phage shift

IISc-Bangalore iGEM 2018

Working with Bacteriophages

A Primer

During the course of our project, we felt that the use of phages as a target for genetic engineering is sparsely documented compared to more frequently used model organisms like *E. coli*. As such, the amount of literature review that we had to undertake for successful execution of even simple protocols was enormous.

To make sure no other teams face similar problems in the future, we decided to draft this document as a guideline for working with phages in a laboratory equipped with basic molecular biology machinery.

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Contents

Where to start?

One of the biggest bottlenecks of any project is knowing where to start your work. If you're going to start working with phages, the first task is obviously to obtain them from somewhere. While a lot of labs all over the world work *with* phages, very few of them work *on* phages i.e. most of the labs use phages as a tool rather than an object of study. As such, while it might be easy for you to find more commonly used temperate phages like P1 or Lambda, obtaining other strains might be a bit more challenging.

Following that, a series of methods need to be followed to prevent contamination or reduction in sample quality while, at the same time, assaying the concentration of the phage sample.

Getting the phage

1. Ask labs in your institution: Try asking your PIs and your instructors about labs in your area that might work on phages.
2. Search online for labs that have used the phage of interest: A simple online search will give you multiple results. Most lab PIs will be more than willing to send you a free sample of the phage if they have it.
3. Search for your phage of interest on the ATCC Website and order it. (www.atcc.org)

Avoiding contamination

One of the reasons labs try to avoid using phages is because of their extreme durability outside a cell. A single phage particle can easily take over a whole bacterial cell culture (in some cases) rendering it unusable.

A series of good lab practices need to be followed to ensure that no such incidences occur in your lab.

1. Use separate glassware and disposal areas for phage related work. Try to keep these separate over the duration of your project.
2. If possible, use a separate working area and biosafety cabinet for all phage related work.
3. Always use gloves while working and keep these separate from your normal bacterial work gloves.
4. If any kind of spillage of phages happens, clean it with 2 M NaOH solution.
5. For cleaning glassware/containers that have been used with phages, use 2M NaOH. *We usually wash our flasks with 2M NaOH followed by detergent and water to ensure removal of both phage and bacteria.*

Storing the phage

Considering how phages are usually extracted from cells, you'll probably get your phages from a full cell extract, suspended in LB or other bacterial media. This kind of sample can be stored at 4C, but it is advised to store your phages at 4C after diluting them in SM gelatine media.

You can safely make a 1:10 dilution in SM for storage if your provided stock is more than 10^{10} pfu/mL.

Recipe for SM buffer (with gelatin)[1]

Reagent	Amount	Final concentration
NaCl	5.8g	100mM
$MgSO_4.7H_2O$	2g	8mM
Tris-Cl (pH 7.5, 1M)	50mL	50mM
Gelatine (2% w/v)	5mL	0.01% w/v
Water	To 1 liter	

Autoclave for 20 minutes at 121C.

Finding phage concentration in your sample

The place where you get your phage sample from will usually tell you what the number of phage particles in the sample is. Phage concentrations are usually measured in terms of pfu/ml, i.e. the number of particles in the sample that can form a plaque on a bacterial lawn plate. Even then, it'll be useful to perform a plaque assay to check the concentration to ensure there has been no decrease in phage titre during shipping and handling.

The *Plaque Assay*is a widely used method that utilises the lytic property of phages to find out the number of virion particles in a sample. The readout is a plate with clear areas (called plaques) on an agar plate where no bacterial growth is observed.

The things required for the assay are:

- Phage stock (10^8 pfu/mL to 10^10 pfu/mL)
- SM Gelatine (See recipe above)
- Overnight culture of wild-type bacteria susceptible to the phage
- Agar plates (1.5% agar concentration with appropriate growth media like LB)

Top Agar (0.7% agarconcentration with appropriate growth media like LB)

Liquid media for bacterial growth (Like LB)

Beaker containing water at ~60C

Procedure:

All these steps need to be carried out in a biosafety cabinet, preferably separate from your usual work area.

Step 1.Make dilutions of the phage stock in SM gelatine down to 10 pfu/mL.

Step 2.To a 1.5mL Eppendorf, add (a) 50uL of phage at the stock concentration and (b) 50uL of overnight liquid culture of bacteria to be tested. Incubate at 37C for 20 mins.

Step 3.Repeat step 2 for all dilutions of phage down to 10 pfu/mL.Don't forget to keep a CONTROL with NO PHAGES in it!

Step 4. During the incubation time, prepare 10mL falcons (for each dilution and the control) with 0.7% Top Agar (can be melted in a microwave, we suggest storing at 60C after preparation) and keep these ready in a beaker containing water at 60C.

Step 5. After incubation is complete, add the 100uL bacteria+phage cocktail to a falcon containing the top agar. Mix quickly and pour on a 1.5% agar plate. Swirl the plate to ensure a uniform spread. Leave for around 15 minutes under the airflow to let it dry completely.

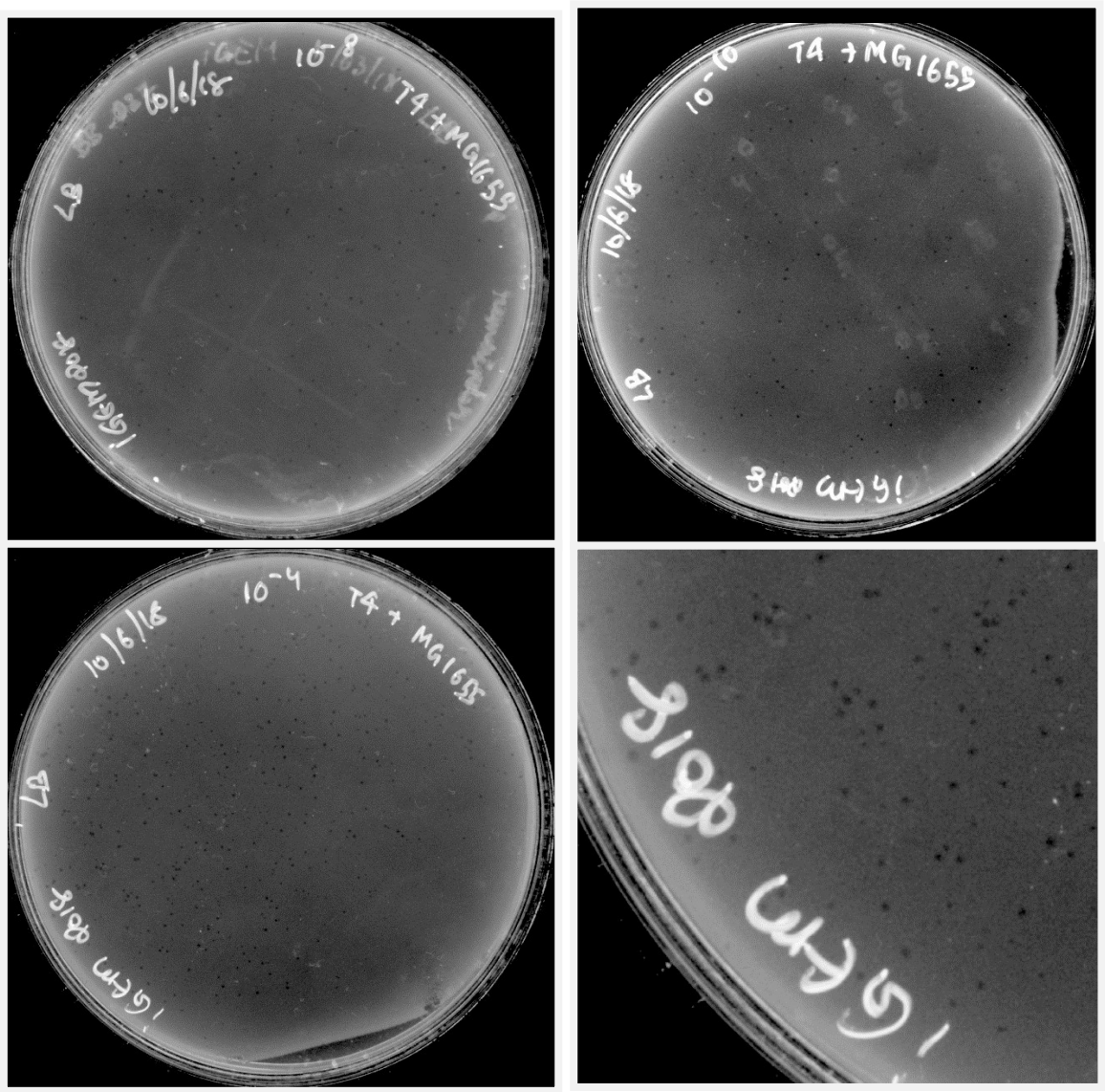
Step 6. Repeat step 5 for all dilutions of phage (and the control).

Step 7. Incubate the plates overnight (~16-20 hrs) at 37C to allow the bacteria to grow.

If everything is done properly, you'll find plates containing plaques on a lawn of bacterial growth. These plaques can be counted to yield an estimate of the number of phage particles in your sample.

We usually get an estimate of the pfu/mL concentration in each dilution and then plot it against the dilution factor on a log-log scale. The place where a linear fit to this curve intersects the x-axis is the phage concentration in your original stock.

Plates from some of our experiments:



What to do next?

The next obvious thing to do after verifying that you do have your virus of interest is to get ready to engineer your phages. Making sure you have enough phage left over for the rest of your experiments is one thing that can be guaranteed by storing a part of your stock. This stored phage can be amplified by propagation in a bacterial host. Most liquid/plate lysate methods give a concentration of 10^6 to 10^8 pfu/mL. These extracts need to be concentrated for further use in recombination-based engineering. Also, an effective strain-phage MOI needs to be calculated to ensure that the infection process is as efficient as possible.

Propagating the phage

The process of amplification for phage is as simple letting them grow inside a host for a long enough time. The rest of the purification process consists of making sure there are no remaining bacteria in your phage sample.

The *culture lysis* method can be used to obtain a large amount of phage from a small liquid sample.

Things required:

- Bacterial growth media (Like LB, we suggest 100mL volume)
- Host bacterial cells (overnight culture, ~16 hrs)
- Phage stock (10^8 to 10^
- Autoclaved Oakridge tubes
- Centrifuge
- 0.22uM syringe Filter and a 50mL syringe
- Chloroform (AR/MB grade)

Procedure:

Step 1. Make a 1% inoculum of the overnight culture in liquid media and wait for it to reach to ~0.6 OD600 at 37C

Step 2. At this point, add around 10^6-10^{10} pfu of your phage and incubate the culture at 37C for 3-6 hours until it becomes clearer due to phage lysis. The actual amount of phage required for this will depend on the strain and phage itself. As such, you might have to try out multiple concentrations before settling on the perfect amount. You can also do the MOI analysis as suggested in the next section for finding the amount of phage that needs to be added.

Step 3. Transfer the culture to Oakridge tubes and chill on ice for 20 minutes.

Step 4. Centrifuge the culture at 4000xg for 20 minutes at 4C.

Step 5. Collect supernatant after centrifugation in a sterile falcon.

Step 6. Filter the supernatant through a 0.22uM syringe filter into another falcon to remove bacterial contamination.

Step 7. Add 1:10 volumes of chloroform to the lysate. Vortex and incubate at room temperature for 10 minutes. This step gets rid of any extra bacterial cells in the lysate.

Step 8. Check the concentration of the lysate using a plaque assay and store appropriate dilutions in SM for further use. The lysate itself can be stored at 4C for a few weeks.

With this method, we were able to obtain 100mL of lysate with concentrations of 10^8-10^{10} pfu/mL.

The *plate lysis* method can be used to obtain a small amount of phage from plaque plates. This extract can be further amplified sequentially to increase the amount.

Things required:

Plaque plates containing the phage of interest.

SM gelatin

Chloroform

Procedure:

Step 1. Add ~5mL SM buffer to the plaque plate and incubate it at 4C on a shaking platform.

Step 2. Transfer as much of the buffer as possible to an Oakridge tube from the plate.

Step 3. Further add another mL of SM to the plate and repeat step 1, followed by transfer to the same tube.

Step 4. Add 100uL of chloroform to the tube. Vortex and incubate at room temperature for 10 minutes.

Step 5. Centrifuge the tube at 4000xg for 10 minutes at 4C.

Step 6. Transfer the supernatant to a sterile falcon. Add a drop of chloroform and store at 4C.

Concentrating the phage

The process of concentrating phage is usually done using a MWCO filter. These kinds of filters are available from Sigma Aldrich under the name of Amicon. A 30kDa cut-off filter is sufficient for phage concentration.

If your lysate concentration is around 10^{11} pfu/mL, it is usually sufficient to proceed with engineering protocols without any concentration.

Things required:

30kDa MWCO spin-column filter

SM Buffer

Procedure:

Step 1. Spin the lysate in the 30kDa filter at 4000xg for 5 mins. Discard the flow-through.

Step 2. Add more lysate until all of it has been passed through the column. Discard flow-through.

Step 3. Add 10-15mL of SM and centrifuge at 4000xg for 5 mins to wash the phages.

Step 4. Add appropriate amount of SM to upper reservoir (depending on concentration factor) and resuspend by vortexing and pipetting. Take out the SM (with resuspended phage) and store in a sterile falcon.

Calculating the MIC for a phage-strain combination

The conventional definition of Minimum Inhibitory Concentration for an antibiotic can be safely applied to a phage given a proper assay that can be used to measure it. During our project, we tried to correlate the phage concentration with an equivalent amount of antibiotic to standardize the dosage required for phage therapy. In the process, we obtained data on how phage concentrations affect bacterial growth. The following assay uses a cell viability dye called resazurin (sold under the name alamarBlue)to detect the relative amount of dead and live cells inside a culture after phage treatment.

Things required:

96 well plate

Bacterial growth media (like LB)

1.5mL microfuge tubes

Resazurin (0.02%, make it from a 1% stock in water)

Host bacterial overnight culture

Phage dilutions (made in SM down to 10 pfu/mL from known stock concentration)

Procedure:

Step 1. Make a 1% secondary inoculum of the overnight culture in 5mL media. Let it grow to ~0.6 OD600.

Step 2. To three (replicates) 1.5mL microfuge tubes add- 470ul growth media, 5uL of phage stock, 25ul of bacterial culture at 0.6OD. Repeat this for all dilutions of the phage. Don't forget to have three samples containing no phage as a control!

Step 3. Vortex and incubate the tubes at 37C for two and a half hours (with shaking).

Step 4. Remove the tubes from the incubator and pipette out 100ul of each sample into its designated well in the 96-well plate. Make sure to label the wells properly and arrange all the triplicates together. Don't forget to keep a well containing only growth media.

Step 5. To each well, add 20ul of 0.02% resazurin solution. Mix by pipetting up and down. This step is best done in the dark to prevent saturation of the resazurin dye.

Step 6. Cover the plate in aluminium foil and incubate at 37C for 20 minutes.

Step 7. Remove the aluminium foil and take absorbance readings at both 570nm and 600nm on a plate reader.

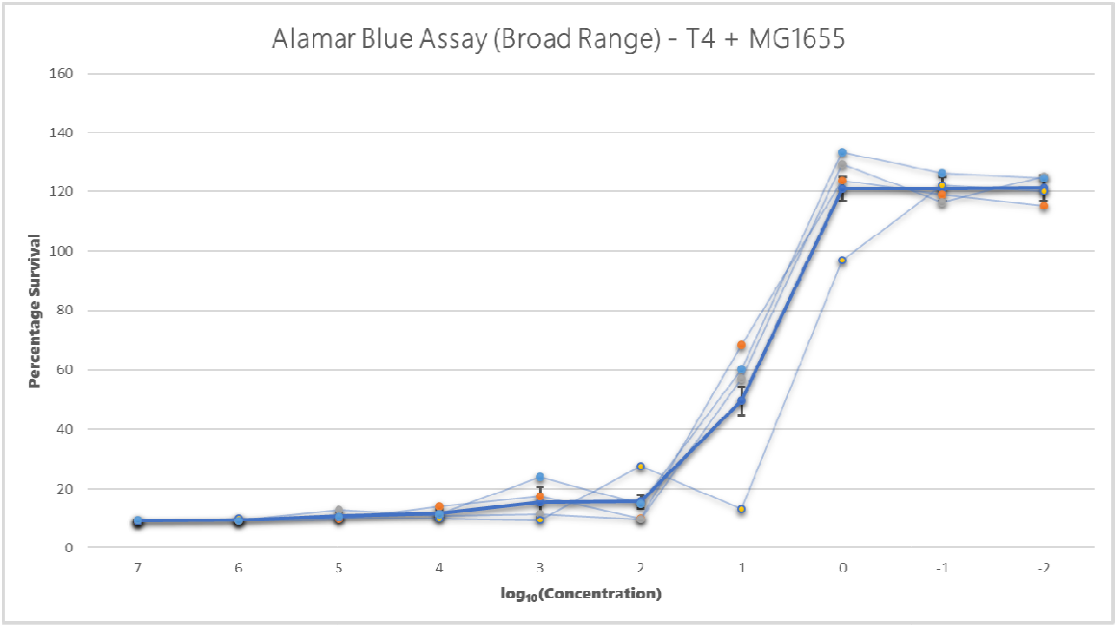
Step 8. Calculate the percentage survival at each antibiotic concentration using the equation.

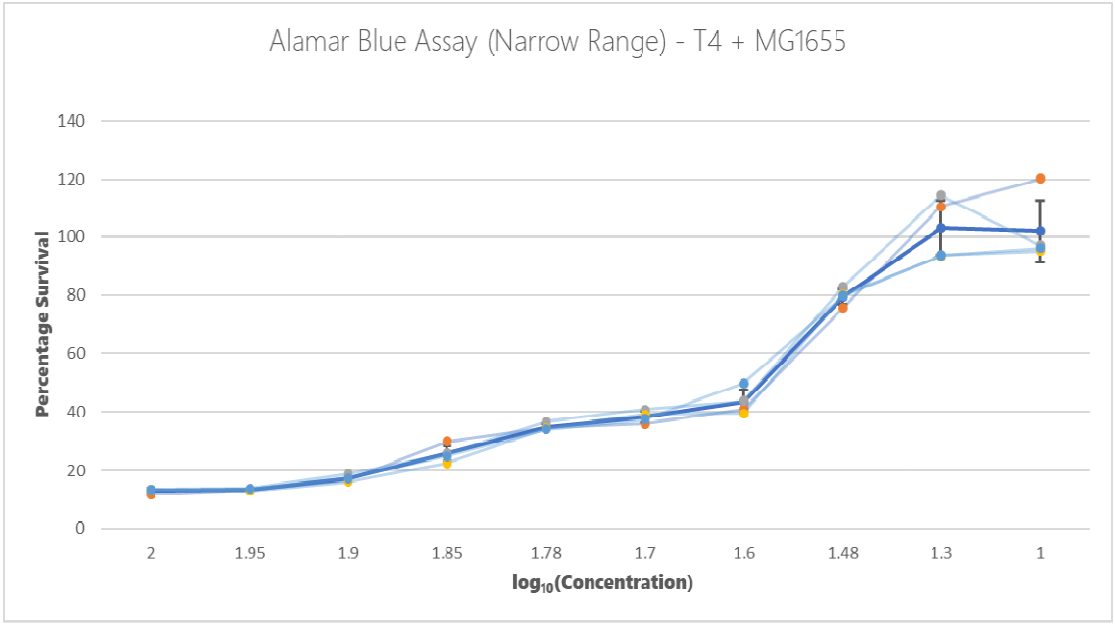
$$\% \text{ survival} = [(E2*A1 - E1*A2) / (E2*P1 - E1*P2)] * 100$$

where,
E1 = molar extinction coefficient of oxidized alamarBlue at 570 nm = 80586
E2 = molar extinction coefficient of oxidized alamarBlue at 600 nm = 117216
A1 = absorbance of test wells at 570 nm
A2 = absorbance of test wells at 600 nm
P1 = absorbance of positive growth control well (cells plus alamarBlue but no test agent) at 570 nm
P2 = absorbance of positive growth control well (cells plus alamarBlue but no test agent) at 600 nm
Note that A1, A2, P1 and P2 are the absorbance values after subtraction from the blank (i.e., medium only control)

To find out the MIC, plot the % survival against the logarithm of effective phage concentration in the incubated sample. The point where the curve falls to zero is the effective MIC of the phage for the host strain.

Our results for T4-MG1655 MIC assay were as follow:





Engineering the phage using recombineering

This is the part where actual principles of synthetic biology are to be applied. The most general protocols in this area are limited to a few and the experiments need to be determined on basis of the actual nature of the problem.

For example, to screen recombinants in our case, we used a endolysin plasmid, a part that changes the host phenotype in presence and absence of e-gene deficient phages. The extraction of pure phage DNA is the first and probably the most difficult task when it comes to phage synthetic biology because of the size of the genome. As such, that is the main protocol that every team should learn and perfect in time.

Phage DNA extraction

For this protocol, the phage lysate obtained from the last few steps is required. After that, the following steps can be followed to extract pure unfragmented phage DNA from the lysate.

1. Take 1 ml of phage lysate in a microfuge tube.
2. Add 10 ul of 0.1 M $MgSO_4$ to the tube (final conc. of 1mM).
3. Add 20 ul of 20 mg/ml lysozyme.
4. Add 2 ul each of 20 mg/ml DNAse and 40 mg/ml RNAse.
5. Incubate at 37°C for 2 hours.
6. Add 20 ul of 0.1 M EDTA (final conc. of 2mM.
7. Add 50 ul of 10% SDS solution (final conc. of 0.5 %)
8. Add 3 ul of 20 mg/ml of Proteinase K (final conc. of 50 ug/ml).
9. Incubate at 50°C for 1 hour.
10. Add equal amount of equilibriated phenol:chloroform (1:1) mixture. Mix it well by rotating the tube slowly.
11. Spin the tube at 13000g for 5 minutes. Pipette out the aqueous layer (usually the top layer) into another microfuge tube. Discard the rest.
12. Add equal amount of chloroform to this tube and Mix.

The Lambda Red system

The Lambda red recombination system is a combination of three enzymes (recombinases) isolated from the Lambda phage that together increase the basal DNA recombination efficiency in the cell by around four orders of magnitude. These recombinases - called Beta, Exo and Gam - can be expressed from a plasmid and ensure efficient recombination of a dsDNA carrier inside the cell to its homologous regions in either the bacterial genome or another target strand inside the cell.

The BioBrick BBa_K1433005 can be used to express these recombinases and perform recombination in vivo by infecting the cells expressing these recombinases with the phage in presence of a recombination cassette containing 50 base pair homologies on both sides of a sequence.