Large-scale Preparation of Bacteriophage λ

Two methods are available for the preparation of large quantities of bacteriophage λ. The first involves infection of bacteria at low multiplicity. The infected culture is then inoculated into a large volume of medium. Initially, the concentration of bacteriophage is low, and uninfected cells in the culture continue to divide for several hours. However, successive rounds of infection lead to the production of increasing quantities of bacteriophage; eventually all the bacteria become infected and complete lysis of the culture occurs.

Care is required with this method because small changes in the ratio of cells to bacteriophage in the initial infection greatly affect the final yield of bacteriophage. Furthermore, the optimum ratio varies for different strains of bacteriophage λ and bacteria. However, with a little effort, the method can be adapted for use with most combinations of virus and host cells.

The second method involves induction of a lysogenic bacteriophage. This technique works most efficiently when the bacteriophage encodes a temperature-sensitive repressor (*cIts*). Lytic growth may then be induced by transiently raising the temperature of the growing bacterial culture. The method is straightforward but of course can only be used for bacteriophages that form stable lysogens. Among this group, however, are several useful strains: for example, *cI857ts*Sam7, which yields large amounts of bacteriophage DNA that may be used to prepare molecular weight markers, and λgt-λC, which has been used as a vector. Most other vectors in current use cannot form lysogens.

INFECTION

There are many variants of this method; however, in our hands, the following protocol gives the best results (Blattner et al. 1977; Maniatis et al. 1978).

To prepare bacteriophage from a 2-liter culture of infected bacteria:

1. Inoculate 100 ml of NZCYM in a 500-ml flask with a single colony of an appropriate bacterial host. Incubate overnight at 37°C with vigorous shaking.

2. Read the OD_{600} of the culture. Calculate the cell concentration assuming that 1 $OD_{600} = 8 \times 10^8$ cells/ml.

3. Withdraw four aliquots, each containing 10^{10} cells. Centrifuge at 4000*g* for 10 minutes at room temperature. Discard the supernatants.

4. Resuspend each of the bacterial pellets in 3 ml of SM.

5. Add bacteriophage and mix rapidly. The number of bacteriophages used is critical. For strains of bacteriophage λ that grow well (e.g., λgtWES-λB), 5×10^7 bacteriophages are added to each suspension of 10^{10} cells; for bacteriophages that grow relatively poorly (e.g., the Charon series), it is better to increase the starting inoculum to 5×10^8. However, there are no hard and fast rules, and you will probably need to experiment to find the multiplicity that gives the best results under your conditions.

6. Incubate at 37°C for 20 minutes with intermittent shaking.

7. Add each infected aliquot of 10^{10} cells to 500 ml of NZCYM, prewarmed to 37°C in a 2-liter flask. Incubate at 37°C with vigorous shaking. Concomitant growth of bacteria and bacteriophage should occur, resulting in lysis of the culture after 9–12 hours.

   A fully lysed culture contains a considerable amount of bacterial debris, which can vary in appearance from a fine splintery precipitate to much larger stringy clumps. If the culture is held up to the light, the Schlieren patterns and silky appearance of a dense, unlysed bacterial culture should not be visible.

8. If lysis is not apparent, check a small sample of the cultures for evidence of bacteriophage growth. Withdraw into glass tubes two aliquots (1 ml) of the infected cultures. Add 1 or 2 drops of chloroform to one of the tubes. Incubate both tubes at 37°C for 5–10 minutes with intermittent shaking. Compare the appearance of the two cultures by holding the tubes to a light. If infection is near completion but the cells have not yet lysed, the chloroform causes the cells to burst and the turbid culture clears to the point where it is translucent. In this case, proceed to step 9.

   If lysis does not occur, the preparation can sometimes be rescued by adding to each of the cultures an additional 500 ml of NZYCM preheated to 37°C. Incubation should be continued for a further 2–3 hours, shaking as vigorously as possible.

9. Add 10 ml of chloroform to each flask and continue incubating and shaking for a further 30 minutes.

10. Proceed to Purification of Bacteriophage λ, step 1 (page 80).

INDUCTION[1]

To prepare bacteriophage λ from a 2-liter culture of induced bacteria:

1. Streak out the appropriate lysogenic bacteria on each of two NZCYM plates. Incubate one at 30°C, the other at 42°C. Colonies should form only on the plate incubated at 30°C.

2. Pick a single colony from the plate incubated at 30°C and inoculate 100 ml of NZCYM in a 500-ml flask. Incubate overnight at 30°C with vigorous agitation.

3. Read the OD_{600} of the overnight culture, and inoculate four 500-ml batches of NZCYM prewarmed to 30°C with sufficient overnight culture to give a starting OD_{600} of 0.05.

4. Incubate at 30°C with vigorous shaking until the OD_{600} reaches 0.5. It is important to induce the culture immediately at this stage, since further growth of the bacteria can result in a significant decrease in the yield of phage.

5. Induce the culture by incubating for 15 minutes in a 45°C water bath with constant shaking.

[1]Pirrotta et al. (1971).

6. Incubate the induced culture at 38°C for 2.5–5 additional hours with vigorous shaking.

7. Check a small sample of the cultures for evidence of bacteriophage growth. Withdraw into glass tubes two aliquots (1 ml) of the induced cultures. Add 1 or 2 drops of chloroform to one of the tubes. Incubate both of the tubes at 37°C for 5–10 minutes with intermittent shaking. Compare the appearance of the two cultures by holding the tubes to a light. If the lysogen was properly induced, the chloroform-treated culture should have become translucent; the untreated culture will remain opaque. If you are satisfied with the result, proceed to step 8. If lysis does not occur, you probably should recheck your lysogen (step 1) and start the experiment over again.

8a. If the phage carries a wild-type *S* gene, lysis of the induced bacteria should occur spontaneously. In this case, add 10 ml of chloroform to each culture and continue incubation for a further 30 minutes. Then proceed to Purification of Bacteriophage λ, step 1 (page 80).

8b. If the phage carries an amber mutation in the *S* gene, lysis will not occur spontaneously. The bacteriophage particles remain trapped within the host bacteria until chloroform is added. The infected bacteria can be concentrated by centrifugation and lysed in a small volume of liquid, thereby eliminating the necessity of handling large volumes of phage lysate.

Collect bacteria by centrifugation at 4000*g* for 10 minutes at 4°C. Resuspend the cell pellet in:

10 mM Tris · Cl (pH 7.4)
50 mM NaCl
5 mM $MgCl_2$

Use 10–20 ml per liter of original culture.

Add 10 drops of chloroform. Vortex well and let stand at room temperature for 30 minutes.

To remove DNA liberated from the lysed cells, add crystalline pancreatic DNase to a final concentration of 0.2 μg/ml and incubate at room temperature for 5 minutes.

Remove debris by centrifugation at 12,000*g* for 15 minutes. Harvest the supernatant, which contains the bacteriophage. Proceed to Purification of Bacteriophage λ (step 8, page 80).

PURIFICATION OF BACTERIOPHAGE λ[2]

1. Chill the lysed cultures to room temperature and add pancreatic DNase and RNase, both to a final concentration of 1 μg/ml. Incubate for 30 minutes at room temperature. Crude commercial preparations of both enzymes are adequate to digest the nucleic acids liberated from the lysed bacteria, which might otherwise entrap bacteriophage particles.

2. Add solid sodium chloride to a final concentration of 1 M (29.2 g/500 ml of culture). Dissolve by swirling. Let stand for 1 hour on ice.

3. Remove debris by centrifugation at 11,000*g* for 10 minutes at 4°C. Pool the supernatants in a clean flask.

4. Add solid polyethylene glycol (PEG 6000) to a final concentration of 10% w/v (i.e., 50 g/500 ml of supernatant). Dissolve by slow stirring on a magnetic stirrer at room temperature.

   Note. Some workers prefer to add the polyethylene glycol at the same time as the sodium chloride (step 2). The centrifugation step (3) may then be omitted. This procedure works well if the bacteriophage grows well and the titer of bacteriophage in the original lysed culture is greater than 2×10^{12}/ml.

5. Cool in ice water and let stand for at least 1 hour to allow the bacteriophage particles to form a precipitate.

6. Recover the precipitated bacteriophage particles by centrifugation at 11,000*g* for 10 minutes at 4°C. Discard the supernatant and stand the centrifuge bottles in a tilted position for 5 minutes to allow the remaining fluid to drain away from the pellet. Remove the fluid with a pipette.

7. Using a wide-bore pipette equipped with a rubber bulb, gently resuspend the bacteriophage pellet in SM (8 ml for each 500 ml of supernatant). Wash the walls of the centrifuge bottles thoroughly since the bacteriophage precipitate sticks to them, especially if the bottles are old.

8. Add an equal volume of chloroform to the bacteriophage suspension and vortex for 30 seconds. Separate the organic and aqueous phases by centrifugation at 1600*g* for 15 minutes at 4°C. Recover the aqueous phase containing the bacteriophage.

9. Measure the volume of the supernatant and add 0.5 g/ml of solid cesium chloride. Mix gently.

[2]Yamamoto et al. (1970).

TABLE 3.1. CESIUM CHLORIDE SOLUTIONS (100 ML) FOR STEP GRADIENTS PREPARED IN SM

Density (ρ)	CsCl (g)	SM (ml)	Refractive index (η)
1.45	60	85	1.3768
1.50	67	82	1.3815
1.70	95	75	1.3990

10. When the cesium chloride has dissolved, carefully layer the bacteriophage suspension onto cesium chloride step gradients that are preformed in Beckman SW41 or SW27 cellulose nitrate centrifuge tubes (or their equivalent) (see Table 3.1).

    The step gradients may be made *either* by layering carefully and sequentially solutions of decreasing density on top of one another *or* by layering solutions of increasing density under one another. Make a mark on the outside of the tube opposite the position of the interface between the ρ 1.50 layer and the ρ 1.45 layer (see Fig. 3.1).

11. Centrifuge in either an SW41 or an SW27 rotor at 22,000 rpm for 3 hours at 4°C.

12. A bluish band of bacteriophage particles should be visible at the interface between the 1.45 and 1.50 g/ml layers. If the yield of bacteriophage is low, placing the gradient against a black background and shining a light from above often helps to detect the band of particles.

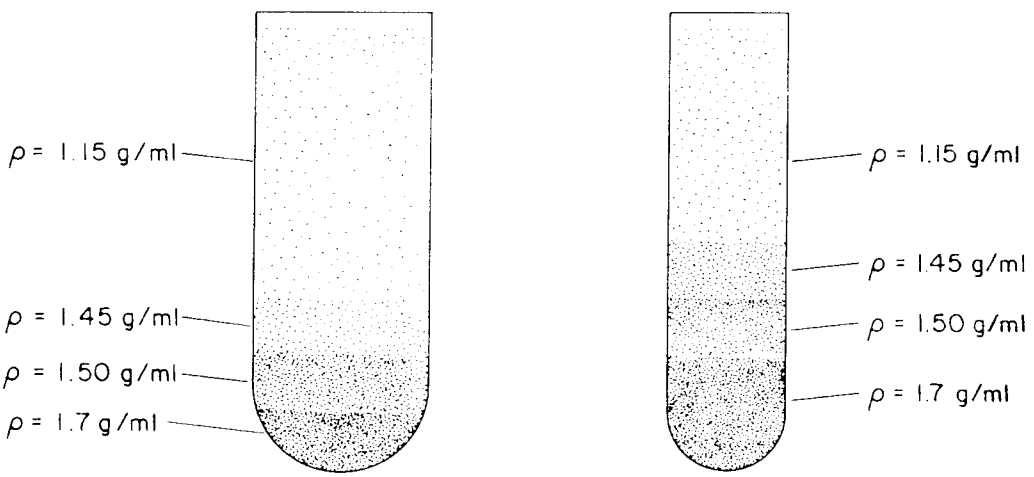

Figure 3.1

Cesium chloride gradients for purifying bacteriophage λ. The bacteriophage will form a visible band at the interface between the 1.45 g/ml and 1.50 g/ml cesium chloride layers.

13. Collect the bacteriophage particles by puncturing the side of the tube. First, place a piece of Scotch tape on the outside of the tube, level with the bacteriophage band. Using a 21-gauge needle, puncture the tube through the tape and collect the band of bacteriophage particles (see Fig. 3.2). Alternatively, the band can be collected from above using a micropipette or a pasteur pipette.

    Be careful not to contaminate the bacteriophage with material from other bands that are visible in the gradient. These consist of various types of bacterial debris and unassembled bacteriophage components.

14. Add enough cesium chloride solution (1.5 g/ml in SM) to the bacteriophage suspension to fill a cellulose nitrate tube that fits either a Type-50Ti rotor or an SW50.1 rotor (or equivalent). Centrifuge at 38,000 rpm for 24 hours at 4°C (Type-50Ti) or at 35,000 rpm for 24 hours at 4°C (SW50.1).

15. Collect the band of bacteriophage particles as described above. Store at 4°C in cesium chloride in a tightly capped tube.

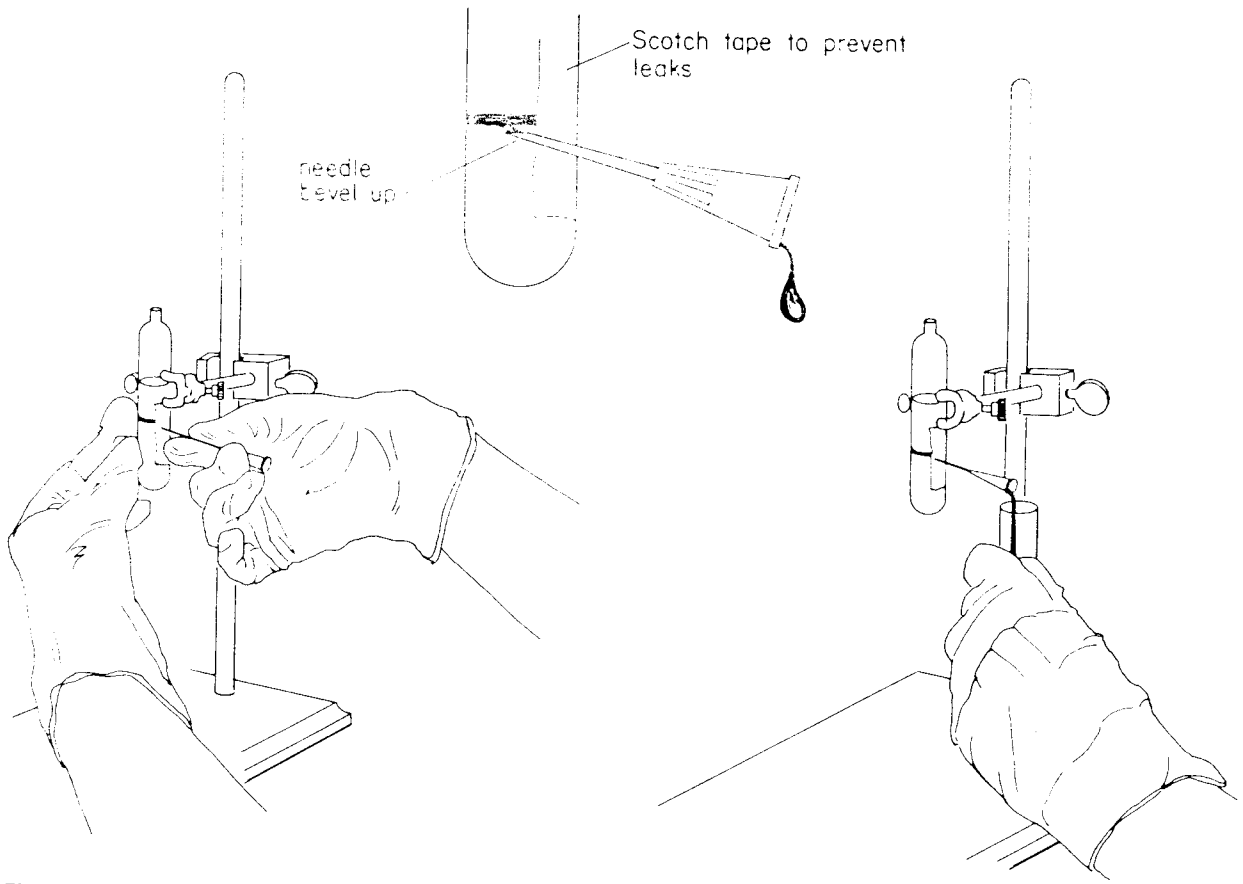

Figure 3.2

Collection of bacteriophage band by side puncture. **Caution:** Keep your fingers out of the path of the needle in case you poke it through the other side of the tube.

Notes

i. Particles of bacteriophage λ are exceedingly sensitive to EDTA, and it is essential that Mg^{++} (10–30 mM) be present at all stages of the purification to prevent disintegration of the particles.

ii. If the yields of purified bacteriophage are low, the number of infectious bacteriophage particles should be determined in samples taken at various stages during the purification in order to determine where losses are occurring.

Alternative Methods of Purification of Bacteriophage λ

The method of purification of bacteriophage particles given on the preceding pages is essentially that of Yamamoto et al. (1970). Over the years many variations and shortcuts have been invented. Three slightly modified procedures are given below.

Pelleting Phage Particles

1. Follow the Yamamoto purification scheme through step 8 (page 80), including the extraction with chloroform.

2. Collect the bacteriophage particles by centrifugation at 25,000 rpm for 2 hours at 4°C in a Beckman SW27 rotor (or its equivalent).

3. Pour off the supernatant. A glassy pellet of bacteriophage should be visible on the bottom of the tube. Add 1–2 ml of SM to each tube and leave it overnight at 4°C, if possible on a rocking platform.

4. The following morning, pipette the solution gently to ensure that all the bacteriophage particles have been resuspended.

Although the preparations obtained in this way are not as clean as those obtained by equilibrium gradient centrifugation, they serve as a useful source of DNA, which can be used in the preparation of bacteriophage λ arms.

Proceed with DNA extraction at step 5 (page 85).

Glycerol Step Gradient[3]

1. Follow the Yamamoto purification scheme through step 6 (page 80).

2. Resuspend the phage pellet in 50 mM Tris · Cl (pH 7.8) and 10 mM $MgSO_4$ (TM buffer) using 5–10 ml per liter of original culture.

[3]Modified from Vande Woude (1979).

3. Extract the bacteriophage suspension once with chloroform.

4. Prepare a glycerol step gradient in a Beckman SW41 cellulose nitrate tube (or its equivalent) as follows:

   a. Pipette 3 ml of a solution consisting of 40% glycerol in TM into the bottom of the tube.

   b. Carefully layer 4 ml of a solution consisting of 5% glycerol in TM over it.

   c. Carefully layer the bacteriophage suspension onto the glycerol solution.

   d. Fill the tube with TM.

5. Centrifuge at 35,000 rpm for 60 minutes at 4°C.

6. Discard the supernatant and resuspend the bacteriophage pellet in 1 ml of TM per liter of original culture.

7. Add pancreatic DNase and RNase to final concentrations of 5 μg/ml and 1 μg/ml, respectively. Digest for 30 minutes at 37°C.

8. Add EDTA from a stock solution (0.5 M, pH 8.0) to a final concentration 20 mM.

9. Proceed with DNA extraction at step 5 (page 85).

Equilibrium Centrifugation in Cesium Chloride

When dealing with small-scale preparations of bacteriophage (1 liter or less), the cesium chloride step gradient can be omitted.

1. After extraction with chloroform (step 8), measure the volume of the aqueous phase and add 0.75 g/ml of solid cesium chloride. Mix gently to dissolve.

2. When the cesium chloride has dissolved, transfer the bacteriophage suspension to an ultracentrifuge tube that fits either an angle or swing-out rotor. Fill the tube with TM to which cesium chloride (0.75 g/ml) has been added.

3. Centrifuge and collect the band of bacteriophage particles as described on page 82.

EXTRACTION OF BACTERIOPHAGE λ DNA

1. Remove cesium chloride from the purified bacteriophage preparation by dialysis at room temperature for 1 hour against a 1000-fold volume of:

   10 mM NaCl
   50 mM Tris · Cl (pH 8.0)
   10 mM $MgCl_2$

2. Transfer the dialysis sac to a fresh flask of buffer and dialyze for an additional hour.

3. Transfer the bacteriophage suspension into a centrifuge tube of a size such that only one third is full.

4. Add EDTA from a stock solution (0.5 M, pH 8.0) to give a final concentration of 20 mM.

5. Add pronase to a final concentration of 0.5 mg/ml, or add proteinase K to a final concentration of 50 μg/ml.

6. Add SDS (stock solution, 20% w/v in water) to a final concentration of 0.5%. Mix by inverting the tube several times.

7. Incubate for 1 hour at 37°C (pronase) or 65°C (proteinase K).

8. Add an equal volume of equilibrated phenol (see page 438). Mix by inverting the tube several times. Separate the phases by centrifugation at 1600*g* for 5 minutes at room temperature. Use a wide-bore pipette to transfer the aqueous phase to a clean tube.

9. Extract the aqueous phase once with a 50:50 mixture of equilibrated phenol and chloroform.

10. Recover the aqueous phase as described above and extract once with an equal volume of chloroform.

11. Transfer the aqueous phase to a dialysis sac.

12. Dialyze sequentially overnight at 4°C against three 1000-fold volumes of TE (10 mM Tris · Cl [pH 8.0] and 1 mM EDTA).