of these dilutions to infect 0.5-mL aliquots of *M. smegmatis;* let sit for 20 to 30 minutes.**

**C. Add TA to each sample and spread onto prewarmed agar plates.**

**D. Count plaques the following day, and, if the titer is $>10^8$ pfu/mL, continue to the DNA-purification protocol.**

## Notes

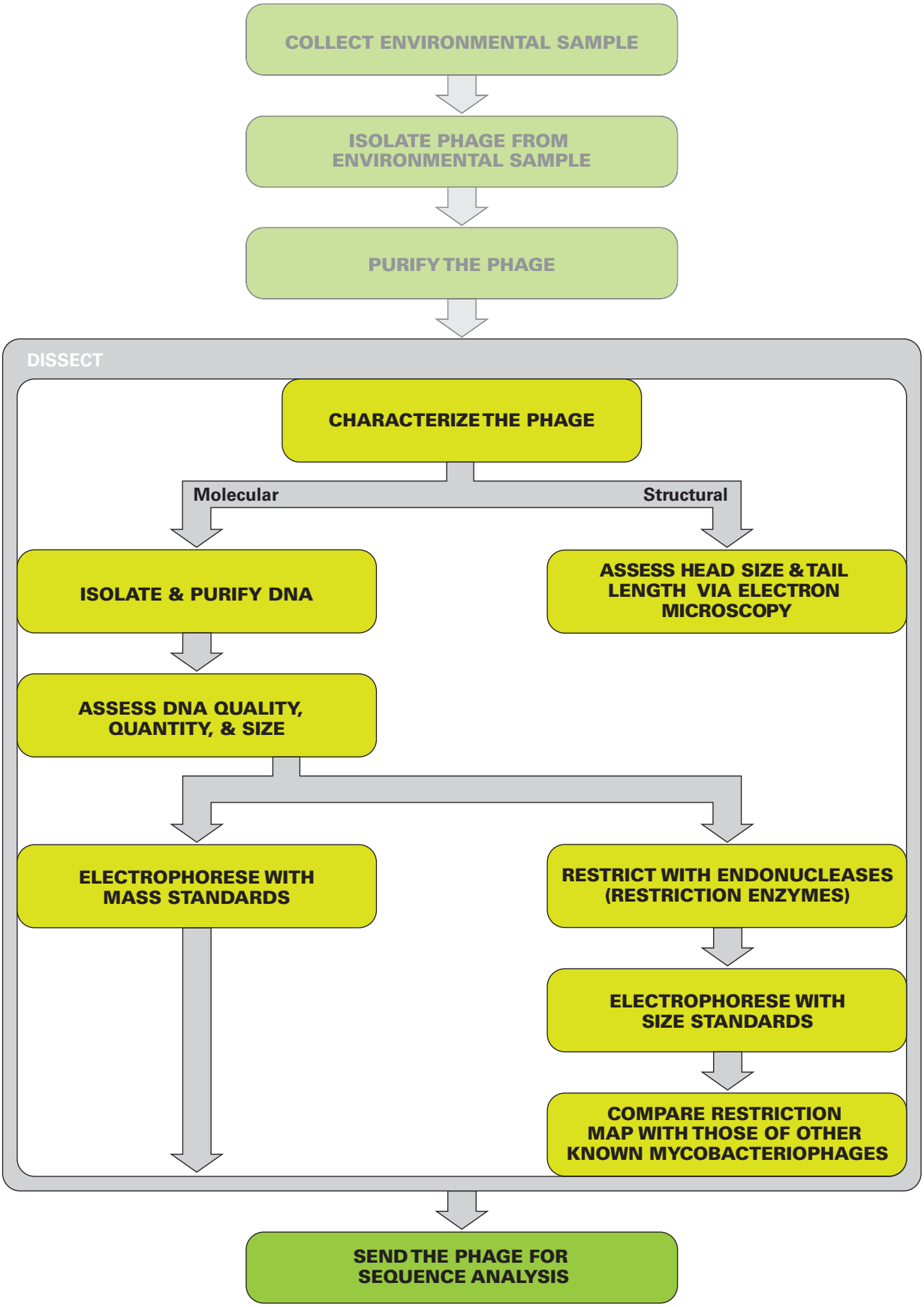
Before starting the 10-plate infection, you must be confident about the volume-dilution combination of lysate that yields a web pattern on an agar plate. *The same subculture of* M. smegmatis *must be used throughout the empirical test and 10-plate infection in order to standardize the conditions*. If the 10-plate infection does not yield a good web pattern, the empirical test will have to be repeated and the 10-plate infection performed again.

It is best to time your lab work to coordinate with your schedule. If you do not plan well, long delays could influence your results. For example, phage titers tend to decrease over time, even when the phage is stored at 4°C. Depending on the characteristics of your particular phage, the age of the bacterial culture can affect the efficiency of phage binding, infection, burst size, and other characteristics. Good planning will increase the likelihood of a good titer and, therefore, a high yield of DNA for sequencing. It will also increase your efficiency in the laboratory and could, potentially, save you a lot of time.

## Questions

1. **Name at least three factors that would decrease the titer of a given phage preparation.**
2. **Knowing what you know about your phage, what are your predictions about the yield that you could expect from a 10-plate harvest?**

## You Are Here


COLLECT ENVIRONMENTAL SAMPLE
ISOLATE PHAGE FROM ENVIRONMENTAL SAMPLE
PURIFY THE PHAGE
DISSECT
CHARACTERIZE THE PHAGE
Molecular
Structural
ISOLATE & PURIFY DNA
ASSESS HEAD SIZE & TAIL LENGTH VIA ELECTRON MICROSCOPY
ASSESS DNA QUALITY, QUANTITY, & SIZE
ELECTROPHORESE WITH MASS STANDARDS
RESTRICT WITH ENDONUCLEASES (RESTRICTION ENZYMES)
ELECTROPHORESE WITH SIZE STANDARDS
COMPARE RESTRICTION MAP WITH THOSE OF OTHER KNOWN MYCOBACTERIOPHAGES
SEND THE PHAGE FOR SEQUENCE ANALYSIS

# Dissect

## Contents

# Part A. Analyze Phage Using Electron Microscopy

## Overview

The objective of this series of procedures is to observe each phage using electron microscopy (EM). The area of the capsid (in nanometers) correlates with the size of the phage genome it contains. Likewise, the length of the tail will correlate with the size (in base pairs) of the tape-measure gene found in all mycobacteriophages. It is important to note that two phages that are very similar in appearance can have vastly different genome organization.

## Objective

To observe individual phages using electron microscopy.

## Supplies

- ☐ A Petri dish
- ☐ Latex or vinyl gloves
- ☐ A plastic-faced paper (diaper)
- ☐ Parafilm, 5 × 5 cm (1 piece)
- ☐ Double-sided disks (PELCO Tabs) or sticky tape
- ☐ Sterile distilled or filtered $H_2O$
- ☐ Fine-point capillary tweezers (1 pair)
- ☐ An EM grid
- ☐ Whatman #3 filter paper
- ☐ A micropipettor and tips
- ☐ Filtered 1% uranyl acetate solution (100 $\mu$L)

## Equipment

- ☐ An electron microscope

## Procedure

### A. Prepare your phage sample.

1. Aseptically transfer 0.5-1.0mL of your high-titer lysate into a sterile microcentrifuge tube.

**Do NOT dilute your high titer lysate before the high-speed centrifugation step!**

2. Balance the tube(s) and centrifuge for one hour at 4°C and at 10,000×*g*.
3. Using the micropipettor and sterile tip, carefully remove all but 20-50 $\mu$L of the supernatant without scraping the bottom of the tube with the pipette tip or otherwise disturbing the pellet.
4. Add 100 $\mu$L of fresh phage buffer and mix gently using the pipette tip.
5. Store this concentrated sample at 4°C for at least one hour, but no more than 48 hours, before staining to allow the phage pellet to completely dissolve.

**Handle the EM grids only with forceps and only by the edges.**

## B. Prepare your work area.

1. Put on a fresh pair of latex or vinyl gloves.
2. Cover the designated lab counter with the plastic-faced paper to create a clean area.
3. Remove the cover paper from a 5 × 5-cm piece of parafilm, and place the parafilm into the *lid* of a Petri dish.
4. Using tweezers, place a PELCO Tab or small (1-to-2-cm) piece of double-sided tape onto the parafilm in the lid of the Petri dish.
5. Remove the liner from the tape or tab to expose the adhesive.
6. Using the pointed tweezers, remove a fresh grid from a box of unused grids, touching only the very edge of the grid.
7. Place the grid, ***shiny side UP***, on the edge of the tab or double-sided tape so that only the very edge of the grid (no more that 0.5 mm) is touching the adhesive.

## C. Mount and stain your phage.

1. Using a micropipettor, place 10 μL of your phage preparation onto the grid without touching the tip to the grid itself.
2. Allow the phage to adsorb (or settle and attach) onto the grid for at least 2 minutes. The approximate times are indicated in *Dissect* table 1.

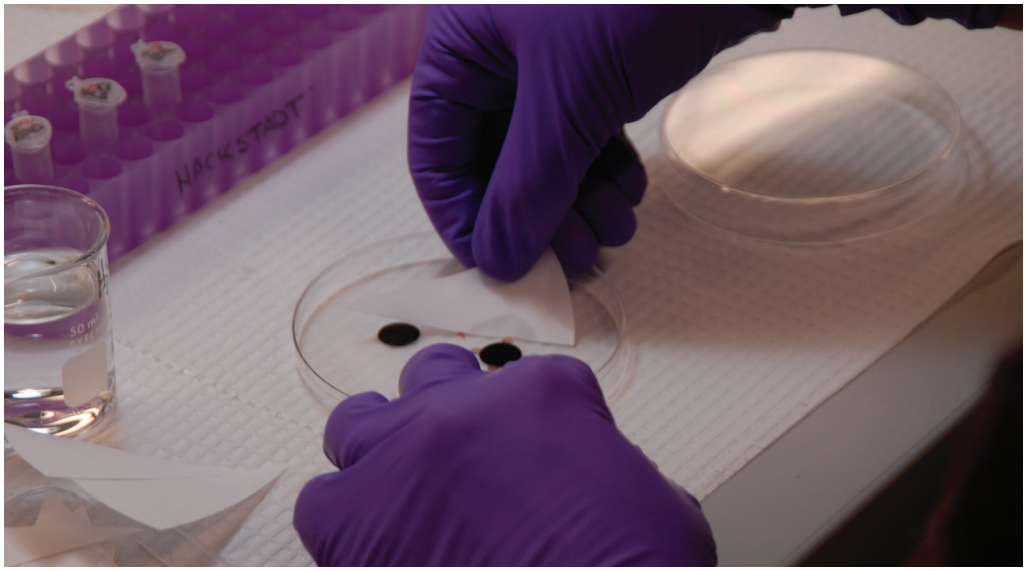
**Dissect table 1**
Approximate times that phage should settle onto the EM grid based on phage titer.

| Phage titer | Approximate time |
|---|---|
| $10^6$–$10^7$ | >5 minutes |
| $10^8$ | 5 minutes |
| ≥$10^9$ | 2 minutes |

3. Using a small (2- to 3-cm) wedge of Whatman #3 filter paper or a low-lint laboratory wipe, wick off the excess fluid (see *Dissect* fig. 1).

**Work quickly (and SAFELY). Do NOT allow the grid to dry out!!**

4. Wash the grid two times by this method:
   a. Carefully pipette 10 μL of sterile water onto the grid.
   b. Allow to sit for 2 minutes.
   c. Wick off water using a fresh wedge of filter paper or a corner of a laboratory wipe.




**Dissect fig. 1**
Wicking liquid off a grid. Touch only the side of the grid and with only a corner of the filter paper or low-lint laboratory wipe. This will prevent contamination of the grid with paper fibers.

5. Add 10 $\mu$L of 1.0% uranyl acetate to the grid.
6. Allow to stain for 2 minutes.
7. Wick off the excess stain and allow the grid to air-dry.

**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.**

## D. Observe your phage.

Use the bottom of the Petri dish to cover your stained phage grids and take it directly to your EM facility. If you are planning to send the phage to a facility, prepare the grid or samples according to the specifications of the facility and send as directed.

### Questions

1. **Why can't you see phages with a standard microscope? What is the resolution of an electron microscope compared with a light microscope?**
2. **What can you learn from your observation of an electron micrograph of your phage? What could the size of the capsid tell you? What could the length of the tail tell you?**
3. **If two phages look very similar by electron microscopy, would you predict that they will have similar genomes? Why?**

# Part B. Isolate and Purify Phage Genomic DNA

## Overview

You will isolate and purify phage genomic DNA from the phage head, which is virtually all protein. Any residual bacterial cell debris and macromolecules (including capsid proteins and bacterial proteins) must be removed. Of all of the bacteria-derived macromolecules, the bacterial nucleic acids, both DNA and RNA, are the most important ones to be removed from solution before the protective coat proteins of the phage head are disrupted. Removing them will reduce the potential complexities of the restriction and sequence analyses.

The initial step is to incubate the intact phage in a solution containing two enzymes. These enzymes, DNase I and RNase A, are nucleases with nonspecific DNA and RNA hydrolytic activities, respectively. Nucleases are enzymes that disrupt the structure of nucleic acids. After the phage is precipitated from the polynucleotide solution, the coat proteins and enzymes are denatured (unfolded and/or inactivated) by the buffer that contains the resin. While the proteins are being denatured, the phage genomic DNA will bind to the resin. Upon removal of the denatured proteins and salts from the resin with an aqueous alcohol, isopropranol, the phage genomic DNA is eluted (removed) from the column with hot (80˚C) buffer and is then ready for analysis.

## Objective

The primary objective is to isolate and purify phage genomic DNA in sufficiently high amounts for restriction analysis and sequencing protocols.

## Supplies

- □ A sterile serological pipette (5-mL)
- □ Isopropanol (80%) (2 mL/10 mL lysate)
- □ Phage precipitant (4 mL/10 mL lysate)
- □ Nuclease Mix (40 μL/10 mL lysate)
- □ Promega DNA Clean Up Kit
- □ 1.5-mL microcentrifuge tubes (6)
- □ A 15-mL clear screw-capped tube (e.g., Oak Ridge tube)
- □ Syringes, 3-mL or 5-mL
- □ Sterile dd$H_2O$ (0.5 mL/10 mL lysate)
- □ TE pre-warmed to 80°C
- □ Gloves (latex or vinyl)

## Equipment

- □ A high-speed centrifuge
- □ A microcentrifuge
- □ 80°C heating block or water bath
- □ 37°C water bath
- □ A refrigerator (or ice)
- □ −20°C freezer
- □ A spectrophotometer, Nanodrop, or fluorometer (and accessories) for DNA quantification

If your lysate has a titer>$10^9$ pfu/mL, you can dilute it e.g., (1:2 or 1:10) with sterile phage buffer

## Procedure

### A. Degrade bacterial DNA that is in 10 mL of phage lysate.

1. Transfer 10 mL of the filter-steriled phage lysate into a Oak Ridge tube. Store the remaining lysate at 4°C.
2. Put on gloves.
3. Add 40 μL Nuclease Mix, screw on cap, and mix gently but thoroughly by repeated inversions. Do *not* vortex!

**When handling the Nuclease Mix, you must wear gloves and work in a hood to avoid contaminating other solutions with the mix. After adding Nuclease Mix to your sample, cap the tubes and discard your gloves before returning to your work area. Otherwise, the enzymes can contaminate all DNA-containing solutions and ruin your sample.**

4. Incubate at 37°C for 30 minutes.
5. Let sit undisturbed at room temperature (RT) for 1 hour.

Remove as much phage precipitant solution as possible without disturbing the phage pellet. Failure to do so will interfere with or clog the column and significantly reduce your DNA yield.

### B. Precipitate phage particles.

1. Put on a fresh pair of gloves.
2. Using a 5-mL pipette, add 4.0 mL of phage precipitant solution to the nuclease-treated lysate, and cap the tube.
3. Mix gently but thoroughly by inversion.
4. Incubate 30 minutes on ice or, for maximum yield, overnight at 4°C.
5. Place the tube in a high-speed centrifuge and spin at 10,000×*g* for 20 minutes.
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.

### C. Re-suspend the phage pellet.

1. Add 0.5 mL of sterile $ddH_2O$ to the pellet.
2. Gently re-suspend the pellet by pipetting up and down.
3. Allow to sit at room temperature for 5-10 minutes (no more than 10 minutes).

**Food for Thought**

Why wouldn't you want to let the resuspended phage particles sit too long in solution?

### D. Obtain the DNA-purification kit from your instructor.

### E. Uncoat the phage genomic DNA.

1. Put on a fresh pair of gloves.

**The resin contains guanidinium thiocyanate, a chemical that denatures proteins. Do *not* get it on your skin!**

2. Add 2 mL of pre-warmed (37°C) DNA Clean Up Resin.
3. Uncoat the phage particles by gently pipetting up and down. Gently swirl to mix.

## F. Isolate the phage genomic DNA.

1. You will need two columns for this procedure. Attach one column supplied with the kit to a syringe. Repeat with the second column.
2. Apply 1.25 mL of water-resin–phage-genomic-DNA solution to each column using a pipette.
3. Use the syringe plunger to push this solution through the column.
4. Wash salts and proteins off DNA. For each column:
   a. Remove the column from the syringe.
   b. Remove the plunger from the syringe.
   c. Re-attach the syringe to the column.
   d. Add 2 mL of 80% isopropanol to the column.
   e. Push the isopropanol through the column with the plunger.
5. Dry each column:
   a. Remove the column.
   b. Centrifuge the column for 5 minutes at maximum speed to remove the isopropanol (do this in a clean microcentrifuge tube that has had its lid removed).
   c. Transfer the column to a clean, lidless microcentrifuge tube.
   d. Centrifuge for 1 minute at maximum speed to remove the residual alcohol.
6. Elute the phage genomic DNA from each column:

**Do *not* allow the TE to cool below 80°C before it is applied to the column.**

   a. Transfer the column to a clean, autoclaved, properly labeled microcentrifuge tube.
   b. Rapidly apply 50μL of pre-warmed, 80°C TE to the resin in the column. Let the TE sit on the column 30 to 60 seconds to dissolve the DNA.
   c. Centrifuge for 1 minute to elute your purified phage genomic DNA.
7. Combine the DNA samples into a single tube.
8. Store your DNA at 4°C until you are ready to quantify and analyze it.

Pushing the solution through sometimes requires a great deal of elbow grease.

## G. Determine the concentration of your DNA.

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

## Questions

1. Drew, who is incredibly conscientious and cares deeply about the environment, decided it would be best to use the same pair of gloves throughout today's procedure. Why is this, although good for the environment, a pretty bad idea for today's procedure?

2. An aqueous alcohol solution is used to "wash" the salts from the column. Why not just use straight alcohol?

# Part C. Restrict and Analyze Phage Genomic DNA

## Overview

The objective is to use restriction analysis of genomic DNA to help identify a unique mycobacteriophage that can be sent for sequence and subsequent bioinformatic analysis. The general strategy is to digest—that is, cut, or *restrict*—the DNA into fragments of manageable size and to separate the fragments using agarose gel electrophoresis.

You will add a tracking dye to the samples that will help determine how long the gel should be run. The dye is a mixture containing bromophenol blue (BPB; a dark blue color) and xylene cyanol (XC; a medium sky blue). The BPB runs at a rate that is approximately equal to a 500-base-pair (bp) DNA fragment; XC's migration is similar to a 4000-bp fragment. The gel should be run long enough to get good resolution of the DNA fragments (bands). Generally, gels are run with DNA standards until the BPB is near the bottom edge of the gel. Once the electrophoresis is complete, a photograph of the gel should be taken to document and record the results. Samples with unique patterns will be prime candidates for further characterization.

## Objective

The objective is to digest the phage genomic DNA sample with restriction enzymes (REs), compare its restriction pattern with those of known mycobacteriophages, and use this information to help decide whether the phage is a viable candidate for bioinformatic analysis.

## Supplies

- □ Microcentrifuge-tube floaters
- □ Latex or vinyl gloves
- □ Restriction enzymes (*Bam*HI, *Cla*I, *Eco*RI, *Hae*III, and *Hin*dIII)
- □ Restriction enzyme buffers
- □ dd$H_2O$ (autoclaved)
- □ 10X bovine serum albumin (BSA)
- □ 1-kb DNA Ladder
- □ Seakem LE agarose gel powder
- □ 1X TBE stock buffer
- □ Ethidium bromide (EtBr, 10 mg/mL)
- □ Tracking dye
- □ A micropipettor and tips

## Equipment

- □ A microcentrifuge
- □ 37°C water bath
- □ A microwave oven
- □ Horizontal midigel (approximately 20 × 20 cm) apparatus with combs
- □ Power supply
- □ Photography equipment and supplies

## Procedure

### A. Digest your phage genomic DNA.

1. Gently mix your DNA sample by either flicking the closed tube with your finger or vortexing it on low.
2. Incubate at 65°C for 10 minutes, then quickly place the tube on ice. Quick spin (i.e., for less than a minute).
3. Set up the reactions shown in *Dissect* table 2 in appropriately labeled microcentrifuge tubes. There will be a total of six tubes. Each has a final volume of 20 $\mu$L.
4. Mix each tube gently but well by flicking it with your finger.

**Dissect table 2**
Reactions to set up for digesting phage genomic DNA.

| Solution: | Tube, amount | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 10X Reaction Buffer | 2 µL | 2 µL | 2 µL | 2 µL | 2 µL | 2 µL |
| Phage genomic DNA | 0.5 µg | 0.5 µg | 0.5 µg | 0.5 µg | 0.5 µg | 0.5 µg |
| 10X BSA | 2 µL | 2 µL | 2 µL | 2 µL | 2 µL | 2 µL |
| *Bam*HI | – | 10 U | – | – | – | – |
| *Cla*I | – | – | 10 U | – | – | – |
| *Eco*RI | – | – | – | 10 U | – | – |
| *Hae*III | – | – | – | – | 10 U | – |
| *Hin*dIII | – | – | – | – | – | 10 U |
| dd$H_2O$ | to 20 µL | to 20 µL | to 20 µL | to 20 µL | to 20 µL | to 20 µL |

5. Quick spin the tubes, then incubate in a 37°C water bath for **at least 2 hours.**
6. Quick spin the tubes and place on ice or store in the freezer until you are ready to perform the next series of steps.

### B. Electrophorese your sample.

1. Set up the gel apparatus according to your instructor's specifications, unless it has been set up for you.
2. Prepare enough 0.8% (weight/volume) agarose gel to fill the gel former.
   a. Weigh out the appropriate mass of agarose powder; transfer the powder to an Erlenmeyer flask.
   b. Add the appropriate volume of 1X TBE buffer to the agarose powder; swirl to mix.
   c. Heat the mixture in the microwave until it boils (usually 1 to 2 minutes).
   d. Using a mitt or heat-resistant glove, very carefully remove the flask from the microwave; swirl to mix. Taking care not to splash the hot liquid or touch the hot flask, examine the solution for small clumps,

often transparent, of agarose. If you see any clumps, return the flask to the microwave oven and heat for a few seconds. Swirl, examine again. If the solution appears to be uniform, allow it to cool slightly. If the solution boils over, you must start over!

e. Check the volume of the solution. If the volume has decreased, bring it back to the original volume using dd$H_2O$. Swirl to mix.

f. Allow solution to cool to between 50°C and 60°C (very warm to the touch but not hot enough to burn).

g. Once sufficiently cooled, put on latex gloves; then, add the EtBr to a final concentration of 0.5 $\mu$g/mL in the agarose solution.

For example, to prepare a 100-mL agarose gel solution containing 0.5 $\mu$g/mL EtBr from a 10-mg/mL EtBr stock:

Total micrograms of EtBr needed in the 100-mL gel solution =

0.5 $\mu$g/mL × 100 mL = 50 $\mu$g EtBr

(10 mg/mL = 10 $\mu$g/$\mu$L)

$$\text{Total volume of EtBr stock needed} = \frac{50\ \mu g}{10\ \mu g/\mu L} = 5\ \mu L$$

**Extreme caution must be exercised when handling EtBr-containing samples. EtBr is a mutagen that intercalates between the bases in DNA. Wear gloves!**

**Food for Thought**

EtBr is a known mutagen and a suspected carcinogen as well. What's the difference between the two?

## The Big Picture

**An entire genome is very large. For example, the human genome contains over 3 billion (3 x $10^9$) base pairs (bp); DNA from mycobacteriophages typically ranges from $10^5$ to $10^6$ bp. These sizes are far too big to be analyzed at one time in their entirety. DNA sequencing takes time, special equipment, other resources that are hard to come by or expensive, and technical expertise that a novice researcher might not have. A special class of enzymes known as nucleases can be used to rapidly compare DNA sequences.**

**DNA can be analyzed in a variety of ways. The general strategy is to digest (cut, cleave, or *restrict*) the DNA into fragments of manageable size using nucleases known as restriction endonuleases or restriction enzymes (REs). REs are bacterial enzymes that cut double-stranded DNA (dsDNA) at specific sequences. Once cut, the mixture of dsDNA fragments is separated, visualized, and analyzed.**

h. Pour the agarose-containing 0.5-μg/mL EtBr onto the prepared gel plate being careful not to introduce bubbles into the solution. Immediately insert the comb.

i. Allow the gel solution to cool and harden (approximately 20 to 30 minutes). During the cooling time, prepare the samples for electrophoresis. Once the gel has hardened, either transfer to electrophoresis chamber that contains 1X TBE buffer or pour enough 1X TBE buffer onto the gel to cover the gel's surface completely so that the agarose does not dry out. (For the appropriate step, ask your instructor.)

3. Prepare the DNA standard by placing the following in a microcentrifuge tube:

   ☐ 0.5 μg 1-kb DNA Ladder,
   ☐ 1 μL 10X Restriction Enzyme Buffer, and
   ☐ dd$H_2O$ to 10 μL.

   Mix gently. Quick spin.

4. Add 2 μL of tracking dye to each of your reaction tubes (Tubes 1 to 6) and to the 1-kb Ladder. Mix gently. Quick spin.

5. Place all samples in a 65°C heating block or water bath for 5 minutes. Immediately place on ice to cool. Quick spin.

6. If you have not yet transferred the gel into the electrophoresis chamber containing buffer (1X TBE), do that now. The gel should be oriented such that the wells are closest to the cathode (black electrode). Very gently remove the comb from the gel.

7. Using a fresh tip on your micropipettor for each sample, carefully load the gel with 10 μL of each sample in the following order (left to right):

   **1-kb Ladder, Tube 1 (undigested DNA), Tube 2 (*Bam*HI), … Tube 6 (*Hin*dIII).**

8. Plug the electrodes into the appropriate slots on the power supply. Turn on the power supply and set to run at 100V.

9. Electrophorese until bromophenol blue (BPB) is within 1 to 2 cm of the end of the gel closest to the anode (red electrode). This will take approximately 30 to 45 minutes. Turn off the power supply.

Watch the gel for 1 to 2 minutes to ensure that the dye runs out of the wells and toward the anode.

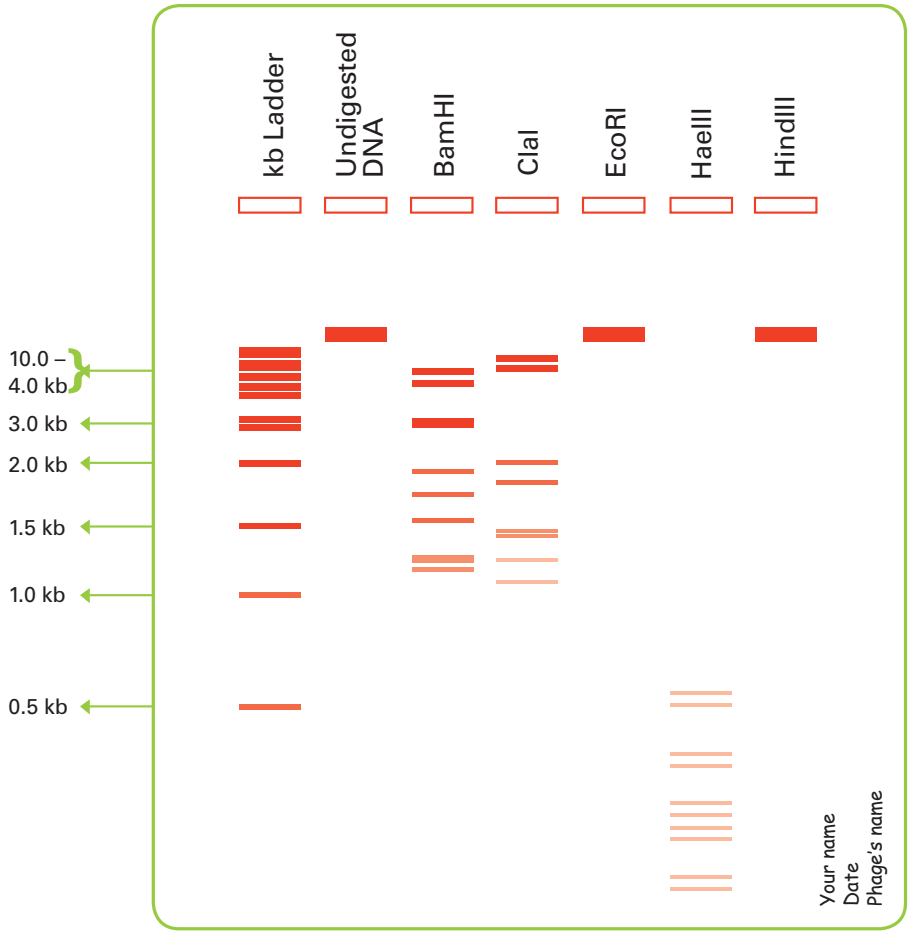
## C. Photograph the gel.

1. Carefully remove the gel plate from the electrophoresis chamber.

2. Following your instructor's directions, photograph your gel.

**Ultraviolet light must be used to visualize the DNA fragments (bands) in the gel. UV burns retinas and skin. Remember to wear eye and face protection and clothes that protect skin.**

3. Use a permanent marker to label your gel photograph as indicated in *Dissect* fig. 2. Include the date and your initials.




**Dissect fig. 2**
A properly labeled illustration of an agarose gel containing restricted DNA.

**Food for Thought**

Why do the shorter fragments move through the gel faster?

## D. Compare the results from your gel with those for other mycobacteriophages in the SEA website to ascertain whether you have discovered a potentially unique phage.

The relationship between size and distance migrated is logarithmic and can be used to estimate the size of the fragments generated with each restriction enzyme.

1. For each restriction enzyme tested, estimate the length of each visible fragment.

   a. For the molecular weight markers:

      i. Open a spreadsheet program on your computer and label the columns "Fragment length (bp)," "Log fragment length (log bp)," and "Distance migrated (cm)."

It is possible that the longer fragments will not be well resolved on your gel. Use data only from fragments that are easily identifiable. Use the sample gel shown in *Dissect* fig. 2 as a guide for identifying the fragments in the standard.

ii. Enter into your spreadsheet the size (bp) of the DNA fragment using the fragment lengths for the appropriate molecular weight markers indicated in *Dissect* table 3.

**Dissect table 3**
Format for recording data on spreadsheet according to DNA-fragment size.

| Fragment length (bp) | Log fragment length (log bp) | Distance migrated (cm) |
|---|---|---|
| 10,108 | | |
| 9,180 | | |
| 8,144 | | |
| 7,126 | | |
| 6,036 | | |
| 5,090 | | |
| 4,072 | | |
| 3,054 | | |
| 2,036 | | |
| 1,636 | | |
| 1,018 | | |
| 506 | | |
| 396 | | |
| 344 | | |
| 298 | | |
| 220 | | |
| 201 | | |
| 154 | | |

iii. Use the appropriate features of your spreadsheet program to fill in the values of the second column ("log bp").

iv. Use the features of your spreadsheet program to plot a graph of log of fragment length (bp, $y$-axis) versus distance migrated (cm, $x$-axis). Determine the **slope** ($m$) and y-**intercept** ($b$) of the generated line.

b. For the restriction-enzyme-generated fragments:

i. Use a ruler to measure the distance (cm) migrated by each fragment, measuring from the bottom of the well to the center of the visible fragment. Enter this information into the spreadsheet.

ii. Estimate the log of the size of each fragment using the equation of a straight line, $y = mx + b$, in the third column under each restriction enzyme in *Dissect* table 4.

iii. Use your spreadsheet to calculate the estimated size of each generated fragment (take the anti-log (i.e., $10^y$) of the $y$ value). This is the fourth column under each restriction enzyme in *Dissect* table 4.

Dissect table 4

| Name of Restriction Enzyme 1 | | | | Name of Restriction Enzyme 2 | | | | Etc. |
|---|---|---|---|---|---|---|---|---|
| Fragment number | Distance migrated (cm) | log fragment length | Fragment length (bp) | Fragment number | Distance migrated (cm) | log fragment length | Fragment length (bp) | |

2. Access the phage genomic DNA restriction analysis data at the SEA website (www.hhmi.org/sea). Record in your notebook the differences and similarities between the results generated from the restriction analysis of your phage genomic DNA and of the phages at the SEA website. These data will help you decide whether your phage is a good candidate for the genomics portion of the course.

**E. Using Decision Tree #3 as a guide, decide whether your phage is a viable candidate to submit for sequencing and subsequent bioinformatic analysis, and prepare to present your argument to your colleagues.**

*It is possible that not all of the phages isolated by your class will advance to the next procedure.*

## Notes

To choose the sample to be sent for sequencing, you can, as a class, add criteria to those listed in Decision Tree #3.

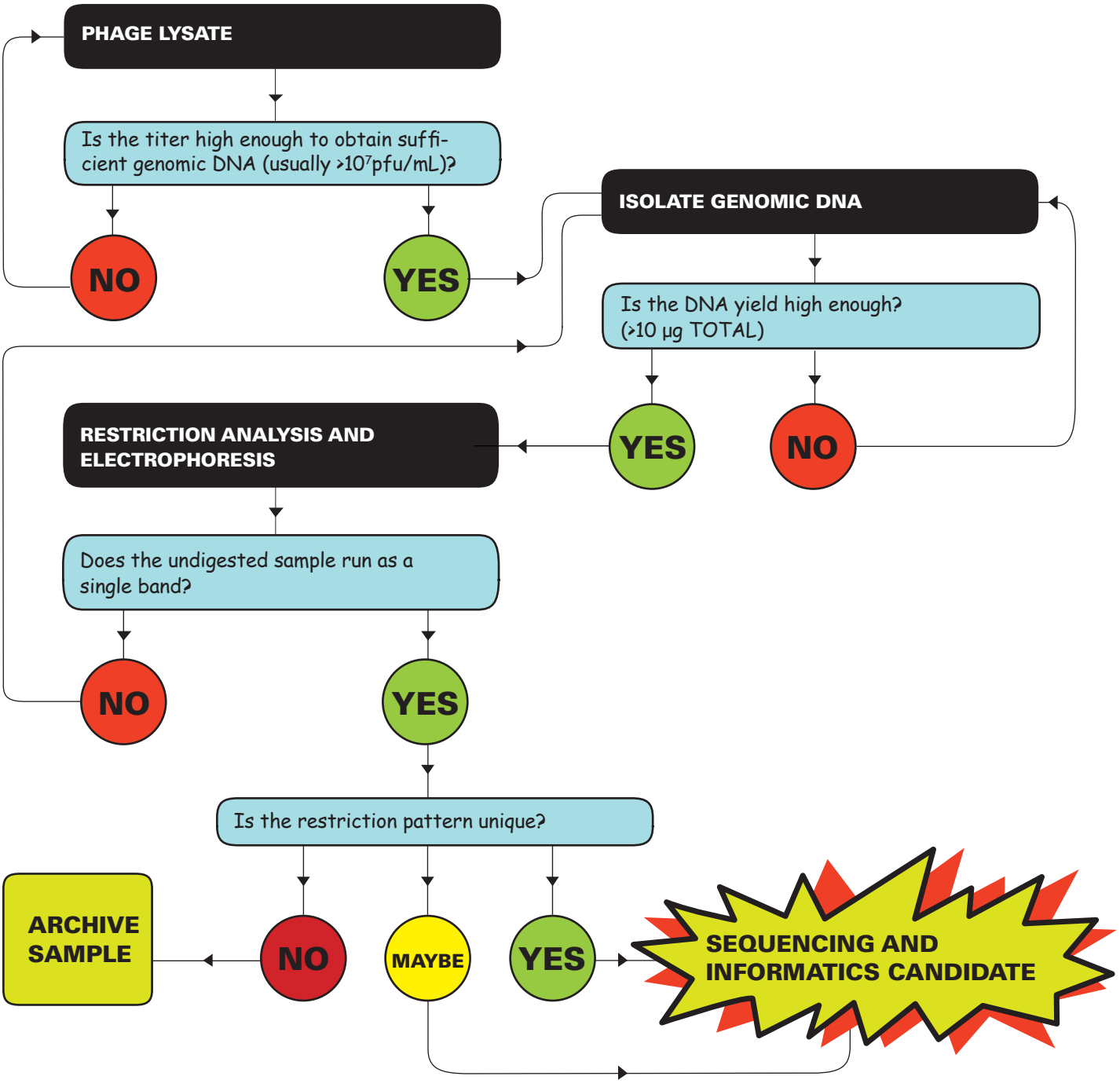
As an individual, you are encouraged to "lobby" for your phage, but your argument must be based on the scientific guidelines outlined in Decision Tree #3 and any additional criteria you have come up with.

## Questions

1. **The photograph of Jesse's gel looked exactly like the photograph of the gel depicted in step C.3 (*Dissect* fig. 2).**
   a. **Should Jesse's sample be selected for sequencing? Explain.**
   b. **From what you can see in the figure, are there any restriction enzymes that do not cut Jesse's phage genomic DNA? Explain.**

2. **Cam added 0.40 g agarose to 50 mL 1XTBE buffer. Will this result in a 0.8% gel? Explain.**

## Decision Tree #3
## From DNA to Informatics



# Part D. Evaluate Genomic DNA Quality and Send DNA to Sequencing Center

## Overview and Objective

The purpose of this protocol is to assess the quality of the genomic DNA samples selected as candidates for sequencing. The response from your assigned sequencing center, in addition to the criteria listed in Decision Tree #3, must be used to decide which sample to send.

## Supplies

- ☐ Microcentrifuge tubes
- ☐ Nuclease-free dd$H_2O$
- ☐ Seakem LE agarose gel powder
- ☐ 1X TAE buffer
- ☐ Ethidium bromide (EtBr, 10 mg/mL)
- ☐ A micropipettor (20-$\mu$L) and tips
- ☐ Loading dye
- ☐ Glycerol
- ☐ Ice
- ☐ QC DNA Standard Kit containing Marker 2 Lambda *Hin*dIII Size Standard and Lambda DNA Mass Standards (15, 31, 63, 125, 250, 500 ng/5 $\mu$L

## Equipment

- ☐ A microcentrifuge
- ☐ An agarose gel electrophoresis apparatus with a 1.5-mm comb
- ☐ Power supply and electrodes
- ☐ Photography equipment and supplies
- ☐ 65°C heating block or water bath

## Procedure

### A. Prepare a 1% agarose gel in 1X TAE buffer containing 0.15-$\mu$g/mL EtBr.

**Although this is a slightly different electrophoresis protocol from that used for restriction analysis, the same precautions must be taken when handling the ethidium bromide and to limit exposure to ultraviolet radiation.**

### B. Prepare the genomic DNA sample for electrophoresis.

1. Place your genomic DNA sample in the 65°C heating block or water bath for 5 minutes. *Immediately* place on ice for 1-2 minutes. Quick spin.
2. Transfer 1 $\mu$L of your DNA to a clean microcentrifuge tube.
3. Bring the volume to 5 $\mu$L with 1X loading dye.

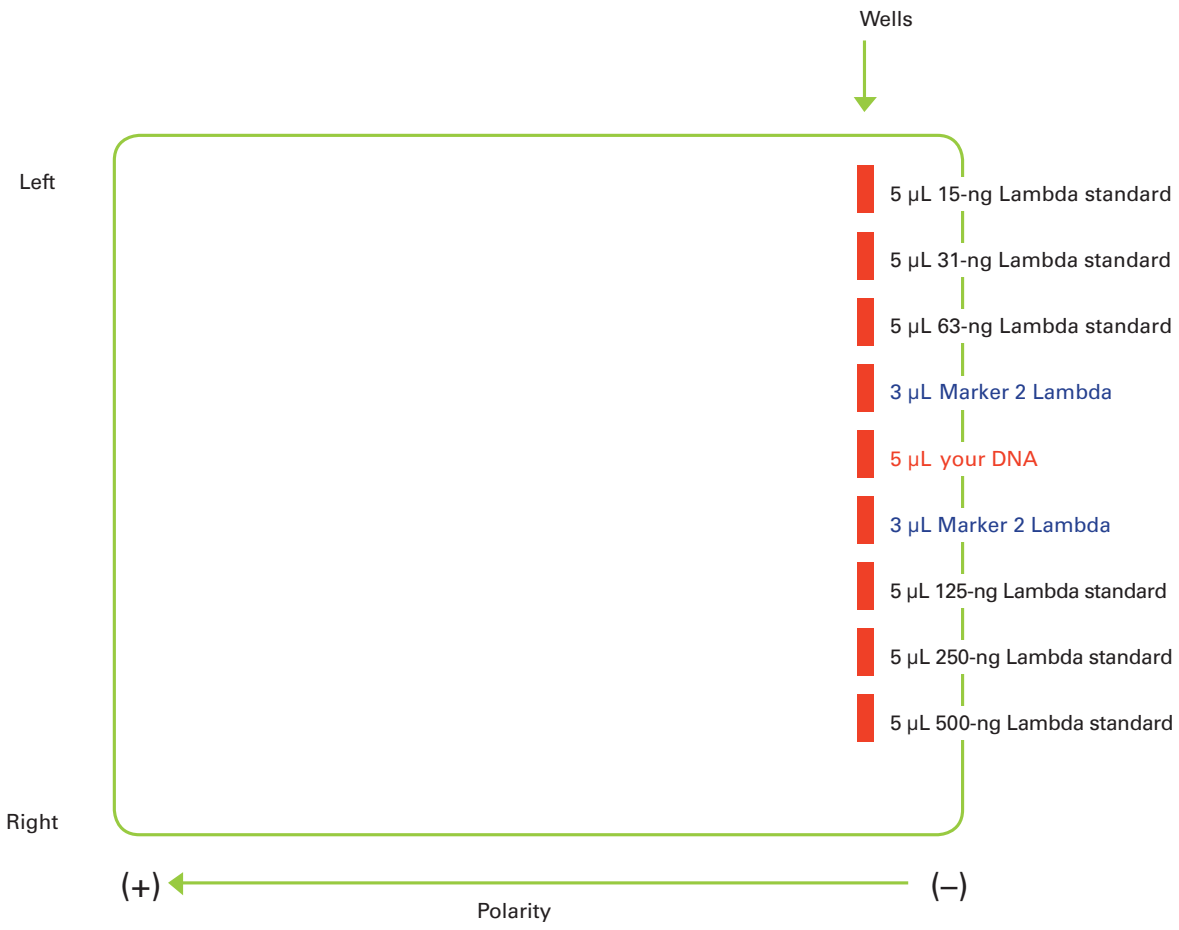
**C. Retrieve QC DNA Standard kit from –20°C freezer. Place the samples in a 65°C heating block or water bath for 5 minutes. *Immediately* place on ice to cool. Quick spin..**

**D. Electrophorese the samples.**

1. Fill gel chamber with 1X TAE buffer.
2. Mix all samples by flicking the closed tubes with your fingers.
3. Quick spin all tubes.
4. Load the gel with the specified sample in the precise order indicated in *Dissect* fig. 3.
5. Run the gel for approximately 40 minutes at 120V.
6. Turn off power supply.

**E. Photograph the gel and save the image in the appropriate file format.**

**F. Analyze the genomic DNA for quality, molecular weight, and quantity using the guidelines delineated in Decision Tree #4.**




**Dissect fig. 3**
The order in which the samples should be loaded into the agarose gel.

**G. Using Decision Tree #4, determine, as a group of collaborators, the phage you will send to your assigned sequencing center for quality-control approval. For sequence submission, use the sequencing center's decision and the information gathered from Desision Tree #3 to determine which sample should be submitted for sequencing. Only one can be submitted for sequencing.**

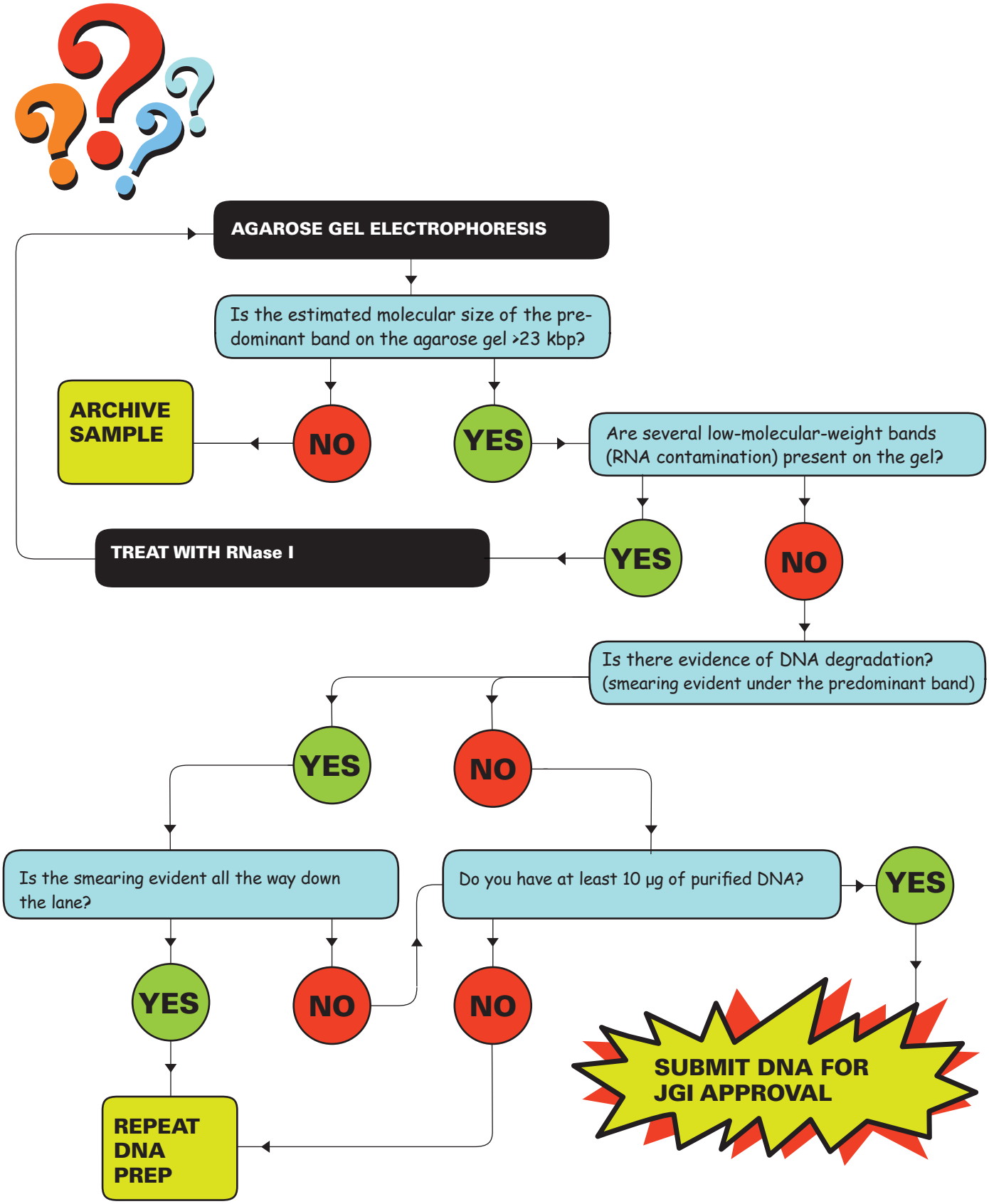
**H. Send DNA to the sequencing center according to instructions on the SEA website.**

## Questions

1. **Using the information presented in Decision Tree #4, why do you think multiple lanes of lambda DNA standards must be run on your gel?**

2. **Nucleases are enzymes that degrade nucleic acids. If your genomic DNA has nuclease contamination, what type of pattern would you expect to see on the gel?**

## Decision Tree #4
DNA to Sequencing Center



# Glossary for Part 1

**ablate.** To remove or destroy.

**adsorb (*noun* adsorption).** To take up and hold; adhesion in a thin layer of molecules to the surfaces of solid bodies or liquids with which they are in contact.

**agar.** A gelatinous colloidal extract of a red alga used in culturing media.

**agarose.** A polysaccharide derived from agar.

**agarose gel electrophoresis.** A method used to separate DNA, RNA, or protein molecules by size; a technique in which an electric field is applied to move charged molecules through an agarose matrix.

**aliquot.** To dispense a solution in exact fractions or quantities.

**amino acid.** A relatively small organic molecule consisting of an amino group on one end and a carboxyl group at the other; the protein building block.

**analyte.** A substance or chemical constituent that is determined in an analytical procedure.

**anode.** A positively charged electrode; the electrode toward which nucleic acids migrate in an electric field.

**aseptic technique.** A method used to maintain sterility and prevent contamination by unwanted organisms.

**assay.** A procedure for measuring a property or concentration of an analyte.

**autoclave.** To sterilize using superheated steam and high pressure; *noun*, an apparatus for doing the aforementioned.

**autonomous replication.** Capable of independent reproduction.

**bacteriophage (*also* phage *or* phages).** A virus that infects bacteria.

**biodegradable.** Capable of being broken down in nature.

**bioinformatics.** The field of science in which biology, computer science, and information technology merge to form a single discipline. The ultimate goal of the field is to enable the discovery of new biological insights as well as to create a global perspective from which unifying principles in biology can be discerned.

**biological database.** A large, organized body of persistent data, usually associated with computerized software designed to update, query, and retrieve components of the data stored within the system.

**capsid.** The protein shell of a virus.

**carcinogen.** A substance or agent that causes cancer.

**cloning.** The process of creating an identical copy of something.

**codon.** A specific sequence of three consecutive nucleotides that is part of the genetic code; it either specifies a particular amino acid or it starts or stops protein synthesis.

**contaminate (*noun* contamination).** To infect.

**contractile.** Capable of contracting.

**cos.** Abbreviation for "cohesive."

**covalent bond.** A chemical bond formed by the sharing of electrons between atoms.

**culture (*e.g.*, bacteria culture).** Bacteria grown in a prepared medium.

**dd$H_2O$.** Abbreviation for "double-distilled water." In the SEA project, 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).

**ddNTP.** Abbreviation for "dideoxynucleotide triphosphate."

**decant.** To draw off without disturbing the sediment or lower layers.

**denaturation (protein/DNA).** To modify the molecular structure, especially by heat, chemicals, UV radiation, or force, so as to destroy or diminish some of the original properties and/or biological function.

**deoxynucleotide triphosphate.** A building block used in deoxyribonucleic acid (DNA) synthesis.

**desiccate (*noun* desiccation).** To dry up; dehydrate.

**dideoxynucleotide triphosphate.** A modified DNA building block that prevents nucleic acid chain elongation during synthesis; a chemical used as a chain terminator during DNA synthesis.

**digest.** To break down by chemical action using enzymes.

**disinfectant.** A chemical that destroys harmful microorganisms.

**dNTP.** Abbreviation for "deoxynucleotide triphosphate."

**electron microscopy.** A type of microscopy that uses electrons to illuminate a specimen and create an enlarged image.

**electroporation.** The process of using an electric current to produce pores (or holes) in an organism's membrane through which molecules can pass.

**elute.** To remove adsorbed material from an adsorbent by means of a solvent.

**empirical assay or test.** An assay used to determine the amount of phage lysate needed for an optimal infection that generates a high-titer lysate.

**filter sterilization.** A technique used to sterilize solutions by excluding contaminants on the basis of size.

**fission.** Splitting or breaking up into parts.

**flagellum (*plural* flagella).** An elongated appendage that projects from a cell and is the primary organ of motion of many microorganisms.

**gel electrophoresis.** A technique for separating deoxyribonucleic acid, ribonucleic acid, or protein molecules using an electric current applied to a gel matrix.

**genome.** An organism's whole hereditary information. It is encoded in the DNA (or, for some viruses, RNA). This includes both the genes and the noncoding sequences of the DNA.

**genomics.** The study of an organism's entire genome.

**genotype (*adverb* genotypically).** All or part of the genetic constitution of an individual or group.

**homolog (*or* homologue).** Have the same relative position, value, or structure.

**icosahedron.** Any polyhedron having 20 faces.

**incubate.** To cause or aid the development of.

**inoculate.** To introduce a microorganism into.

**isolate.** To distinguish from, purify.

**light microscope (*or* optical microscope).** A microscope that uses visible light and a system of lenses to magnify images of small samples.

**lipids.** Important components of membranes; molecules that are generally insoluble in water and soluble in nonpolar organic solvents.

**lysate.** A solution containing the contents of disrupted cells.

**lyse (*adjective* lytic).** To cause to undergo lysis.

**lysis (*plural* lyses).** Refers to the death of a cell caused by cell membrane disruption; act of loosening.

**lysogen.** Bacterium that contains a prophage.

**lysogeny.** The mechanism by which a naïve bacterial cell becomes infected with a prophage.

**macromolecules.** Large, covalently bonded chemical structures.

**magnification.** The apparent enlargement of an object by an optical instrument.

**media.** Liquid or semisolid solutions in which microorganisms or cells can experience growth.

**micron.** A unit of measure equal to one-millionth of a meter, or one one-thousandth of a millimeter.

**morphology.** The appearance of an organism (e.g., size, shape, color, pattern, structure).

**MSDS.** Abbreviation for Material Safety Data Sheet; information accompanying chemicals that describes toxicity, flammability, solubility, hazards, etc.

**mutagen.** A physical or chemical agent that changes the genetic information (usually DNA) of an organism and thus increases the frequency of mutations above the natural background level.

**mutation.** A significant and basic alteration; change.

**mycobacteriophages.** Phages that specifically infect different species of bacteria called mycobacteria.

***Mycobacterium* (*plural* mycobacteria).** A bacterial genus. The Latin prefix "*myco-*" means both *fungus* and *wax*. In general, these bacteria have "waxy" compounds in the cell wall.

***Mycobacterium smegmatis* (*also M. smegmatis*).** An acid-fast bacterial species in the genus *Mycobacterium*. It is a Gram-positive bacterium that is generally considered nonpathogenic to healthy humans.

**myoviridae.** A family of bacteriophages.

**naïve bacterial cell.** A bacterium that has not been infected by a phage.

**negative control.** An experimental sample used to generate an outcome in which nothing happens.

**nomenclature.** Name or designation.

**nuclease.** An enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids.

**nucleic acid.** A macromolecule consisting of covalently linked nucleotides. These molecules carry genetic information or form structures within cells.

**nucleotide.** An organic molecule consisting of a sugar molecule, a nitrogenous (i.e., nitrogen-containing) base, and a phosphate group; the building block of nucleic acids.

**open reading frame.** A segment of DNA between the initiation and stop codons that potentially encodes a protein.

**orf.** Abbreviation for "open reading frames."

**PB.** Abbreviation for "phage buffer."

**pfu.** Abbreviation for "plaque-forming unit."

**phenotype (*adverb* phenotypically).** A visible physical characteristic or behavior.

**photoelectronmicrograph.** A photograph or similar image taken through an electron microscope.

**pilus (*plural* pili).** Hairlike structure on the surface of a bacterium.

**plaque assay.** A protocol used to determine the number of plaque-forming units (pfu) in a given solution.

**plaque-forming unit (pfu).** A viral particle that infects a cell and propagates itself radically to form a plaque; also called an infective center. It is used to estimate the number of viral particles in a solution; however, all the particles are not capable of infection, so this number is lower than the actual number of viral particles.

**plaque morphology.** The way the plaque looks (such as round or oblong, clear or cloudy, large or small).

**plaque titer.** A method of enumerating (counting) phage particles on the basis of the number of plaque-forming units per unit volume.

**podoviridae.** A group of bacteriophages characterized by virions with short noncontractile tails and isometric or elongated heads (there are no known mycobacteriophages in this group).

**polymerase chain reaction.** A chemical reaction in which DNA is synthesized in vitro using repeated cycles of DNA denaturation (i.e., strand separation), primer annealing, and extension (DNA synthesis). The reaction employs a high-temperature DNA polymerase (an enzyme), deoxynucleotide triphosphates (building blocks), cofactors (to make the reaction run efficiently), and buffer (to maintain constant pH).

**polynucleotide solution.** Solution containing nucleotides that have been hydrolyzed by the enzymatic activity of nucleases.

**polysaccharide.** A polymer consisting of covalently linked simple sugars (monosaccharide building blocks).

**positive control.** An experimental sample used to demonstrate the positive result of an experimental outcome.

**pristine (*e.g.,* pristine bacterial lawn).** Original state; not spoiled, corrupted, or polluted.

**prokaryotes (prokaryotic).** Usually refers to bacteria; single-celled organisms that usually contain simple circular DNA chromosome(s), lack a nuclear membrane, and have few organelles other than ribosomes.

**prophage.** A phage genome incorporated into bacterial DNA.

**proteins.** Macromolecules consisting of covalently linked amino acids. These molecules perform a diverse array of functions and include enzymes (e.g., endonuclease), transport molecules (e.g., hemoglobin), and structural molecules (e.g., collagen).

**putative.** Tentative, supposed; assumed to exist.

**quality control.** Inspection for defects or errors.

**reagent.** A substance used because of its chemical or biological activity.

**recombination.** A genetic combination not found in either parent but present in the offspring.

**replicate.** Duplicate; repeat.

**repress.** To hold back, dampen, restrain.

**repressor.** A protein that binds to DNA and prevents its transcription.

**ribosome.** A macromolecular structure consisting of RNA and protein that is found in the cytoplasm of living cells and serves as the site of protein synthesis.

**saturated.** Unable to hold or contain more.

**serial dilution.** A common method of preparing a series of a particular solution containing progressively lower solvent concentration. Each subsequent sample is prepared from the previous sample of higher concentration.

**sheared DNA.** Deoxyribonucleic acid that has been "broken" or "torn" into smaller pieces by the action of applied force.

**spot test (*or* chemical test).** A qualitative or semiquantitative procedure designed to indicate the existence of an agent (e.g., a phage), a chemical compound, or a chemical group with the aid of a specific reagent.

**standard microscope.** *See* "light microscope."

**sterile field.** An area devoid of microorganisms.

**subculture.** A culture (as of bacteria) derived from another culture.

**supernatant.** The liquid portion of a mixture from which the particles have been pelleted by centrifugation.

**temperate phage.** A phage that can exist as a prophage in infected cells and that rarely causes lysis.

**thermocycler.** A machine that repeatedly heats and cools samples to specific temperatures over a defined period of time; a laboratory machine used for the polymerase chain reaction (PCR) process.

**titer.** The concentration of phage particles in a given solution.

**titration.** A method or the process of determining the concentration of a dissolved substance in terms of the smallest amount of a reagent of known concentration required to bring about a given effect in reaction with a known volume of the test solution.

**turbidity.** Thickness or cloudiness.

**vortex (*noun* vortexer).** To mix.

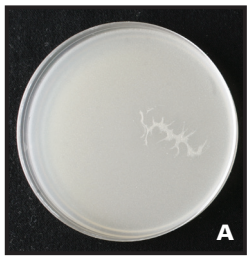
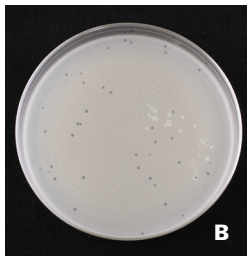
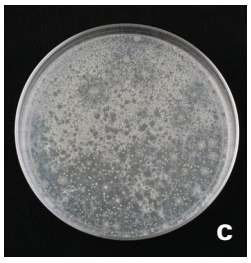
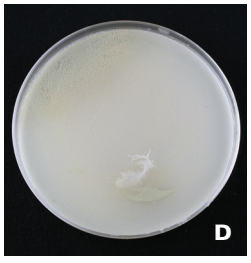
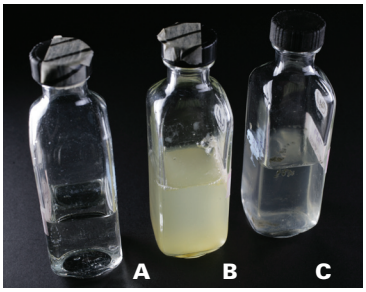
**web lysis or web pattern.** The appearance of a threadlike pattern on the surface of an agar plate resulting from almost complete lysis of a bacterial lawn by phage.

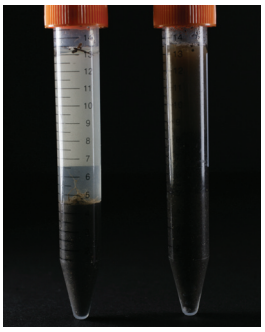
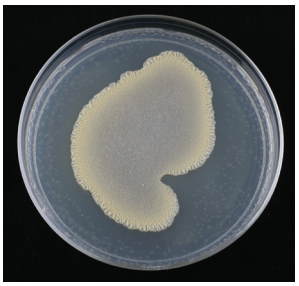
**wicking.** The ability of a substance to draw another substance into it.

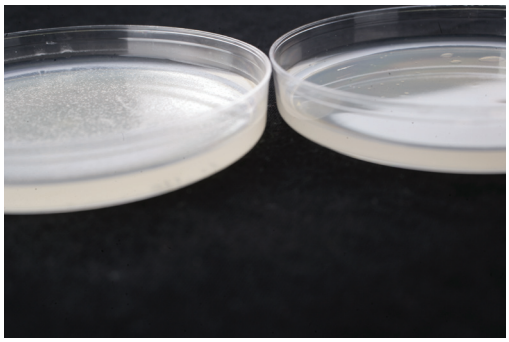
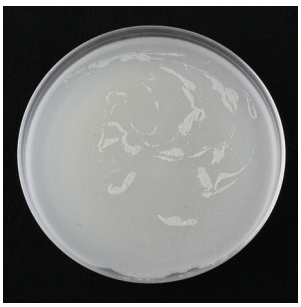
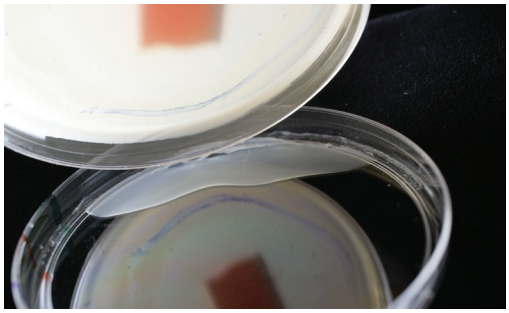
# Appendices for Part 1

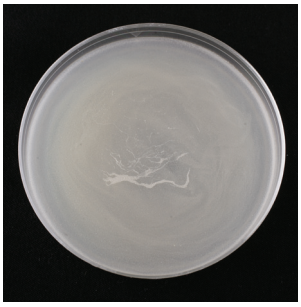
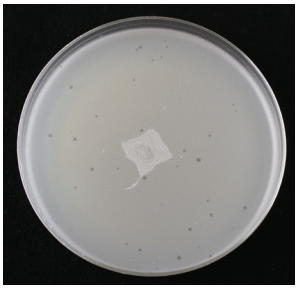
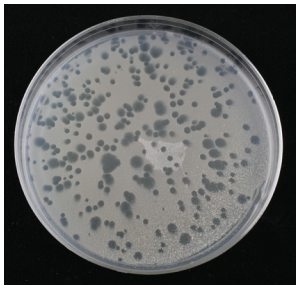
## Contents

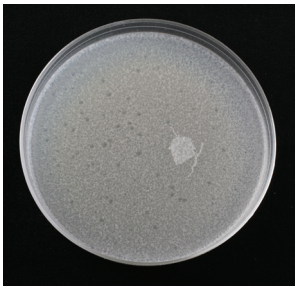
# Appendix 1. The SEA-PHAGES Troubleshooting Guide

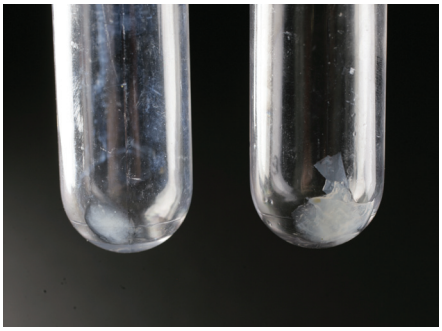
| Problem | Notes, suggestions, and solutions |
|---|---|
| Contamination of bacterial culture by<br>☐ Phage<br>☐ Bacteria<br>☐ Fungus<br><br>  <br><br>Pristine bacterial lawn.<br><br>  <br><br>Bacterial culture contaminated with phage.<br><br>  <br><br>  <br><br>Bacterial culture contaminated with bacteria (**C**) and with fungus (**D**). | Any solution suspected of being contaminated should be streaked out immediately. In other words, when in doubt, streak it out! If colonies form in 24 hours or less, they are probably not *M. smegmatis.*<br><br>In the meantime, always replace your supplies with a *full* complement of fresh media and reagents.<br><br>One control that will address several potential points of contamination is the preparation of a plate on which bacteria in top agar are spread out. A 5-$\mu$L spot of PB is then put onto the TA bacterial lawn (be sure to mark the spot!). If the spot yields plaques, the PB is contaminated with phage. Bacterial contamination of the PB will also be apparent (it will be cloudy). If the TA or the bacterial cultures are contaminated with other bacteria, phages, and/or fungi, it will be apparent over the entire surface of the agar (rather than in the marked-spot area).<br><br>Never underestimate the power of negative controls!<br><br>Treat contaminated media with broad-action disinfectant (e.g., Lysol or CiDecon) and discard. |
| Contamination of a bottle of liquid medium by<br>☐ Phage<br>☐ Bacteria<br>☐ Fungus<br><br>  <br><br>Liquid medium contaminated with phage (**A**), bacteria (**B**), and fungus (**C**). | This is likely to happen with already-opened bottles of media that were contaminated by previous use. When in doubt, set the medium aside and open another bottle. If the "contamination" worsens over time, the medium should be discarded.<br><br>Precipitates in media or other solutions can appear to be contamination even though the solutions are sterile! |

| Problem | Notes, suggestions, and solutions |
|---|---|
| A bottle of TA "looks" contaminated:<br>  <br>Top agar that looks contaminated (**left**) and that *is* contaminated (**right**). | Could be precipitated $CaCl_2$ ***or*** contamination. Do not discard TA if there is precipitate that "might" be contamination. Rather, use a new aliquot, and leave the "suspect" bottle of TA in the 55°C water bath for a day. If the TA is truly contaminated, it will be obvious by the next day. |
| A soil sample won't "settle":<br>  <br>Too much soil in the tube (**right**); correct amount (**left**). | Soil samples should fill about one-half the volume of the tube. If there is too much soil in a sample tube, it will be difficult, if not impossible, to aspirate enough liquid to filter-sterilize.<br><br>If this happens, rather than obtaining new samples, the contents of the tube should be diluted 50:50 into fresh PB and allowed to resettle. |
| If you see this:<br>  | The TA was not added. |

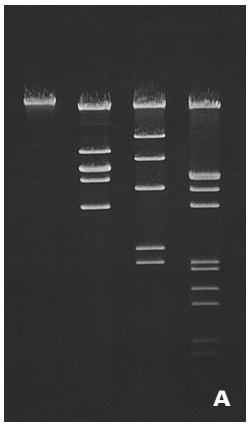
| Problem | Notes, suggestions, and solutions |
|---|---|
| If you see this:<br>  <br>Plates with no bacteria (**right**) and with bacteria completely lysed by phage (**left**). | A completely "clear" TA layer can be either the result of complete lysis *or* a plate to which the bacteria have not been added.<br><br>Do a range of lysate concentrations in an infection experiment, and *always* prepare a negative (no-phage) control. |
| If you see this:<br>  | When the TA was added, the culture tube was poorly mixed. Bacteria will "drift" to one side of a plate, and the lawn will not be homogeneous.<br><br>Be sure to mix the culture well after TA addition. |
| If you see this:<br>  | The TA was not allowed to completely solidify before the plate was inverted.<br><br>Do not move plates until the TA is completely solid. Before inverting the Petri dishes, check the TA by gently tipping the plate. It is a good idea to test with the negative control! If the TA is not solidified before the inversion of the plate, the agar will slide or fall onto the lid of the Petri dish, as shown in the photo.<br><br>No rule of thumb applies; the time that it takes for the TA to completely solidify depends on many factors, including room temperature and humidity. |

| Problem | Notes, suggestions, and solutions |
|---|---|
| If you see this:<br>  | Plates were allowed to solidify on a slanted surface.<br><br>This is not a problem. |
| If you see this:<br>  | This is an *artifact*. When the top agar is poured onto the plate, the "seams" (where the TA comes together) have a different appearance.<br><br>This is not a problem. |
| If you see this:<br>   | A (presumably) single plaque appeared to yield different morphologies when plated out.<br><br>If single plaques of both or all morphologies yield the same assortment of plaque morphologies each time, the phage is likely pure.<br><br>Pick from a plate that has fewer than 10 plaques, where plaques are far from each other. This increases the likelihood of picking only one plaque.<br><br>Perform several rounds of purification, noting the different morphologies yielded from *each* morphology type to be sure that only one phage has been isolated. |

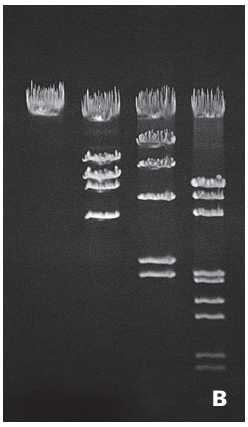
| Problem | Notes, suggestions, and solutions |
|---|---|
| If you see this:<br>  | When performing the empirical assay, you may see a clearing zone within a turbid plaque web plate.<br><br>This happens relatively often, and it does not indicate contamination. A turbid plaque morphology indicates a temperate phage. A clearing zone within an area of turbid plaques is likely due to a phage mutation ***or*** the fact that a prophage has excised from the bacterial genome and entered the lytic life cycle.<br><br>This is not a problem, but you should record the information and take a photo. |
| If you're not getting enough aspirant volume off flooding 10-plate preps, do this:<br>  | Much of the liquid can be ***under*** the agar.<br><br>Carefully flip up a corner of the agar or put pipette under the agar. Do this without spilling phage lysates, because this can contaminate the entire bench.<br><br>Use a large pipette to avoid sucking bubbles (and liquid) into the Pipette-Aid filter and thus clogging the pipettor. |

| Problem | Notes, suggestions, and solutions |
|---|---|
| The phage lysate is viscous, or "goopy," after PEG precipitation:<br>  <br>A pellet that is diffuse and that precipitated unsuccessfully (**right**); a compact pellet and successful precipitation (**left**). | Too much PEG in the phage precipitation mixture was left behind after centrifugation.<br><br>Decant off as much PEG as possible. Let the tubes sit upside-down to drain excess PEG. If necessary, perform another low-speed spin to compact the pellet, and use a Pasteur pipette to pull off excess PEG. |
| *M. smegmatis* culture is clumpy<br>  <br>Baffled fiask growth (**left**) and clumpy, nonbaffied fiask growth (**right**). | The culture should not be used. THROW IT OUT. Start a fresh culture in a baffled flask, for efficient aeration, by inoculating 1 L of fresh medium with no more than 100 $\mu$L of a saturated culture. |

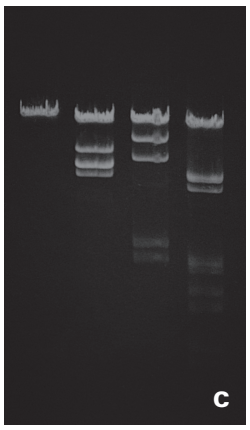
| Problem | Notes, suggestions, and solutions |
|---|---|
| Your DNA gel looks like this: | The solution to each of these problems is to pour another gel. Take care to load less sample (B), prepare the gel with buffer rather than water (C), use a lower voltage (D), and avoid bubbles when pouring (E). |



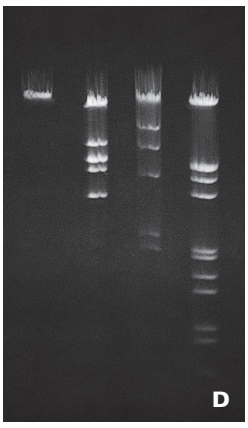

Ideal.



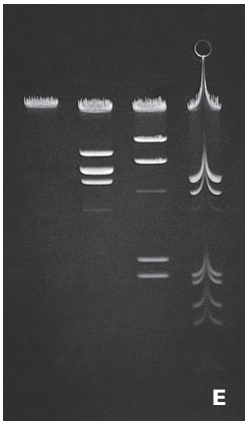

Samples overloaded.




Gel prepared with water rather than buffer.




Voltage too high.




Bubble in lane.

# Appendix 2. Additional Resources for SEA-PHAGES Participants

For more information about bacteriophages, mycobacteriophages, and phage biology and ecology, please see the following resources. This is certainly not an exhaustive list, but it will give you a place to start learning more about bacteriophages. If you find a particularly good resource that is not listed, please notify the SEA staff, and we will add it to our list.

## Websites

Phages DB
http://phagesdb.org/

American Society for Microbiology Bacteriophage Group Site:
www.asm.org/division/M/M.html

The Virology Journal "All the Virology on the WWW":
www.tulane.edu/~dmsander/garryfavweb.html

Cells Alive! Oh Goodness, My *E. coli* Has a Virus:
www.cellsalive.com/phage.htm

The Bacteriophage Ecology Group:
www.mansfield.ohio-state.edu/~sabedon

## Books

Birge EA. 2006. ***Bacterial and Bacteriophage Genetics***. New York: Springer. (ISBN 0387239197.)

Cairns J, Stent GS, and Watson JD, eds. 2007. ***Phage and the Origins of Molecular Biology, the Centennial Edition***. New York: Cold Spring Harbor Laboratory Press. (ISBN 0879695951.)

Calendar R, ed. 2006. ***The Bacteriophages***. 2nd ed. New York: Oxford University Press. (ISBN 0195148509.)

Kutter E, and Sulakvelidze A. 2004. ***Bacteriophages: Biology and Applications***. Boca Raton, FL: CRC Press. (ISBN 0849313368.)

## Book Chapter

Sarkis GJ, and Hatfull GF. 2001. Mycobacteriophages. In T Parish and NG Stoker, eds. ***Methods in Molecular Biology, Vol. 101: Mycobacteria Protocols***. Totowa, NJ: Humana Press. (ISBN 0896034712.)

## Primary Literature and Reviews

Hanauer DI, Jacobs-Sera D, Pedulla ML, Cresawn SG, Hendrix RW, and Hatfull GF. 2006. Inquiry learning. Teaching scientific inquiry. ***Science*** 314(5807): 1880–81. (PMID: 17185586.)

Hatfull GF, Pedulla ML, Jacobs-Sera D, Cichon PM, Foley A, Ford ME, Gonda RM, Houtz JM, Hryckowian AJ, Kelchner VA, Namburi S, Pajcini KV, Popovich MG, Schleicher DT, Simanek BZ, Smith AL, Zdanowicz GM, Kumar V, Peebles CL, Jacobs WR Jr, Lawrence JG, and Hendrix RW. 2006. Exploring the mycobacteriophage metaproteome: phage genomics as an educational platform. ***PLoS Genetics* 2**(6): e92. Epub 2006, June 9. (PMID: 16789831.)

Bacteriophages are the most abundant forms of life in the biosphere and carry genomes characterized by high genetic diversity and mosaic architectures. The complete sequences of 30 mycobacteriophage genomes show them collectively to encode 101 tRNAs, three tmRNAs, and 3,357 proteins belonging to 1,536 "phamilies" of related sequences, and a statistical analysis predicts that these represent approximately 50% of the total number of phamilies in the mycobacteriophage population. These phamilies contain 2.19 proteins on average; more than half (774) of them contain just a single protein sequence. Only six phamilies have representatives in more than half of the 30 genomes, and only three—encoding tape-measure proteins, lysins, and minor tail proteins—are present in all 30 phages, although these phamilies are themselves highly modular, such that no single amino acid sequence element is present in all 30 mycobacteriophage genomes. Of the 1,536 phamilies, only 230 (15%) have amino acid sequence similarity to previously reported proteins, reflecting the enormous genetic diversity of the entire phage population. The abundance and diversity of phages, the simplicity of phage isolation, and the relatively small size of phage genomes support bacteriophage isolation and comparative genomic analysis as a highly suitable platform for discovery-based education.

McNerney R. 1999. TB: the return of the phage. A review of fifty years of mycobacteriophage research. ***Int J Tuberc Lung Dis* 3**(3): 179–84. (PMID: 10094316.)

The first mycobacteriophage was isolated in 1947, and since that time, more than 250 of these viruses have been identified. Phages have made a significant contribution to our knowledge of mycobacteria over the past 50 years, and following the development of typing techniques in the 1960s and 1970s, they were widely used in epidemiological studies of tuberculosis. Unfortunately, attempts to use lytic phages therapeutically during tuberculosis infection have so far failed to elicit cure in experimentally infected animals. During the past decade, phages have become important in molecular studies of mycobacteria, both in terms of studying phage biology and as tools in recombinant DNA technology, thus facilitating the investigation of mycobacterial pathogenesis. Today their potential as diagnostics reagents is also being realized with the development of exciting new techniques for rapid bacterial detection and drug susceptibility testing. This review outlines the history of these remarkable organisms, from their discovery 50 years ago to the current developments in rapid diagnostic techniques.

McNerney R, and Traoré H. 2005. Mycobacteriophage and their application to disease control. *J Appl Microbiol* **99**(2): 223–33. (PMID: 16033452.)

The resurgence of tuberculosis and emergence of drug-resistant disease has stimulated fresh research into mycobacteriophage. Studies are under way to develop phage-based tools for therapeutic and diagnostic use. Previous attempts at mycobacteriophage therapy in experimentally infected animals were not successful and alternative strategies of phage delivery that enable killing of intracellular bacteria are required. Replication of mycobacteriophage provides a simple means of detecting viable bacteria and good progress has been made toward the development of new phage-based diagnostic tools. When screening isolates for resistance to the major anti-tuberculosis drug rifampicin phage-based tests have been shown to have high sensitivity. For the diagnosis of pulmonary tuberculosis evaluation studies indicate that current phage tests are not as sensitive as traditional culture methods. Further trials are needed to determine whether they might have a role in the detection of smear negative tuberculosis. A second generation of phage tests are under development following the construction of luciferase reporter phage. Preliminary data suggests they may offer rapid detection of mycobacteria and simple screening for drug resistance. The potential of mycobacteriophage to detect and treat other mycobacterial diseases remains largely unexplored.

Pal C, Maciá MD, Oliver A, Schachar I, and Buckling A. 2007. Coevolution with viruses drives the evolution of bacterial mutation rates. *Nature* **450**(7172): 1079–81. (PMID: 18059461.)

Bacteria with greatly elevated mutation rates (mutators) are frequently found in natural and laboratory populations, and are often associated with clinical infections. Although mutators may increase adaptability to novel environmental conditions, they are also prone to the accumulation of deleterious mutations. The long-term maintenance of high bacterial mutation rates is therefore likely to be driven by rapidly changing selection pressures, in addition to the possible slow transition rate by point mutation from mutators to non-mutators. One of the most likely causes of rapidly changing selection pressures is antagonistic coevolution with parasites. Here we show whether coevolution with viral parasites could drive the evolution of bacterial mutation rates in laboratory populations of the bacterium ***Pseudomonas fluorescens***. After fewer than 200 bacterial generations, 25% of the populations coevolving with phages had evolved 10- to 100-fold increases in mutation rates owing to mutations in mismatch-repair genes; no populations evolving in the absence of phages showed any significant change in mutation rate. Furthermore, mutator populations had a higher probability of driving their phage populations extinct, strongly suggesting that mutators have an advantage against phages in the coevolutionary arms race. Given their ubiquity, bacteriophages may play an important role in the evolution of bacterial mutation rates.

Papke RT, and Doolittle WF. 2003. Phage evolution: new worlds of genomic diversity. *Curr Biol* **13**(15): R606–7. (PMID: 12906814.)

A recent comparative survey of genomes of phages infecting mycobacteria reveals a vast combinatorial network of gene rearrangements and may provide general models for pattern and process in genome evolution.

Pedulla ML, Ford ME, Houtz JM, Karthikeyan T, Wadsworth C, Lewis JA, Jacobs-Sera D, Falbo J, Gross J, Pannunzio NR, Brucker W, Kumar V, Kandasamy J, Keenan L, Bardarov S, Kriakov J, Lawrence JG, Jacobs WR, Hendrix RW, and Hatfull GF. 2003. Origins of highly mosaic mycobacteriophage genomes. *Cell* **113**(2): 171–82. (PMID: 00928674.)

Bacteriophages are the most abundant organisms in the biosphere and play major roles in the ecological balance of microbial life. The genomic sequences of ten newly isolated mycobacteriophages suggest that the bacteriophage population as a whole is amazingly diverse and may represent the largest unexplored reservoir of sequence information in the biosphere. Genomic comparison of these mycobacteriophages contributes to our understanding of the mechanisms of viral evolution and provides compelling evidence for the role of illegitimate recombination in horizontal genetic exchange. The promiscuity of these recombination events results in the inclusion of many unexpected genes including those implicated in mycobacterial latency, the cellular and immune responses to mycobacterial infections, and autoimmune diseases such as human lupus. While the role of phages as vehicles of toxin genes is well established, these observations suggest a much broader involvement of phages in bacterial virulence and the host response to bacterial infections.

Pham TT, Jacobs-Sera D, Pedulla ML, Hendrix RW, and Hatfull GF. 2007. Comparative genomic analysis of mycobacteriophage Tweety: evolutionary insights and construction of compatible site-specific integration vectors for mycobacteria. *Microbiology* **153**(Pt. 8): 2711–23. (PMID: 17660435.)

Mycobacteriophage Tweety is a newly isolated phage of *Mycobacterium smegmatis*. It has a viral morphology with an isometric head and a long flexible tail, and forms turbid plaques from which stable lysogens can be isolated. The Tweety genome is 58,692 bp in length, contains 109 protein-coding genes, and shows significant but interrupted nucleotide sequence similarity with the previously described mycobacteriophages Llij, PMC, and Che8. However, overall the genome possesses mosaic architecture, with gene products being related to other mycobacteriophages such as Che9d, Omega, and Corndog. A gene encoding an integrase of the tyrosine-recombinase family is located close to the center of the genome, and a putative attP site has been identified within a short intergenic region immediately upstream of int. This Tweety attP-int cassette was used to construct a new set of integration-proficient plasmid vectors that efficiently transform both fast- and slow-growing mycobacteria through plasmid integration at a chromosomal locus containing a tRNA(Lys) gene. These vectors are maintained well in the absence of selection and are completely compatible with integration vectors derived from mycobacteriophage L5, enabling the simple construction of complex recombinants with genes integrated simultaneously at different chromosomal positions.

Rohwer F. 2003. Global phage diversity. *Cell* **113**(2): 141. (PMID: 12705861.)

Ten new mycobacteriophage genomes presented by Pedulla et al. (2003) show that most phage diversity remains uncharacterized. Extrapolation suggests that less than 0.0002% of the global phage metagenome has been sampled. The new genomes also contain a number of potential virulence factors that may be important in pathogenesis.

# Appendix 3. Archiving and Reporting Your Phage

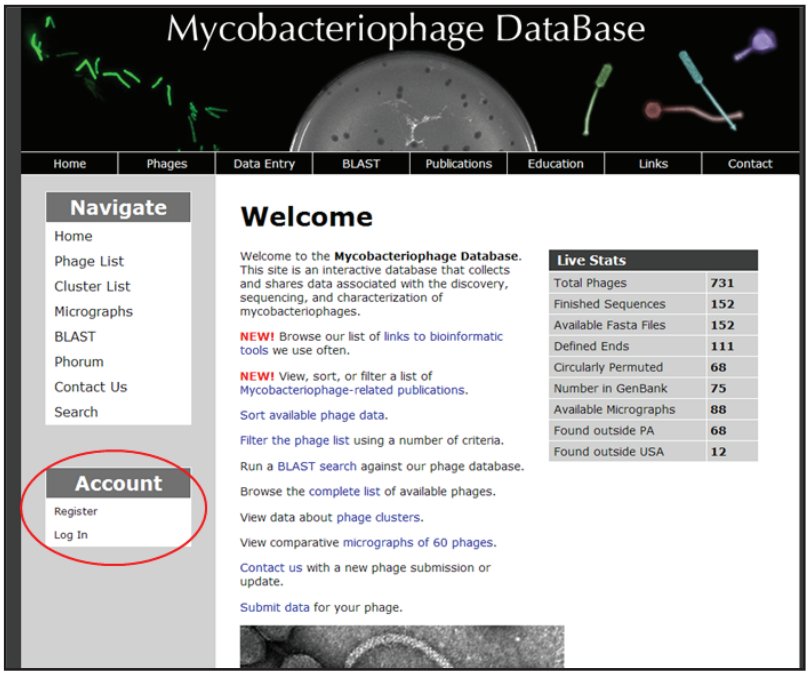
## Overview

Two sets of samples of each phage lysate must be archived. One set will be sent to the University of Pittsburgh; one set will be held at your institution for your archiving. *You must enter information for all phages isolated, whether or not they are sequenced, onto the PhagesDB database site. Do not enter phages into the database without archiving, and do not archive phage samples without entering their complete information to phagesdb.org.*

**Please check the SEA Wiki for the most up-to-date archiving protocol information.**

## I. Create an account at PhagesDB

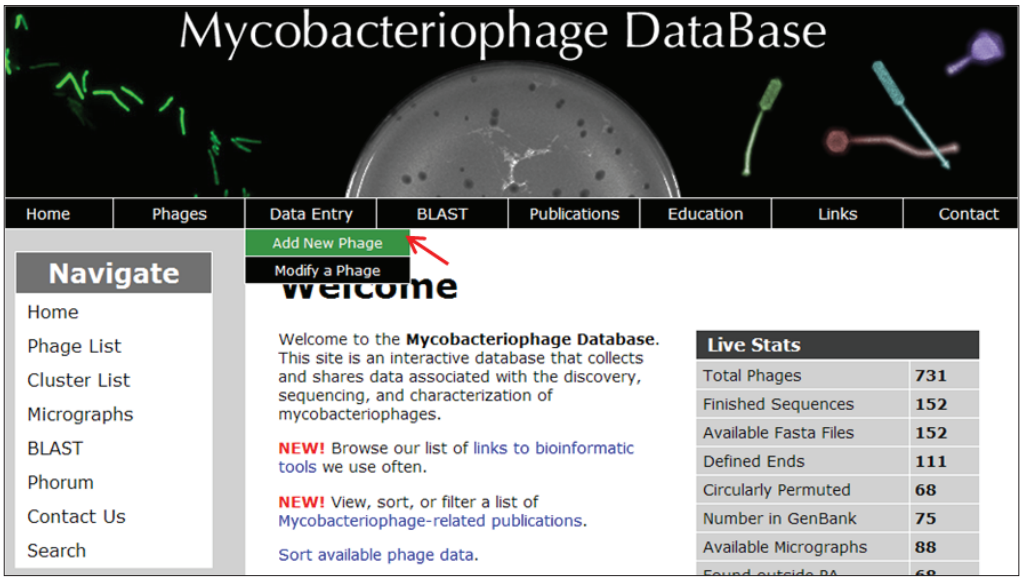
1. Go to http://phagesdb.org/
2. On the "Account" pane to the left, click "Register". (See *Appendix* 3 fig. 1)
3. Fill out the information required to establish an account.
4. You MUST follow the link in the email you receive to activate your account (Emails are sent immediately. If you do not receive the email soon after registering, check to make sure that no-reply@phagesdb.org is not being filtered into a "spam" file by your email program).



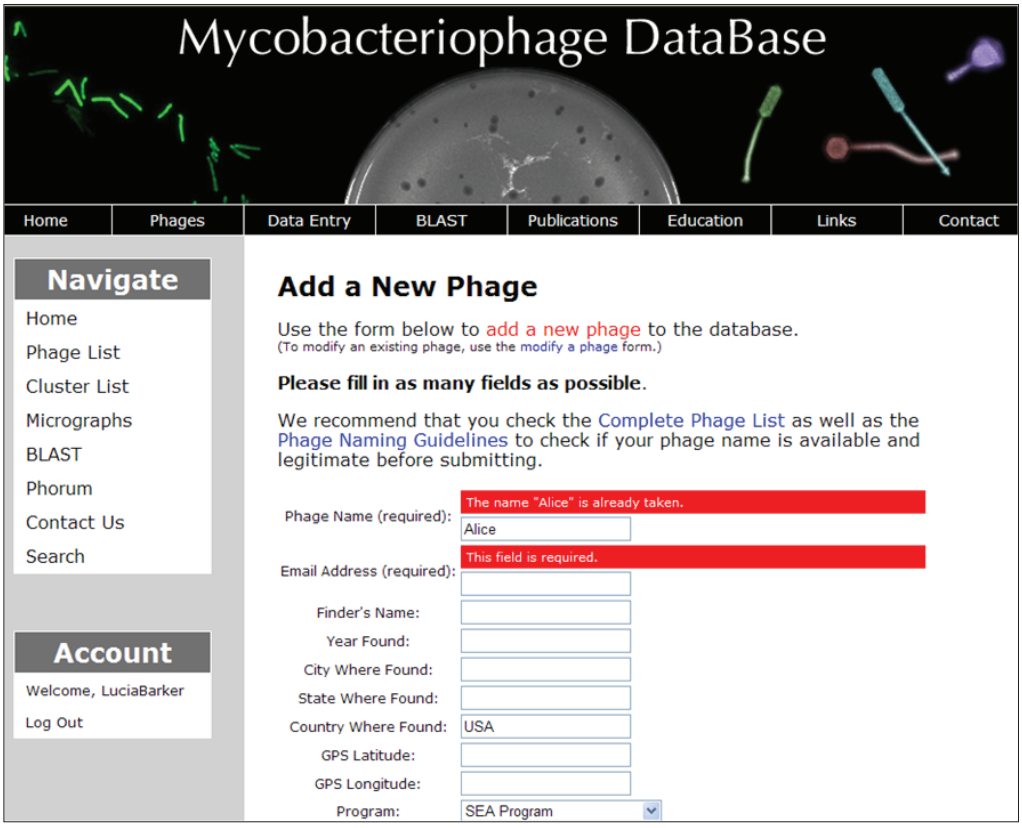

**Appendix 3 fig. 1**
The "Account" tab. Use this to create a new account or to log into PhagesDB.

## II. Check that your phage name has not yet been used

1. Go to http://phagesdb.org/
2. On the "Account" pane to the left, click "Login". (See *Appendix* 3 fig. 1)
3. Log in using your new account information.
4. Using the "Data Entry" Tab, pull down to "Add new phage". (See *Appendix* 3 fig. 2)
5. Enter your phage name into the "Phage Name" field.
6. If you receive notification that the name has already been used, you must modify/change the name (see *Appendix* 3 fig. 2).
7. Once your phage name has been decided, assign a Phage Designator as outlined below and assemble your phage information.




**Appendix 3 fig. 2**
The "Data Entry" tab. Use this to check on your phage name and to enter information about your phage.




**Appendix 3 fig. 3**
Adding a new phage. If you enter a phage name that has already been used, you will receive a notification in red.

## III. Assign a Phage Designator

This will include, without spaces:

- ☐ The year the phage was isolated from the soil
- ☐ Your university's four-letter designation (upper case; see the SEA Wiki for your school designation)
- ☐ The name of the phage (lower case)
- ☐ The student's initials (upper case)

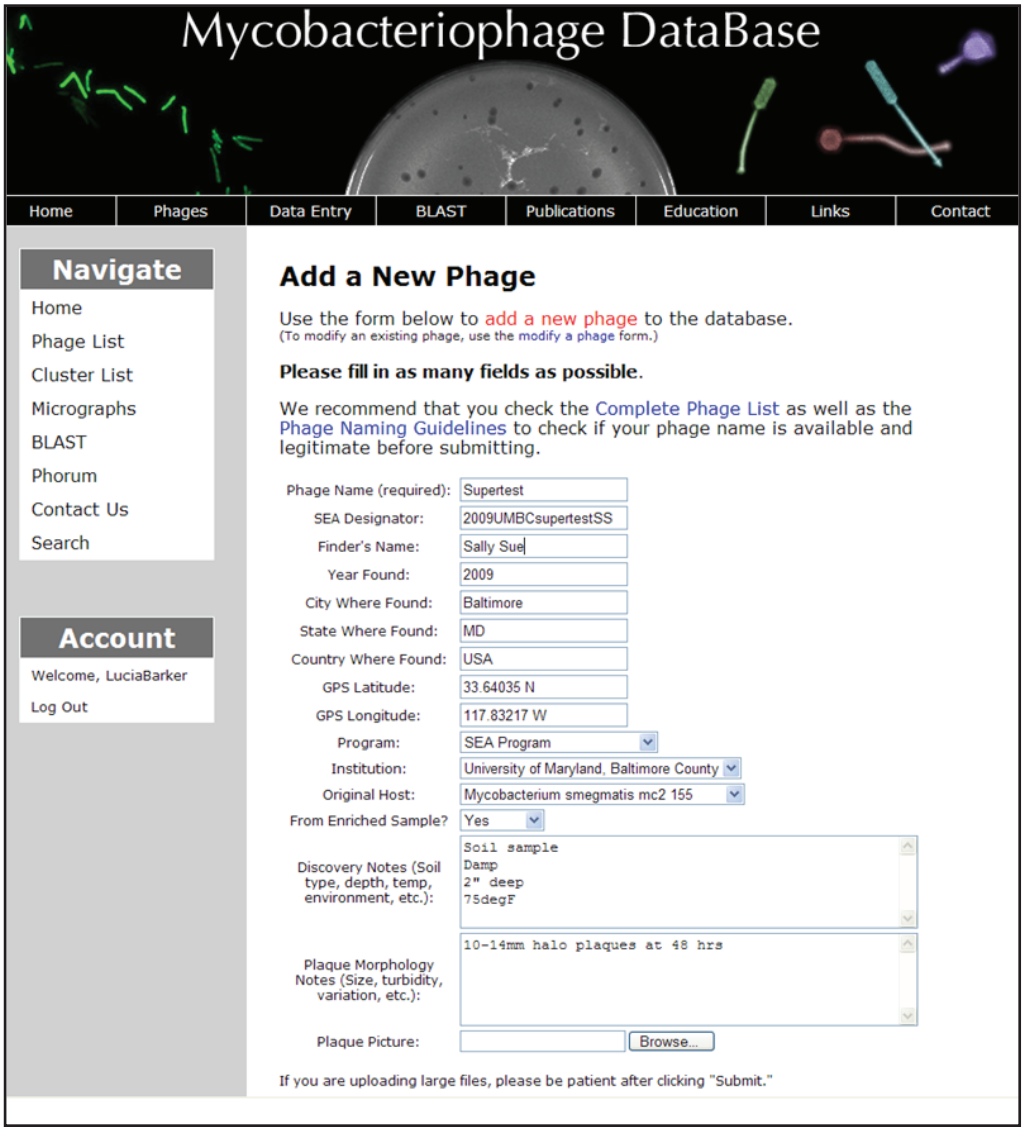
*For example:* Mary Jane Davis is sending her archived sample in the spring of 2011, but the phage was isolated in September of 2010. She goes to the University of North Kansas and the name of her phage is Henry. The phage designator is **2010UNKAhenryMJD**.

## IV. Complete your phage report on PhagesDB

1. Log in to http://phagesdb.org/ as above.
2. Using the "Data Entry" tab, pull down to "Add new phage". (See *Appendix* 3 fig. 2)
3. Enter your phage name into the "Phage Name" field.

4. Enter your Phage Designator into the "SEA Designator" field.
5. As directed, enter all other available information into the fields (see *Appendix* 3 fig. 4).

**Note: Do not hit "enter" or "Submit" until you are done with your submission. Once you are notified that your phage is added to the database, you can edit and/or add more information using the "Modify a Phage" menu from the "Data Entry" dropdown.**




**Appendix 3 fig. 4**
Adding your phage to the database. After logging in to PhagesDB, enter all of your available phage information. As more data and images become available, you can add these using the "Modify a Phage" tab.

## V. Prepare to archive your samples

1. Download the "Freezer Box Inventory" ("FBI")from the SEAWiki at: http://www.hhmi.org/seawiki/display/EduRes/SEA+Research+Archive
2. Assemble the following materials from the archiving kit that you obtained from the SEAlab:
   - □ Sterile 75% glycerol
   - □ Freezer boxes (2 per school—*one to send to the University of Pittsburgh, one to keep*)
   - □ Sterile cryotubes (2 per phage)
   - □ Styrofoam shipping box
   - □ 2 plastic bags
   - □ 1 FedEx shipping label
3. Assemble from your laboratory, for each phage:
   - □ At least 300 $\mu$L of high-titer phage lysate (>$10^9$ pfu/mL)
   - □ A sterile microcentrifuge tube

   Note: As of Fall 2010, the SEAlab is no longer accepting DNA samples for archiving

## VI. Prepare phage lysates for archiving:

1. Label two cryotubes for each phage lysate with **the phage designator, date and titer.**
2. Place 300 $\mu$L of lysate into *a microcentrifuge tube*.

   NOTE: Before transfer, be sure to mix the lysate gently but well, especially if it has been stored for more than a few days.
3. Add 600 $\mu$L of 75% glycerol to the lysate and mix well by gently pipetting up and down. This yields a final glycerol concentration of 50% in PB.
4. Distribute 300 $\mu$L into each of the two cryotubes.

   NOTE: The titer on the tube label is the titer BEFORE glycerol addition.
5. Place one of each replicate phage sample into the same position in two separate freezer boxes.
6. Record the phage designations and titer on the "FBI" sheet.
7. **Refrigerate the freezer box until the box contains all of the samples. Do NOT freeze/thaw! Once the box is ready, ship on ice to the University of Pittsburgh.**

## VII. Send and store freezer boxes

1. Place one freezer box into Ziplock® bags and seal.
2. Using the Styrofoam shipping box with which the archiving kit was sent and the provided FedEx label, please mail the one cryoboxes to the University of Pittsburgh on wet ice OR with cold packs. ***See shipping checklist below.***
3. Prepare a sealed plastic bag containing a hard copy of the ("FBI").
4. Place the bag on top of the Styrofoam insert.

   NOTE: Do NOT ship archive samples on dry ice.
5. Place the other freezer box into a freezer at your institution (at –80°C) for long-term storage.
6. Double check all of the items on the "Archiving Checklist" below.
7. Do not forget to send an electronic version of the "FBI" to the University of Pittsburgh at phagearchive@gmail.com.

## Archiving checklist:

1. Styrofoam shipping box (mailed to the University of Pittsburgh):
   - □ One freezer box (sealed in plastic bag) with all phage lysates
   - □ Ice and/or gel packs
   - □ One CD containing an electronic version of the freezer box inventory (or confirmation that you sent the list via email.)
   - □ A sealed plastic bag containing a hard copy of the freezer box inventory and the CD (on top of the Styrofoam insert)

     NOTE: Please be sure to send a hard copy of the freezer box inventory with the freezer box!
2. Stored at your institution:
   - □ A replicate freezer box (at –80°C) with all phage lysate and DNA samples
   - □ Electronic versions of the freezer inventory file

## Important notes:

- □ Students MUST generate a Phage Designator and use this to physically label the tubes containing the phage lysate samples that will be archived!
- □ An electronic copy of the Freezer Box Inventory must be sent to the University of Pittsburgh!
- □ All of the phage information will be permanently stored on PhagesDB.

# Appendix 4. Format for Research Papers

One of the most important steps in any research project is effective communication of the findings. Work buried in a laboratory notebook is not going to inform or inspire our collaborators or other researchers who might benefit from the discoveries we have made. To truly contribute to our body of scientific knowledge, the content must be well organized, effectively summarized, and soundly interpreted. Examples of properly formatted papers are on the SEA website, www.hhmi.org/sea. Papers should be double-spaced, have 1-inch margins, and use a font size of at least 10 points and black ink. Acceptable font choices are Times New Roman, Arial, Helvetica, Garamond, and Courier. Symbols can be used when appropriate.

## Sections to Include in SEA-PHAGES Research Papers

### Title
A short descriptor (10 words or fewer) that gives the reader an idea of the paper's content.

### Author
A list of all individuals who made a scientific contribution to the described work. If there is more than one author, the primary author (the person who did the majority of the experiments) should be listed first. The senior author (usually the faculty member) should be the last name in the list. All other contributors should be listed in the order of the amount of work contributed to the publication (in order of most to least). If their contributions are equal, they should be listed alphabetically.

### Institutional address
Course name and number, department, institution, city, state, zip code.

### Abstract
A summary of the data presented in the paper. This usually consists of a short introductory statement (one to three sentences), a brief description of the presented data, and a single sentence describing the relevance of the presented work.

### Introduction
An overview of known information that nicely summarizes the state-of-the-field as it relates to the presented work. It should be focused and concise. A well-written introduction should guide the reader through previously published work in such a way that the work the author presents appears to be the next logical step in the scientific process.

### Materials and methods
A compilation of all of the reagents and specialized equipment used in the described experiments and a description of all of the methods employed to generate the data. It is acceptable to reference an already-published procedure if it was used without modification. If any modifications were made, these must be described after the original work is referenced in sufficient detail to allow the reader to repeat the experiment under the precise experimental conditions used in the paper with the same reported results.

## Results
A description of each of the experiments performed with a brief statement of rationale and the resulting data. Data can be depicted in tables, graphs, charts, photographs, and micrographs. Some data that serve to substantiate a point can be simply described along with the phrase "data not shown."

## Discussion
A summary of the presented data, interpretation of the data from each experiment, the relationship of the presented data to other published work, and speculation about the potential impact on the field. The discussion should also provide a hint of future directions.

## Acknowledgments
A list of people and why they are being acknowledged. Generally, people are acknowledged for reading and commenting on drafts of the paper, providing technical support and intellectual stimulation, etc.

## Funding
Agencies that provided funds to support the described work.

## References
A list of references cited. The format should be: Authors (last name and initials, each separated by a comma). Year published. Title of article. *Name of journal* **volume number**: pages. An example:

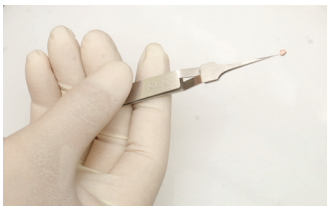
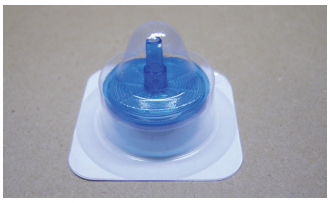
> Summer EJ, Gonzalez CF, Bomer M, Carlile T, Embry A, Kucherka AM, Lee J, Mebane L, Morrison WC, Mark L, King MD, LiPuma JJ, Vidaver AK, and Young R. 2006. Divergence and mosaicism among virulent soil phages of the *Burkholderia cepacia* complex. *J Bacteriol* **188**: 255–68.

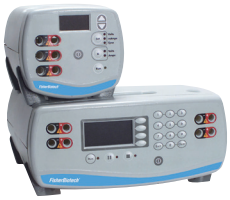
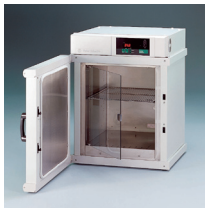
## Figures
Should be legible and appropriately labeled (content, axes, symbols, etc.) and presented in the order in which they appear in the manuscript. Figure numbers should be on all figures, and captions and legends should be included.

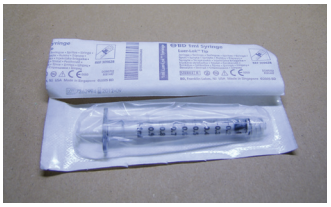
## Tables
Should be legible, appropriately labeled, and presented in the order in which they appear in the manuscript. Tables do not have legends, so all the information required to interpret the data should be included in the body of the table. Table numbers should also be included.

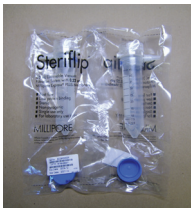
# Appendix 5. SEA-PHAGES Equipment and Supplies

| | Name | Alias | Equipment Purpose/Use |
|---|---|---|---|
|  | Automatic pipettor | Pipette-Aid, automated pipettor | Aspirating (drawing up by suction) large volumes |
|  | Baffled flask | Special growth flask | Aerating and growing bacterial cultures |
|  | Bunsen burner | Flame, burner | Generating aseptic field; heating samples |
|  | Capillary tweezers | Really, really small tweezers | Manipulating EM grids |
|  | Centrifuge, bench-top | Bench-top 'fuge, bench-top | Separating substances of different densities using centrifugal force |
|  | Centrifuge, high-speed | High-speed 'fuge, Sorvall | Separating substances of different densities using centrifugal force (generates more centrifugal force than a bench-top centrifuge) |

| | Name | Alias | Equipment Purpose/Use |
|---|---|---|---|
|  | Centrifuge, micro | Microfuge, Eppendorf | Centrifuging samples contained within microcentrifuge tubes |
|   | Electron microscopy grid | EM grid, grid | Immobilizing samples to be viewed by electron microscopy |
|  | Filter, syringe sterilization, 0.22-$\mu$m | Syringe filter, 0.22-micron syringe filter | Sterilizing small sample volumes through a filter of small pore size; generally removes all but the tiniest of organisms (e.g., phage) |
|  | Filter, sterilization, 0.22-$\mu$m, 100-mL | Filter-sterilization unit | Sterilizing larger volumes through a filter of small pore size; generally removes all but the tiniest of organisms (e.g., phage) |
|  | Freezer | –20 | Maintaining samples at temperatures below freezing, generally at –20°C (–4°F) |

| | | | |
|---|---|---|---|
|  | Gel apparatus power supply | Power supply | Supplying electrical current to the electrophoresis equipment via electrodes |
|  | Gel apparatus, horizontal | Electrophoresis setup, agarose gel equipment | Casting agarose gel and electrophoresing samples |
|  | Gel-documentation photography system | Gel doc | Visualizing and photographing bacterial growth plates and electrophoresis gels |
|  | Heating block | | Maintaining a constant temperature using dry heat |
|  | Hood, fume | Hood, chemical hood | Removing vapors and odors emitted by chemicals by ventilating them to a designated area |
|  | Incubator, forced-air or radiant-heat | Incubator | Maintaining bacterial growth at a constant temperature |

| | Name | Alias | Equipment Purpose/Use |
|---|---|---|---|
|  | Incubator, shaking | Shaker, shaking incubator | Maintaining liquid cultures at constant temperature with shaking (to aerate the cultures) |
|  | Micropipettor | Pipettman, pipettor | Dispensing small (<1-mL) volumes |
|  | Pipette, serological | Pipet | Dispensing volumes ranging from 0.5 to 50 mL |
|  | Refrigerator | 'Fridge, 4° | Cooling and maintaining samples to 4°C (39.2°F) |
|  | Syringe, tuberculin | 1-mL syringe, TB syringe | Aspirating samples (e.g., through a 0.22-$\mu$m filter) |
|  | Transilluminator | UV light box | Visualizing ethidium-bromide-stained gels |

| | Name | Alias | Equipment Purpose/Use |
|---|---|---|---|
|  | Tube, centrifuge, 15-mL | Oak Ridge tube | Centrifuging samples at high speed |
| 15 mL  50 mL  | Tube, conical, 15-mL or 50-mL | 15-mL Falcon tube or 50-mL Falcon tube | Preparing, manipulating, and storing samples |
|  | Tube, glass culture, 10-mL | Culture tube | Preparing and manipulating bacterial cultures |
|  | Tube, microcentrifuge | Microfuge tube, Eppendorf tube, Eppi | Preparing and manipulating small (up to 1.5-mL) samples |
|   | Tube, Steriflip | | Filter-sterilizing liquid samples (10-to-50-mL volumes) |

| | Name | Alias | Equipment Purpose/Use |
|---|---|---|---|
|  | Vortexer | Genie mixer, Vortex | Mixing samples by gyration |
|  | Water bath | | Maintaining samples at a constant temperature; uses wet heat (i.e., water) |