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Phagehunting with Introductory Biology Students

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Viruses that infect bacteria are the most numerous biological entities on Earth. These bacteriophages (phages) are found everywhere, including the soil in campus flowerbeds, students' backyards, and local parks. By implementing this project, students can isolate novel phages, which can subsequently be purified, characterized, and used to expand our growing scientific understanding of phage genomics. This protocol is designed for the bacterial hosts *Mycobacterium smegmatis* and *Acinetobacter baylyi*, but other species could be used. Unlike many introductory level biology labs, this project can and has produced results from student experimental work worthy of inclusion in articles in the scientific literature.

Keywords: bacteriophage, *Mycobacterium, Acinetobacter,* phagehunting, phage, discovery

Introduction

There is growing interest in the scientific community about the genomic diversity of one of the most numerous biological entities on the planet: bacteriophages. These tiny (<0.2 μm) viruses play a critical role in gene exchange between bacteria, which can influence pathogenicity. Luckily, this important area of active research is extremely accessible to introductory biology students of all ages. The protocol included here focuses on the process of isolating novel bacteriophages from soil samples brought in by students. Students enjoy the possibility of finding a completely novel bacteriophage in their backyard. Once novel phages have been isolated, protocols are available at **www.phagesdb.org** to further purify them, to conduct morphology analysis, to isolate their DNA, and to conduct genomic sequencing and bioinformatic analysis. These further analyses are appropriate for independent research projects and/or more long-term laboratory work. Phagehunting can be used to introduce the concepts of viruses, aseptic technique, bacterial diversity, and the ubiquitous nature of bacteria and viruses to beginning students, as well as concepts of genome organization and gene exchange. The lab also provides an excellent way for faculty to link together their individual pedagogical goals with the research goal of extending our knowledge about the genome sequences of some of the $>10^{31}$ bacteriophages that exist on Earth.

This discovery-based laboratory program has been successfully conducted with students at a wide range of levels, from middle school through to upper-level undergraduates and beyond. Environmental soil samples collected by the students are processed and used to infect laboratory strains of bacteria. The potentially infected bacteria are plated to allow visualization of plaques resulting from phage activity. We use here our favorite host bacteria, *Mycobacteria smegmatis* and *Acinetobacter baylyi,* but the procedure can be modified to suit other bacteria typically found in soil that can be grown in the lab. Selection of the bacteria to be used and preparation of appropriate media must be done in advance, and will vary depending on the organism. The procedure consists of three steps that can be completed within a 50 minute class period (although more time is better!), and resulting plaque formation can be observed the next day after incubation at an appropriate temperature. The first step is mixing of students' soil samples with buffer. The second step is filtering the settled mixture through a 0.2 μm filter to isolate phage particles. Finally, the isolated phage is incubated with the bacteria and plated in a low percentage top agar.

After incubation, plates are observed and students look for clear plaques, regions in a thick lawn of bacterial growth where phages have destroyed the bacteria. Not every student, or even every classroom of students, will discover a phage that infects the bacterial strains utilized. For students who DO find a phage, the next steps really depend upon the student and the instructor. It is important to note that due to the huge number of phage particles and the dynamic nature of phage genomes, any phage isolated by a student is novel to the scientific world as well. This is truly discovery science! Of course, before one calls the press, the result must be confirmed. The confirmation process and other steps to continue investigation of a newly discovered phage are de-

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scribed in Appendix C and through the PhageHunters Website (**http://phagesdb.org**/.)

The protocol presented at the 2010 ABLE conference and included here in the Student Outline describes the first step in a research endeavor for entry-level students to engage in research. This research program includes not only the microbiology described here, but uses molecular biology and bioinformatic techniques and tools to characterize and analyze phages and their genomes. With sequencing costs becoming affordable, the potential for more students to be involved is now a reasonable consideration. Individual instructors and departments are welcome to adapt these protocols to use in their courses; participation in a specific program is not required.

Colleges and universities from across the country are using this program as their introductory biology courses. These schools participate in Howard Hughes Medical Institute's Science Education Alliance's (SEA) first initiative, the National Genomics Research Initiative. You can find more information at **www.hhmi.org/sea**.

For scientific publications built upon the results of student phagehunting, see Hatfull et al. (2010), Hatfull et al. (2006), and Pedulla et al. (2003). For a more thorough consideration of pedagogical and assessment aspects of this exercise, see Hanauer et al. (2006) and Hanauer, Hatfull and Jacobs-Sera (2009).

Student Outline

Introduction

For this lab, each of you will have the chance to discover a new virus living in your own backyard. You have likely heard of bacteria and viruses primarily as infectious agents responsible for annoying colds as well as serious illnesses. Hopefully, you have also heard that bacteria are actually found everywhere - on your skin, in your gut, in every house (clean or dirty) and even in the soil. What you may not know is that those ubiquitous bacteria are also susceptible to viruses. There are viruses that infect all types of living organisms from humans to mice to insects to worms to yeast to bacteria. Viruses are tiny particles containing genetic material (DNA or RNA) typically surrounded by a simple coating of protein. They are not cells, but they infect cells and use cellular machinery to reproduce. For historical reasons, viruses that specialize in infecting bacterial cells are called bacteriophages, or simply phages for short. Phages, bacteria, and humans all share the same genetic code, many similar proteins, and many similar strategies for survival.

In this lab, we will be searching for phages that infect two different types of bacteria, *Mycobacterium smegmatis* and *Acinetobacter baylyi*. Both are non-pathogenic soil bacteria. However, *M. smegmatis* is related to the pathogenic *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and *A. baylyi* is related to *Acinetobacter baumannii*, a common pathogen of growing concern in hospital-acquired infections. Phages that we find that can infect the lab strains may also be able to infect these pathogens, thereby potentially providing new tools for research and potential treatment. Further, basic biological understanding of the diversity of phages may have applications we cannot even imagine today. It has been estimated that there are 10^{31} phage particles on earth. This number is nearly incomprehensible. We guarantee that there will be phage particles in the soil sample you bring with you to class. However, only a subset of those phages will be able to infect *M. smegmatis or A. baylyi*, so the outcome of our investigation will be a surprise to all of us.

Let's go do some phage prospecting!

Materials

Soil samples	Microcentrifuge tubes
Phage buffer	0.2 µm filters
Pipettes	Syringe
Automatic pipettor	Top agar (0.35% agar)
Micropipettors, tips	Bottom agar (7H10)
Collection bags	*Acinetobacter baylyi*
Conical tubes	*Mycobacterium smegmatis*
Centrifuge tubes	Microwave and incubator

Procedure

1. Place 3 – 5 g of soil sample in 15 mL capped conical tube. Add 10 mL of phage buffer. Seal tube and invert, mixing well, to release phage into the buffer.

2. Allow the sample to settle.

3. Remove ~ 1 mL of the supernatant (the liquid portion) into a syringe. Connect the syringe to a 0.2 µm filter. Push the fluid into a sterile microcentrifuge tube that is labeled with the specimen number, your initials, and the date. Record specimen number and source, and your name on the appropriate chart. Specimen is now considered sterile and ready for further testing.

4. *Sterilely* add 50 µL of sample into a tube of 1.0 mL of bacterial culture. Mix well and wait 10 minutes. This time will allow the bacteriophage and bacteria to interact. You will prepare two tubes, one with *M. smegmatis* and one with *A. baylyi*.

5. Starting with one tube of phage-bacteria mixture, sterilely pipet 4.0 mL of 55°C 0.35% top agar into the tube containing the phage-bacteria mixture. Immediately pour the mixture onto a labeled 7H10 agar plate and disperse it evenly. To disperse the top agar over the plate evenly, rotate the plate both clockwise and counter-clockwise while it is flat on the table. This entire process must be done ***quickly*** so that the top agar is smooth.

6. Allow this plate to **REMAIN STILL** until the agar has cooled and hardened.

7. Once cooled, invert the plate and incubate at 30°C for *A. baylyi* and 37°C *M. smegmatis* for 24 – 48 hours.

8. Observe the bacterial lawn for plaques. Plaques are clearings in the lawn of bacteria in the top agar. They are evidence of phage infection.

9. If plaques are observed, phages must be purified through additional rounds of infection before further characterization by electron microscopy, DNA purification, restriction digest analysis, genome sequencing, and bioinformatic analysis. Information on newly found mycobacteriophages is constantly being added to **www.phagesdb.org.**

Rationale

In steps 1 and 2, phages present in your soil sample are being moved from the surface of the soil particles into the aqueous solution of phage buffer. Heavy soil particles are allowed to settle so that phages may be collected from the solution. Because phages are so tiny, they diffuse very quickly, and do not settle with the larger particles.

In step 3, the phages, typically 50-100 nm in diameter, are separated from bacterial cells (typically 1-10 µm in diameter) and eukaryotic cells (typically 10-100 µm in diameter.) Only particles smaller than 0.2 µm can move through the filter into the microcentrifuge tube.

In step 4, the bacteria and potential phages are allowed to mix, giving the phage particles time to recognize and begin to infect the bacterial cells.

Steps 5-7 describe plating the bacteria/phage mixture onto solid media. You may be familiar with solid media for growing bacteria, typically made by mixing specific nutrients with 1.5% agar, which solidifies the nutrient media. Often bacteria are grown on solid media at low concentration to allow a single cell to divide into a large, visible colony on the surface of the agar. For this assay, we use a very concentrated sample of bacteria so that the whole surface of the plate will be covered with a cloudy bacterial lawn. To get the bacteria to spread evenly, they are mixed with a small amount of "top agar," which is made with the same nutrients but a lower concentration of agar (we use 0.35%.) This thin layer of top agar/bacteria/phage mixture must be spread evenly and completely over a typical agar plate and allowed to solidify. The plates are then incubated at an optimal temperature for growth of the particular bacterial strain used.

Step 8 is where the magic happens! Okay, so it's really where the science happens, but it's sometimes just as exciting as a magic show. Remember that phages are viruses that reproduce in and usually destroy their bacterial host as they release hundreds of copies of themselves into the environment. If a phage is able to infect a bacterium in our bacterial lawn, it will kill that bacterium and spread to other nearby bacteria. The phages will then kill those bacteria and spread to other nearby bacteria. The spreading process occurs in a way that leads to circular regions where no bacteria are present in the bacterial lawn. Basically, what we will be looking for on our plates of happily growing bacterial lawns are ... ZONES of DEATH. Officially, these regions where phages have killed the bacteria are called plaques. One important observation to make about any newly discovered phage is its plaque morphology. Plaques can be small or large, clear or turbid. They are often easiest to see by holding the plate up so that light shines through the bacterial lawn. If there are circular regions where the light shines through more clearly compared to the rest of the lawn, you just may have found a phage!

Step 9 describes some of the ways in which newly isolated phages can be further characterized, ultimately contributing to the body of scientific knowledge. Specific next steps will be varied.

Post-Lab Questions

1. What is a phage?

2. What method did we use to isolate bacteriophages from other components of soil?

3. How does a plate of medium alone differ from a plate of medium upon which a lawn of bacteria is growing?

4. How could we detect the presence of phages on a plate containing a lawn of bacteria?

Materials

- Soil sample, each student should bring in a soil sample from home
- Phage buffer, 10 mL per soil sample/student
- 0.2 µm filters, 1 per soil sample/student
- 1 mL syringe, 1 per soil sample/student
- Top Agar (0.35% agar), 4 mL per plate/8 mL per student
- Bottom Agar (7H10), 2 plates per sample/student, one for each bacterial host used, more if controls are used
- *Mycobacterium smegmatis* – 1 mL liquid culture per sample
- *Acinetobacter baylyi* – 1 mL liquid culture per sample
- 5 mL sterile pipettes, 1 per plate/2 per student, can be omitted if instructor dispenses both phage buffer and top agar
- Automatic pipettors, 1 per class
- P-200 micropipettor, 1 per 2-4 students
- Sterile micropipettor tips, 2 tips per student
- Sterile test tubes, 2 per sample/student
- Sterile 15 mL conical tubes, 1 per sample/student
- Sterile microcentrifuge tubes, 1 per sample/student
- Incubator (30°C for *A. baylyi*, 37°C for *M. smegmatis*. Room temperature incubation is also feasible)
- Microwave
- Internet access for bioinformatics extension exercises

Notes for the Instructor

Bacterial Strains and Media

- Cultures of host bacteria are required for the lab. Use of bacteria requires early planning, dependent on the organism.
  - *Acinetobacter baylyi* ADP1 (ATCC # 33305) should be prepared fresh for the protocol, inoculating M9 growth media (recipe in Appendix A) the night before and shaking at 30°C overnight.
  - *Mycobacterium smegmatis* mc^2 155 (ATCC # **700084)** liquid cultures can be used for weeks after growth of the culture. Note that *M. smegmatis* is a fast growing mycobacterium. Its doubling time is about 2 ½ - 3 hours, so a colony would form in 3-4 days. Overnight liquid cultures can be grown. The mycobacterium's cell wall contains mycolic acids, long chain fatty acids. Mycolic acids provide a terrific matrix for biofilm formation, thereby clumping the bacteria in liquid cultures. Growth conditions must be strictly followed to ensure proper growth of an appropriate liquid culture for phagehunting.
- The growth media used for both bacteria is 7H9.
- A full listing of media recipes can be found at **http://phagesdb.org/**

Safety Concerns

Both *Mycobacterium smegmatis* and *Acinetobacter baylyi* are BSL-1 organisms. They are not pathogenic and can be used safely in a teaching laboratory. Care should still be taken to avoid introducing these lab-reared organisms into the environment. All bacterial cultures and plates, as well as any plastic items or glassware that contacts bacteria should be autoclaved before disposal. Students should be cautioned to disinfect any spills, disinfect their workspace after the procedure, and wash hands after finishing their work.

Soil Samples

- Note that the point of the project is to check for bacteriophages in as many different soil samples as possible. It is vital that students bring in samples from a variety of places. If a student forgets their soil, they could be allowed to collect a sample from the area surrounding the building. If that is not possible, simply have the student use another student's purified phage to do a second infection. The expensive materials (syringes and filters) are used to prep the phage isolate, and should not routinely be used to make multiple isolates of the same soil.
- To remind students to collect the soil samples, the sterile 15 mL conical tubes can be given out at the class meeting before the experiment is to be run. The students enjoy having the "fancy" tubes. One can also post a reminder for the soil mentioning that collection in a plastic baggie is also appropriate.

Observing and Interpreting Results

- Plates need to be checked for plaques after overnight incubation, although leaving longer can still work. Students should carefully hold plates up to the light, opening the plates and looking only through the agar if condensation is present on the lid. The bacteria should form a uniform cloudy lawn on the plate, although it is common to find light, opaque streaks of *M. smegmatis* on plates. Phage presence on the plate will be marked by the appearance of plaques on the plate, usually circular regions of clear or turbid spots in the bacterial lawn. Light will shine more clearly through these plaques, making them appear lighter when held up to a light, but darker when held over a black surface like slate (as pictured in Figure 1.)
- Students may miss plaques that are present on the plate. Plaques that are turbid, rather than clear, are difficult to identify. Similarly, small plaques are sometimes disregarded by students. The instructor can circulate and check student plates as they make their observations.
- Students may identify plaques where there are none. Bubbles in the media are often mistaken for plaques. These potential plaques can be subjected to a spot test (see Appendix C) for confirmation.

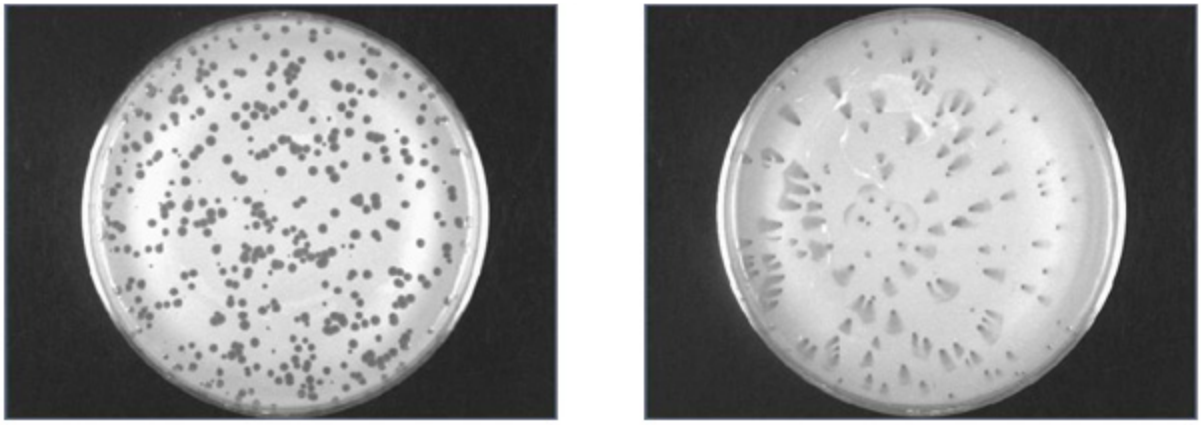

Figure 1. Two examples of mycobacteriophage plaques on *M. smegmatis*.

- Having a known phage as a positive control can be helpful, but steps must be taken to be sure the negative controls and soil samples do not get contaminated.
- Frequency of finding phages varies and some classes may not find any. An estimate for mycobacteriophage finding is about 1 in 30 samples. Negative results may be frustrating, but can inspire important discussions about the nature of biological research.

Modifications for Different Student Levels

- Please note that the Student Outline as presented is geared toward high school or introductory college biology students. DJS often uses the protocol with high school and even middle school students, while PLC uses it with introductory biology majors as an early first semester lab.
- The procedure can be modified depending on the existing skills of the students and learning goals for the lab.
  - To save time or reduce costs of materials, the instructor can pre-aliquot the bacteria into tubes, dispense the phage buffer into the soil samples, and add the molten top agar to students' phage-bacteria mixtures.
  - To emphasize the importance of controls, students can be organized into groups of four, with each student preparing 1 phage sample and plating one infection. Pairs of students add 50 µL of both phage samples to the same infection tube, resulting in 2 experimental infections per group. This process leaves enough material to prepare negative (bacteria + phage buffer rather than specimen) and positive (bacteria + known phage) controls, modeling properly controlled experiments. If a combination infection results in plaques, each student's saved phage isolate can be used separate to reinfect the bacteria and determine which original sample contained the phage.

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Appendix A

Media and Reagents for Growing *Mycobacterium smegmatis* **mc² 155**

The following text is the media and reagent recipes and methods of the Hatfull Lab at the University of Pittsburgh. We suggest consulting the PhageHunter Program website (http://phagesdb.org/) to find a link to the most current protocols available as well as other key information relating to mycobacteriophages. Product ordering information (vendors and their catalog numbers) is also available at the website.

Media and Reagents for Growing Mycobacterium smegmatis mc² 155

Mycobacterium smegmatis mc² 155, "smeg," is used as the host bacterium. It is a non-pathogenic, common soil organism that forms colonies on an agar plate in ~ 4 days. Because it is resistant to carbenicillin (CB), CB is added to the growing medium to kill other bacteria. To inhibit molds and yeasts in the growing medium, cycloheximide (CHX) is also added. In liquid culture, *M. smegmatis* tends to clump, thus 0.05% Tween®80 is added to the initial cultures to minimize the clumps, however, it is excluded from subsequent subcultures because it may inhibit phage infection. Smeg has a wide temperature range, but is typically grown in baffled flasks at 37ºC on a shaker, allowing aeration of the culture. The bacteria are plated as a lawn for optimal visualization of cell lysis, *plaques*. Since calcium is required by most phages for their successful proliferation, $CaCl_2$ is always added to the medium. To ensure optimal phage diffusion the final concentration of the top agar is 0.35%.

ADC (NO HEAT, filter sterilize)
- 60 g dextrose
- 25.5 g NaCl
- 150 g albumin
- 2850 ml ddH_2O

20% Tween®80 (50°C to dissolve, filter sterilize)
- 20 mL Tween® 80
- 80 mL ddH_2O

MBTA (Middlebrook Top Agar) made at 0.7%, melted, diluted to 0.35% with 7H9 (plus 2 ml $CaCl_2$ *)
- 4.7 g 7H9
- 7 g agar
- H_2O to 900 mL

Phage Buffer (autoclave or filter sterilize, add 0.1 mM $CaCl_2$ * prior to use)
- 10 mL 1M Tris, pH 7.5
- 10 mL 1M $MgSO_4$
- 4 g NaCl
- 980 mL ddH_2O

7H9 (autoclave, add antibiotics, ADC, and calcium prior to use)

4.7 g 7H9 broth base
5 mL 40% glycerol
900 mL ddH_2O

7H10 Plates (autoclave, cool to ~55° C and add 100 mL ADC and 10 mL 0.1 mM $CaCl_2$, 1 mL CB, 1 mL CHX, and then pour.)

19 g 7H10 agar
12.5 mL 40% glycerol
890 mL ddH_2O

$CaCl_2$ *

Calcium chloride is added to the mycobacterial growth media to ensure adequate calcium supply required for necessary cellular metabolic processes. Note that in most cases, we add calcium chloride to obtain a 1 mM concentration in the final solutions. (Therefore we will add 1 mL of the 0.1 M $CaCl_2$ stock solution to 100 mL of phage buffer, but 2 mL of $CaCl_2$ to 100 mL 7H9 because it will be used to dilute top agar by 50%). It is added after agar is melted, because it can precipitate out of solution upon heating.

7H9/CB/CHX/ADC/$CaCl_2$ - for liquid culture of *M. smegmatis* mc^2 155

90 mL 7H9
10 mL ADC
100 µL of CB
100 µL of CHX
1 mL of 0.1 M $CaCl_2$

250 µL Tween®80 (For initial sub-culture ONLY!)
See directions below.

Working Media

Buffer/$CaCl_2$ = 100 mL of Phage Buffer + 1 mL of 0.1 M $CaCl_2$

7H9/$CaCl_2$ = 90 mL of 7H9 +1 mL of 0.1 M $CaCl_2$ (for growing *smeg* in liquid culture)

7H9/2x CaC_{l2} = 100 mL of 7H9 + 2 mL of 0.1 mL $CaCl_2$ (for diluting top agar)

Top Agar = 50 mL of MBTA + 50 mL of **7H9/2x CaC_{l2}**
**Note:* Final agar concentration in top agar is 0.35%.

Microwave and completely melt a bottle of 50 mL 0.7% agar MBTA (stop the microwave and swirl the MBTA intermittently to prevent uneven melting). To a 100 mL bottle of 7H9, add 2 mL $CaCl_2$ Add **50 mL** of this 7H9/$CaCl_2$ mixture to the melted MBTA and shake well. ALWAYS include the components you added on the label of the bottle. The temperature of the top agar is critical for success and 55ºC is the desired temperature. If the agar is too hot you can kill bacteria and/or phages, if too cool the agar will start to solidify (you see "chunks"). Chunky agar will make plaque visualization extremely difficult.

Growing *Mycobacterium smegmatis* mc^2 155

1. **When culturing *M. smegmatis* mc^2 155 ("smeg") on a plate:** Start by retrieving a sample from the frozen stock in the -70°C freezer and streaking it for isolation on a 7H10/ADC/CB/CHX/$CaCl_2$ plate. Allow to grow for several days. The goal is to produce isolated colonies. "Smeg" has a distinct colony morphology. Colonies are wrinkled, gray-white, dry, and irregular in appearance. When sub-culturing from a plate, choose well-separated colony and pick a tiny (smaller than what you can see) inoculum from its center to grow in liquid culture (see 2 below).

2. **For growing smeg in liquid culture — Initial transfer from plated smeg**

   Use the 7H9/ADC/CB/CHX/$CaCl_2$ media preparation stated above. For the stock solution of Tween®80-medium add **250 µL** of 20% Tween®80 to **100 mL** of 7H9/ADC/CB/CHX/$CaCl_2$ medium and store at 4°C (fridge). For growing smeg from plate in liquid culture use a 'touch' of the culture from your plate into 1-2 mL of this Tween®80-media, vortex well, and place on a shaker at 37°C for at least 24 hours. Tween®80 is added at this step to minimize the clumping of the initial bacterial growth (Tween®80 acts as a detergent). Once a homogenous culture is obtained, subculture in media without Tween®80 (see 3 below).

3. For growing smeg for phage infections (7H9 without Tween®80)

   Prepare 7H9/ADC/CB/CHX/$CaCl_2$ media as stated above. Transfer a small amount (**1–100 µL** of the smeg grown in Tween®80-medium into **100 mL** of 7H9/ADC/CB/CHX/$CaCl_2$ (same concentration as above but **NO Tween®80**). Use a flask that holds five times the amount of media you are using to ensure adequate space for the media to move on the shaker. Place securely on shaker at 37°C for 24 hours.

Appendix B

Media and Reagents for Growing *Acinetobacter baylyi* ADP1

In general, the same reagents used for *M. smegmatis* (Appendix A) can be used to grow *A. baylyi* ADP1 ("ADP1"). We typically use a lower concentration of $CaCl_2$ (0.1 mM in working solutions rather than 1 mM), but have not seen any ill effects from using either more or less $CaCl_2$. However, we grow liquid cultures of ADP1 in liquid M9 media, rather than in 7H9. Additionally, work in the Connerly Lab has shown that carbenicillin may be required for some *Acinetobacter* phages to form plaques on solid media, in addition to its role in preventing contamination by other bacteria.

M9

384 mL deionized water
0.8 g dextrose
Autoclave
Aseptically add 8 mL of Part A 50x M9 Minimal Media Salt Solution
Aseptically add 8 mL of Part B 50x M9 Minimal Media Salt Solution

Part A 50x M9 Minimal Media Salt Solution

45 g M9 Minimal Media Salts (Part A)*
Add deionized water to 100 mL total
Autoclave to sterilize.

Part B 50x M9 Minimal Media Salt Solution

8.44 g M9 Minimal Media Salts (Part B)*
Add deionized water to 100 mL total
Autoclave to sterilize.

*Part A and Part B 50x M9 Minimal Media Salt Solutions made using Qbiogene Powder for 1000 mL 50x M9 Salts (Catalog #3035-022, 450 g)

Growing ADP1 cultures for infection

Fresh ADP1 cultures should be made the night before phage infection. A large chunk of cells from a streak of bacteria on M9 agar (liquid M9 recipe + 1.5% Bacto agar) should be added to a volume of M9 media and incubated with shaking overnight at 30°C. Cultures grow fine in sterile Erlenmeyer flasks, but special flasks with baffles for increased aeration might give more robust growth. The culture can be used anytime the next day for phage infections. We have had limited success with infections using grown culture stored at room temperature for a couple of days - we see fewer plaques over time, so we currently use only fresh cultures for experiments.

Appendix C

Procedures for Isolation, Purification, and Concentration of Mycobacteriophages

The following text is the procedures for isolation, purification and concentration of phages of the Hatfull Lab at the University of Pittsburgh. We suggest consulting the PhageHunter Program website (**http://phagesdb.org/**) to find a link to the most current protocols available as well as other key information relating to mycobacteriophages.

Procedure for the Isolation, Purification, and Concentration of a Phage

A. Sample Collection and Preparation

1. **Collect environmental samples in 15-50 mL screw-cap conical tubes.**

   Liquid or solid samples can be collected from virtually anywhere, particularly where decay is occurring. Good starting places include compost, sewer, garden, soil, bark, stagnant ponds, fecal matter, etc… (Avoid samples of human waste.)

2. **If the sample is solid, add phage buffer with calcium and mix/vortex well. Allow phage to diffuse into buffer for about 20 minutes.**

   Add 5-10 mL of phage buffer, or enough to cover the sample and form a liquid layer that can be pipetted off. Mix well.

   If the sample is liquid, it will just be filtered and processed.

3. **Allow sample to settle to bottom of tube, then remove 1 mL of the supernatant with a sterile 1.0 mL syringe.**

   As an alternative you can remove 1.0 ml with a micropipettor and place in a microcentrifuge tube. Centrifuge the sample to pellet the debris. Depending on your centrifuge, two minutes at medium speed is suitable. Make sure that you balance the tubes evenly.

4. **Filter sterilize the removed volume with a 0.22 µm filter**

   Attach a 0.22 µm filter to the syringe. Note that the syringe twists into the filter. (Do not just jam it into place.) Push the sample out of the syringe through the filter into a sterile microcentrifuge tube. Cap and label. Well-labeled samples can be stored in a refrigerator for short periods of time.

B. Plaque Screening

1. **Infect 0.5 mL *Mycobacterium smegmatis* mc²155 with 50 µL of your filtered sample.**

   In sterile tubes (one for each sample plus one for a

negative control), pipette 0.5 mL of *M. smegmatis* mc^2155 that was grown in 7H9/ADC/CB/CHX/Leu/$CaCl_2$. To each 0.5 mL aliquot of *M. smegmatis*, add 50 µL of the filtered sample. Mix well by vortexing. **Be sure to prepare an uninfected control tube that contains the bacteria and buffer but does not contain any of the phage sample.** As you prepare the top agar, the tubes can sit at room temperature for 15-30 minutes to allow phages in the sample to infect the bacteria.

2. **Add 4.5 ml top agar (see recipe) and plate**

   While the phages are infecting the bacteria, make the top agar following the directions stated in the media and reagents pages. Using a sterile 5 mL serologic pipette, dispense 4.5 mL of top agar to the first tube. With that pipette draw the entire contents of the tube back into the pipette and then dispense onto the surface of a labeled 7H10/ADC/CB/CHX/Leu/$CaCl_2$ plate. Be sure to use a sterile pipette for each sample. Gently swirl the liquid over the surface of the plate (by moving the plate flat on a surface in a clockwise, then counter-clockwise motion).

   Note: If the agar has begun to harden, it will fill the plates with chunks intermixed with the liquid agar. This will interfere with the visualization of plaques tomorrow. If your agar is chunky, reheat (and cool) the agar before continuing.

   Continue with all of your sample tubes.

   Remember to finish by setting up a negative control.

3. **Allow the top agar to cool and harden at room temperature before disturbing the plates.**

4. **Incubate at 37°C overnight for 24 hours.**

   After the samples harden, turn the plates upside-down. (The amount of time required for this cooling process depends on how fresh the plates were, how warm they were, and the relative humidity and temperature in your lab.) By inverting the plates, you prevent condensation from dropping onto the agar surface. Place in the 37°C overnight.

   Incubation times can vary. You may check your plates as early as 18 hours and may keep them incubating for 48 hours. Good description of the plaque morphology always includes incubation time, because morphology will change over time. Some phages are best seen at 36 hours, others must be picked at 18 hours incubation. As you purify your phage, you will get to know the specific 'growing' characteristics of your phage and its plaque morphology. Record all pertinent information in your notebook.

5. **Check plates for plaques**

   Allow 24 hours for bacterial growth and phage infection. Plaques may be difficult to see due to small size or turbidity, so look carefully. The best way to look for plaques is to remove the lid from a plate, allow any condensation to drip onto a clean paper towel, and hold the plate up to a light, allowing the light source behind the plate. Look for cleared zones – zones of bacterial cell death. **Plaques** are areas where LESS bacteria is present in a perfect circular configuration. Areas of increased density are typical of bacterial or fungal colony formation. Any perfect circle of clearing is a putative plaque. A confirmation test, a spot test, is done to demonstrate the presence of phage. If appropriate, photograph your plates. Print a copy for your notebook, save an electronic version for future use.

   If no putative plaques are observed, leave the plates at 37° C for another 24-hour period, then check them again. If there are no putative plaques, don't be discouraged, but collect some new samples and repeat the procedures until you observe plaque formation.

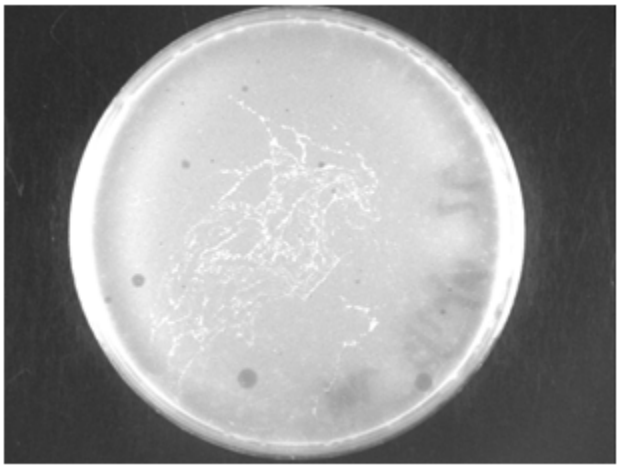

Figure 2. Plaque Formation - Plaques come in many sizes and degrees of turbidity. This plate is an example of at least 3 plaque morphologies on a plate generated from a single soil sample. Plaques range in size from large to pinpoint. Check all plates carefully for plaque formation.

NOTE: If you are certain that your putative plaque is the result of phage infection, there is no need to perform Step C. Go directly to Step D. The goal of the Spot Test process is to determine whether putative plaques are indeed plaques, and a spot step can be skipped if there is no doubt.

C. Verification of Putative Plaques (Spot Test)

1. **Use a pipette tip to pick a single putative plaque into 100 µL phage buffer + calcium**

   Sterilely aliquot 100 µL of phage buffer + calcium into enough microcentrifuge tubes for each of your putative plaques. Just touch the center of the plaque ONCE with the end of a tip, then put the end of that tip into the liquid and gently shake the tip in the liquid to release the phage. Do your best to avoid picking up the neighboring bacteria. **Discard the tip** and mix the liquid well by vortexing. Pick all possible plaques and place in separate microcentrifuge tubes with 100 µL of aliquotted phage buffer as outlined above. Be sure to label each tube based on the plate where the plaque originated. A great place to label the pick is on the plate itself. **Refrigerate these samples until the presence of phage is verified.**

2. **Making plates for Spot Testing**

   This is just like your negative control done previously. Add 4.5 mL of top agar to 0.5 mL of smeg. **Do not infect the cells with any putative phage samples.** Remember to swirl the plated top agar.

   Allow the plates to cool and harden. As a short cut, you can prepare you plates for spot tests the day before (when you are processing your samples). HOWEVER, the plates must be stored in the refrigerator overnight.

3. **Use a marker to draw a grid with enough blocks for each putative plaque that was picked**, **labeling each square with the sample number.**

4. **Spot 5 µL of each phage sample on the plate in the appropriate grid.**

   Using the appropriate volume micropipettor, draw up 5 µL of each of the prepared samples and hold the tip above the plate at the labeled grid. Don't touch the pipette tip to the agar. Avoid making bubbles, as these will scatter phages across your plate.

5. **Allow the liquid from the spots to absorb into the agar, then invert. Incubate at 37° C overnight.**

   Remember to refrigerate your putative phage samples in the microcentrifuge tubes!

6. **The next day, check spot plate for plaques**

   A positive spot test will appear as complete obliteration of the entire drop area. Typically, the concentration of phage allows plaques to 'grow' together to form a bacterial death zone the size of drop size.

   Positive Spot Test: Complete bacterial cell destruction in the area of the dropped sample is observed. This confirmation that phage is present. Based on the grid, identify where the phage sample originated and, using the refrigerated sample from C-1, continue on with step D-2.

   Negative Spot Test: No effect of the bacterial growth in the area of the dropped sample. No phage is present. Collect more dirt samples and return to step A-1.

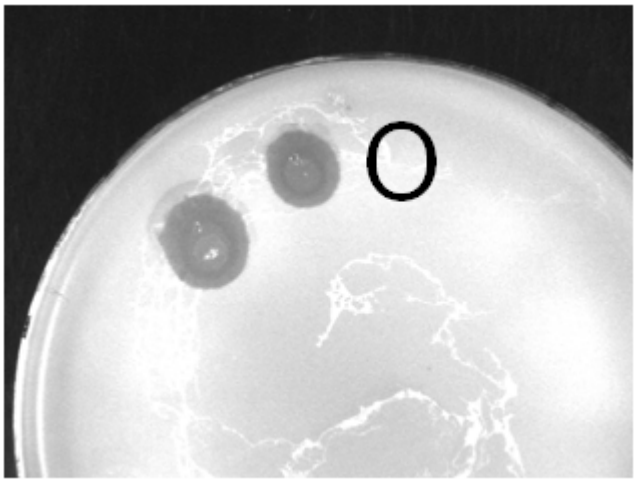

Figure 3. Three Spot Tests - two spots are positive, with complete cell destruction, the third is outlined because, no cell lysis occurred, i.e. a negative spot test.

D. Plaque Purification

When purifying phage, be aware of the following considerations:

a. **Pick** putative plaques as soon after plating as possible. The longer the phage is on the plate, the further it diffuses across the plate.

b. **Check** plates with **high phage concentration** (a lot of plaques) for uniform plaque morphology.

c. **Check** plates with **low phage concentration** (a few plaques) for uniform plaque morphology and to pick your plaque.

Record all of this information in your notebook. Remember to photograph and place a copy of your photograph in your notebook. Save the electronic file in a well-named manner. Record in your notebook where that file is located.

1. **Use a pipette tip to pick a single plaque into 100** µL **phage buffer + calcium**

   Aseptically aliquot 100 µL of phage buffer + calcium into microcentrifuge tubes. Just touch the center of an isolated plaque with the end of a tip (just ONCE), then put the end of that tip into the liquid and gently agitate. Throw away the tip and mix the liquid well using the vortex.

2. **Serially dilute sample to 10^{-1} ,10^{-2}, 10^{-3}, 10^{-4} in phage buffer + calcium.**

Make 10-fold serial dilutions of your picked sample. This is done to ensure 2 goals: 1) to see a lot of plaques (you need enough plaques to represent the sample) and 2) to have well isolated plaques to pick to continue purification. (Well separated plaques are your best opportunity to have no cross-contamination.)

Aliquot 90 µL of buffer + calcium into each of 4 microcentrifuge tubes. Label the tubes as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} . Remove 10 µL of the neat sample and carefully dispense it into the 10^{-1} labeled tube. VORTEX WELL. Next, remove 10 µL of the 10^{-1} sample and dispense it into the 10^{-2} labeled tube. VORTEX WELL. Next, remove 10 µL of the 10^{-2} sample and dispense it into the 10^{-3} labeled tube. VORTEX WELL. Finally, remove 10 µL of the 10^{-3} sample and dispense it into the 10^{-4} labeled tube. VORTEX WELL.

Keys to success include:

- Careful, accurate pipetting
- VORTEX WELL
- Aliquot all buffer into tubes before dispensing phage samples.
- Keeping your place in the series by using 2 racks and moving finished dilutions to the second rack

Figure 4. Serial Dilutions - The solution becomes less concentrated in each consecutive round of dilution, demonstrated by the fading purple coloration above. In this process, a solution is successively diluted from a neat (undiluted) sample to form a series of dilutions that each originates from the previous dilution.

3. **Infect 0.5 mL *M. smegmatis* mc²155 with 10 µl of 10^{-2}, 10^{-3}, and 10^{-4} dilutions**

   Aliquot three tubes of 0.5 mL of smeg mc²155 per sample, plus the negative control. (A negative control is required EVERY time you plate.) **Allow the phage to infect the bacteria for 15-30 minutes**.

4. **Continue the steps listed in Section B: 2-4.**

5. **The next day, ALL plates are observed for plaques.**

   You may need to adjust the dilutions you use to obtain a series of plates that reflect two components. First, observe the plate that has MANY plaques on it. Check to see that all plaque morphologies look uniform. Second, observe the plate with the fewest plaques present. This is the plate to pick a plaque for the next round. For purification, well-isolated plaques are best!

   Note: Be cautious of a plate with just one plaque on it. Is it a contaminant?

6. **Repeat steps D: 1-4 several times to insure plaque purity**

   When observing a large number of plaques on any one plate, always check for homogenous plaque morphology. Some plates may include multiple types of phages that are difficult to isolate and purify. It is of utmost importance that the final round of plaque purification contains only a single plaque morphology. (A minimum of 3 rounds of purification is required.) Continue to pick isolated plaques, make dilutions, infect *M. smegmatis*, plate samples, and incubate overnight until plaque morphology and other characteristics remain consistent. **This may require 5-10 subsequent rounds of purification to isolate your phage. Be patient.**

Notes:

- If plaques disappear on subsequent rounds of infection, you can:
- Grow fresh smeg
- Decrease your concentration of smeg
- Increase your concentration of phage
- If you have any doubt, continue to purify. 2 phages will never sequence!
- Extended incubation times may be necessary to get optimal concentrations of phage. Titer the phage to determine what is optimal.
- Picking plaques at 18 hours may be required to promote isolation of a single phage.
- When more than one plaque morphology is obtained from a sample, purify only ONE at a time.

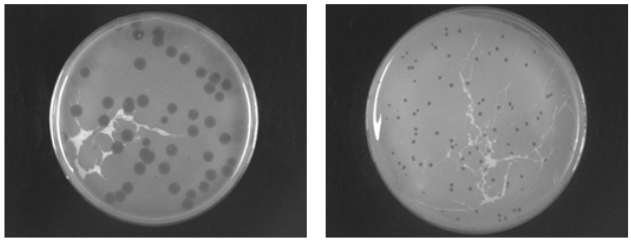

Figure 5. Plaque Purification - Two examples of purified phages are shown above. Note that the plaques within each of the two plates are consistent in size, but quite different from the plaques on the other plate.

E. Plate Lysate Production

1. **To a nearly cleared plate from your last round of purification, add 5.0 mL phage buffer + calcium, and swirl gently.**
2. **Let sit at room temperature for 2-3 hours (or overnight at 4°C)**

   Occasionally, swirl the phage buffer on the plate gently. Do not splash.
3. **Siphon liquid lysate and filter it through a 0.22 μm filter**

   Remove the liquid with a 5 mL syringe. Filter the lysate, collecting the filtrate into ONE 15 mL orange capped conical tube. Label this tube well with your phage name, your name, date (including year), and titer. From here on, the lysate must be stored in a refrigerator. This tube is stored in the master racks for lysates. There it receives a unique number for identification and storage. Record all information in your notebook.

   The next step is to determine the concentration, or titer, of the lysate. Once a titer is obtained, the phage is amplified using larger volumes and bigger plates.

F. Titers

There are two ways to determine a titer. The small plate titer allows for easier and more accurate plaque counting, but it requires more plates and time than the "quick and dirty" spot tests. Either method can lead to successful results. The best titer calculation is always obtained from a plate with 20 – 200 plaques. (Which can ONLY be obtained from the small plate titer.) Use this step as a way to establish that you are confident with your technique. Do both the small plate titer and spot test and compare your results. Discuss the differences with your mentor.

Small Plate Titer (The Preferred Method for New Phagehunters.)

1. **Serially dilute lysate to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} in phage buffer + calcium.**

   See section D-2. In this process, you will prepare several more dilutions than you previously prepared. Remember to save the last dilution if you have to take the titer out higher than 10^{-10} .
2. **Infect 0.5 mL *M. smegmatis* mc²155 with 10 μL of all dilutions, 10^{-1} to 10^{-10}.**

   Aliquot eleven tubes of 0.5 mL of smeg mc²155 per sample (including the negative control). **Allow the phage to infect the bacteria for 15-30 minutes**.
3. **Continue with plating steps B: 2-4**.
4. **The next day, many plaques will be visible.**

   Look for the dilution plate with 20-200 plaques to calculate the titer of phage sample. Count all plaques on that plate. Verify your number by recognizing that there are 10x fewer on the plate from the dilution one greater than that plate, and 10x more plaques on the plate from the dilution one lesser than that plate.

"Quick and Dirty" Spot Tests (The NOT-Preferred Method for New Phagehunters)

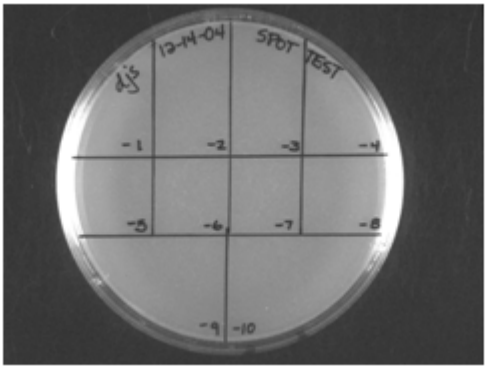

Figure 6. Spot Test Grids - The use of a grid allows for the appropriate documentation of the origin of each spot on a plate. A marker is used to form the necessary blocks, and each block is labeled with the identity of the spot that is present within its lines.

1. **Mix 0.5 mL *M. smegmatis* mc²155 and 4.5 mL Top Agar and Plate.**

   This is prepared just like the negative control. See section B-2.
2. **Use the serial dilutions made for the small plate titers.**

3. **Spot 5 µL of each dilution on the plate after the top agar has cooled and hardened completely.**

   As you place the drops on the agar, do not touch the pipette tip to the agar. Just hold the tip slightly above the top agar, and push the droplet out slowly.

4. **Allow the spots to absorb into the agar, then invert. Incubate at 37° C overnight.**

G. Calculation of Titer

1. **Find the plate with 20 – 200 plaques on it. Count the plaques.**

   Upon examination of the plates, choose a plate with roughly 20-200 plaques. Count each plaque, paying careful attention to the number of plaques in the adjacent plates. For example, if you count 50 plaques on the 10^{-6} plate, you would expect to see 5 plaques on the 10^{-7} plate. Remember that it is statistically possible to diverge from the expected number, but be wary of error. If you have more than one plate with countable numbers of plaques (20-200), you can average the two values.

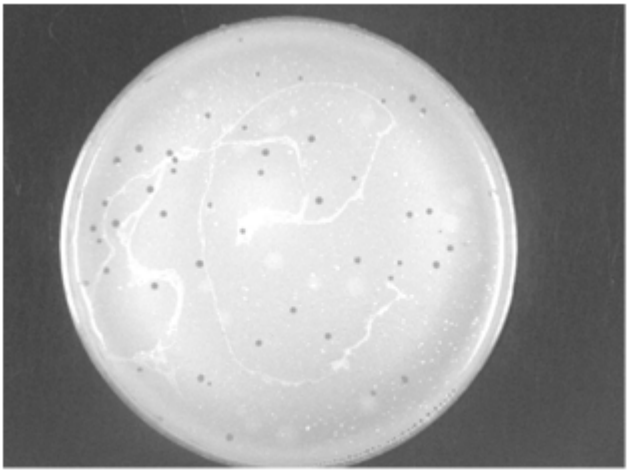

Figure 7. Calculation of Phage Titer - Count the plaques in this photo. There are 50 plaques on this 10^{-7} dilution plate.

2. **To calculate the titer (the concentration of phage in the lysate), reported in plaque forming units per milliliter (PFU/mL), multiply the number counted by the dilution factor appropriate for the plate you are counting.**

   Multiply the number of plaques that you counted by the reciprocal of the dilution used to make that plate. Divide this number by the volume of the phage sample used (5 or 10 µL), and then convert µL to ml to obtain the titer in plaques/ml. Use the example calculation below, based on counting 50 plaques in the 10^{-7} dilution plate:

Titer calculation:

$$\frac{50 \text{ plaques}}{10\ \mu L} \times 10^{7} \times \frac{1000\ \mu L}{1\ mL} = 5.0 \times 10^{10} \text{ PFU/mL}$$

The titer for this lysate is 5.0 x 10^{10} PFU/mL.

3. **The numbers obtained from the "quick and dirty" titer are much cruder than those of the small plate titer.** Counting plaques in a 5 µL spot is not easy. In addition, the small sample size has a greater error associated with it. **It is preferable to use the small plate titer method to calculate the titer**.

$$\frac{28 \text{ PFUs}}{5\ \mu L} \times \frac{1000\ \mu L}{1\ mL} \times 10^{7} = 5.6 \times 10^{10} \text{ PFUs/mL}$$

Note: The point of determining a titer is to produce enough phage in your big plate infection for the subsequent procedures you will perform. A final concentration of 10^{13} phages/ml is desirable. To accomplish this task without wasting resources, an empirical test is essential. The goal is to produce 30 plates with maximum production of phage. We have estimated that having 6000 – 10,000 plaques appear on a big plate yields the desired numbers. The appearance of plates with maximum phage production yields a plate with only remnants of bacterial growth present. A web pattern of bacteria is the desired observation. This is only an estimate and your phage may require a higher or lower concentration. Factors that influence the numbers are concentration and age of the bacterial culture, size of plaque, and incubation time (for both 'infecting' and growing). By now, you have learned how to optimize the growth of your phage. All of this information is used to produce an abundant yield for phage harvest.

H. Empirical Test of Lysate Concentration

Before starting the 30-plate infection, you must be confident that you have identified a volume and dilution of lysate that yields a web pattern on a large plate. Also, be sure that you have a large quantity of *M. smegmatis* prepared for this step. **Use the same subculture of *M. smegmatis* throughout the empirical test and big plate infection in order to standardize the conditions.** Check that there are enough plates for the 30 plate infection so that the empirical test is done on same batch of plates as those used for the 30 plate prep. This is to ensure that the water content, etc. will be the same for the empirical test and for the 30 plate prep.

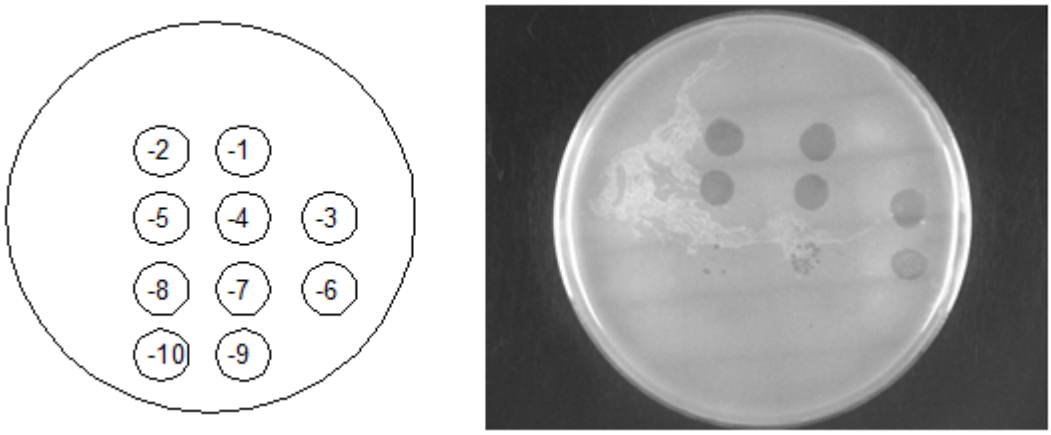

Figure 8. Calculation of Titer from Spot Test - The above spot plate contains spots of 10^{-1} through 10^{-10} dilutions. The 10^{-1} through 10^{-5} dilutions form cleared spots, and no plaques are evident on the 10^{-9} or 10^{-10} spots, report as TMTC (too many to count). However, individual plaques are visible on the 10^{-6}, 10^{-7}, and 10^{-8} dilution spots (-6, -7, and -8 above). The 10^{-7} dilution spot above shows 28 plaques and the 10^{-8} dilution shows 3. Both spots give you approximately the same titer.

1. **Use the calculated titer to determine the amount of lysate necessary to infect one large plate.**

   The goal of the empirical test based on the titer calculation is to determine the dilution of lysate necessary to form a web pattern of *M. smegmatis* growth (the appearance of a nearly cleared plate). This web requires about 6000 plaques per large plate for an average-sized plaque. For very large or very small plaques, adjust the number up or down (<6000 for very large plaques and >6000 for very small plaques). This calculation is performed by dividing 6000 (or adjusted value) by the calculated titer. For example:

   6000 plaques per plate / 5 x 10^{10} plaques per mL =

   1.2 x 10^{-7} mL lysate per plate

   1.2 x 10^{-7} mL lysate per plate =

   1.2 x 10^{-4} µL lysate per plate

   Based on the above calculations, add 1.2 µL of a 1:10,000 (or 10^{-4}) dilution of the lysate per plate. Depending on your preference, you may choose 12 µL of a 1:100,000 (or 10^{-5}) dilution or 120 µL of a 10^{-6} dilution.

2. **Choose additional volumes and dilutions of lysate to infect *M. smegmatis* for the purpose of creating a web pattern on a large plate.**

   Because of the vast number of uncontrollable variables, we suggest that you 'box' your calculated amount of titered lysate. By doing so, you can see a broader range of empirical tests, so your 30 plate is not a guess. The cost and time of the materials is considerable. Continuing with the example started above, the 5 plate empirical test would look like this:

   a. 10 µL of 10^{-5} dilution of lysate (based on IX-1 calculation)

   b. 10 µL of 10^{-4} dilution of lysate (one order of magnitude above)

   c. 60 µL of 10^{-4} dilution of lysate (about half way between the first two dilutions)

   d. 10 µL of 10^{-6} dilution of lysate (one order of magnitude below)

   e. 60 µL of 10^{-5} dilution of lysate (about half way between the first and fourth dilutions)

3. **Serially dilute your lysate in order to produce the dilutions necessary for each of the 5 volume/dilution combinations defined above.**

   See section B-2. Take meticulous care in pipetting and vortexing each subsequent dilution.

4. **Infect 1 mL aliquots of *M. smegmatis* mc^{2}155 into 5 separate large test tubes with the appropriate amount of diluted lysate.**

   After ensuring that the lysate is well-mixed (vortex) with the *M. smegmatis*, allow 20 – 30 minutes for infection in the large test tubes. Take careful note of the conditions of the infection so that you can recreate the same environment when your 30 plate prep is prepared. For instance, note the time allowed to infect and pay attention to which subculture of *M. smegmatis* is used. This will create consistency in the empirical test, and between the empirical test and 30 plate prep.

5. **Add 9 mL top agar.** Same procedure as with the smaller plates.

6. **Plate on pre-warmed 150 x 15 mm 7H10/CB/CHX/ADC/$CaCl_2$ plates.**

   Swirl gently to spread top agar.

7. **After plates harden completely, incubate at 37°C overnight.**

   Plates do not need to be incubated upside down as in the past.

8. **Determination of Desired 'Web" plate.**

   The next day, check for the plate that yielded the best web pattern of phage-infected *M. smegmatis* growth. You are looking for the plate that yields the highest possible titer. If in doubt, titer all plates. The web pattern that is most desirable is one that shows as little bacterial growth as possible in as many places as possible. The web pattern is a fine lacey growth of smeg where plaques have almost totally merged into one another.

   When you have found the desired web pattern, identify the amount of phage put on the plate. Multiply that amount times 30, for the next 30 plates you will infect.

   If no web patterns form, repeat the empirical test using different lysate volume/dilution combinations.

I. Big Plate Infection

Remember: You want to continue to maintain (keep the same) as many variables of the empirical testing as possible. That means use the same smeg, incubate sample/smeg for the same time, plate in the same manner, use the same batch of plates, etc.

1. **Using the volume of phage that yielded the best web pattern in the empirical test, calculate the volume and dilution of lysate necessary for a 30-plate infection.**

   Multiply the volume of phage lysate used to create a web on 1 plate by 30 to get the volume needed for all 30 plates. For example, if 60 µL of a 10^{-5} dilution of lysate formed the 'perfect web pattern' on 1 plate, 60 x 30 = 1800 µL for all 30 plates. Instead of using 1800 µL of a 10^{-5} dilution, you could use 180 µL of a 10^{-4} dilution or 18 µL of a 10^{-3} dilution for the infection.

2. **Pre-warm 30 large 7H10/CB/CHX/ADC/Ca plates.**

3. **In a 500 mL flask, infect 30 mL *M. smegmatis* mc^2155 with the appropriate volume and dilution of lysate.**

   Be sure to mix the bacteria and lysate well by swirling the flask. Allow phage to infect for 20 minutes (Use the same amount of time used to infect for the empirical test).

4. **While the phage is infecting, make 270 mL top agar.**

   Be careful that your temperature is not too hot, but definitely not too cool.

5. **After completion of the appropriate incubation time, add all 270 mL top agar to the 500 mL flask with the infected *M. smegmatis.***

6. **Quickly plate on 30 pre-warmed 7H10/CB/CHX/ADC/$CaCl_2$ large plates.**

   It is most desirable to recruit a partner to help plate all 30 plates as quickly as possible. Using a pipette gun and a 50 mL pipette, pull up 60 mL of the mixture at a time. Dispense 10 mL per large plate (6 plates per 60 mL pipette load). Swirl plates well. Work quickly so that the agar doesn't cool too much before finishing all 30 plates.

7. **Allow plates to harden, and then incubate at 37° C overnight.**

   Plates do not need to be inverted for incubation.

8. **Check plates at 24 hours** (or when you determined the best yield incubation time was.)

   Once the perfect web is detected, flood the plates with 10 mL of phage buffer + calcium. Swirl well. Allow the buffer to sit on the plates for 2-3 hours (or overnight in the cold room).

9. **Siphon off all buffer with large pipettes.**

10. **Collect all buffer in 250 ml centrifuge tubes.**

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