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Phagehunting Program Introduction

Welcome! You are now a member of a group of students who have embarked on the quest to find and identify bacteriophages. During the process, you will find yourself doing independent research to uncover the secrets of your own phage. And we don’t want you to miss a beat! This manual will guide you through the process. Though you will be using techniques used by many, remember, your phage hasn’t read the manual. You will always need to adapt the basic techniques to your individual phage. An example is the Big Plate Infection. In this technique, the goal is to cover the plate with a ‘web’ of phage-infected bacterial lawn. Six thousand plaques/plate is the suggested number. However, your phage’s plaques may be so tiny en thousand plaques are needed to accomplish you goal. Just make the necessary adjustments to your dilutions.

As you get started, a few paperwork, workflow, and housekeeping items require your attention.

**Photo permission:** Please sign (or have your parent/guardian sign if you are <18 years of age) and return to Debbie Jacobs-Sera.

**Safety:** To work in the university lab, you must complete the following modules.
- Hatfull Lab Safety (HHMI Lab Safety Training video) - this is available from Mrs. Jacobs-Sera
- Chemical Hygiene Module – schedule is found on-line at [http://www.ehs.pitt.edu/training/training.htm](http://www.ehs.pitt.edu/training/training.htm)

**Ethics:** Your integrity is very critical to the lab and science. It may be easy to say “Thou shall not cheat or steal”, but there are many ethical considerations in research conduct. You are required to read two Sigma Xi pamphlets: “The Responsible Researcher: Paths and Pitfalls” and “Honor in Science”. When completed, please sign and return the form that acknowledges that you have read them.

**Lab Notebook:** You will be given a bound notebook to record the work you do in the lab. There are two key points about your notebook

1. ALL that you do in the lab needs to be recorded. Recording dates, (BE SURE TO INCLUDE THE YEAR IN THE DATE – we keep things for quite a long time and need to locate your results) times, places, and exactly what you did is critical to doing good science.
2. Your lab notebook is actually the property of the Hatfull lab. It should be kept at the lab. When you have finished working with us, the lab notebook stays here. You are free to photocopy pages for future reference.
Protocols: You have been given a binder with current protocols. It is always your job to read upcoming protocols. These protocols are designed to promote efficient execution of the necessary procedures. In addition, following the set procedures will enhance safety in the lab.

Time Log: We need to have an accurate record of when you are in the lab. Please record the times you are here and have your mentor co-sign your entry.

Plaque Picture Book: A copy of a picture demonstrating the plaque morphology of your phage needs to be entered. Please include the file name (& location) with your entry.

EM Picture Book: A copy of your photoelectronmicrograph needs to be entered. Please include the file name (& location) with your entry.

Digest Book: As we discover more phages, it is important to document that each phage is different from the others. The DNA digest is a great way to compare phages to identify that uniqueness. It is important that lanes be labeled, and the same enzymes be put in the same lanes. As a rule of thumb, when applying samples, start from the left, apply the ladder, uncut DNA, the enzyme digested DNA by alphabetical order of enzyme. Be sure the gel picture is well labeled in the book.

Written Summaries: Refer to the document “Research Summary Report Requirements” for instructions. The goal of this document is to prepare your work as a publishable manuscript.

Presentations: To reinforce that science is not science until it is reported, you are expected to present your findings to the lab, at poster conferences, and at general meetings. They are all excellent ways to learn to communicate your science to others.

Notebook Meetings: It is required that mentors attend the weekly notebook meetings. They are useful for two reasons. One, you can confirm that your research is going in the best direction. If you are stuck, suggestions will be made to help you understand the science and make modifications to the process. Two, you will become aware of the other undergraduates’ concerns and can help them in their endeavors.

Computers: Access codes are available in the lab. Server space is available for phagehunters. It is located on the BIOSCI server, with the folder name of ‘phages’. If you have a Pitt username, you should contact Debbie Jacobs-Sera to be added to the access list. If you do not, see Debbie for instructions on how to gain access.

The server location is afp://136.142.54.170
From a PC in the Hughes Phagehunting Lab::
Right click on My computer
Choose Search
Choose Computer or People
Choose a Computer on the Network
Enter 136.142.54.170 (as soon as you type in 1, a box will appear with the number there, click on it)
If it asks for a username and Password, enter UPITT-_USERS\[your Pitt username in lowercase], then enter your Pitt password
Access will be to a folder named Phages
**Sign-off Sheets:** Be sure that all paperwork is completed. Permissions and requirements of the program (like the safety courses) need the appropriate documentation. All hard copies are kept with Debbie Jacobs-Sera.

**Keep in touch:** We would like to know how this research experience impacts your life. That means a longitudinal study of YOU! The only way we can accomplish that is if you stay in touch. Please continue to contact us with what you are doing and your current (i.e. ACTIVE) e-mail address. Thanks!

**Submit Phage stock:** Once you make a stock of your phage, you are required to submit an archive sample of that phage to the Genome Center (see Making Phage Stock Protocol). It is imperative that we secure a pristine aliquot of each phage for reference.
PHAGE CHECKLIST

As each phage is isolated and characterized, the following items will need to be recorded!

Phage Name
Date first isolated
Date, location, temperature, description of type sample (dirt/pond water, etc) sample was collected
School/Class/Teacher
Individual who collected sample
Primary PBI contact/Phage Discoverer
Host bacterium on which it was isolated
Other bacterial species it infects
Other bacteria it is known to NOT infect
If it was sent to us: By WHOM, When, ANY INFORMATION!!!!
Describe the plaques (halos, clear/turbid, approximate size and size range), and attach photos

Dates of Titer of High Titer CsCl and Dialyzed Phage Stocks for storage
Do Not Proceed until stock has been archived.

Date of and Titer of High concentration DNA Stock for sequencing and storage
Do Not Proceed until stock has been archived.

Date and file name and attach photo of Restriction Digest Gel

Dates and results of attempts to isolate lysogens

Dates, file names of Electron Microscopy (attach micrograph)
Estimated Diameter of head

Estimated Length & nature (contractile/noncontractile) of tail

Date, file name and attach Protein Gels
N terminal sequences from John Hempel

<table>
<thead>
<tr>
<th>Band</th>
<th>SEQ</th>
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Dates/description of library (attach photos of sheared DNA, digested minipreps)

HOW MANY Blocks in the sequencing project?

How many Primers were run?

Ace file, date, contig of final DNA SEQUENCE (and description of how this was cut into unit length)

Date of finished sequence

How were the ends trimmed? How was base one defined?

Name and date of FASTA file

Genome Length

%GC Content

Nature of ends (circularly permuted, cohesive, terminally redundant), 3’ extension, 5’ extension

tRNA genes report by tRNA Scan (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/)

Date/name of Genemark output file (and what organism was used as the profile)

Date and Name of Glimmer

Date and Name of DNA Master File

Annotate genes (How Many Protein coding genes)

Date.name of Protein file

Frameshift searches run and where the frameshifts are found in genes

Tapemeasure is gene _________________________________

Size, bacterial homolog domains_______________________

Name/Date Excel file of ORFs
Promoters

Terminators

Morons

Dotter against other phages, against self

Signal Sequences search

tmRNA search

ANY Notable gaps in the sequence? (Regions lacking proteins or RNA coding region calls)

Long Stretches of one Base or Repeats?

Date, xml file of BLAST P against Genbank

Date, xml file of BLAST P against PBI Database

Canvas files/Dates of ANNOTATED MAPS (single tier and single page)

Dotters (vs what??)

Dates, Names of GenBank File made submitted (and record the Accession Number)

Manuscript Publication
Student Agreements

**Photos, etc.**
I give permission for myself to be surveyed, photographed and/or filmed while participating in the phagehunting experience at the University of Pittsburgh and for the Hatfull Lab phagehunting program to use these images on the internet and in other promotions or evaluations of the program.

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**Photos, etc.** (if under 18, must have a parent or guardian sign this)
I give permission for ____________________________ to be surveyed, photographed and/or filmed while participating in the phagehunting experience at the University of Pittsburgh and for the Hatfull Lab phagehunting program to use these images on the internet and in other promotions or evaluations of the program.

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**Ethics**
I have read and understood “The Responsible Researcher: Paths and Pitfalls”
I have read and understood “Honor in Science”
I agree to conduct scientific research in a responsible and ethical manner and will bring any ethical or safety issues that arise with my research or that of my mentees or coworkers to the attention of Debbie Jacobs-Sera or Graham Hatfull.

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**Pre-Assessment Survey** (Check with Debbie for the correct survey to use.)
I have completed the assigned Pre-Assessment survey at [http://www.getfast.ca/](http://www.getfast.ca/)

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2005 HHMI Summer Phagehunter Program
Research Summary Report Requirements

A research summary report is a printed and electronic submission (in MS Word or a pdf) containing the following in journal format (full sentences and paragraphs using standard scientific writing.) A draft of this report will be due as designated at the beginning of the term. The final summary for each semester is due at the end of the semester.

I. **Introduction/Background:** Why is the project important? What are the broad and specific goals? What is the current state of the field?

II. **Methods/Procedures:** How, in great detail, were the experiments done?

III. **Results:** What are the results (figures, gels, numbers, etc, etc)? Please include pictures and words.

IV. **Discussion:** What does it mean? What is the big picture?

V. **Bibliography:** Relevant published works cited in the earlier sections.

VI. **Figures and Tables** You can put these at the end or interweave them, with detailed figure legends (captions)!

VII. **Next step:** What further experiments are suggested by your data? What do you propose to do next?
Background

The following is from the American Society of Microbiologists website on bacteriophages. Other reference articles are available on the server.

Division M: Bacteriophage

Division M is composed of researchers and teachers dedicated to the study of bacterial viruses. Current topics of interest are: assembly and structure, genome structure, initiation of infection, regulation of transcription and translation, replication, recombination, repair, virus-host interactions, new phage systems, molecular cloning technology, and bacteriophage evolution.

What is a bacteriophage, anyway?

Members of Division M certainly know what bacteriophages are, but other readers of this page may wonder what it is about these tiny microorganisms that would make so many scientists devote their professional lives to understanding how they work. This part of the site provides a brief primer for the uninitiated about what bacteriophages are, what they do, and what they do for us; and gives a quick refresher for those who once knew something about bacteriophages. Teachers who wish to include bacteriophages in their curricula will find some useful links in our "Resources for teachers", "Books", and "Links" pages.

- What is a bacteriophage— the basics
- Abundance & variety of bacteriophages
- History of bacteriophage research
- The practical phage— Human uses of bacteriophages

What is a bacteriophage?—the basics

Bacteriophages ("phages" for short) are viruses whose hosts are bacterial cells. Like all viruses, phages are metabolically inert in their extracellular form (the "virion"), and they reproduce by insinuating themselves into the metabolism of
the host. The mechanisms by which phage virions infect their host cells—described in more detail below—vary among the different types of phages, but they all result in delivery of the phage genome into the cytoplasm of the bacterial host, where it interacts with the cellular machinery to carry the phage life cycle forward. The result of infection can be, and often is, total devastation for the cell. A good example of this is infection by the *E. coli* phage T4, the *Tyrannosaurus rex* of phages, which commandeers the material and energetic resources of the cell and turns them toward making more virions, after which it causes violent lysis of the cell and release of the progeny virions. At another extreme, the large group of phages known as *temperate* phages have the option when they infect of setting up a state of coexistence with the host ("lysogeny") in which the genes that would harm the host are prevented from being expressed, while a small set of genes that provide benefit to the host are expressed. Both scenarios result in replication and perpetuation of the bacteriophage.

Follow this link for guidance on pronouncing the words ‘bacteriophage’ and ‘phage’.

**Abundance and variety of bacteriophages**

There are probably more individual bacteriophages in the biosphere than there are of any other group of organisms, including all the *prokaryotes*. Until recently nobody knew how to get even an approximate estimate of how many phages there are, but a little over ten years ago, it occurred to someone that at least for the best studied group of phages, the tailed phages, their shape is so distinctive that their numbers in aquatic environments could be estimated simply by centrifuging them onto an electron microscope sample grid and counting them. Astonishingly, in coastal sea water there are typically as many as $10^7$ (ten million) tailed phages per milliliter. In some fresh water sources there are up to $10^9$ (a billion) per milliliter. Although there is still little information about how uniform is the distribution of phages around the globe, these numbers give at least a rough basis for calculating the global population. The remarkable result of such a calculation is that there may be as many as $10^{30}$ tailed phage globally. For people who like their numbers written out, that’s 1,000,000,000,000,000,000,000,000,000,000. If you were to gather them all up and weigh them, they would outweigh the world population of elephants by a thousand-fold or more.

The tailed phages are also known as the dsDNA tailed phages because their genomes are molecules of linear double-stranded DNA. Their genomes are relatively large for viruses, with most of them in the vicinity of 50 kbp (50,000 base pairs). However, some are less than 20 kbp, the common and well studied ‘T4-like’ group is more like 160 kbp, and bacteriophage G is the largest virus on record, with a genome of nearly 500 kbp—bigger than the smallest bacterial genomes.

Although the dsDNA tailed phages account for about 95% of all phages reported in the scientific literature, and may in fact make up the majority of phages on the
planet, there are other phages that occur abundantly in the Biosphere sporting very different virions, genomes, and lifestyles. These are listed in more detail in the "Phage Facts & Portraits" pages on this site, but they include phages with ssDNA, ssRNA, or segmented dsRNA genomes, virions with and those without membrane components, and many other differences. In fact, the diversity of phages is at least as great as the diversity of plant and animal viruses, in keeping with the probability that phages and viruses of eukaryotes share common ancestry.

**History of bacteriophage research**

Bacteriophages were discovered a little over 80 years ago—in 1915 by the Englishman Frederick Twort and independently in 1917 by the French Canadian Félix D’Herelle. Initial research on phage was concerned with defining the nature of the bacteriophage—the two leading theories being that it was a filterable virus, like the Tobacco Mosaic Virus that had been discovered some 20 years earlier, or a self-perpetuating enzyme whose expression caused destruction of the bacterial cell.

Regardless of the exact nature of the bacteriophage, it was quickly realized that bacteriophages had the potential to kill the bacteria that cause many infectious diseases in humans, as well as in agriculturally important plants and animals. This idea formed the basis for much research as well as for the Pulitzer Prize-winning 1924 novel *Arrowsmith* by Sinclair Lewis (still a good read). Félix D’Herelle in particular was a champion of the potential for therapeutic uses of phage, which he promoted vigorously. In 1933, D’Herelle co-founded an institute for phage research in the Soviet Republic of Georgia, together with Georgian microbiologist George Eliava. Although Eliava was killed in one of Stalin’s purges in 1937, and D’Herelle never returned, the G. Eliava Institute of Bacteriophage survived and continued to supply phage for therapeutic uses to the entire Soviet Union until the recent breakup of the Soviet Union. In the West, research on such ‘phage therapy’ was dropped when penicillin and other chemical antibiotics were discovered starting in the 1940’s, though there has been some renewed interest in phage therapy in recent years as antibiotic resistance of pathogenic bacteria has become a more prominent threat to public health.

Meanwhile, bacteriophage research continued. The viral nature of the bacteriophage was clearly established, the chemical composition of the virions (the extracellular virus particles) was measured and shown to be protein and DNA, new phages infecting a variety of bacterial hosts were isolated, and some rudimentary progress was made in understanding the virus life cycle. The first electron micrographs of phages, showing a tadpole-like shape, were obtained in 1942 by Tom Anderson.

The ‘modern’ era of bacteriophage research is usually dated from 1938 when the expatriate German physicist, Max Delbrück, began his work on phages at the California Institute of Technology. Salvadore Luria, an Italian expatriate at Indiana University (later at MIT) and Al Hershey, an American at Vanderbilt
University (later at Cold Spring Harbor) soon joined Delbrück in pursuing bacteriophage research as a route to understanding the most fundamental features of biological life.

Phages soon became central players in the foundation of the discipline that later came to be known as molecular biology (the Journal of Molecular Biology started publication in 1959). Through the 1950’s and 1960’s, phage research had a dominant role in elucidating the most fundamental facts about what genes are and how the information in genes is read out to determine the properties of an organism. An underlying assumption (and justification) of the early phage molecular biologists was that the principles of life that could be learned from phages would also apply to other forms of life. As it has become clear in subsequent decades just how remarkably correct that assumption was, it has become similarly clear that the history of phage biology is a major and essential part of the modern history of biology as a whole.

The astonishing success of bacteriophage research over the 25-30 years prior to about 1970 in revealing the fundamental ‘secrets of life’ can be attributed largely to the fact that phages are so tractable as experimental systems. That is, they are genetically and structurally simple, they have a short life cycle that can be synchronized in a population, and genetic, biochemical, and structural approaches can be applied synergistically. The fact that phages interact intimately with their bacterial hosts means that virtually everything that is learned about phages is also informative about the bacterial cells they infect, and often about even broader biological questions.

Around 1970 the world of biological research began to be transformed by the ‘recombinant DNA revolution’, with which it becomes possible to effectively change a gene from any organism—no matter how complex or how eukaryotic—into a phage gene. The suite of laboratory techniques that made this revolution possible was developed largely through research on phages (with, of course, major contributions from research directed at their bacterial hosts and their genetic cousins, the plasmids). The recombinant DNA revolution has produced some profound changes in bacteriophage research, as in all other areas of biological research. For one thing, the number of researchers working primarily on phages decreased precipitously as it became possible to study the genes of more complex—particularly eukaryotic—organisms with nearly the same ease as had been possible previously primarily with phages and bacteria. At the same time, the number of biological researchers using some form of phage in their research has increased substantially, since many of the tools of modern molecular biological research are phages or phage-derived (see The Practical Phage, below). And just as recombinant DNA and other ‘modern’ techniques have made it easier to study the molecular biology of fruit flies, elephants, and sea slugs, they have also greatly increased the sophistication of the experiments that can be done with phages. Thus for those scientific problems where phages provide advantageous experimental systems, bacteriophage research is still vigorous and in many cases leading the field.
The Practical Phage
Because phages attack bacteria, and bacteria are sometimes harmful to people, many phage biologists believe it is possible to use phage or phage products (such as phage-encoded enzymes) as disease therapy or in other ways to solve our bacterial problems. At this writing, with a few exceptions, these techniques are in research stages rather than in actual use. Examples include treatment of particular bacterial infections or infestations with specific phage, treatment of bacterial infections with phage products, and use of phage-encoded toxins to combat cancer. Among the issues that need resolution before application of these methods becomes practical are how the phage-based therapeutic agent is to be delivered to the patient, how to protect the phage-based agent from immune attack by the patient's body, and how to handle the expected development of phage-resistant pathogens.

On the other hand, phages are and have for decades been widely used as tools in recombinant DNA technology: important in applications and developing applications ranging from medical diagnostics and forensics to basic research. See above, History of bacteriophage research, and our page Major discoveries made with bacteriophages.

Messages from Division M Chairs:
“The Phage Manifesto”, by Ry Young 2003 (PDF file)
“Bacteriophage Annals”, Fall 2002" by Mike Feiss (PDF file)

Officers of Division M
Meetings & events
Information about the ASM General Meeting, and other meetings of interest.

Resources for teachers
Instructions on assembling icosahedra, links to websites about teaching, books, and more.

Phage facts & portraits
Information about and micrographs, diagrams, or other images of specific phages.

Links
Links to other sites on the World Wide Web that are primarily about bacteriophages or generally about viruses.

Oddments
The hot topics of the day, and information about phage books, phage art, and phage history.
Phage Genome Analysis Background

Sequencing strategy

You are going to use a shotgun sequencing strategy that involves the following steps:

1) **Shearing.** Mechanically breaking the DNA into relatively small segments. You don’t really know how big each genome is to start with, but it’s a good bet that it is somewhere between 50,000 and 100,000bp. You’ll break it into pieces that are about 2000 bp long. The method for doing this is hydrodynamic shearing in which a solution of DNA is passed through a very small hole. One reason for choosing this method is that the breaks in the DNA are at essentially random positions, such that each of the resulting pieces is of a different segment of the genome. The shearing is done with a computer-controlled system that regulates the degree of shearing. Note that there is always a range in the sizes of DNA segments that are generated, but we expect the bulk of the DNA to be within the range of 1000-3000 bp.

2) **Repair.** When the DNA is sheared, it doesn’t always shear such that both DNA strands are broken between the same base pairs. If you imagine a broken ladder, the sides are not always broken between the same two rungs. For your subsequent steps, it is important that the DNA ends don’t have single-stranded extensions, since this will make it difficult to clone. You will therefore treat the DNA with enzymes that will convert the ragged ends into blunt ends.

3) **Size-fractionation.** Since there is a range of DNA sizes, and you would rather have a more narrow range of sizes, you will separate the DNA fragments on an agarose gel, which the DNA fragments move from one end to the other with smaller fragments moving faster than bigger ones. This spreads out the DNA according to size, and you can literally cut out a block of agarose that contains the desired size fraction of choice and purify the DNA from it.

4) **Cloning.** From the steps above, you now have a solution of DNA that contains DNA fragments that are all of a similar size (about 2,000bp) but are all different. You may have a microgram or so of DNA, but this represents a large number of individual molecules (about a billion). What you need to do is join together individual molecules with another DNA molecule (a vector) that will carry the DNA around. Basically, the vector has all the components necessary to ensure that the DNA is maintained and replicated in the bacteria that we going to use to prepare large amounts of that particular DNA molecule. To make these joint (recombinant) molecules, you will mix together your phage DNA fragments with a prepared form of the vector DNA and join them together using an enzyme called DNA Ligase that sews the DNAs together.

Here’s a way to think about the vector DNA. It starts out as a circle and is fairly small (just a few thousand bps). This is the way that it replicates inside of cells, and the way that you isolated it. To prepare it for your experiments you will cut it with a restriction enzyme that acts like a pair of **HHMI**
scissors and will cut the circular DNA just once. The result is still one piece of DNA, but now it is long and linear (no longer a circle), and it has two ends. Because the enzyme that you use to cut it, cuts it at a very specific location, all the DNA molecules are identical.

5) **Transformation.** After the ligation reaction, you will have a solution that contains many joined molecules. Some of these may be vector molecules joined to other vector molecules and some may be phage DNA fragments joined to other phage DNA fragments, but there will also be molecules that have one end of a vector molecule joined to one end of a phage DNA fragment and the other end joined to the other end of the same phage DNA fragment so that you again have a circle, albeit a rather larger one than with just vector alone. These are the ones you want. To recover these you need to get them into a bacterium where they will replicate and grow. You will add the DNA mixture to a sample of prepared *E. coli* cells and give them a large electric shock. In this process (called electroporation), the cells are encouraged to take up DNA molecules. However, this is overall a rather inefficient process, and only a few of the cells take up DNA. You need to have a way to differentiate between cells that have taken up DNA and those that haven’t. The trick is to use a vector DNA that contains a gene that confers resistance to an antibiotic such as penicillin, and *E. coli* cells that are penicillin-sensitive. After the electroporation you will plate out the cell mixture onto agar plates that contain penicillin. The cells that have not taken up any DNA will be killed, since they are sensitive to the drug. However, cells that have taken up either vector DNA, or one of the recombinant molecules will grow into colonies, since they are now drug resistant. These colonies contain either vector DNA molecules (i.e. not containing any phage DNA insert) or a joint or recombinant molecule. A key point to recognize is that each colony recovered from this transformation is derived from the uptake of a single DNA molecule. This plasmid will then replicate within the bacteria until there are hundreds of copies of the molecule in each cell, and you can grow many, many cells easily (up to a billion cells per milliliter). But at this point, all of these will be the same (and replicas of the original single DNA molecule that was taken up). One more important point concerns how you can distinguish between those cells that have taken up a vector DNA molecule and those that took up a recombinant molecule. This is simple. The vector DNA has been designed so that the site where we cut the DNA is inside a gene that can turn the cells dark blue or black. Thus cells that have taken up vector DNA will be blue or black, whereas those that took up recombinant molecules will be white, since the addition of the insert DNA destroys the integrity of the color-forming gene. The white colonies are therefore the ones that you are after. If your procedure has worked well, about half or more of your colonies will be white.

6) **DNA preparation.** Next, you need to prepare DNA from several hundred of these white colonies. You could grow up as much as you need. You know that if you grow about 1ml of cell culture until it is dense that you should be able to isolate many micrograms of DNA, which is enough for several sequencing reactions. You will grow up ‘blocks’ of clones, in which there are 96 wells in each block. Each well has about 1ml of broth and you will inoculate each well with a white colony. These cultures are then grown overnight until they are saturated cultures. The BioRobot is then used to break open the cells and isolate the DNA. Normally, you will prepare anywhere from 5-10 blocks for each phage genome.

7) **DNA sequencing.** Remember that each of your DNA clones contains the same piece of vector DNA but a different segment of phage DNA. The sequencing reactions can be thought of as sequential determination of the order of insert base pairs, and you can therefore perform this from both ‘sides’ of the vector DNA. The reactions work by annealing a short DNA primer (typically about 17 bases long) to the vector DNA in one particular position. The sequencing reactions involve extending this primer by use of a DNA polymerase, which is the type of enzyme that makes DNA. It makes the
DNA chains longer by adding one base at a time, and the base (either A, T, C, or G) is chosen as one that is complementary to the base of the template strand that is being copied. If we didn’t change anything, the enzyme would just copy the template to give long newly-made DNA strands. However, these reactions contain small amounts of each nucleotide (A, C, G, or T) altered in two ways. First, the base is altered so that it carries a chemical group that fluoresces at a particular wavelength; each base has a different dye – think of it as blue, black, green and red dyes. The second change is that these nucleotides have had their 3’OH removed, so that they stop any further lengthening of the chain – they are chain terminators. Therefore, the reaction makes a group of DNA chains of different lengths, and their precise lengths will differ by single base pairs. The base at the end of the chain will be determined by the color of the base that was added last. Since this is the only fluorescent dye in the entire chain, this chain will fluoresce that particular color. Therefore, if after you separate the chains by virtue of their length, then you see a series of different colored products, and the order of the different colors defines the order of the base pairs. To actually do this, you will add all the reagents together and run the reaction in a thermocycler. The products are then cleaned up (filtered to remove the unincorporated nucleotide dyes) and loaded into the sequencing machine. The machine takes 48 tiny reactions at a time and separates the chains in each reaction by running them through a matrix within a fine capillary. As they separate, a laser excites the dyes, and a detector recognizes which dye is fluorescing.

8) Assembly. You hope to obtain about 900bp or so from each primer run. Each clone can be sequenced from both ends and you will use two different primers for this, which we refer to as ‘forward’ and ‘reverse’ primers. Every clone thus gets sequenced twice, so that 5 blocks will be about 1000 sequencing reactions in total. Remember also that each of these clones has a random segment of phage DNA in it. The trick now is to take all of these pieces and put them together like a jigsaw puzzle, since many clones will represent overlapping segments. It’s important to note that the sequencing reactions only give the sequence of one strand and it can be either strand. You therefore anticipate that you will sequence each base pair many times (an average of perhaps 7 or 8 times) and hopefully at least once on each strand. Note also that the sequences of both strands within a single segment of DNA are different, but they are related by the pairing rules: i.e. G pairs with C, and A pairs with T. We have computer programs that perform these assembly functions.

9) Clean-up. The sequencing reactions and protocols are not perfect, and the assembly often generates regions of some ambiguity. Also, there may be small areas that were not sequenced at all. To resolve these, you will make a small collection of primers that are specific to a particular segment of phage sequence. These are then used with phage DNA as a template to sequence the weak areas.

10) Analysis. There are a variety of different methods of analysis once the sequence is complete. The first goal is often to identify the genes.

This step involves several pieces of data, and there are a few key facts about the way DNA is “read” that you need to know.
THERE ARE 6 TRANSLATIONAL FRAMES TO BE CONSIDERED

First, **double stranded DNA**, has two antiparallel strands each with opposing 5’ and 3’ ends.

For our example

\[
5' \ldots \text{ATCGGTCAGGCTT}\ldots 3'
\]
\[
3' \ldots \text{TAGCCAGTCCGAA}\ldots 5'.
\]

Second, **transcription** always occurs with the new chain growing from the 5’ to 3’ direction. (Think of the DNA strands as one-way tracks and the RNA polymerase as a vehicle that can only latch onto the track with its headlights in one direction and its tail in the other, and it can only go forward so there are two “directions”.) Remember, that the RNA polymerase is making a message, a mRNA copy of the DNA that will be read (“translated”) by the ribosome into the protein that is the “gene product”.

Third, **translation** occurs when the ribosome reads the message RNA (the words or “codons” are three bases long, each three specifying one amino acid, which is then added to the next amino acid specified by the next three bases.). A protein is a chain of amino acids whose identity was determined by the order of bases of the gene’s DNA. Now if you think of a chain of bases, for our example

\[
5' \ldots \text{AUCGGUCAGGCUU}\ldots 3',
\]

it could be broken into triplets of

- AUC-GGU-CAG-GCU-U.. or
- UCG-GUC-AGG-CUU-... or
- CGG-UCA-GGC-UU-...

The protein depends on where the ribosome starts “reading”. These are called the “frames” of translation. For each strand of the DNA, there are three frames.

Remember there is another strand that would be 3’.....UAGCCAGUCCGAA.....5’. It is almost always written 5’ to 3’ so it would be 5’......AAGCCUGACCGAU......3’

And the three frames would be

- AAG-CCU-GAC-CGA-U....
- AGC-CTUG-ACC-GAU...
- GCC-UGA-CGG-A

Together these six frames describe six different sequences of amino acids, so each piece of DNA potentially has the information for six amino acid sequences. The way nature works in phages is that only one of these six frames is actually part of a gene. (In other words, genes don’t really significantly overlap. One part of DNA is generally part of only one gene.)

Interesting exceptions to some of this are known, but can be saved for another discussion!!

Some of the codons say “START” and some of the codons say “STOP” to the ribosome, and that determines where the message reading begins and ends. In other words, what frame is used to start the translation depends on where the “START” signal is (preceded by another signal called a Ribosome Binding Site (RBS) or Shine-Delgarno sequence that tells the ribosome to latch on), and then that frame is translated into a protein that ends when the ribosome encounters a “STOP”.

The beauty of it is that some really smart hard working phage scientists in the last forty years worked out the details of this code!!!! You just open a book (or a computer) and the DNA sequence can be translated immediately into all six frames. Your job is to then look at the DNA where the computer tells you there are “START” and “STOP” and figure out if the stuff between these signals is a gene. You have several tools to help you figure this out.
The first fact is that phages like to conserve fuel on their superhighway. So, there is not a lot of “junk” DNA that is not part of a gene. In fact, there is darn little such DNA (unlike your own cells, but that is another topic). So, the fact that most of the DNA is “coding” and the earlier fact that genes don’t really significantly overlap tells you that the genes should be generally much right next to each other along the whole phage genome.

Second, once you know the DNA sequence, the computer can tell you instantly what sequence of amino acids the DNA in between would tell the ribosome (via the message RNA of course) to string into a protein. Maybe you think, “Big deal what does Methionine-alanine-guanine-cytosine-alanine-lysine-etc.etc.etc. mean to me???

You can take that sequence and “BLAST” it. This means to enter it as a search query into Genbank. What the BLAST program does is to take this sequence and compare it to all possible sequences that have been identified in the billions of bases of DNA sequences that have entered into the public database. These sequences are from all sorts of organisms, from phages, bacteria, plants, animals, etc. Anytime you have a significant match to something that is in the database, it is a good bet that, indeed, you have the right frame (this START……..STOP region is a bona fide gene, encoding a protein product with similarity to something in the database). You can record it and move on to the next one!

But, if you remember that one reason why we actually do all this is because phages have NEW and DIFFERENT gene products, that means that a lot of phage genes, when BLASTED, come up with “No Database Matches”. How do you identify these genes? A lot of it is based on how we know the phages typically pack their genes rightly, so you call genes that make sense in this pattern. More importantly the “coding potential” of the gene sequence you want to call is evaluated.

Coding potential comes from some more of the basic facts from molecular biology. Remember that there are 4 bases. This means there are 64 possible codons for the ribosome to read. The ribosome, however, has only twenty amino acids (words) in its language to put into the growing protein (plus three that say STOP). What this means is that some of the 61 codons that code for amino acids have to mean the same word (the code is degenerate: more than one codon specifies certain amino acids.) Different organisms have different preferences for the triplets that they use for certain amino acids, and by studying enough of any one organisms’s genes and gene products, these preferences can be determined. Then, a stretch of DNA can be scanned for its “coding potential,” (are the preferred codons used? Then it has high “coding potential”! Is the stretch full of rarely used codons? Then it has low coding potential.) Again, thanks be to those who worked all this out! There are programs that can graphically output the coding potential of all six frames of the whole phage genome (along with marking the STARTs and STOPs.

You can take all of these tools, (six-phase-translation, coding potential output, BLAST results, general knowledge of how phages pack their genomes with genes) and call all the genes.

The final and highest level of the analysis, which really never ends, is comparing these genes to other genes and the overall organization of the phage to other phages and to bacterial sequences and to try to understand how Nature is assembling these jewels. These are the product of natural selection acting on a whole slew of phages in the world. You can find the results of successful mixes and matches. The unsuccessful ones never propagate to wind up in your dirt samples!
PHAGE FACTS

PHAGE FACT: As many seconds as if the entire human population of the world counted 1 number per second for 50 trillion years.

PHAGE FACT: As many words spoken if every person in the world spoke their daily quota for 1.487 trillion years.

PHAGE FACT: As many as the miles of the distance of 5.8 hundred trillion times around the planet Jupiter.

PHAGE FACT: As many as the dollars it would take for each person in the entire world population to pay back the national debt 250 billion times.

PHAGE FACT: As many as the number of metric tons of 1.8 billion earths.

PHAGE FACT: As many as the miles of 425 billion round trips to the nearest galaxy, the Andromeda Galaxy.

PHAGE FACT: As many as milliliters of water as the daily amount of water to pass through a human intestine of the entire world population at the rate of 9 liters/day for 50 trillion years.

PHAGE FACT: As many as the number of words if you typed 1 quadrillion words/minute for 3.29 quadrillion years.

PHAGE FACT: As many as the number of base pairs as found in all human cells in the total world population.
Media and Reagents for Growing *Mycobacterium smegmatis*

*Mycobacterium smegmatis* mc² 155, smeg, is the host bacterium. It is a common soil organism, non-pathogenic. It grows slowly (colonies in ~ 4 days). It is resistant to carbenicillin (CB), so we always include CB at 50 µg/ml to kill other bacteria. Cyclohexamide (CHX) at 10 µg/ml is added to the media because it kills most molds and yeast. In liquid culture, *M. smegmatis* tends to clump. We grow it from a colony from a plate in 7H9 media with calcium, CB/CHX/ADC, and 0.05% Tween® 80, then subculture this to media without the Tween® for cells for phage infection. We grow it in standard test tubes placed at an angle on a shaker, but for bigger volumes, we use baffled flasks. Most of the phages have a calcium requirement. We always put 1mM CaCl₂ in media and top agar. We standardly use 0.35% top agar in our screens, hopefully to identify bigger phages that might not form plaques on the standard 0.7% agar.

**ADC** (NO HEAT, filter sterilize)
60 g dextrose
25.5 g NaCl
150 g Albumin
2850 ml ddH₂O

**20% Tween®** (50°C to dissolve, filter sterilize)
20 ml Tween® 80
80 ml ddH₂O

**MBTA** (Middlebrook Top Agar) made at 0.7% melted, diluted to 0.35% with 7H9 (plus 2 ml CaCl₂ *)
4.7 g 7H9
7 g agar
H₂O to 900 ml

**Phage Buffer** (autoclave or filter sterilize, add 0.1mM CaCl₂ * prior to use)
10ml 1M Tris, pH 7.5
10 ml 1M MgSO₄
4 g NaCl
980 ml ddH₂O

**7H9** (autoclave, add antibiotics, ADC, and calcium prior to use)
4.7 g 7H9 broth base
5 ml 40% glycerol
900 ml ddH₂O

HHMI
HOWARD HUGHES MEDICAL INSTITUTE PROFESSORSHIP PHAGEHUNTING PROGRAM
7H10 Plates (Autoclave, cool to 55° C and add 100 ml ADC and 10 ml 0.1 mM CaCl₂, CB, CHX and then pour.)
19g 7H10 agar
12.5 ml 40% glycerol
890 ml ddH₂O

CaCl₂ * Calcium is added to Mycobacterial growth media to ensure adequate calcium is available for necessary cellular metabolic processes. Note that for the most part, we add calcium to obtain a 0.1mM concentration in the final solutions of each media or reagent we use. (Therefore we will add 1ml of the 1mM CaCl₂ stock solution to phage buffer, but 2ml of CaCl₂ to 7H9 (because it will be used to dilute top agar by 50%).)

7H9 + CB + CH + ADC + CaCl₂ - for liquid culture of smeg
100ml 7H9
10 ml ADC
100 µl of CB
100 µl of CHX

250 µl Tween® (For intial sub-culture ONLY!) See direction below.

Growing Mycobacterium smegmatis mc²155

When culturing Mycobacterium smegmatis mc²155: Start by retrieving a sample from the frozen stock in the -70°C freezer and streaking it on a 7H10 ADC CB CHX plate. Allow to grow for several days. The goal is to produce isolated colonies. Smeg has a distinct colony morphology. When sub-culturing from this plate, pick a tiny (smaller than what you can see) piece from the center of the colony to grow in liquid culture. The liquid media (7H9 ADC CB CHX) has Tween® added to minimize the clumping of the bacterial growth. Once a homogenous culture is obtained, subculture in media without Tween®.

For growing smeg: Initial transfer from plated smeg
(Autoclave, for each L, add 100 ml ADC enrichment and CaCl₂ to 1mM, 100 µl carbenicillin (CB) and 100 µl cycloheximide (CHX). Store in fridge after adding ADC.) When growing smeg from plate, add 250 µl 20% Tween® 80 to 100 ml bottle. It is advisable to use a ‘touch’ of the culture from your plate into 1-2 ml of this Tween®-media. Vortex well. Place on shaker for at least 24 hours. Sub-culture from this culture into media without Tween® for phage infection.

For growing smeg for infections (7H9 without Tween®)
Transfer a small amount (1 – 100 µl of the smeg grown in Tween® into a flask that is only filled ~1/5 full of 7H9 + CaCl₂ + ADC + CB + CHX (same concentrations as above but no Tween®). Place securely on shaker for 24 hours.
Instructions for growing *Mycobacterium smegmatis* mc² 4518

When culturing *M. smegmatis* mc² 4518 from an agar plate. (Tween® is added because it dissuades the bacterium from clumping. *M. smegmatis* mc² 4518 should not be used for testing with the Tween® additive (it will inhibit plaque formation.)

To grow *M. smegmatis* mc² 4518 (pantothenate negative mutant) from an agar plate make this media. Refrigerate any unused part. Be sure to label with name, date, and additives.

One bottle (90 mls) 7H9
100 µl CB
100 µl CHX
10 mls ADC
1 ml CaCl₂
250 µl Tween® 80
100 µl* of stock solution of pantothenate (25mg/ml) (This solution is light sensitive, so keep in a dark (foiled-wrapped) container.) *200 µl if using it for top agar prep.

Pick a colony into about 2 mls of this media, vortex well. Shake at 37°C overnight or if necessary, longer until saturated.

When culturing *M. smegmatis* mc² 4518 for phagehunting.

Once *M. smegmatis* mc² 4518 is smoothly into broth culture, it can be transferred to flasks for larger volumes. Limit the number of serial transfers as much as possible. Routinely, go back to the initial stock culture. Remember, smeg can sit on the bench at room temperature for weeks. Just check that it resuspends smoothly.

To Subculture from this

- Make the media the same as above, with no Tween®
- Subculture 1:100 or 1:1000, depending how soon you need the cells
- Grow shaking at 37°C in a baffled flask overnight, after which you can sit this culture at room temperature for an extended period without inhibiting the growth of mycobacteriophages.
- Typically, we use a flask 5 times the volume of cells you want to grow. This ensures a neat, oxygenated, well contained environment for growth of the bacteria on the shaker.
- An alternative to using a shaker: use a stir bar in an unbaflled flask and incubate at 37°C overnight.
- *Mycobacterium smegmatis* can also be grown at room temperature; it will just take longer.
Outline of Procedure for the Isolation and Purification of a Phage Stock

I. Sample Collection and Preparation
   1. Collect environmental samples in 15-50 ml conical tubes
   2. If solid, add phage buffer with 1mM CaCl₂ and vortex
   3. Remove 1 ml aliquot and spin to pellet debris
   4. Filter sterilize supernatant with 0.22 µ filters

II. First Round of Infection: Plaque Screening
   1. Infect 0.5 ml *Mycobacterium smegmatis* mc²155 with 50 µl of filtered sample
   2. Add 4.5 ml MBTA top agar/CaCl₂
   3. Plate on 7H10/CB/CHX/ADC/CaCl₂
   4. Incubate at 37° C overnight
   5. Next day: Check for plaques

III. Spot Test for Verification of Putative Plaques
   1. Pick putative plaques into 100 µl phage buffer/CaCl₂
   2. Mix 0.5 ml *M. smegmatis* mc²155 with 4.5 ml MBTA/CaCl₂
   3. Plate on 7H10/CB/CHX/ADC/CaCl₂
   4. Allow top agar to dry completely
   5. Spot 5 µl of each sample into appropriate labeled grid box
   6. After spots dry, incubate at 37° C overnight
   7. Check spot plate for plaques

IV. Additional Rounds of Infection for Plaque Purification
   1. Use a pipette tip to pick a single plaque into 100 µl phage buffer plus calcium
   2. Serially dilute sample to 10⁻², 10⁻³, 10⁻⁴
   3. Infect 0.5 ml *M. smegmatis* mc²155 with 10 µl of each sample dilution
   4. Add 4.5 ml MBTA/CaCl₂
   5. Plate on 7H10/CB/CHX/ADC/CaCl₂
   6. Incubate at 37° C overnight
   7. Twenty-four hours later, many plaques should be visible
   8. Repeat steps IV-1 through IV-7 several times to isolate and purify a single phage
V. Final Plaque Purification
1. Use a pipette tip to pick a well-isolated plaque from one of the dilution plates into 100 µl phage buffer plus calcium
2. Serially dilute phage sample to $10^{-1}$, $10^{-2}$, $10^{-3}$
3. Infect 0.5 ml *M. smegmatis mc²155* with 10 µl of neat (undiluted) and dilutions
4. Add 4.5 ml MBTA
5. Plate on 7H10/CB/CHX/ADC/CaCl₂
6. Incubate at 37° C overnight

VI. Plate Lysate Production
1. To a nearly cleared plate, add 4.5 ml phage buffer/CaCl₂, swirl gently
2. Let sit at room temperature for 2-3 hours
3. Siphon liquid lysate and filter through a 0.22 µM filter

VII. Titer Determination

“Quick and Dirty” Spot Tests
1. Mix 0.5 ml *M. smegmatis mc²155* and 4.5 ml MBTA/CaCl₂
2. Plate on 7H10/CB/CHX/ADC/CaCl₂
3. Serially dilute lysate $10^{-1}$ through $10^{-10}$ in phage buffer/CaCl₂.
4. Spot 5 µl of each dilution on the plate after it has cooled and dried
5. After the spots evaporate, incubate at 37° C overnight
6. Calculate titer and compare to small plate results.

Small plate titer
1. Serially dilute lysate $10^{-1}$ through $10^{-10}$ in phage buffer/CaCl₂.
2. Infect 0.5 ml *M. smegmatis mc²155* with 10 µl of each dilution.
3. Add 4.5 ml MBTA
4. Plate on 7H10/CB/CHX/ADC/CaCl₂
5. Incubate at 37° C overnight
6. Review your plates at 24 hours and choose a plate with between 20 to 200 plaques. Since the counting error is proportional to the square root of the number counted, the greater number that you count the smaller, the error. 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%. At the other end, it becomes cumbersome to count more than 300 plaques on a plate without losing count, so again you begin to introduce a significant counting error into the mix.
7. Calculate titer and compare to “quick and dirty” results.

IX. Empirical Test of Lysate Concentration
1. Use the titer to calculate amount of phage lysate necessary to infect one large plate
2. Choose additional volume/dilution combinations of lysate to test which dilutions will form a web effect of plaques that almost cover the bacterial lawn.
3. Serially dilute lysate to create necessary dilutions
4. Infect 1 ml *M. smegmatis mc²155* with each volume/dilution combination
5. Add 9 ml MBTA/CaCl₂
6. Plate on large-size 7H10/CB/CHX/ADC/CaCl₂
7. Incubate at 37° C overnight
8. Determine lysate volume/dilution that yields the best web effect of *smeg* growth
X. Big Plate Infection
  1. Calculate volume/dilution of lysate needed to infect 30 plates based on your empirical test
  2. Prewarm 30 large 7H10/CB/CHX/ADC/CaCl\(_2\)
  3. Infect 30 ml *M. smegmatis* mc\(^2\)155 with appropriate amount of freshly diluted lysate
  4. Make 300 ml 0.35% MBTA/CaCl\(_2\)
  5. Add 300 ml top agar to infected *M. smegmatis* mc\(^2\)155 and swirl
  6. Quickly plate on large-size 7H10/CB/CHX/ADC/CaCl\(_2\)
  7. Allow plates to harden, then incubate at 37° C overnight
Procedure for the Isolation and Purification of a Phage

I. Sample Collection and Preparation

5. 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… (You can wear gloves to keep the bacteria from your hands out of the sample and vice versa!)

6. If the sample is solid, add phage buffer with 1mM CaCl$_2$ and mix/vortex well. Allow phage to diffuse into buffer for about 20 minutes.
   Add enough phage buffer to flood sample and form a liquid layer that can be pipetted off. Phage buffer is 10 mM Tris, pH 7.5, 10 mM MgCl$_2$, 68 mM NaCl, (remember to add 1ml CaCl$_2$!).

7. Allow sample to settle to bottom of tube, then pipette a 1 ml aliquot from the top liquid layer of phage buffer into a sterile 1.6 ml eppendorf tube. Centrifuge the sample to pellet the debris.
   Depending on your centrifuge, two minutes at medium speed should be suitable. Make sure that you balance the tubes evenly (Put tubes directly across from each other in the centrifuge. If you have an odd number, make a blank with water to balance the odd tube.)

4. Filter and sterilize 1 ml supernatant with 0.22 µ filter
   After centrifugation, pipette the liquid portion of the sample above the pellet into a syringe fitted with a .22 µ filter. The syringe-filter device should already be set up over an open 1.6 ml eppendorf tube. Then, plunge the liquid slowly into the eppendorf. From here onward, treat the phage sample steriley.

II. First Round of Infection: Plaque Screening

1. Infect 0.5 ml *Mycobacterium smegmatis mc$^2$155 with 50 µl of your filtered sample
   In sterile tubes (one for each sample plus one for a negative control), aliquot 0.5 ml of *M. smegmatis* mc$^2$155 that was grown on 7H10/CB/CHX/ADC/Ca. (*M. smegmatis* tends to clump when grown, so use a baffled flask and shake well. Do not add Tween® 80, as this can inhibit phage infection. *M. smegmatis* grows well at 37° C on a shaker. If you don’t have access to a heated shaker, it can be grown at room temperature in a sterile flask on a stir plate with a sterile stir bar.) To each 0.5 ml aliquot of *M. smegmatis*, add 50 µl of the filtered sample to infect the
bacteria. Mix well by vortexing. **Be sure to do an uninfected control tube that contains the bacteria and buffer but does not contain any phage sample.** As you prepare the top agar, the tubes can sit at room temperature for 15-30 minutes to allow phage in the sample to infect the bacteria.

2. **Add 4.5 ml MBTA top agar/CaCl₂**
   While the phages are infecting the bacteria, 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 and boil over). Cool the MBTA by steriley adding 50 ml 7H9 with 2ml CaCl₂ and mixing well. This will bring the final concentration of agar to 0.35%. This top agar must be stored at 55° C or used before it hardens. It must be cooled enough so the bacteria are not killed...(i.e. feels warm in your hand, but not scalding). Using a 5 ml pipette, add 4.5 ml MBTA top agar with Ca to each tube of infected bacteria one at a time. Then, immediately pull the mixture back up into the pipette. Be sure to use a fresh pipette for each sample.

3. **Plate total volume of 5.05 ml (4.5 ml top agar + 0.5 ml M. smegmatis + 50 µl of filtered sample) on 7H10/CB/CHX/ADC/CaCl₂**
   Immediately expel the cells/top agar mix onto a 7H10/CB/CHX/ADC/Ca plate (plates should be prewarmed to 37°C if possible). Swirl each plate to evenly spread the top agar before it cools and hardens. Allow the top agar to dry and harden before flipping the plates.

4. **Incubate at 37°C overnight**
   After the samples harden (how long that takes depends on how fresh the plates were, how warm they were, and the relative humidity and temperature in your lab!), put the plates upside-down at 37°C to prevent liquid condensation drops from forming on the lid and dripping onto the plate.

5. **Check plates for plaques**
   If you see plaques the next day….great!….continue on!! If you don’t see any possible plaques, leave the plates at 37° C another day, then check again. Plaques may be difficult to see due to small size or turbidity, so look carefully. Test anything that could possibly be a plaque by continuing with step III. Be warned that an air bubble in the agar can look a lot like a plaque. Be sure that there are no plaques on the control plate! If there are no possible plaques, don’t be discouraged, but select some new samples and repeat the procedures until you obtain plaque formation.
**Figure 1: Plaque Formation**- Plaques come in many sizes and degrees of transparency. This plate is an example of at least 3 plaque morphologies on a plate generated from a single soil sample. Plaques may be large or almost microscopic pinpricks. They may also appear either clear or turbid. Check all plates carefully for plaque formation, and if in doubt, pick a putative plaque and test it with a spot test.

### III. Spot Test for Verification of Putative Plaques

9. **Use a pipette tip to pick a single putative plaque into 100 μl phage buffer + CaCl₂**
   Sterilely aliquot 100 μl of phage buffer into eppendorf tubes. Just touch the center of the plaque with the end of a tip, then put the end of that tip into the liquid and gently tap it on the wall of the tube. Throw the tip away and mix the liquid well by vortexing. Pick all possible plaques and place in separate eppendorf 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. **These samples should be refrigerated after the spot test until the presence of phage is verified!**

2. **Mix 0.5 ml *M. smegmatis* mc²155 and 4.5 ml MBTA + CaCl₂**
   This is just like your uninfected cell controls done above. **Do not infect the cells with any putative phage samples!**

3. **Plate on 7H10/CB/CHX/ADC/CaCl₂ with grid**
   Prior to plating, use a marker to draw a grid with enough blocks for each putative plaque that was picked. Remember to swirl the top agar evenly over the plate before it cools.

4. **Allow top agar to harden completely or the spots will diffuse through the plate!**

5. **After the plate hardens, spot 5 μl of each phage sample on the plate in the appropriate grid.**
   Using the phage samples from above, pipette 5 μl and hold the tip above the plate. Don’t touch the pipette tip to the agar! Just hold the tip slightly above the agar, and push the droplet out slowly to avoid splattering. Avoid making bubbles, these burst, scattering phage across your plate.

6. **After the spots evaporate, incubate at 37° C overnight**
   Be completely sure that there is no liquid left before you move the plate. Remember to refrigerate your putative phage samples in the eppendorf tubes!

7. **The next day, check spot plate for plaques**
   If a spot develops: The plaque you picked was a genuine phage! Based on the grid, identify where the phage sample originated and, using the refrigerated sample from III-1, continue on with step IV-2.
   If no spot develops: You probably picked an air bubble. Don’t be discouraged because most people collect many (sometimes dozens) of samples before the find a phage. Collect more dirt samples and return to step I-1.
IV. Additional Rounds of Infection for Plaque Purification

When purifying phage, be aware of the following considerations:

a. Pick as soon as possible. The longer the phage is on the plate, the further it diffuses and cross into an adjacent plaque.

b. Check plates with low dilutions (a lot of plaques) for uniform plaque morphology.

c. Check plates with high dilutions (a few plaques) for uniform plaque morphology and to pick your plaque.

1. Use a pipette tip to pick a single plaque into 100 µl phage buffer plus calcium

   Aliquot 100 µl of phage buffer sterilely into eppendorf tubes. Just touch the center of an isolated plaque with the end of a tip, then put the end of that tip into the liquid and gently tap it on the wall of the tube. Throw the tip away and mix the liquid well using the vortex.

10. Serially dilute sample to $10^{-2}$, $10^{-3}$, $10^{-4}$ in phage buffer with calcium.

   The keys to successful dilution technique are to mix each sample well before removing the next aliquot and to change tips between each aliquot. A $10^{-2}$ dilution means 1 µl of your undiluted plus 99 µl of phage buffer. Then to do $10^{-3}$ you add 10 µl of the $10^{-2}$ with 90 µl phage buffer. To make $10^{-4}$, add 10 µl of the mixed $10^{-3}$ to 90 µl phage buffer. To make it easy, aliquot all the phage buffer for the dilutions into labeled eppendorfs first. A good way to avoid errors is to have two tube-holding racks, and when you have added the phage to one tube, put it in the second rack.

   ![Serial Dilutions](image)

   **Figure 2: 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 originate from the previous dilution.

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

   Aliquot three tubes of 500 µl mc²155 per sample, plus an uninfected control. (See section II-1.) Allow the phage to infect the bacteria for 15-30 minutes.

12. Add 4.5 ml MBTA

   See section II-2.

13. Plate on 7H10/CB/CHX/ADC/CaCl₂

   See section II-3

14. Incubate at 37° C overnight

   See section II-4.

15. The next day, many plaques should be visible and the numbers of phages should be relative to each plate’s respective dilution.
16. Repeat steps IV-1 through IV-6 several times to insure plaque purification
Evidence of many plaques insures that a genuine plaque was picked and not an air bubble in the agar. Some plates may include multiple phages that are difficult to isolate and purify. It is of utmost importance that the final plaque purification contains 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. **This may require 5-10 subsequent rounds of purification to isolate your phage!**

![Figure 3: Plaque Purification](image)

**Figure 3: Plaque Purification** - Successive rounds of picking isolated plaques, making dilutions, infecting *M. smegmatis*, and plating allow for the purification and isolation of a single phage. The plaque purification steps must be repeated until a purified phage is obtained, reflected by a single plaque morphology. Two examples are shown above.

**V. Final Plaque Purification**

7. **Use a pipette tip to pick a well-isolated plaque from one of the above dilution plates into 100 µl phage buffer plus calcium**
   See section IV-1.

8. **Serially dilute the picked plaque in phage buffer to 10⁻¹, 10⁻², 10⁻³**
   10⁻¹ is 10 µl of the undiluted into 90 µl phage buffer with CaCl₂. 10⁻² is 10 µl of 10⁻¹ in 90 µl phage buffer with CaCl₂. 10⁻³ is 10 µl of 10⁻² in 90 µl phage buffer plus CaCl₂. See section IV-2.

9. **Infect 0.5 ml *M. smegmatis* mc²155 with 10 µl of neat (undiluted) and 10⁻¹, 10⁻², and 10⁻³ dilutions.**
   Aliquot 500 µl of *M. smegmatis* mc²155 into each of 4 tubes per sample, plus an uninfected control. See sections II-1 and IV-3.

10. **Add 4.5 ml MBTA/CaCl₂**
    See section II-2.

11. **Plate on 7H10/CB/CHX/ADC/CaCl₂**
    See section II-3.

12. **Incubate at 37° C overnight**
    See section II-4.
VI. Plate Lysate Production

4. To a nearly cleared plate from step V, add 4.5 ml phage buffer plus calcium, and swirl gently.

5. Let sit at room temperature for 2-3 hours (Or longer at 4°C)
   Occasionally, swirl the phage buffer on the plate gently. Do not splash.

6. Siphon liquid lysate and filter it through a 0.22 µm filter
   See section I-4 except use one 5 ml syringes, and filter the lysate separately into 2-3 eppendorf tubes. From here on, the lysate should be refrigerated when stored.

VII. Titers

There are two ways to do 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.

Small Plate Titer

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 with 1 mM CaCl$_2$.
   See section IV-2, continues out several more dilutions!

2. Infect 0.5 ml *M. smegmatis mc²155* with 10 µl of $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions
   Aliquot three tubes of 500 µl mc²155 per sample, plus an uninfected control. (See section II-1.) Allow the phage to infect the bacteria for 15-30 minutes.

3. Add 4.5 ml MBTA
   See section II-2.

4. Plate on 7H10/CB/CHX/ADC/CaCl$_2$
   See section II-3

5. Incubate at 37° C overnight
   See section II-4.

6. The next day, many plaques should be visible and the numbers of phages should be relative to each plate’s respective dilution.
   You will find the dilutional plate with 20 -200 plaques to calculate the titer of phage sample.
“Quick and Dirty” Spot Tests

Figure 4: Spot Test Grids- The use of a grid allows for the appropriate labelling of the origin of each each spot on a plate. A marker is used to form the necessary blocks, and each block is labelled with the identity of the spot that is present within its lines.

1. **Mix 0.5 ml** *M. smegmatis mc²155 and 4.5 ml MBTA + CaCl₂**
   This is just like your uninfected cell controls done above. See section III-2.

2. **Plate on 7H10/CB/CHX/ADC/CaCl₂**
   Swirl to spread before it cools. See section III-3.

3. **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 with 1 mM CaCl₂.**
   See section IV-2, continues out several more dilutions!

4. **Spot 5 µl of each dilution on the plate after the top agar has cooled and hardened completely.**
   Don’t touch the pipette tip to the agar. Just hold the tip slightly above the top agar, and push the droplet out slowly. See section III-5.

5. **After the spots evaporate, incubate at 37° C overnight**
   Really be sure that there is no liquid left before you move the plate. Gently turn the plates upside-down to prevent condensation from dripping on the plate.

VIII. **Calculation of Titer**

1. **The quick and dirty titer will give you fast numbers to work with.** However, it is difficult to count the plaques in a 5µl spot. **It is preferable to use the titer plates prepared above to calculate the titer.**
Figure 5: 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. 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.

$$28 \text{ PFUs/} 5\mu l \times 1000 \mu l/1 \text{ ml} \times 10^7 = 5.6 \times 10^{10} \text{ PFUs/ml}$$

2. **On the small plate that was incubated overnight, 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 should 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, you can average the two values.

Figure 6: Count the plaques in this photo. There are 50 on this $10^{-7}$ dilution plate.

3. **To calculate the titer (the concentration of phage in the lysate, measured in plaques per ml), multiply back up based on the dilution of the spot.**
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 \( \mu l \)), and then convert \( \mu l \) to ml to obtain the titer in plaques/ml. Use the example calculation below, based on counting 50 plaques in the \( 10^{-7} \) spot:

**Titer Calculation:** 50 plaques/10 \( \mu l \) \( \times \) \( 10^7 \) \( \times \) 1000\( \mu l/ml = 5.0 \times 10^{10} \) plaques per ml

**The number of plaques per ml calculated is the titer (concentration of PFU/ml).**

The point of determining a titer is to produce enough phage in your big plate infection for the subsequent procedures you will do. A final concentration of \( 10^{13} \) phages/ml is desirable. To accomplish this task without wasting the big plates, an empirical test is essential. The goal is to produce 30 plates with maximum production of phage. We have estimated that 6000 plaques on a big plate yield the desired numbers. This number is only an estimate and your phage may require more or less. Factors that influence the numbers are concentration and age of the smeg culture, size of plaque, incubation time (for both ‘infecting’ and then growing). Your phage didn’t read this manual. By now, you have learned how to optimize the growth of your phage. All of that information is used to produce an abundant yield for phage harvest.

**IX. Empirical Test of Lysate Concentration**

Before starting the 30-plate infection, you must be confident with the volume/dilution combination of lysate that yields a web pattern on a large plate. Also, be sure that you have a large quantity of \( M. smegmatis \) ready to go for this step. The same subculture of \( M. smegmatis \) must be used 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 because the empirical test should be on the same age plate (water content, etc.) as the 30 plate infection.

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, this number should be adjusted (<6000 for large plaques and >6000 for small plaques). This calculation is performed by dividing 6000 (or adjusted value) by the calculated titer. For example:

\[ \frac{6000 \text{ plaques per plate}}{5 \times 10^{10} \text{ plaques per ml}} = 1.2 \times 10^{-7} \text{ ml lysate per plate} \]

\[ 1.2 \times 10^{-7} \text{ ml lysate per plate} = 1.2 \times 10^{-4} \mu l \text{ lysate per plate} \]

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

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

Pick a total of 5 lysate volumes and dilutions combinations with which to infect large plates in order to find the combination that yields the best web pattern of \( M. smegmatis \) growth. One combination of lysate volume and dilution refers to the calculation in step IX-1 above. Of the
remaining 4 combinations, 2 lysate concentrations should be above the IX-1 calculation, and 2 should be below the IX-1 calculation. Continuing with the example started above, the 5 plate empirical test would look like this:

1. 10 µl of $10^{-5}$ dilution of lysate (based on IX-1 calculation)
2. 10 µl of $10^{-4}$ dilution of lysate (one order of magnitude above)
3. 60 µl of $10^{-4}$ dilution of lysate (about half way between the first two dilutions)
4. 10 µl of $10^{-6}$ dilution of lysate (one order of magnitude below)
5. 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 IV-2. Meticulous care should be taken in pipetting and vortexing each subsequent dilution.

4. **Infect 1 ml aliquots of *M. smegmatis* mc²155 in 5 separate large test tubes with the appropriate amount of diluted lysate corresponding to the calculations shown above.**
   After insuring that the lysate is well-mixed into solution with the *M. smegmatis*, allow 20 – 30 minutes for infection in the large test tubes. Take careful note of the conditions of the infection (time allowed to infect and same subculture of *M. smegmatis* used) in order to create consistency in the empirical test.

5. **Add 9 ml MBTA top agar + CaCl$_2$.**
   See section II-2 (only difference is larger volume of top agar).

6. **Plate on pre-warmed 150 x 15 mm 7H10/CB/CHX/ADC/CaCl$_2$ plates.**
   Swirl gently to spread top agar. See section II-3 (only difference is large plates).

7. **After plates harden completely, incubate at 37° C overnight.**
   Plates do not need to be incubated upside down as in the past.

8. **The next day, analyze the plates for the purpose of determining which volume/dilution combination yielded the best web pattern of phage infected *M. smegmatis* growth.**
   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 30-plate infection. If no web patterns form, repeat the empirical test using different lysate volume/dilution combinations.

**X. Big Plate Infection**

1. **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 30-plate infection.**
   Multiply the volume of phage 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. Prewarm 30 large 7H10/CB/CHX/ADC/Ca plates.

3. In a 500 ml flask, infect 30 ml *M. smegmatis mc²* 155 with the appropriate volume and dilution of lysate.
   Be sure to mix the bacteria and lysate well. 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 with .35% MBTA and 1 mM CaCl₂.
   Top agar must be between 55 and 60°C (not boiling or cooler).

5. Add all 270 ml top agar to the 500 ml flask with the infected *M. smegmatis*.

6. Quickly plate on 30 prewarmed 7H10/CB/CHX/ADC/CaCl₂ large plates.
   Work with a partner if necessary. 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 incubated upside down.
Procedure for the Harvest, Precipitation, and Dialyzation of phage

Harvest and Precipitation
1. Add 10 ml phage buffer plus CaCl\(_2\) (use the larger volume of prepared buffer, a 500 ml volume, with 5ml CaCl\(_2\) added) to each plate. Make sure the buffer covers the whole surface of the agar. The plates should be almost clear, but a web of cells should ideally remain. Store at room temperature a couple hours or longer at 4 degrees (up to overnight)
2. Siphon off the buffer with a pipette gun and 10 ml pipette into 2 conical centrifuge bottles.
3. Centrifuge 3500 x G for 5 minutes to pellet debris. Be sure to balance tubes and set the rotor code correctly. Ask if you need help!!!
4. Transfer the supernatant to a 250 ml flat bottom plastic bottle. Remove 10 ml and filter sterilize for a lysate stock. Be sure to determine the titer of this stock in the near future. Check the remaining volume. Add 10% polyethylene glycol (PEG 8000) and NaCl to 1M.
   e.g. If you have 265 ml (0.265 L), add 26.5 grams PEG 8000
   Add 0.265 L x 58 g/mole x 1 mole/L NaCl= 15.4 g NaCl.
   Stir this at 4ºC overnight or longer.

Titer you phage.

Resuspend Phage
1. In 2 balanced (by volume or weight) conical centrifuge bottles, centrifuge the precipitated phage for 5 minutes 3500 x G at 4ºC. Be sure to balance tubes and set the rotor code correctly. Ask if you need help!!!
2. Resuspend the phage pellets in 10 ml phage buffer with calcium. Gently combine both pellets in the 10 ml buffer. Pipette gently up and down, rock at 4 degrees for several hours.
3. Centrifuge at 3500 G at 40º C, save the clear supernatant.
4. Add 8.5g cesium chloride (CsCl). Mix.
5. Adjust the density to 1.5 g/ml using a refractometer. Measure, adjust, and repeat until you are there. Refer to the density/refractive index in the appendix.
6. Seal ultracentrifuge tube (be sure to get rid of all air bubbles in the tube).
7. Ultracentrifuge at 38,000 RPM for 16 hours. Calculate the speed X G depending on the centrifuge and rotor used.
Remove and Dialyze Phage

1. Carefully remove your tubes from the rotor. Wash the ultracentrifuge rotor with water. If you’re not sure exactly how to do it, ask someone who knows.
2. Put the tube in ring stand firmly.
3. Cut off the top of the tube with a hot scalpel.
4. Remove phage band with a syringe and needle. Insert the needle below the band, beveled edge facing close to the band, and slowly pull back the plunger.
5. A 100µl sample of undialyzed phage is to be submitted to Jen Houtz (Genome Center) for the Archives. Each sample must be a screw capped (O-ring) microcentrifuge tube labeled with your name, phage name, date (including year), and titer*. Be sure to seal well.
6. Dialyze phage: Put the remainder of your phage in a dialysis bag. Dialyze at 4°C in 500 ml pre-chilled phage buffer plus calcium for several hours, changing the buffer at least once.
7. Titer your phage. The titer must be at least $10^{13}$ phages/ml. An additional 100µl sample of dialyzed phage is to be submitted to Jen Houtz (Genome Center) for the Archives. Each sample must be a screw capped (O-ring) microcentrifuge tube labeled with your name, phage name, date (including year), and titer. Be sure to seal well.
8. Make DNA, do electron microscopy, etc.

*Record the titer of dialyzed phage. It may be difficult to obtain plaques with the CsCl present. However, this sample is most stable and needed for long-term storage.
Mycobacteriophage Genomic DNA Preparation Protocol

The phage particle is basically a tough protein shell around a single wonderful DNA chromosome. We want to purify the DNA away from the proteins. We start with 100,000,000,000 or so phages (and chromosomes) and isolate the DNA. Phenol is a chemical that denatures proteins. It also has the properties of being immiscible (doesn’t mix) with water or aqueous liquids and is more dense than water. We use these properties to sequentially break up the phages and separate the DNA from the denatured proteins.

NOTE: Be sure to remove the phenol from below the phenol/buffer interface in its container.

SAFETY NOTICE: Since phenol denatures proteins you must wear gloves and be sure not to spill any on yourself. It is poisonous if absorbed. Phenol will produce contact burns and acts as an anesthetic.

General note about mixing: To avoid mechanical damage to the DNA, use tube gentle inversion, rather than a vortex. Don’t use small-bore pipettes when transferring DNA mixtures.

1. Add 600 µl dialyzed CsCl phage to eppendorf Tube #1
2. Add an equal volume buffer equilibrated phenol and mix be gentle inversion. The liquid should turn white and goopy upon mixing.
3. Spin at 14K RPM at Room Temperature for 5 minutes.
4. Transfer aqueous (top) phase to fresh Tube #2, avoiding the white interface. There will be two layers with some white interface (denatured phage protein) between them. Use a 1000 µl pipettor to pipette up slowly.
5. Add 500 µl phenol to Tube #2 and gently mix.
6. Add 600 µl TE to Tube #1 and gently mix.
7. Spin Tube #1 and Tube #2 at 14 K RPM at Room Temp for 5 minutes.
8. Transfer the aqueous phase from Tube #2 to the fresh Tube Tube #3, avoiding the white interface.
9. Transfer the aqueous phase from Tube #1 to Tube #2, avoiding the white interface, and gently mix Tube #2.
10. Add 500 µl phenol to Tube #3 and gently mix Tube #3
11. Spin Tube #2 and Tube #3 at 14 K RPM at Room Temp for 5 minutes.
12. Transfer the aqueous phase from Tube #3 to fresh Tube #4, avoiding the white interface.
13. Transfer the aqueous phase from Tube #2 to Tube #3, avoiding the white interface, gently mix Tube #3.
14. Add 500 µl phenol to Tube #4 and gently mix Tube #4.
15. Spin Tube #3 and Tube #4 at 14 K RPM at Room Temp for 5 minutes.
16. Repeat steps 12-15 (adding one to each Tube #X) until there is no white goopy interface.
17. Transfer the final aqueous phases to a fresh tube(s). If the total volume of this aqueous phase is >700µl, two tubes will be needed. At step 31, they may be combined in one tube.
19. Spin at 14K RPM Room Temperature for 5 minutes.
20. Transfer the aqueous phase to a fresh tube.
21. In a fume hood, add an equal volume of chloroform and gently mix.
22. Spin at 14K RPM at Room Temperature for 5 minutes.
23. Transfer the aqueous phase to a fresh tube.
24. Add a 1/10 volume of 3 M sodium acetate (NaOAc) and 2.5 Volume of 95% ethanol (EtOH).
25. You should see a fine ball of string (DNA!) appear upon mixing.
26. Mix very well.
27. Freeze on dry ice. After freezing, you may continue with the protocol or leave the tube in the freezer to finish later.
28. Spin at 14K RPM at 4ºC for 30 minutes
29. A white pellet should have precipitated. Remove (decant or pipette) the EtOH and wash the pellet with 1 ml of 70% ethanol. Be careful that the pellet stays in the tube!
30. Air dry the pellet but do not over dry. A pellet that is too dry will be difficult to dissolve.
31. Dissolve in 200 µl TE.
32. You can gently pipette up and down to mix, and incubate at 37ºC or 42ºC to help it dissolve. Be careful not to accidentally aspirate and remove the pellet in this process. The final solution should be viscous but homogeneous. You can leave the DNA in TE at room temperature or 37ºC overnight to dissolve completely with no mixing. (You can also do the final dissolve step at 65ºC for 10 minutes, then put on ice.
DNA Restriction Enzyme Analysis and Electrophoresis

You have a dialyzed cesium chloride banded phage stock from which you have isolated DNA by phenol-chloroform extraction and ethanol precipitation. CONGRATULATIONS!!!! Now you want to check the DNA for quantity, quality, a first estimate of the genome size, and comparison with other phage genomes. Where would you start? One choice is restriction enzyme digestion, followed by agarose gel electrophoresis!

The Digests
1. Gently mix your DNA, warm it to 65°C for 10 minutes, then chill it quickly on ice to separate the DNA. Soin briefly to collect the DNA solution at the bottom of the tube.
2. To eppendorf tubes, add
   a. 13 µl H2O
   b. 2 µl appropriate enzyme buffer (see the NEB catalog!)
   c. 2 µl 10X BSA (diluted from 100X with ddH2O)
   d. 2 µl DNA
   e. 1 µl unit of enzyme (use BamHI, ClaI, EcoR, HaeIII, and HindIII)
      Mix gently. Never vortex the mixture once the enzyme is added.
3. Incubate at 37ºC in a waterbath for two hours or overnight.
4. Run a gel or freeze the samples until you have time to run the gel.

The Gel
1. Fully melt 0.4 g agarose in 50 ml 1X TBE in the microwave in a 250 ml flask. Weigh the flask. Heat this in the microwave until boiling (~1 minute, keep an eye on it). Make sure the agarose is completely melted by swirling the flask. Look for small lumps of agarose floating in the melt or stuck to the glass. Simmer until all grains of agarose are dissolved.
   *Use Seakem LE agarose from the chemical shelf. 1x TBE is in the carboy near the electrophoresis area.
2. Weigh the flask gain and add pure water to return the liquid that evaporated. Briefly heat the melt again.
3. Cool the melt to 50-60 degrees, either at room temperature or by holding it under running cold water. It is cool enough when you can hold the flask in your hand without getting burned.
4. Wearing gloves, add 2. \( \mu l \) of 10 mg/ml ethidium bromide. Ethidium bromide is a **carcinogen!!!** It is located in a small red bottle above the electrophoresis bench. **WEAR GLOVES WHEN HANDLING THE GEL FROM HERE ON!**

5. Put an inner tray in a casting tray. Pour the gel in to the casting tray.

6. Put a comb with the appropriate number of wells in the liquid gel.

7. Add 5 ml 1X TBE, allow the gel to cool and harden. Then remove the comb and pour the buffer into the gel box and remove the gel from the casting tray. Rinse off the comb and casting tray, and put them on the test tube rack to dry.

8. Put the gel on the tray into an electrophoresis chamber (gel box) with the wells to the left side.

9. Add enough 1X TBE with 0.5 g/ml Ethidium Bromide to cover the wells.

10. Add 2 \( \mu l \) ficoll dye to each sample. The ficoll dyes are located above the electrophoresis area.

11. Mix 2 \( \mu l \) uncut DNA with 10 \( \mu l \) TE and 2 \( \mu l \) ficoll dye in an eppendorf tube.

12. Also mix 1 \( \mu l \) 1kb DNA Ladder with 9 \( \mu l \) TE and 2 \( \mu l \) ficoll dye. The 1 kb DNA ladders are in the freezer next to Dr. Hatfull’s office in the drawer next to “Communal DNA Regrows, etc.” Make sure the ladder is completely thawed and mixed before using.
13. Load the samples in the gel in the following way:

![Image of gel with bands for HindIII, HaeIII, EcoRI, Clal, BamHI, Uncut, and 1 Kb Ladder]

**Note:** Gel is loaded ladder, uncut, alphabetically ordered enzyme digest name. When reading gels, the gel’s orientation is that the wells are placed at the top and the order can be read left to right.

14. Gently place the top on the electrophoresis chamber so your samples don’t rock out of the wells. Make sure the black (negative) electrode is on the left (DNA is negatively charged, so it will move towards the red (positive) electrode). Make sure black and red wires are connected to the power supply correctly.

15. Turn the power supply on and set it to constant voltage. Put it on the low setting and turn the dial until the voltmeter reads 100 volts. Run the gel at this voltage until you have good separation. There are two dyes in ficoll dye and they will separate as you run the gel. Both dyes should be on the gel when you are done. This will take ~30-45 minutes.

16. Turn the power source off and remove the chamber lid. **(If another person is running a gel on the same power source quickly unhook your cables and turn the power supply back on!)**
17. Place the gel on saran wrap and take it to the gel doc. (Do not touch the gel doc or the computer with an ethidium bromide buffer contaminated glove!!!!) Push the gel out of the inner tray into the gel doc and close the door. Hit “Live Focus” on the software and focus on the gel with the knob on top of the gel doc. Frame the gel so that the gel fills the screen. Push the trans-luminator button on and adjust the intensity if needed.

18. Video print two copies of your gel (one for your notebook and one for the digest book) and save the file on the Hatfull lab folder in a subfolder with your name. Be sure to label the file accurately.

19. Remove your gel from the gel doc. Wipe down the surface with DI water and KimWipes.

20. The picture of your gel should be taped in the digest book as shown here with well labeled wells and your name, your phage’s name, and the date.

The Hints!
1. The enzymes are in the -20º C freezer…THEY MUST STAY COLD… (Therefore, prepare the other ingredients the digest uses at your bench and keep on ice if necessary.)
   The enzymes are in blue racks in the freezer. Take out the enzymes you need and put them in your ice bucket and return promptly to minimize the time they are out of the freezer!!!!
2. Mix the reactions well, but don’t vortex once the enzyme is added!
3. Make sure the tubes are fully sealed before putting them in the water bath
4. Wear gloves with ethidium bromide gels!!! It is a carcinogen!
5. Note which manufacturer of DNA ladder you use.
6. Keep track of what is loaded in each lane – diagram it in your notebook.
7. If you are running more than one phage, load the gel with the 2 phages with the same enzymes in lanes next to each other, not all one phage with its enzymes then the other phage.
8. If gel doc is not up and running, select the Quantity One icon from the desktop.
9. Make sure you clean everything. Rinse off all electrophoresis equipment, wipe off gel doc platform, throw the gel in the proper chemical waste disposal bag, etc.
EM Grid Preparation

1. Cover the lab counter with the plastic faced paper to create a clean area.
2. Using the pointed forceps, remove a fresh (unused) grid from the grid box of unused grids. Handle the grids by the edges with forceps only.
3. Place the edge of the grid beneath the parafilm in the Petri dish used for cleaning grids. Close the front (gray) valve.
4. Turn the valve to connect to pump (Figure 1). Pump out the vacuum desiccating chamber.
5. Using the vacuum tester, clean the grids (blasting the grid to remove dirt and oil).
   "NOTE: Touch the tester to the metal piece at the top for ~ 45 seconds. You should see a purple glow (ionized gas) above the Petri dish."
6. **Bleed vacuum before turning it off.** (Figure 2) "NOTE: This is to prevent backwash of oil into the pump."
7. Slow vent the vacuum to open (gray valve).
8. Remove the Petri dish containing the now cleaned grids.
9. With forceps, remove one grid.
   "NOTE: To keep the forceps closed, push the rubber ring down toward the bottom."
10. Lay the closed forceps holding the grid on top of the Petri dish.
11. Using a Pasteur pipette, spot the phage onto the grid. Use your thumb pressure on the back of the pipette to expel the drop.
12. Let stand ~ 1-2 minutes (depending on phage titer).
   "NOTE: Too much phage will burst the frame on the grid."
13. Using the pipette, remove the phage spot by capillary action.
14. Do not let the grid dry out.
15. Wash the grid by spotting with water. (6-7 times) To remove the water, turn the forceps on its side and gently tap on the Petri dish.
16. Using the Pasteur pipette, add a spot of stain. Let stand ~1 minute.
17. Remove the stain by knocking the drop off then wicking it off each side with Whatman paper. Be sure to wick, touching only around the edges to avoid contamination with paper fibers.
REPAIRING SHEARED DNA

90 µl sheared DNA, estimate quantity
16 µl 10mM dNTPs
16 µl T4 10X buffer
1.6 µl 100X BSA
7 µl T4 Polymerase
29.4 µl dH2O
160 µl TOTAL VOLUME

If you use a larger or smaller volume of sheared DNA, adjust the water volume.
Incubate at Room Temperature for 15 minutes.
Add 5 µl Klenow (5U/µl), mix carefully and incubate at RT for another 15 minutes.
Add 35 µl dH2O and proceed with phenol extraction/EtOH precipitation.
Wash with 70% EtOH.

Your final pellet should be dissolved in 10 µl TE.

This entire volume of DNA needs to be run on a 0.7% agarose gel. Take a photo of the gel and carefully size-fractionate between 1-3 kb. Minimize the amount of time your DNA is exposed to the UV light from the transilluminator. UV light damages DNA.

LIGATIONS

Total ligation volume is 15 µl.
Constants:
1.0 µl pBlue Script vector (We use pBluescriptII SK+, cut with EcoRV/CIAP) (Stored in -20C freezer)
1.5 µl 10X Buffer
0.75 µl ATP
1.0 µl T4 DNA ligase

At least 2 ligations per phage DNA fragments preparation should be done. Examples are: 1.0 and 2.0 µl, or 0.5 and 1.0 µl, or 2.0 and 4.0 µl.
The remaining volume should be filled with dH2O.
Check with Jen/Alexis for suggestions about DNA fragments volume.
Mix ligations carefully and incubate at RT for 1 hour.
Add 15 µl water and heat kill at 65°C for 15 minutes.

The ligations can be transformed directly. If you aren't going to transform them right away, the ligations may be frozen in the -20C freezer. **Make sure everything is labeled: contents, date, ligation number, initials, phage name.**

**TRANSFORMATIONS**

You will be using *E.coli* XL1-Blue F’ competent cells for transformations. The stock in the Hatfull Lab was made by students and when the stock gets low, students need to replace them. This is not Molly's job, so DO NOT ask her to make cells for you. When it is time to make more competent cells, 2 or 3 students need to set up a time with Jen and Alexis and they will help you. The whole procedure takes a couple of days, so ample warning time is necessary.

Each frozen tube of cells is good for 5 transformations. The cells shouldn't be re-frozen once they are thawed.
Set everything up on ice. Label the electroporation cuvettes and set them on ice as well.

1. Put 1.0 µl of each ligation into a cuvette. You will also use 1.0 µl of pBlue (uncut, stored in cold box). Keep on ice.
2. Once the competent cells are thawed (use a fresh aliquot for your phage library), aliquot 40 µl of cells into a clean cuvette for each transformation. Each ligation gets its own transformation. You will also need 40 µl for a pBlue control and 40 µl for the ‘no DNA’ control. Make sure you label the transformation cuvettes.
3. Transfer the cell/ligation mix into the electroporation cuvettes. Be careful not to introduce any air bubbles into your sample. You may need to flick the cuvette several times to get any bubbles out.
4. Keep the cuvettes with sample on ice while you get the recovery tubes ready.
5. You will need a sterile culture tube for every ligation plus 1 tube for no DNA and 1 tube for the pBlue control. Aseptically add 1ml SOC broth to every culture tube. Make sure you label everything.
6. Have Jen or Alexis show you how to use the electroporator. (We use the one in the Lawrence Lab)
7. Recover your samples for 1 hour on a shaker in the 37ºC room. After plating, the remaining cells, may be stored in the cold box for a couple of days.
8. They should be plated on S-GAL Amp/Tet plates. (Amp=100 mg/ml, Tet= 12.5 mg/ml)
9. Plate 100 µl cells/plate. Also plate 100 µl of No DNA cells. For pBlue, Plate 100 µl LB and Place 1 µl cells in puddle to spread.

**TEST COLONIES**

1. Aseptically aliquot 2 ml LB/Amp into several test tubes. Pick a white colony with a pipet tip and inject directly into the tube.
2. Test 8-12 white colonies per ligation.
3. Grow the cultures overnight (about 16 hours) in the 37ºC room on a shaker.
4. The next day, take 1.5 ml from each tube and put it into a clean eppendorf tube.
5. Spin tubes for 30-60 seconds and pipet off the supernatant.
6. Follow the protocol in the Qiagen mini-prep kits.
RESTRICTION DIGESTS

Once the DNA from your test colonies has been mini-prepped, restriction digests need to be set up. We typically use EcoRI and HindIII.

MASTER MIX
1X Restriction Digests
1.0 µl 10X React 2 Buffer
7.0 µl dH2O
always add enzymes last
0.5 µl EcoRI*
0.5 µl L HindIII*

*Volumes of enzymes are given in this protocol, however enzymes are sold in units. A unit of enzyme is the amount of enzyme necessary to digest 1 µg of DNA/hour. You will need enough enzyme to digest the sample. Check with Jen and Alexis for accurate conversions.

Each tube will have 9 µl of your master mix. Add 1.0 µl of DNA to each tube. Digest for 3 hours at 37C. A thermocycler can be used, or you may put your samples in the water bath or warm room.

Once the digest is complete, run 10 µl of each digested DNA sample on a gel with a 1KB ladder. Show the picture to Jen or Alexis.

If you cannot run your digests on a gel that same day, place them in the cold box or -20 ºC freezer.

PICKING BLOCKS

You will need large quantities of LB broth for picking blocks. Make 500 ml at a time. This isn't Molly's job either; she will give you the recipe, but it is your job to make and autoclave it. Once the LB is cool, add ampicillin (100 mg/ml).

Obtain blocks from Jen or Alexis. If they need to be sterilized, one of them will tell you how to do it. Aseptically aliquot 1ml LB/amp per sample well. Pick white colonies with a pipet tip and inject one colony directly into each well. Cover the block with Qiagen Airpore tape and shake at 37ºC for 24 hours. There are special rectangular clamps on all the shakers for this purpose. The next day, spin your blocks down (5 minutes at 1500 G) Use the centrifuge in the Genome Center. Dump liquid and tips into the sink, then put the tips into the trash. Cover the blocks with 3M tape and store them in the -20ºC freezer in the Genome Center. **Make sure everything is labeled properly. Name, Date, Sample Name, etc.**

USING THE BIOROBOT

The preparation of DNA templates for the sequencing reactions is accomplished using the BioRobot 96, an efficient way to get quality high out-put sequencing reactions.
To use the biorobot

1. Schedule a time with Jen and Alexis well ahead of the time (at least one day in advance) you will need to use it. The time will be confirmed and written on the Genome Center board.
2. You must use the Biorobot under Jen and Alexis’ direction.

General outline of what occurs:

1. Spin down the blocks of cultures. The blocks of cultures need to grow 20-24 hours and then the cells are harvested in our centrifuge for 5 minutes at 1500 RPM.
2. Decant the liquid into the sink with paper towels. If you are not going to prep the DNA right away, put a piece of freezer tape over the block, put the lid back on and store in our -20 ºC freezer. If you are going to prep the DNA right away, it can go on the robot.
3. The computer that runs the robot goes through the protocol, but it will be reviewed with you.
4. Once the DNA templates are recovered, you will set up sequencing reactions.

SEQUENCING REACTIONS

MASTER MIX 1X
4.5 µl dH2O
1.0 µl Big Dye
1.5 µl ABI 5X Buffer
0.016 µl primer (SK or KS)

Make one master mix for forward and another for reverse reactions. (100X/each usually works)
This master mix should be kept on ice. Distribute 7 µl of master mix to all 96 wells. You will need two 96 wells to plate for each block.
Aliquot 3 µl DNA into plates. Jen/Alexis will show you how to do this. Seal the plates.
The cycling takes 2.5 hours.

CLEAN UP REACTIONS
Again, see Jen and Alexis for specific direction. The following is the manufacturer’s protocol for cleaning up reactions.
CENTRI•SEP 96
96 WELL GEL FILTRATION PLATE
Catalog Number CS-965
Box of 50 plates

Description:

CENTRI•SEP 96 plates are prepacked with a hydrated, cross-linked gel suitable for removing excess terminators and nucleotides from Dye Terminator sequencing reaction mixtures. The purified reaction mixtures are suitable for sequencing on an automated DNA sequencer such as the ABI 377, 3100 or 3700. The plates are sealed top and bottom with an adhesive foil seal to prevent drying of the gel beds. Products may be collected into standard 96 well format collection plates (not supplied) for subsequent concentration and denaturing steps.

The procedure consists of removing the interstitial fluid from the CENTRI•SEP 96 plate by spinning for 2 minutes in a centrifuge equipped to handle deep well plates. The samples are applied to the individual wells and the plate is spun again to collect the purified product in a 96 well plate. See reverse side for additional usage considerations.

Materials Provided:

- CENTRI•SEP 96 Plates (50)

Materials / Equipment Required:

- Reusable 96 well wash plates
- 96 well collection plates
- Sealing film (optional)
- Centrifuge with rotor and carriers capable of handling stacked plates (5.1 cm height) at 1500 x g
- Multi-channel pipettor and tips

Storage and Stability:

The CENTRI•SEP 96 plates are stable until the indicated expiration date when stored at 2-8°C.

Protocol:

1. Important! Allow CENTRI•SEP 96 plate to come to room temperature before use.
   Remove the adhesive foil from the bottom and then from the top of the CENTRI•SEP 96 plate.

2. Stack the CENTRI•SEP 96 plate on top of a 96 well wash plate and centrifuge at 1500 x g for 2 minutes. Use an external timer and start timing when the rotor has reached the set speed. Discard the liquid by shaking the wash plate dry. The gel matrix in the wells should appear opaque at this point.

3. Transfer the samples (20µL or less) to the individual wells in the CENTRI•SEP 96 plate, taking care to place the samples in the centers of the gel beds.

4. Stack the CENTRI•SEP 96 plate on top of a 96 well collection system and centrifuge at 1500 x g for 2 minutes.

5. Remove the 96 well collection plate containing the cleaned samples and dry in a speed-vac equipped with the appropriate rotor. Alternatively the plate can be sealed for storage.

NOTE: This product is intended for research use only.
Centrifugation:

Most centrifuges, either bench or floor models, that accept microplate rotors may be used with the CENTRI•SEP 96 protocol. However, the rotor must accept a plate stack approximately 5.1 cm in height (combined height of CENTRI•SEP 96 plate and wash plate) as the carrier swings 90° from its horizontal position to the vertical position.

Timing

It is very important to control both the centrifuge speed and the duration of the run. Centrifuges vary by manufacturer in exactly when the internal timers start. Some models begin counting down as soon as the centrifuge run is started so that the ramp up to speed is included in the run time. If the ramp up is slow, the total time at the selected rpm is reduced, thus reducing the total g-force on the plates. We recommend the following procedure:

Use an external timer to monitor the centrifuge run. Start the timer after the rotor has reached the set speed. Set the brake on maximum. Switch off the centrifuge after 2 minutes at 1500 x g.

As a visual check on the effectiveness of centrifugation, the matrix in the wells should appear opaque and slightly pulled away from the wall after the initial spin prior to sample application. If the matrix appears translucent or shiny, the initial centrifugation conditions are incorrect. Re-spin the plates at 1500 x g for 2 minutes.

Cushions:

Cushions supplied with the centrifuge should be used under the wash plates at all times.

g-force

Speed settings required for each centrifuge to reach 1500 x g will vary with the radius of the rotor used. The centrifuge manufacturer usually supplies a table or nomogram relating rpm to g force. Alternatively, the following table may be used. Values for fractional radii (i.e., 9.5cm) may be determined by interpolation.

<table>
<thead>
<tr>
<th>Radius (cm)</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpm required to reach 1500xg</td>
<td>4375</td>
<td>4093</td>
<td>3860</td>
<td>3660</td>
<td>3490</td>
<td>3342</td>
<td>3211</td>
<td>3094</td>
</tr>
</tbody>
</table>

Manual Sample Application:

CENTRI•SEP 96 plates are manufactured using precision filling equipment. This method ensures the extremely uniform gel bed heights required for robotic sample application. Since many users will be loading samples with multi-channel pipettors rather than robots, the following practices should be followed:

Samples should be loaded onto the centers of the matrix beds, without touching the pipet tips to the beds.
Allow the sample to "touch-off" onto the gel bed rather than "blowing-out" the pipet tips.
Place the forefinger of your non-pipetting hand alongside the plate row to which the samples are to be applied. Rest the pipet tips on this finger as they are being guided to the center of the gel beds.
Mycobacteriophage Protein Gel Electrophoresis

1. Transfer 50 µl dialyzed phage to a clean eppendorf tube.
2. Spin at 14K RPM for 4 minutes to get rid of dust and other debris.
3. Transfer the supernatant to a fresh tube.
4. To the new tube with the supernatant, add 75 µl distilled water and vortex.
5. Freeze/thaw on dry ice 3 times, vortexing in between.
6. Incubate at 37°C for 4 minutes.
7. Add 2 µl (20 units) of DNA I.
8. Incubate at 37°C for 1 hour.
10. Boil for three minutes.
11. Place on ice.
12. When the sample is cool, run the sample on a polyacrylamide gel, loading 5-20 µl per lane, filling blank lanes with 20ul 1x sample buffer.

To purify proteins further, instead of step 9 above, continue here.
9. Add 20% TCA to 1:1 to the sample to get 10% TCA (total volume ~125ul). Vortex and incubate on ice for 5 minutes.
10. Centrifuge at 14K RPM for 5 minutes.
11. Wash with 1 ml ice coldacetone. Incubate for 2 minutes at room temperature.
12. Centrifuge and pour off the supernatant.
13. Repeat steps 11 and 12 twice more.
14. Air dry the pellet.

Note: For 1-D gels, resuspend the phage protein pellet in 50 µl 4x SDS page sample buffer. Boil this for 3-5 minutes. Store in the freezer. For 2-D gel samples, air dry and store in the fridge as pellets.

Running the Gel
1. Obtain a precast polyacrylamide gel and remove it from its pouch. Remove the comb and rinse the wells with distilled water or running buffer.
2. Cut along the dotted line at the bottom of the gel cassette with a razor blade. Pull the clear tape at the bottom of the cassette to expose the bottom edge of the gel.
3. Place the precast gel into the electrode assembly with the short plate facing inward. Since only one gel will be run, and there are two places to run gels in the apparatus we’re using, use the mini cell buffer dam for the side that has no gel.
4. Slide the gel cassette and electrode assembly into the clamping frame and press down on the electrode assembly while closing the two cam levers of the clamping frame. Now, lower this into the mini gel tank.

5. Fill the inner chamber with ~125 ml running buffer. The buffer level should be between the tops of the taller and shorter glass plates of the gel cassettes.

6. Add ~200ml running buffer to the mini-gel tank (lower buffer chamber).

7. Make sure the wells are filled with buffer and load 5-20 µl per lane using flat tips, filling any blank lanes with 1x sample buffer. Be careful not to puncture the gel with the pipette tip.

8. Run the protein gel at 200V until the dye runs to the bottom of the gel.

9. Turn off the power supply, remove the tank lid, lift out the inner chamber assembly and pour the running buffer into the sink.

10. Open the cams of the clamping frame and pull the electrode assembly out. Remove the gel cassettes.

11. Remove the gels from the two plates, and transfer it to a small Tupperware container.

12. In the Tupperware container, add gel stain to cover the gel. Let this sit for ~10 minutes. Pour off the stain.

13. Rinse off all electrophoresis apparatuses with ddH₂O and set on a paper towel to dry.

14. Add destain to the container and pour it off. Add destain again to cover the gel and put a kimwipe in the container. Set on the rocker near the geldoc. Change the Kimwipe or the destain if needed. This may take up to a day. Photograph the gel and save the result in the geldoc computer folder and in the sever Phage Folder. Carefully label your photograph for your notebook.
Protocol: Checking Lysates for Lysogens

1. **Spot Test of Diluted Lysate**
   A. Make serial dilutions of your lysate from $10^{-1}$ to $10^{-10}$. A $10^{-1}$ dilution is 9 parts phage buffer plus CaCl$_2$ and one part undiluted lysate. A $10^{-2}$ dilution is 9 parts phage buffer plus CaCl$_2$ and one part of the $10^{-1}$ dilution and so on.
   B. Put 0.5 ml *M. smegmatis* mc$^2$155 in a test tube. To this test tube add 4.5 ml 0.35% MBTA. Plate this mixture on 7H10/CB/CHX. Allow the liquid to cool and solidify, then spot 5 µl of each dilution so that none of the spots touch. Before moving on, allow this to dry so that the spots evaporate completely on the plate.
   C. Invert and incubate your plates at 37° C overnight.

2. **Purifying the Lysogen**
   A. With a sterile inoculating loop, pick a nearly cleared section from your spot test. Be sure not to touch the lawn of mc$^2$155 with the loop. Use the clear spot made with the most dilute sample possible.
   B. Streak this out on a new 7H10/CB/CHX plate. To streak, start at the top of the plate and move back and forth horizontally until you are about one third of the plate. Heat your loop and cool it. Then move vertically on the lower left side of the plate until you reach the middle. Heat the loop again and then cool it. Next make a 3rd pass. With each pass, the number of cells will decrease, hopefully spreading far enough away from each other that isolated colonies form.
   C. Incubate the plate at 37° C for as long as needed (probably 3-4 days).
   D. If there is bacterial growth, use a sterile inoculating loop to pick a well-isolated single colony.
   E. Repeat steps B, C, and D several times (3-5) to ensure that there is nothing but lysogen on your plate. (You are streaking for isolation of a pure colony.)

3. **Inoculating the Lysogen**
   A. With a sterile inoculating loop, pick a well isolated single colony from the most recent streaking. Swirl this into a test tube with 2 ml of 7H9/Tween/CB/CHX/ADC/CaCl$_2$. Vortex to ensure that there are no clumps of bacteria. Incubate at 37° C overnight or until there is noticeable growth.
   
   B. Subculture this lysogen 1:50 to 1:1000, (depending on how long you want it to grow before using it, several hours later that day or overnight) in 5 ml 7H9/CB/CHX/ADC/Ca. Shake at 37ºC.

4. **Testing for immunity**
   A. Dilute your phage and an unrelated mycobacteriophage (for control) 10$^1$ to 10$^9$. Spot on both lawns of both *M. smegmatis* mc$^2$155 and on the putative lysogen. Also spot some of the lysogen culture supernatant (see below) on both lawns.

5. **Testing for phage release**
   A. Spin 1 ml of the culture of lysogens at 14K RPM for 1 minute to pellet the cells.
   B. Transfer the Supernatant to a fresh tube and repeat A.
   C. Spot the dilutions of this supernatant on prepared lawns of *M. smegmatis* mc$^2$ 155 and your lysogen.
END DETERMINATION

Your sequence is in one contig. Primers have been run and coverage is sufficient. There are no weak areas (gray colored bases in the consensus).

The Consed screen shows two possible choices for ends: defined ends or circular (these represent more than two possible physical realities, but for the purposes of annotation, these two are all we consider). Read up on circularly permuted, cohesive, and terminally redundant ends to find out what is happening in the phage and bacterial infection.

Defined ends are characterized by a pile-up of clones that begin at each end (they are overrepresented). On the left end on rightward reads, one can sometimes find the “GAT” half of the EcoRV cloning site, and on the leftward reads at the right end one can sometimes see the “ATC” half of the EcoRV cloning site.

For defined ends, in the actual phage particle, there is usually a “sticky” overhang. Each end is single stranded and its sequence is complementary to the other end. There can be 5’ or 3’ extensions. Because the polymerase that does the sequences only adds bases 5’ to 3’ direction, this means that the sequence “falls off” the 3’ extensions and all of the bases are not read. A false “A” is added to rightward reads and a “T” to leftward

```
5' ______________________________________________ 3'
3' ______________________________________________ 5'
```

Remember the polymerase is using the one strand as a template, but producing something equivalent to the other strand. In the diagram above, the leftward facing arrow is copying the top strand, but generating a sequence that is found on the bottom strand. Except it reaches an end of the top strand and “falls off” without making the few bases on the lower left. Only by ligating the DNA (joining the sticky ends) and then sequencing can you learn what those bases are (10 is a common number of bases present in the overhang.)
The situation is different with 5' overhangs. The polymerase reads to the ends and then falls off. Ligating and sequencing yields no new bases, the read just “goes around the block” and picks up reading the other end, which will show up, right under the false A or T.

5'  
   <---  
3'  
   ---->  
3'  
   ---->  
5'

The second possibility of Consed is that you see the same sequence at both ends. This can mean your genome is circularly permuted (each genome has somewhat more than one unit length genome, and where these begin is not the same for each phage particle) or terminally redundant (actually has the same sequence at each end, and difficult enough, this may or may not be the sequence that is shown as the ends on Consed). In practice, we usually treat these cases the same, unless we know it is terminally redundant and then we do biochemistry (DNA Digests, etc) to figure out what the repeated sequence actually is. So what do we do with this type of genome? First Jen or Alexis cuts it to “unit length” essentially throwing away the repeats at each end leaving one phage genome. Now…where do we define base one? It depends! Try to find a terminase. Take the DNA sequence as a text Fasta file and blastX it against the terminase database. Also, see if it is similar to other phage genomes. (BlastX chunks of it against genbank and the pbi database, then if you find a similar phage, try aligning two sequences in Blast or dottering those two sequences.) For highly similar phages, define your base 1 according to similarity with their base 1. (Cut off the preceding bases and paste them to the end)

If there are no highly similar phages, use terminase as gene 1. If you can find a large subunit, search around the surrounding open reading frames and blastp their products to see if one is a small terminase subunit. If so, use it as base 1.

Some other things to consider: try to not end the genome with a “wrap-around” gene. This becomes a pain later. It’s a lot easier to annotate rightward facing orfs, so look at the overall pattern before finalizing base 1. You might want to flip the whole genome around before getting going! To address these, it is good to run glimmer before printing your 6 frame to be sure you like where you have called base 1.

First choice for end: physical end if one is known. Three prime sticky ends are by convention placed at the right end. Five prime sticky ends can go at the left end. Only one copy of the extensions should be in the fasta text file of unit length genome.

Second choice for end: near identity to a highly similar phage

Third choice for end: terminase small subunit

Fourth choice for end: terminase large subunit if you cannot identify a small subunit

Finally: orientation or architecture (leftward arms typically go on the right of the genome and rightward arms go on the left end of the genome.) Not all phages have two opposing transcriptional units.
Annotating the Genome of a Bacteriophage - Part 1

The process of annotating a genome is a file-manipulation-rich endeavor. Understanding where your files are and what you are doing to them will be very helpful. The genomes of most phages range in length from 40,000 bp to 400,000 bp (40Kb – 400Kb), so being able to manipulate them using computer programs is essential.

A folder of all pertinent files needs created in the Master files. Please see Debbie to do this. Save each and every document as you go, labeling them carefully. Record how you label them in the annotation folder and your notebook. Also, create and Old Stuff folder in that phage folder. As you generate an updated version of a file, place the older version in the old stuff folder. This will avoid confusion in the future.

Once base 1 is established, e-mail the FASTA sequence file to Dr. Jeff Lawrence. He will post it on the PBI website at http://pbi.bio.pitt.edu/

Example of a simple FASTA file

> seq1 This is the description of my first sequence.
AGTACGTAGTACGTGCTGCTACGTGCGCTAGCTAGTACGTCA
CGACGTAGATGCTAGCTGACTCGATGC
> seq2 This is a description of my second sequence.
CGATCGATCGTACGTCGACTGATCGTAGCTACGTCGTACGTAG
CATCGTCAGTTACTGCATGCTCG

FASTA is probably the simplest of formats for unaligned sequences. FASTA files are easily created in a text editor. Each sequence is preceded by a line starting with >. The first word on this line is the name of the sequence. The rest of the line is a description of the sequence (free format). The remaining lines contain the sequence itself. You can put as many letters on a sequence line as you want, and a FASTA file can have as many lines as necessary to include your phage genome sequence.

Blank lines in a FASTA file are ignored, and so are spaces or other gap symbols (dashes, underscores, periods) in a sequence. Any other non-amino or non-nucleic acid symbols in the sequence should produce an appropriately strident string of warnings on your terminal screen when you try to use the file.
Once a unit-length genome has been generated, the file needs to be formatted for use by DNA Master.

1. Open the Fasta-formatted file of your genome sequence
   **Phage name.txt**

2. DNA Master has special format for its input
   - replace the Fasta description line (>blahblahblah) with two periods
   ```
   ATGATCGGATTTGATGCGGATGACCTGGAGCTTTAA
   ```
   continuing to the end of the sequence
   - Save this file as **Phage nameDNAmas.txt**

3. Open DNA Master, Then open your input file in DNA Master
   - **Open File ==> Degenerate DNA**

4. What is the length of the Genome? __________________________ bp

5. Check out the base composition
   - **DNA ==> DNA Composition ==> Table**

6. What is the percentage of GC in this genome? _______________________

7. What is the significance of that percentage? __________________________

Each program that is used detects specific features of the genome. The first program we will run is tRNA Scan. Like its name implies, it is used to detect putative tRNA producing sequences. Instead of making proteins, these are copied into tRNA molecules. This program will identify whether your genome has any tRNAs and give you output (printouts) of what each one looks like.

8. We’ll search for any tRNA genes using the tRNAscan server:
   - Notice that it only accepts up to 100,000 bases – If your phage genome is larger than 100Kbp, you’ll need to copy/paste only the first 82 Kb or so (anything less than 100kb) region of your genome. (When you cut the genome, be sure to overlap, so as not to miss any tRNAs. Also, RECORD exactly the base pairs you cut & paste so that you can calculate exactly where the tRNA is located when you are working with the entire genome.)
   - Select search mode other (browse for your FastA file)
   - Select **bacterial tRNA**
   - **Run** program

   tRNAs found __________________________

Send the FastA file to Craig Peebles and he will check for tRNAs and tmRNAs using a different program, Agarorn. **cpeebles@pitt.edu**

Another program you will need to use is Frameshift Programmed Frames. Written by Jun Xu, this program will identify areas of possible -1 frameshifts. This is based on his earlier work that found g and t
ORFs directly preceding the tape measure gen in ___.? The research found that the code of g sometimes undergoes a frame shift to make a protein gt. The ratio of g/gt can predict tail assemble efficiency. It is a web-based program that uses the FASTA sequence. Copy and paste the sequence in the appropriate place. Always change the output format to coordinates. Use L5 (or closest choice) as the coding potential model. The RBS(ribosomal binding site) model is M. tuberculosis (is annotating a Mycobacteriophage). All of these can be deselected (and should be) and run. Print the output. This information will go into the final map and be explained in the analysis.

The next programs (GeneMark and Glimmer) try to predict where the protein-coding genes are ==> make sure to use the FASTA sequence (see step 1) as input. GeneMark is a program that highlights protein-coding regions in a graph-analysis fashion. The gene sequence is identified numerical with ‘up-ticks’ on the lines to denote starts and down-ticks’ to denote stops. (Note: TTG is not used in this program as a start, so “leucine starts” can be missed.) The peaks on the graphs represent good coding potential predictions, according to a model organism, and the highlighted areas signify where this program might call genes. The GeneMark Program calls genes based on similarities to the closest species (which you specify).

9. Open the GeneMark server:
   - hit Browse to select your input file (you will use a FASTA file)
   - Select the closest species of organism or host as the model (M. tuberculosis for the Mycobacteriophages)
   - Under graphics export options, select everything except “generate postscript” & “mark putative exons”. In the second column choose only ‘list open reading frames’ and ‘list regions of interest’.
   - Run Genemark (Start)

10. You should see a text output of your GeneMark results
    - hit the view PDF graphic link
    - save this file with a new name*****

Glimmer is another computer program used as a tool to predict genes. It calls the genes based on their coding potential. It gives you a text file that identifies the genes, using large predicted orfs in your phage genome as a model gene. The following are directions on how to use the program.

11. Glimmer is a command-line program, so your input file (your phage genome FASTA file) needs to be in the same folder as the Glimmer program.
    - Place a copy of your input file in this folder:
      C:\Program Files\glimmer2.02_Win
    - Open glimmer (Start ==> Programs ==> Biology)
    - Run glimmer with the syntax:
      Run-glimmer2 [input file name]

Go to command prompt
Go to H drive
Cd downloads
Cd gliimer
Cd glimmer 2.02
Run glimmer2 [input file]  (*.txt)

Output comes back as XXX putative genes extracted, results will go into the folder of the original file named g2.cord. All records come back with that file name, so immediately go to the folder and change the name to your phage. Copy that file back in to your phage’s folder.

12. Your output should be in the glimmer2.02_Win folder, called g2.coord
  • Open your results in Word
  • Adjust Margins and Save a copy
  • Print out a copy

Helpful HINTS!!
DNAMaster
Save DNA Master file often in case of crashes.
In the DNA Master frames, a full vertical line is a stop, a partial line is start.
Everytime you open DNA Master you must tell it that TTG is a start!
The frame number on DNA Master is not necessarily the same frame in Genemark or Glimmer.

Glimmer
In Glimmer, Gene 1 can be at the end of the list (if so, move it to the top).
Glimmer almost always calls the longest orf which is not necessarily the best choice.

GenMark
The Genemark output and graph shows no TTG starts.
An uptick on the Genemark graph is start (the longer uptick denotes ATG, and the smaller one denotes GTG).
A downtick on Genemark graph is a stop codon(TAG, TGA, TAA).

General
The numbering of Genemark and DNA Master protein sequence predictions includes stop codons,
Glimmer does not include the number of base pairs for the stop codons.
A few extra genes may be in the Glimmer and Genemark outputs.
A few genes may be missed by Glimmer and Genemark.
Remember, genes are tightly packed in phage genomes, so there should be few gaps.
Small (a few amino acids) gene overlaps are OK. Big gene overlaps are not OK.
Often the stop codon and the start codon of adjacent genes overlap.
Annotating negative frame genes is somewhat more difficult, it may be helpful to look for the stop of the next gene before you call the start of the current gene.

Annotating the Genome of a Bacteriophage – Part 2

Armed with all the information you have from part 1 (you have those printouts in front of you, right?),
you’re ready to start calling genes. Stops are stops, so you can rest assured that when you run into an asterisk, you are at the end of that gene. (This is assuming that no nonsense suppressor or tRNAs are present.) However, picking gene start codons can be tricky. Gene start calls are based on input from Glimmer, GeneMark, how closely the ends fit, the length of the gene, and the Shine Delgarno score. This
score represents the nucleotide sequence (AGGAGG) that is present in the 5'-untranslated region of many prokaryotic mRNAs. This sequence serves as a binding site for ribosomes.

1. **Open** up your entire phage sequence in DNA Master (see part 1, step 3)

2. **Save** a copy of this file with today’s date in your folder [Phage name Today’s date.seq]

3. Make sure DNA Master recognizes all three of the possible phage start codons:
   - **Select** any piece of sequence
   - **DNA ==> ORF ==> Cues**
   - Make sure ATG, GTG, and TTG are selected as start codons
   - Hit the red check-mark to save changes

4. **Select** the first 5000 bases of your sequence, and display all six reading frames
   - **DNA ==> FRAMES**
   - In each reading frame, the long vertical lines represent stop codons, and the smaller vertical lines indicate start codons.

5. Consulting your Glimmer printout, Genemark printout and the Frame analysis (in DNAMaster) information, **select** your first gene
   - Clicking in the Frame Analysis window selects an open reading frame (green line from start codon to first stop).
   - Notice that the ORF coordinates show at the bottom left corner of the window.
   - To erase the green lines, hit the exclamation point icon in bottom right of the Frame Analysis window.

6. Once you’ve highlighted your first gene in the Frame Analysis window, switch back to your sequence view
   - **Window ==> Sequence**
   - Notice your ORF is highlighted

7. Now you have to choose the best start codon for this gene:
   - **DNA ==> ORF ==> CHOOSE START**
   - Using the scores here, coupled with your Glimmer and GeneMark results, choose the best start site. The usual rule of thumb is to choose a start site with a high score that is early in the ORF and that minimizes gene overlaps.

8. Now you’re ready to document this first gene (annotate)
   a. Make sure the correct ORF with the best start codon are selected in the Choose ORF Start window, and hit the **Document** button
   b. Document the Gene as ‘1”
   c. Document the Product as “gp1”
9. Also document (highlight in color) this gene on your 6-frame translation printout, Glimmer printout, and Genemark printout. Going from top to bottom on the six frame, use the following colors:
   - Fr1=purple
   - Fr2=pink
   - Fr3=orange
   - Fr-1=blue
   - Fr-2=yellow
   - Fr-3=green

10. Also put the number of this gene next to the highlighted info on all these printed documents.

11. Go back to your Frame analysis window, and move on to your next gene.
   - Note that each gene can be in a different reading frame, but the genes will not usually overlap each other.
   - Repeat steps 5-10 above, using the next consecutive number (2) for gene/product name.

12. Call all the genes in your DNA segment.

13. Save the DNA Master file. (Save often as you work!)

**Annotating the Genome of a Bacteriophage – Part 3**

**BLASTing the predicted gene products**

Once you have called all of your phage genome genes, you may want to ask if you might be right. Without a gene product (an isolated protein or RNA), you are calling putative genes (ones you think that are there, based on all the factors you have learned). One way to check your prediction is to compare them to other genes that have been called in other genomes. Do they match base-pair for base-pair or nucleotide for nucleotide? How close of a match is it? Is the function of the gene known? What kind of genes does your phage genome have?

**IN DNA MASTER:**
- parse the orfs
- Select gene 1
- Copy as translation
- Go on the web to NCBI Blast Proteins, BlastP
- Paste in the amino acid sequence
- Blast and Record Results

Individually Blasting each predicted protein sequence one at a time is slow, and faster “batch” Blasting is a good way to proceed once you have mastered the slow one-by-one approach. Each technique has its own merits, you may Blast one gene when calling, but then Blast all protein coding sequences at the end to compare genomes.
Dotter Plots
Dotter plots are great ways to compare the genomes. The program allows a comparison of two genomes are as many genomes as you can to string together. The time it takes to run is related to the square of the size of the files.

This program uses a sliding window to compare DNA code. We typically use a 25 bp window for ‘sliding’.

You will need to move a copy of your FASTA file into the Dotter folder. In the command prompt window, change directories to dotter
H:\dotter>dotter your file.txt your comparison file.txt
Unlike other DOS programs, even though the next prompt automatically appears, it DOES NOT mean that the program is completed.

Once done, you want to open the file. You can remove crossbar (right click on image) and adjust the grayscale. We typically print two versions, one light and one dark, to ensure the lack of manipulation of data. To print, be sure to deselect mail and select copy, then hit OK. Once you close the program you can no longer manipulate it. Be sure you do all the things you want to so, otherwise you will run it again.
How To Blast Your Sequences Against The PBI Database

First, make a FASTA text file of your DNA or Protein (yourfile.txt). This can be copied directly from the edit_dir folder as output of your contigs from CONSED or exported from DNA Master as proteins, depending on where you are in the process.

Copy the file into the PBI Blastall Folder. (Use the house icon at the bottom of the LINUX screen, click on your folder, click on the edit_dir folder, click on the most recent fasta.screen.contigs file, hit copy, then paste into Blastall

From PBI Home, go into the Blastall folder (>cd Blastall)

Type the following command (and exchange blastp for blastx if your file contains protein sequences, not DNA sequences) at the prompt. The –b and –v options limit the number of hits to keep file sizes reasonable. Typically 10 is used, but you can use 20. A database called phage7.25.05.txt is the most recent as of now, but look in the Blastall folder for the name of the most recent version. (Files are located in the phage folder on the server.) The –m7 gives the output in xml format which is what you need.

>blastall –p blastx –i yourfile.txt –d phage7.25.05.txt –m7 –b10 –v10 –o yourfilenamepbi.xml –e.001

This command will translate your phage DNA sequence in all 6 frames and will check the possible proteins against all of the proteins listed in the PBI Database.

After it runs, type at the prompt

>formatxml.pl yourfilenamepbi.xml yourfilenamepbi.today’sdate.xml

This formats the file so it can be opened by Jun Xu’s program, Blast Inspector. You need to give it a new name, and it is useful to include the date here for keeping things somewhat organized.

Then go to the BlastInspecctor folder

>cd
>cd BlastInspector

type
>java –jar BlastInspector.jar

Click on the folder Icon to open a folder. Select the Blastall folder, then select your file, and it should open
A few notes and warnings:

The computer cares about upper and lower case and spelling.

Blasts against this database should only take a minute or two, so if it is taking a long time, something has gone wrong.

Just ask for help if you don’t understand what to do! It seems confusing at first, but it is not really too bad once you get used to using the command prompt.

The Command prompt is nice in that the arrow keys bring you to the previous history of commands (arrow up and arrow down), so if you miss one character you don’t have to retype the whole line.

You can’t use the mouse to move around within a command prompt line, but instead you can use the left and right arrow keys.

Here’s the short-hand for the above directions:

Left click on “Start”, “All Programs”, Find “Command Prompt” (usually in Accessories)
>cd H: (or the drive containing BLAST folder)
>H:
>cd BLAST
>blastall –pblastx (or whatever blast you want to run) –dphage7.25.05.txt (the latest file you have available to compare to) –iyourphage.txt (FastAfile) –e.001(the option that cuts the search results to a reasonable size, giving only ‘good’ matches) –m7 –b20 –v20 –oyourphagepbi.xml

WAIT, Then:
>formatxml.pl yourphagepbi.xml yourphagedatepbi.xml

To see your BLAST Results
Go to Blast Inspector
Open, go to Blast folder

GenBank BLAST

>cd H:
>H:
>cd blastcl3
>blastcl3 –pblastx (p for proteins or n for DNA) –iyourphage.txt –e.001 –m7 –b20 –v20 –oyourphagegb.xml

PLAN TO WAIT A LONG TIME
>formatxml.pl your phagegb.xml yourphagedategb.xml

Note: A whole phage is too large to blastx. It will need to be segmented or translate it to proteins via Glimmer/DNAMaster to complete the blast against GenBank.
How to Make A GenBank File

Go to DNA Master
Parse all your ORFs
Go to DNA>Genome>Profile
Deselect all windows on left
Select Export non orfs
Select Load into XCEL

Copy start and stop coordinate columns
Reverse starts and stops for reverse frame genes
Copy one of these two fixed columns into Word
Convert table to text
Replace paragraph marks with seven paragraph marks
Copy all back to XCEL
Copy it again into another column
Add two spaces above the second column
Copy the second column
Paste Special into the first column, skipping blanks

Repeat for stops

Find template file (Final DNA Master files, GenBank files, template.xls)
Copy these into the template
Change the tRNA genes as appropriate (CDS becomes tRNA)
Delete all numbers below your last gene
Save as tab-delimited text file
Change the Top line to
>Feature Bacteriophage[your phage name]
Save as text file

Final DNA Master file is a sequin file “Sequin.exe”
Open Sequin (Double Click)
Fill in all data
Give one year for release
Tentative title “Bacteriophage Genomics”
Institution: University of Pittsburgh
Department: Pittsburgh Bacteriophage Institute and Department of Biological Sciences

Other Authors
Me (Marisa L. Pedulla)
You and your partner
Jennifer M. Houtz
Alexis L. Smith
Graham F. Hatfull
(Valerie Oke for N3)

Location of sequence: Virion
Scientific Name: Bacteriophage MP##
Genetic Code: Standard

Import Fasta file of your sequence
Check the no of bases is correct

Save all this
Then hit open
Open your “features” text file

Save it with a new name
Proof it
Change all GTG and TTG starts translations to “M”
Double click on the orf
Edit protein sequence
Change to M
Close the back window
Choose “accept changes”

Go in to the sequin file to add notes for each gene (Blast matches, other data, frameshift annotation, etc etc)
Appendices
GENERAL SAFETY NOTES

Safety in a laboratory is mostly common sense, and thinking before acting. If you are not sure, ask first!!!!!

A few general rules include:

- No opened-toed shoes
- No eating, drinking, smoking, applying make-up, or removing/inserting contact lenses.
- Keep your workspace clear of clutter
- Don’t wear baggy clothing
- Tie back long hair
- Minimize loud talking and other distractions
Guidelines for Ethidium Bromide Disposal

Although ethidium bromide is not regulated as a hazardous waste, its mutagenic properties may present a human health hazard if it is placed in the trash or poured down the sanitary sewer system. Use the following procedures when disposing of ethidium bromide solutions, gels, and ethidium bromide contaminated materials.

Ethidium Bromide Solutions

- Do not discard ethidium bromide gels or ethidium bromide solutions containing organic solvents or alcohol down the sewer. Aqueous solutions containing < 10 µg/ml (10 ppm) may be released to the sanitary sewer.
- Aqueous solutions containing > 10µg/ml ethidium bromide must be treated (S&S extractor with charcoal filter, or the Green Bag method) prior to sewer disposal or the solutions may be disposed of via the University's Chemical Waste Program. If the material is going to be treated, the solution can be filtered. Once the solution is filtered, pour the filtrate down the drain, place the filter in a sealed plastic bag (do not use red bags) and place a WASTE CHEMICALS label on it. The filter and the ethidium bromide should be disposed of through the Chemical Waste Program (call 4-8952 to schedule a pick up).

Gels Containing Ethidium Bromide

- Place gels in sturdy plastic bags, then place the bag in a cardboard box. Use an orange Chemical Waste label, identify as “Ethidium Bromide gel”, and process through the Chemical Waste Program.
- Dry gels should also handled in this manner

Gloves and Contaminated Debris

- Gloves and paper towels that are visibly contaminated with ethidium bromide should be placed in a bag (do not use red bags) or box. Label the container to identify the material and dispose through the Chemical Waste Program.

Glassware

- Test tubes contaminated with ethidium bromide should be emptied prior to disposal. Dispose of the liquid according to the above procedures. Visibly contaminated test tubes should be washed with bleach prior to disposal in a broken glass box.

Sharps

- Contaminated sharps and needles contaminated with ethidium bromide should be disposed into sharps containers. An orange CHEMICAL WASTE label must be placed on the sharps container to identify the contents. Note: no hazardous materials should be poured down the drain or placed in the trash.

Revised: 3-15-2004
Math and Dilutions

Microbiology is full of Math and Dilution Terminology. Luckily, once you become accustomed to using it, you will find that it is not difficult!

**Exponents: powers of 10**

\[10^0 = 1\] (All numbers to the zeroth power equal 1)

\[10^1 = 10\] (Ten to the first)

\[10^2 = 100\] (Ten to the second)

\[10^3 = 1,000\] (Ten to the third)

\[10^4 = 10,000\] (You get the idea now, right?)

\[10^5 = 100,000\]

\[10^6 = 1,000,000\]

\[10^{-1} = 1/10 = 0.1\] (Ten to the minus one or one tenth)

\[10^{-2} = 1/100 = 0.01\] (Ten to the minus two or one hundredth)

\[10^{-3} = 1/1,000 = 0.001\] (Ten to the minus three or one thousandth)

\[10^{-4} = 1/10,000 = 0.0001\] (You see the pattern!)

\[10^{-5} = 1/100,000 = 0.00001\]

\[10^{-6} = 1/1,000,000 = 0.000001\]

**Dilutions**

A \(10^{-2}\) ("ten to the minus two") dilution means a “one to one hundredth dilution”, so to make this:

Mix 1 in 100 total (1 of your stuff plus 99 of diluent)

or 5 in 500 total (5 of your stuff plus 495 of diluent)

or 10 in 1000 total (10 of your stuff plus 990 of diluent)

You could, also, dilute \(10^{-1}\) and then dilute that \(10^{-1}\) dilution again tenfold to make a serial dilution of \(10^{-2}\) overall.
## Density and Refractive Index for Cesium Chloride at 25 degrees C, MW=168.37

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HATFULL LAB GUIDELINES

I. Introduction: As Phagehunters, you will be interacting with others: other high school students, other undergraduate students, other doctoral students, other post-docs, other professors. The consideration you learned in kindergarten applies here. Be kind to those around you, be courteous, and treat others as you would have them treat you.

II. Lab Etiquette
   A. Socializing
      1. Have fun and socialize with your fellow phagehunters but do so at a low volume and be considerate of others.
      2. Music should be kept at a low volume.
   B. Lab Protection
      1. If you are the last one to leave, lock all doors and turn off the lights.
      2. Check the other lab rooms, and close them if no one else is around.
   C. Break Room
      1. This is shared space provided to ensure a safe place to eat and store food.
      2. Bring in a box (or obtain one from the lab manager) to store non-perishable items.
      3. Label items in the frig with your name and date. DISCARD items in a reasonable time frame. Do not let food go bad.
      4. Wash items when done; do not leave dishes in the sink.

III. Safety/Clean-up
   A. Sanitation
      1. Wash your hands when you start your work, when you end your work, and as necessary as you do your work.
      2. Wipe down lab bench with ethanol before & after use.
   B. Dishware Upkeep
      1. Dump all contents into sink with running water & use bleach to rinse out test tubes, flasks, etc. Add bleach to live cultures before pouring down the drain. Rinse before placing the glassware in the dish bins.
      2. Remove all labels and tape before placing dishware into bins.
C. Waste Disposal
   1. Dispose of all plates & melted agar in the biohazard bag.
   2. Remove a full biohazard bag, tape it shut and place in the appropriate area (please ask).
   3. Dispose of sharps (needles, scalpels, etc.) in the sharps containers on top of the lab benches.
   4. Dispose broken glass and Pasteur pipettes in the broken glass containers. (Please do not put anything but glass in these containers).

IV. Equipment
   A. Lab Bench
      1. Your bench is assigned to you, and it is your responsibility to maintain it. At times, more than one person will be assigned to the same area.
      2. To maintain sterility of items, follow aseptic techniques at all times.
      3. Be sure to include controls to help in resolving discrepant results.
      4. If you contaminate equipment or supplies, it is always better to be safe than sorry. Discard contaminated reagents when appropriate and re-autoclave supplies that have been contaminated.

   B. Gel Bench
      1. Discard any gels, tips or gloves contaminated with ethidium bromide into an appropriately labeled EtBr chemical waste bag.
      2. When placing a wet gel casting tray on the drying rack, put the dry ones away.
      3. Remove all tape from gel comb after use.
      4. If sharing a power source, turn it back on if other gels are still running when you remove your gel.
      5. Only use the communal gel bench (or your own) pipettes & pipette tips.

   C. Shaker/Warm Room & Cold Room
      1. Turn shakers back on after placement & removal of samples.
      2. Use appropriate clamps for a tight fit to ensure your flask will stay on.
      3. Make sure the doors are tightly closed when entering or leaving.
      4. Discard old plates in the biohazard bag.

   D. Centrifuges
      1. Sign up a day ahead if planning to spin samples. Include name, date, start time and length of spin on dry erase board. When using the ultracentrifuge, ask when you can sign-up.
      2. Balance all tubes before spinning. Please ask if uncertain about how to do this.
      3. Use the appropriate rotor codes & speeds when spinning samples in the large centrifuge. Refer to the list & chart next to the centrifuge. Please ask!

   E. Genome Center
      1. When using the tools and equipment in the Genome Center, it is best to ask ahead of time. Do not wait till the minute you need to do something to ask if you can.
F. Computers
   1. Log-in: Access codes are available in the lab.
   2. Server space is available for phagehunters. It is located on the BIOSCI server, with the folder name of ‘phages’. If you have a Pitt username, you should contact Debbie Jacobs-Sera to be added to the access list. If you do not, see Debbie for instructions on how to gain access.
      i. The server location is afp://136.142.54.170

V. Materials
   A. When using freezer stocks, keep them on ice at ALL times. Only remove them from the freezer when you are absolutely ready to use them.
   B. Glycerol stocks are frozen on dry ice.
   C. Cold blocks, enzymes, buffer & antibiotics are to be kept on ice.
   D. Plates should be restock if supplies are running low.
   E. Recipes/proper procedures are available upon request. See area next to scales for lab recipes.
   F. Label all materials with initials, date (including year), and content. Record where you put lysates and stocks in your notebook.
   G. Inform a lab technician if any supply is running low.
   H. Sharing is great, but use aseptic techniques and also have your own stocks if possible.

VI. High School Phagehunters
   A. A mentor must be present while you are in the lab.
   B. Follow protocols and instructions of your mentor.
   C. Ask a lot of questions.
   D. Time Log Book: Record name, date, and time every day and period of time when you are present in the lab. Have your mentor initial the entry.
   E. Enjoy doing science!
1) General

a) Intro. The Genome Center is a collaboration between several labs. As a result, there is large amount of shared equipment and consumables in the Genome Center from the Hatful, Hendrix, and Lawrence Labs, although the physical space itself is part of the Hendrix Lab. Some of the equipment is used exclusively in the Genome Center and stays there permanently. Other items belong to a specific lab and should be returned to that lab promptly after being used in the Genome Center. You should always ask someone before using shared items.

b) Planning. Proper planning is essential to successful lab research. A detailed protocol makes any experiment or procedure proceed smoothly and reduces errors. If you need to use equipment, instruments, supplies, or knowledge in/from the Hendrix Lab or its members, please do not simply show up at the moment that you need it and expect to receive instant gratification. We are here to do serious research and are willing to help and teach you, but we do not exist solely to serve you. Making the effort beforehand to schedule time to discuss your experiment or receive instruction can alleviate potential conflicts. If you plan ahead of time for the supplies and help that you will need, everyone, including you, will be happier and healthier.

c) Introduce yourself. Please do not simply walk into lab and begin pulling items from the shelves or using equipment. Hopefully, someone will ask you if you need assistance. If not, introduce yourself to someone and let that person know why you're here. Contrary to rumors, those of us who work in the Hendrix Lab are not mean and grumpy, merely bitter and jaded.

d) Ask questions. Ask for help. It is part of everyone's job in the lab to instruct and assist others. We are all here to learn and to teach. We may not always be cheerful or civil in our response, so please don't let this deter you from asking further questions. If you are in any way unsure or nervous about: operating a piece of equipment; performing a protocol; using an enzyme, reagent, or chemical; how to clean or dispose of something; where an item is located or where to return it; ask for help. It is always better (and safer) to ask rather than guess. Doing something without knowing how to do it properly can be dangerous, wasteful of supplies, wasteful of your time, and cost considerable time and effort for those who will inevitably have to deal with your mistake.

e) Be patient. People may not be immediately available to assist you. We may be in the middle of our own work or assisting others. It is not always possible for someone to stop what she/he is doing at that moment. We will help you as soon as we are able.

f) Clean Up. After you are finished using equipment, dishware, or a bench area, be sure to clean up properly. It is rude to other members of the lab to leave your mess for someone else to deal with. Some equipment needs to be cleaned or shut down in specific ways. Ask for assistance/instruction if you are unsure how to do it properly.
Dishware

g) Once you are done using a piece of dishware (bottles, flasks, beakers, tubes, trays, etc; glass or plastic) return it to the lab from whence it came. It is your responsibility to know where you got the dishware that you are using and to return it to the proper lab. There are any number of items that are always in short supply in any given lab and the lab managers don't need the extra work and frustration of having to deal with returning and tracking down dishware.

h) The Hatful and Hendrix Labs have different procedures for dealing with dirty dishware. It is your responsibility to know what to do with dirty dishes from both labs.

i) Hendrix Lab dishware is handled in several different ways, as described below.

i) **Glass and plastic beakers, flasks, bottles, caps, low-speed centrifuge tubes and bottles, funnels, and graduated cylinders** are placed in the large bins located (a) next to the sink in the main lab or (b) next to the small sink in the Genome Center. All of these items are to be rinsed thoroughly with tap water before being placed in the bins. Residual media, reagents, and cultures tend to leave pungent smells floating through the lab if glassware is not rinsed properly.

ii) **Glass test tubes** are placed in the small bin next to the sink in the main lab. Once again, rinse thoroughly, particularly if the tubes were used for growing cultures. The caps are placed in the large bin with the rest of the dishware.

iii) **Magnetic stir bars** are rinsed and then placed in the plastic beaker labeled "dirty stir bars" on the sink in the main lab.

iv) **Pyrex bowls** (often used for staining and destaining gels) are washed by hand and then placed on the drying cart (located next to the ultracentrifuge in the main lab).

v) **Side-arm vacuum flasks** (not to be confused with nephelometer flasks) are washed by hand and then placed on the drying cart.

vi) **Dialysis clips** are rinsed and then placed on the drying cart.

2) **Consumables**

a) Empty **pipet tip boxes and microcentrifuge tube containers** are placed on the stand next to the ultracentrifuge in the main lab. They can't be filled if we don't know that they're empty.

b) **Disposable glass pipettes** are placed in the broken glass containers located in both labs.

c) **Reusable glass pipettes** are placed in the dirty pipette cylinders located in both labs.

d) If you use the **last of an item** or take the last container from a shelf (petri plates, tryptone media, microcentrifuge tubes, buffer, dialysis tubing, etc.) it is your responsibility to notify someone (Brian, Bob, Crystal, Jen, Alexis etc) so that it can be replaced. This may mean that you will have to fill, make, prepare and/or autoclave more of the item.

e) The Hendrix Lab provides only a small number of **media and reagents for common use.** These include LB (small volume), LB soft agar, sterile deionized water, various Tris buffers, EDTA, reagents for pouring gels and gel buffers, and some other salt reagents. If you are using these reagents in large volumes you will be expected to replace them. All other media, reagents, and buffers that you require you will have to make yourself.
f) **Enzymes.** We have a large selection of restriction endonucleases. If you want to use them you must follow proper asceptic technique. NO DOUBLE DIPPING! A tip should be used to pipette the enzyme and then place it into your tube, then be discarded. If you are using more than 1 enzyme or are adding it to more than one tube, change tips every time. The same rule applies to enzyme buffers. Otherwise, whatever is in your tube gets into the enzyme and buffer. It doesn’t matter how careful that you think you are; this is not an acceptable risk.

3) **Equipment.** Different equipment and instruments require different care and clean-up procedures. The following is a list of several commonly used items. This list is not all-inclusive. You may need to use something in the Hendrix Lab that is not discussed below. If so, please ask to be instructed on its use.

a) **Beckman Optima L-90K Ultracentrifuge.** This is one of the most frequently used pieces of equipment in the lab. It requires simple, yet careful, operation and attention. Improper operation leads to expensive repairs and when something goes wrong it tends to happen loudly, which means everyone will know when you mess up.

i) Schedule sheet: There is a schedule sheet located on the L-90K. If you wish to use the centrifuge you should check the sheet to see if it is available for the day & time that you want to use it. In addition, before signing up on the sheet, you need to check with Brian, Bob, or Crystal in order to be sure that no one from the Hendrix Lab needs to use it at that time. Then you can sign up for use of the block of time that you require, being sure to include your name (not your initials—we don't know you—first name is sufficient if not shared by someone else in your lab) and initials of your lab (GH or MP—we do know them).

ii) Before you actually use the L-90K, you should find out which rotor, tubes, reagents, and other supplies you need. By “before” I mean a day to several hours before, not 5 minutes.

iii) Loading: The tubes must be balanced against each other and then loaded into the rotor (or into the buckets that attach to the rotor) in a symmetrical fashion. It is useless to balance the tubes then load them improperly. Heat sealed tubes must be capped and buckets must have their caps properly attached. Your tubes must be clean and dry on the outside, too. The lid must be properly attached to fixed angle rotors or the O-ring will not seal properly. The rotor must then be properly loaded onto the spindle of the L-90K.

iv) Setting the vacuum, run parameters, and beginning the run. After the door is closed, the vacuum must be engaged. The L-90K reaches very high speeds and a considerable vacuum must be achieved for proper operation. You should then set the length of the run, speed, temperature, acceleration, and deceleration. You cannot start the run until the vacuum is at least 200 microns. If you need to run the centrifuge at cold temperatures, be sure to turn on the centrifuge and set the temp at least 30 minutes ahead of time and to chill the rotor for at least several hours. You need to wait until the rotor accelerates to your set speed before you can leave. If something goes wrong or the centrifuge aborts the run for any reason (i.e. rotor imbalance) this usually happens before full speed is reached.

v) After your run is complete the speed will read 0 rpm. Disengage the vacuum. When the hissing has ceased, the vacuum has been released, and the door can be opened. After removing the rotor from the centrifuge and your tubes from the rotor, you must clean up. The inside well of the centrifuge should be wiped out with a dry paper towel. The rotor/buckets should be rinsed with warm water, then with deionized water and set aside to dry. This is especially important when CsCl₂ or any other salt is used, because salt is highly corrosive. Just because you can't see a leak doesn't mean that it didn't leak.
vi) Supervision: These instructions are not a substitute for direct supervision. Brian, Bob, Marisa, Crystal or someone else knowledgeable in the operation of the centrifuge must show you how to operate the centrifuge and supervise you for the first several times that you operate it. Another high school student or undergraduate student does not qualify as knowledgeable in this circumstance. We will notify you if and when we believe you have attained the level of competence necessary for unsupervised operation of the centrifuge.

b) Balances/Scales. There are two located in the main lab. The AE63 is an analytical balance for measuring <10g. The P1200 can measure up to 1kg.

i) The P1200 operates with a lamp. Please turn it off when you are done to preserve the life of the bulb.

ii) Clean the balance and bench when you are done. We do not keep track of what every individual has measured on the balance, so we don't know what it is that you left behind. I don't leave neurotoxic acrylamide behind after weighing it out and I don't expect you to leave behind whatever it is that you were measuring out. This is just common courtesy.

c) Thermocycler. There is one located in the main lab. Ask if you need to use it. Be sure to have your cycle settings ready to program into it.

d) Milli-Q water. There is a Milli-Q water system in the main lab, located above the main sink. There are several simple steps that must be followed to operate it.

i) Turn the water flow on by turning the orange valve open.

ii) Check the water supply by briefly opening the dl water tap in the sink. If there is no water flow from this tap, the tank is empty and the Milli-Q system should not be used.

iii) Turn the pump on. Without the pump the water will not flow through the cartridges.

iv) Wait until the resistivity meter reads nearly 18Ω before turning on the output flow.

v) The white valve on the right side controls the output water flow.

vi) Shut off the output flow, pump, and water valve when you are done.

e) Agarose Gel Boxes. The Hendrix Lab owns several Hoeffer electrophoresis systems for running agarose gels, as does the Hatful lab and the Genome Center. For procedures on using those owned by the Hatful lab ask someone from that lab that is knowledgeable (remember definition of knowledgeable from centrifuge operation). The following rules apply only to Hendrix Lab and Genome Center electrophoresis equipment.

i) Location. Hendrix Lab gel boxes, casting trays, running trays, comb backs, combs, and lids are only to be used in the Hendrix Lab and the Genome Center. It is not to be used in any other lab in this building. The equipment is expensive and has a tendency to "walk" out of labs. It doesn't need further assistance from you.

ii) Pouring gels. Contrary to popular belief, the running trays were not designed to have boiling or near-boiling hot agarose poured onto them. That is an urban myth. Allow the agarose to cool to 55°C before pouring onto the tray. There is a water bath in the main lab that is ideal for this purpose. A good rule of thumb is that if the flask or bottle is too hot to hold in your hand, it is too hot to pour.
iii) **Excising bands.** If you are gel-purifying bands of DNA do not cut out the bands on the running tray. This destroys the trays very quickly. We have hundreds of disposable UV-transparent plastic lids in the Hendrix Lab that gels will fit on so that you can cut out your bands.

iv) **Retribution.** If I discover anyone in the act of performing or evidence of having performed either of the two previous forbidden actions, that person will be witness to a verbal assault that would make a steelworker blush.

v) **Cleaning.** After using the electrophoresis apparatus you are required to clean it in a prompt manner. Prompt does not mean 2 days or even 2 hours later. Rinse the box, trays, combs, and lid thoroughly with warm tap water and deionized water, being careful not to break the platinum wires.

vi) **Return Location.** After rinsing, all electrophoresis equipment is to be returned to either the main lab or the Genome Center. Place it on the drying cart in the main lab. This allows me to keep track of our inventory.

f) **Pipetters.** These are very useful and expensive instruments.

i) The pipetters located in the Genome Center and the Hendrix Lab are never to leave those labs. People always say that they will bring them right back, but inevitably something interferes with the completion of this task. Pipetters from the Hatful Lab should not be brought down to these labs for use. Pipetters follow the same migration habits as gel boxes. Few situations are more annoying than going to use a pipetter from your set and finding that it is missing.

ii) Pipetters do not have to be slammed into a pipette tip or shaken violently to ensure proper sealing of the tip. This damages the shaft and can affect the accuracy and precision of the instrument. Calibration and repair not only costs money, but is also inconvenient because of the loss of a tool.

g) **Water bath.** There is a relatively large water bath set to 60°-65°C in the main lab. It is often used for cooling agar and agarose. Water evaporates quickly from this bath. If you are using it you are responsible for checking on the water level, even if someone else has something in it. If the level is low add dl water from the faucet in the sink. A water bath that is turned on without water is a fire hazard. If you remove the last item from the bath, turn it off.

h) **Autoclave.** The autoclaves are not located in our lab but they are commonly used devices. It is very inconvenient to a large population in this building when one or more is not working. Someone should supervise you the first 6-8 times that you use them. The controls are slightly different for the two models and there are different settings for different types of loads. Ask before guessing.

i) If you are sterilizing liquids in the autoclaves, be sure to put your flasks, bottles, or other containers on a tray. There are a few metal baking trays in the autoclave room. Evidence of an overflow is not always apparent, especially to the novice. Remember that autoclaves sterilize by steam and pressure. Liquids can boil over from their containers. The exhaust cycle acts as a drying cycle then burns the overflowed liquid to the surface it is on. Water simply evaporates. Media and agar dries as black carbon and salt flakes. If not cleaned up after a spill, during the next use this mess is hydrated, flows all over the chamber, and then is dehydrated again and dries on anything else in the chamber. Your mess is now all over somebody else's stuff. This is unacceptable.
4) **Conclusion.** We have many cool pieces of equipment and instruments that make lab work easier and more fun. We are happy to allow you and others to use them. However, you must use it properly and respectfully. *Using equipment, supplies, etc from another lab is a privilege, not a right.* If you fail to follow the guidelines and rules of our lab, even if they are different from those of your lab, your privilege will be revoked. Remember: ask questions, be patient, and plan ahead. Those 3 simple ideas will avoid a great deal of conflict, misunderstanding, and lost time.
First of all, you must be aware that the Genome Center has plenty of work to do and we cannot drop everything to accommodate you immediately. **You must check with us in advance, especially if you want to use any of the equipment (cyclers, robot, hydroshear, elutrap, etc) in our lab.** There will be sign up sheets for the cyclers as well as the robot if necessary. Check with Jen or Alexis before signing up to make sure they have time to fit you in on the equipment. We will try to accommodate on a first come/first served basis, but we can’t always promise that and many times our own work will take precedence.

When it’s time for you to work with Jen and Alexis in the Genome center, there are certain things you need to be aware of.

**S-GAL plates:** Molly has ordered packets of S-GAL, if you need to know where they are, ask her. If the Hatfull lab is running low, inform Molly and she will take care of it. You need to pour your own plates. We add ampicillan(100mg/ml) and tetracycline(12.5mg/ml) to the media before pouring the plates.

**Fast-link Ligase:** Molly has also ordered this for use in the Hatfull Lab. She will tell you where to find it.

**E.coli XL1-Blue cells:** There is a stock in the Hatfull lab. Keep an eye on it; you are responsible for making more.

**LB:** You need to make this in 500mL bottles. Be sure to make enough for your experiments at least 1 day in advance.

**SOC:** You will also be responsible for making this. See Jen/Alexis for a protocol.

**T4 Polymerase/Klenow:** Molly will have a stock of this for you, check with her to see where it is.

**Order of Events:**
When you make DNA from a large phage prep, you need to have at least 200-300uL of good quality (~1ug/uL) DNA before we will even talk to you. Run 1uL of your DNA on a gel and bring the picture to us before we discuss your sequencing options. Make sure that you have also digested your DNA with a few restriction enzymes.

**Shearing DNA:** Check with Jen or Alexis before beginning this procedure. Once you have done that, take a tube of your DNA and heat it in a 37C water bath for at least 30 minutes. Aliquot between 15-30uL of DNA to a clean eppendorf tube. (Jen or Alexis will give you an exact amount) Dilute the DNA with TE so the total volume equals 100uL. Put this new tube in a 37C water bath for 30 minutes and in 5 minutes intervals vortex vigorously for several seconds. After 30 minutes of heating/vortexing, spin sample briefly and transfer contents to a clean eppendorf tube. You may now shear your DNA under the watchful eye of Jen or Alexis. Run 5uL of sheared DNA on a gel with a 1kB ladder.
**DNA repair:** Once sheared, your DNA now needs to be repaired. Check with Jen/Alexis for the protocol. The final volume should be resuspended in 10uL TE. This entire volume needs to be run on a 0.7% agarose gel. (It is best to tape 2 wells together)
Take a photo of the gel and carefully size-fractionate between 1-3kB. **ONCE AGAIN, CHECK WITH JEN/ALEXIS.**
**NEVER CUT GELS IN CASTING TRAYS!!!!!!!**
**NEVER CUT GELS ON LIGHT BOXES!!!!!!!**

**Elutrap:** Once you have a DNA slice, you are ready to run the elu-trap. The elutrap does not leave the genome center. Do Not take it to another lab. Jen/Alexis will help you set it up. The elutrap can run for 3 hours at 200V or overnight at 100V. Keep this time constraint in mind when it comes time for you to use the elutrap.

After extracting your sample from the elutrap, the sample needs to be phenol extracted/ EtOH precipitated and resuspended in a final volume of 10uL. If you have 2 tubes of the same sample, resuspend each tube in 5uL TE, then heat for 15 minutes at 37C and combine the tubes. The elutrap apparatus needs to be taken apart and soaked in DI water overnight. Come back the next day to dry it and put it away. Be sure to remove the membranes when taking it apart.

**Ligations:** You are ready to ligate. Check with Jen/Alexis for a protocol. Ligations must be stored long-term in the freezer.

**Transformations:** There is a stock of *E. coli* XL1-Blue cells for common use in the Hatfull Lab. It would be wise to check this stock periodically, because as a group, you are responsible for making more. Each tube is good for 5 transformations. Always do a positive and no DNA control. See Jen/Alexis for a protocol. Transformations need to recover on the shaker in the 37C room for 1 hour in 1mL SOC broth. They can then be plated on S-GAL with Amp/Tet.

You are also responsible for making sure you have S-GAL/Amp/Tet plates. Plate 100uL of each ligation. 100uL no DNA and 1uL (in a puddle of 100uL LB) pBlue control. The plates need to grow overnight at 37C. Your plates will need to grow anywhere from 16-24 hours. Check with Jen or Alexis if you are unsure about the growth on your plates. The colonies should be well-formed and the black/white color selection should be distinguishable.

Hopefully the next day you have white and black colonies. If this is the case, pick 8-10 white colonies per ligation and grow them up overnight in test tubes with 2mL LB/AMP. See Jen/Alexis for protocol. The next day mini-prep your colonies. Molly has qiagen mini-prep kits, check with her. Restriction digests must be set up with EcoRI and HindIII. See Jen/Alexis for protocol. They need to digest at 37C for 3 hours. Run 5uL of the digest on a gel with a 1kB ladder.

**Show picture to Jen/Alexis.**
If all is well, Jen/Alexis will tell you to pick blocks. See them for a protocol. Blocks grow overnight and need to be spun down the next day in the Genome Center centrifuge. Check the bottom of the blocks for good size pellets before dumping out the liquid and tips. If you feel the cultures need to grow more, put them back in the 37C room. If you are unsure if your pellet size is acceptable or not, check with Jen or Alexis. If you can’t deal with them right away, they can be frozen. All blocks must be frozen in the genome center –20C freezer.
Blocks need to be prepped on the biorobot in the genome center. See Jen/Alexis for instructions and check with them to find out when the robot is available for your use. **Remember to fill out the logbook.** When finished on the robot, be sure to leave it in the same condition you found it and run the flush procedure.

All plasmid preps must be labeled according to protocol and kept in the genome center cold box. There is a diagram for proper labeling on the side of the cold box.

**Sequencing reactions:** See Jen/Alexis for a protocol. Also, there is a sign-up sheet for both thermocyclers in the genome center. You must first check with Jen/Alexis to see if it is ok to use the thermocyclers, then you must sign up. **DO NOT SIGN UP WITHOUT ASKING JEN/ALEXIS FIRST!!!!!**

Take 2 princeton plates out as soon as your reactions are getting cycled if you are going to clean up your reactions the same day. The plates must be warmed to room temperature before they can be used and should not be inverted.

Once the reactions are cleaned up (See Jen/Alexis for a protocol), they are ready to be sequenced. Jen/ALexis will do that.

Jen/Alexis will tell you when to expect results. Come back then to extract your data from the computer and start your project on the computer.

**Do Not Touch the Sequencing Computer** without Jen or Alexis present. You will have no reason to mess with this computer or the sequencer. It is a VERY expensive piece of equipment! You should not be using any equipment in the Genome Center without Jen or Alexis present and they should be made aware of when you wish to use the equipment.