Protocol: Purifying phage by CsCl gradient ultracentrifugation

Materials required:

- Large volume (e.g., 4 liter) culture flasks and an appropriate incubator
- Visible-light spectrophotometer
- DNase I and RNase A, powder or stock solutions
- Polyethylene glycol 8000, NaCl, chloroform (for optional PEG precipitation)
- Large high-speed centrifuge with large capacity (e.g. GSA, GS-3 or JA10) and small-capacity (e.g., SS-34) rotors, centrifuge bottles and tubes
- Gelatin-free SM buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.5, 8 mM $MgSO_4$)
- CsCl
- Ultracentrifuge, rotor and appropriate disposable tubes (tubes should be clear and able to be punctured by a standard 20 ga. needle)
- 10 cc syringes, 18 – 20 ga. needles
- Dialysis cassettes or tubing, 3 kDa – 50 kDa NWCO cutoff

Lysate preparation

1) Use 1-2 liters of phage liquid culture, phage titer should be at least 1 x 10^9 PFU/ml for best results. Having too few phage will make the band in the CsCl gradient difficult to find. In a 70.1 Ti quickseal tube, about 5 x 10^{10} PFU are required to see a band.
    a. Lysate propagation for virulent phage varies from phage to phage and host to host. A basic starting protocol is to subculture your host into 1 or 2 liters of fresh broth medium (starting OD_{550} should be about 0.05) and incubate until the culture reaches and OD_{550} equal to about 5 to 10% of the host's stationary phase OD_{550} (e.g., if a saturated culture of your host has an OD_{550} of ~5, wait until the culture reaches and OD_{550} of about ~0.25). At this point, inoculate the culture with your phage so that the final MOI is about 0.025, and monitor the culture growth at OD_{550}. Ideally, you should see the OD of the culture grow, plateau and then drop off. When the culture OD stops dropping (it will probably not clear completely), move on to the next step and harvest the lysate. If the lysate titer is too low, you can try changing the point at which you inoculate the culture, or changing the amount of phage you add.
2) Centrifuge the liquid culture at 12,000 x g, 15 min, 4 ºC.
3) Carefully decant the supernatant into a clean Erlenmeyer flask. The supernatant should be optically clear (OD_{550} of about 0.05 or less); centrifuge again if it is not. Filtration of the supernatant to remove cell debris (0.45 µm) is a good idea if practical, although this is not strictly required.
4) Add DNase I and RNase A to the cleared lysate to a final concentration of 1 µg/ml each. Stir or gently shake the lysate at RT for 1-2 hr. Lysates usually can be stored for up to 48 hrs at 4 ºC.

Phage concentration

You can concentrate the phage by one of two methods: high-speed centrifugation, or precipitation in the presence of NaCl and polyethylene glycol. The centrifugation method usually provides a cleaner prep with less chance for phage inactivation.

Centrifugation method

1) Tailed phages have sedimentation coefficients of about 300-1200; for example, lambda is about 400 while T4 is about 800-1000. Calculate the pelleting time for your phage based on its estimated size, using the formula $t = k/S$, where t is the time in hours, k is the rotor k factor, and S is the sedimentation coefficient. Centrifuge the phage in clean bottles at 4 ºC for a time equal to two to four times the calculated pelleting time. A typical run for a phage like lambda would be 7,000 x g, 4 ºC for 16 – 18 h in a Sorvall GSA or GS-3 rotor.
2) Carefully pour off the supernatant and retain the pellet, which will be glassy or opaque. Do not disturb the pellets.
3) Add enough gelatin-free SM buffer to just cover the pellets and let them soak at 4 ºC for a few hours to overnight, until they loosen up. When loose, gently mix to resuspend thoroughly transfer to a 50 ml centrifuge tube. The final resuspension volume should be about 5 - 10% of the original lysate volume.
4) Centrifuge the phage suspension again at 12,000 x g, 4 ºC for 10 min to pellet any remaining cell debris. Transfer the supernatant to a new tube. Continue to the CsCl gradient procedure.

PEG precipitation method

1) Add solid NaCl to the lysate to a final concentration of 1 M (58.4 g/l). Stir until dissolved.
2) Add solid PEG-8000 to the lysate to a final concentration of 10% (100 g/l). Stir until dissolved, then stir on ice for at least 30 min.
3) Place the lysate flask at 4 ºC (no stirring) overnight.
    a. This is a convenient stopping step; some phages (e.g. BcepIL2, *S. aureus* phage K) can be left in this state for at least several days.
4) Centrifuge the PEG-precipitated lysate at 10,000 x g, 10 min, 4 ºC.
5) Pour off the supernatant and let the centrifuge bottles stand inverted for 3-5 min to drain any excess supernatant from the pelleted precipitate.
    a. Examine the supernatant after pouring off to ensure most of the precipitate remained in the pellets. Centrifuge again if a significant portion of the pellets sloughed off into the supernatant.
6) Resuspend the PEG precipitate in a minimal volume of lambda-diluent (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5).
    a. Start with about 18 ml of lambda-dil per 2 litres of starting lysate, use a greater volume if necessary. Don't vortex; swirling and gently pipetting with a wide-bore 1 ml pipette tip helps.
7) Transfer the resuspended precipitate to a 50 ml Falcon tube, and centrifuge at 3,000 x g, RT, 10 minutes to pellet any insoluble material. Discard the pellet.
8) Transfer the supernatant to a clean polypropylene or teflon Oak Ridge tube. Add an equal volume of chloroform and mix by inverting for 2 min.
    a. Be sure to use a chloroform-compatible tube; clear polycarbonate is not compatible with chloroform.

9) Centrifuge at 12,000 x g, 10 min, 4 ºC. Transfer the supernatant to a new Oak Ridge tube; avoid the chloroform and the white interface layer.
10) Repeat steps 12 and 13 until there is little or no white precipitate at the interface. Retain the phage suspension. Continue to the CsCl gradient procedure.

Isopycnic CsCl gradient centrifugation

1) Carefully measure the volume of the phage suspension and transfer it to a 50 ml Falcon tube. Add solid CsCl at a rate of 0.75 g/ml of phage suspension. Add the CsCl slowly (about ¼ at a time) and swirl gently to dissolve.
    a. The final density of the solution should be about 1.40 – 1.45 g/ml.
2) Load the phage-CsCl solution to ultracentrifuge tubes and run.
    a. Using a Beckman 70.1 Ti rotor: 45,000 RPM, 5 ºC, 24 hrs.
    b. Using a Beckman SW28 rotor: 25,000 RPM, 5 ºC, 24 hrs.
    c. If there is not enough phage suspension to completely fill the tubes, top them up with a solution of gelatin-free SM buffer containing 0.75 g/ml CsCl. Be sure to mix the phage and added buffer thoroughly as the densities of the two solutions will probably not be identical.
    d. Be sure to balance the tubes to within 10 mg of each other before loading into the rotor.
3) The phage should form a grey-white band somewhere in the middle third of the tube. Clamp the tube into a retort stand and carefully insert a 10 cc syringe with an 18 or 20 ga. needle through the wall of the tube, 2-3 mm below the band. Carefully draw out the band with the syringe.
4) Transfer the phage "bandate" to a dialysis cassette. Dialyse against 1 litre of gelatin-free SM buffer containing 1 M NaCl, (add 52 g NaCl per liter buffer) at 4 ºC overnight.
5) Transfer the dialysis cassette to 1 liter of normal gelatin-free SM and dialyze for 2-3 hrs at RT. Repeat.
6) Filter-sterilize the phage prep and store in the dark at 4 ºC.