EXERCISE

# 37

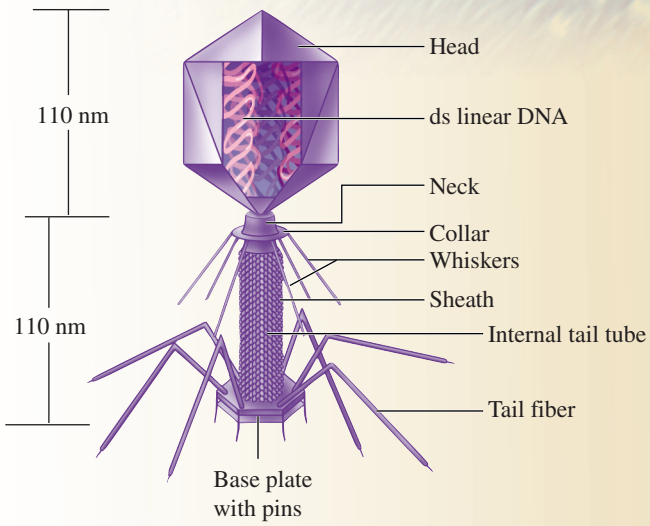
# Isolation of Bacteriophage from Sewage and Determination of Phage Titer

## Background

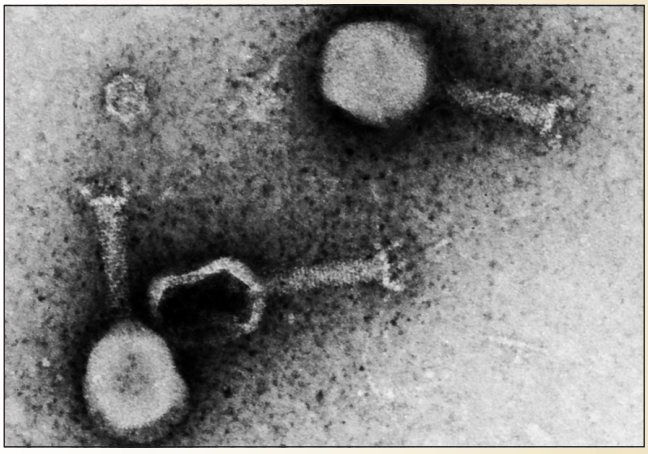
Virtually any type of cell is susceptible to virus infection; viruses cause disease in plants and animals, and can also infect procaryotes and unicellular eucaryotes. Viruses that infect procaryotes are known as *bacteriophages,* or *phages,* because when they were first discovered, they appeared to eat bacterial cells, generating a clearing, or *plaque,* on a lawn of susceptible bacteria. In reality, the bacteria are killed by **lysis** as newly produced phages are released from the damaged cells.

Like all viruses, bacteriophages consist of nucleic acid (RNA or DNA) surrounded by a protein coat, or capsid. Unlike some plant and animal viruses, bacteriophages are not enveloped. Some phages have elaborate structures for attaching to the bacterial surface and injecting nucleic acid into the cytoplasm. A diagram of one such bacteriophage, T4, is shown in figure 37.1.

Most bacteriophages are lytic; that is, each infection event leads to the production of new virions and the death of the cell by lysis. Some bacteriophages—most notably bacteriophage lambda ($\lambda$)—are categorized as temperate. Sometimes $\lambda$ DNA is integrated into the bacterial chromosome, with its genes largely silent. The infected cell survives as a *lysogen*. In some $\lambda$ infections, the DNA remains independent of the host chromosome, and is replicated many times over; its genes are expressed at high levels, and newly assembled phages are released. The "choice" between a lysogenic, nonproductive infection and a lytic, productive infection depends on environmental conditions. For example, UV exposure can cause a $\lambda$ infection to switch from lysogenic to lytic. A typical lytic bacteriophage infection cycle is depicted in figure 37.2.




(a)



(b)

**Figure 37.1** Bacteriophage or "phage" T4, a DNA virus of *E. coli*. (a) Diagram of phage T4. (b) Electron micrograph of phage T4 particles.

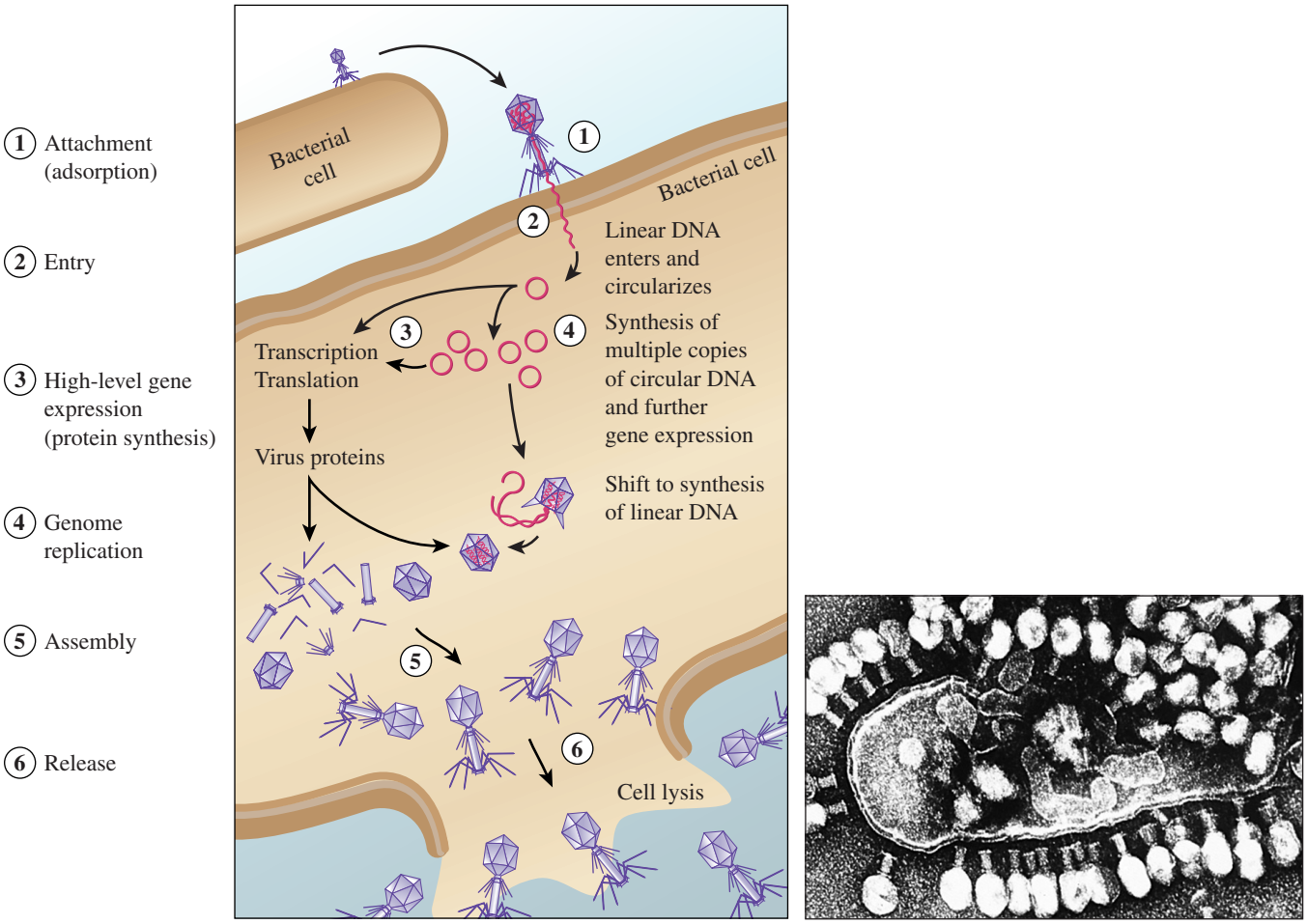



**Figure 37.2** The infection cycle of bacteriophage T4. (a) This kind of infection is "productive" because new viruses are produced. The steps listed on the left are generally applicable to any productive virus infection. (b) Electron micrograph of *E. coli* infected with phage T4 (36,500×).

In this exercise, we will focus on the bacteriophages of coliform bacteria. Coliform bacteria are relatively harmless microorganisms that live in large numbers in the intestines of mammals, where they aid in the digestion of food. *Escherichia coli* is a common fecal coliform bacterium. The presence of fecal coliform bacteria in water indicates that it has been contaminated with human or other animal feces, and that a potential health risk exists for those who use the water. Raw, untreated sewage contains large numbers of *E. coli*. Therefore, we will use raw sewage as a source of bacteriophages that infect *E. coli*.

In this exercise, you have the opportunity to: (1) amplify (increase the numbers of) phages in the sewage sample by allowing them to infect and reproduce within fresh *E. coli*, (2) collect the phages from the culture by centrifugation and filtration, and (3) detect and *titer* the amplified, isolated phages using a *plaque assay*. The assay is based on the fact that each plaque on a lawn of bacteria, although it contains $10^6$ to $10^7$ virions along with bacterial debris, represents a single infecting phage that entered one cell at the start of the culture. The infection then "spread" as the viruses reproduced and cells lysed, eventually forming a visible plaque (figure 37.3). The titer of a phage suspension, therefore, is determined by counting the number of plaques that form from a given volume of suspension. Phage titer is expressed as plaque-forming units (PFU) per milliliter (ml).

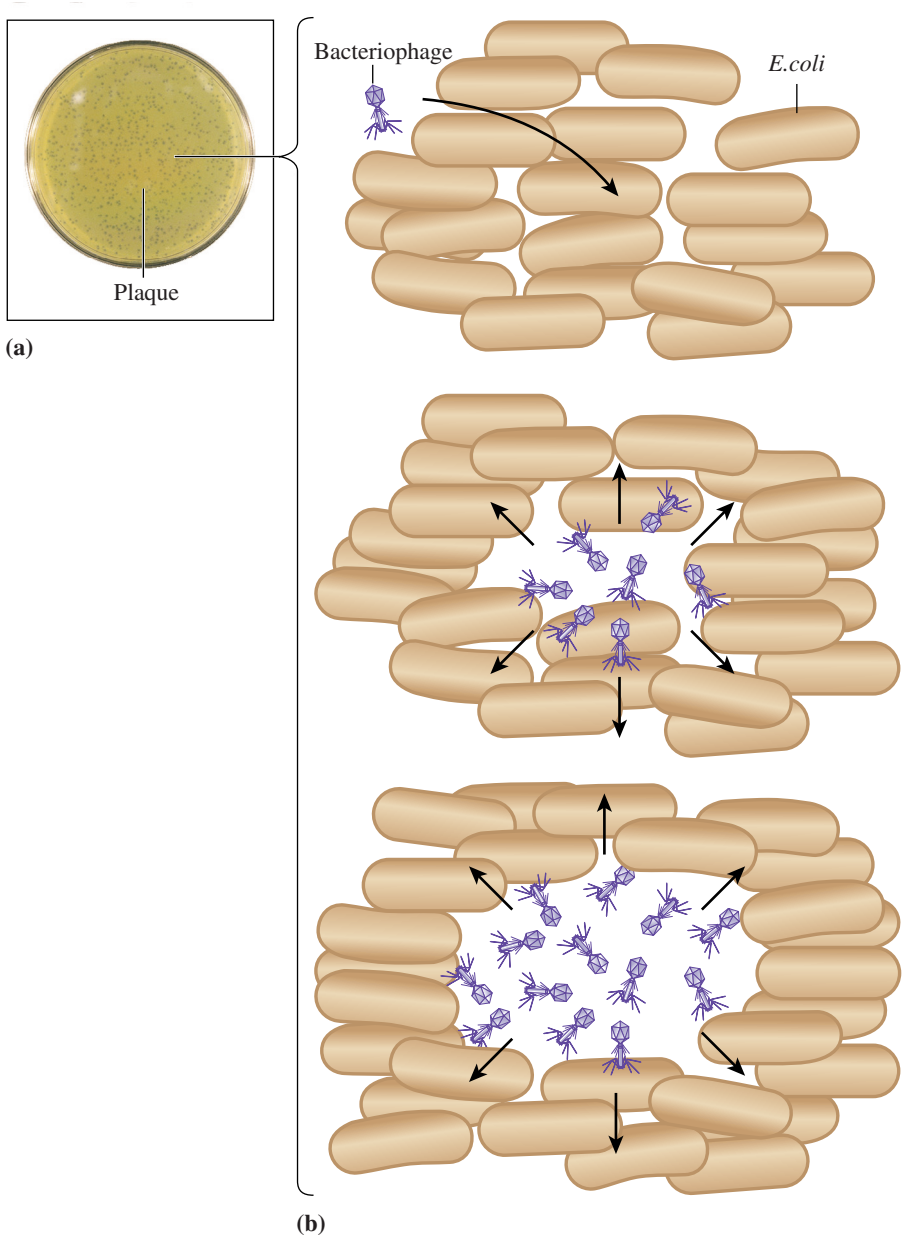



**Figure 37.3** Phage plaques. (a) A lawn of *E. coli* B containing plaques. (b) Each clearing or plaque contains $10^6$ to $10^7$ bacteriophages and bacterial debris, but represents a single phage that infected one cell at the approximate center of that site.

## Materials

Cultures
- Overnight culture of *E. coli* B
- 1 (125 ml) Erlenmeyer flask containing 40 ml of raw sewage

Media
- Nutrient broth (1 g Peptone, 0.5 g yeast extract, 0.25 g NaCl, 0.8 g potassium phosphate, dibasic in 100 ml distilled water)
- 10× strength nutrient broth (Peptone 20 g, yeast extract 10 g, NaCl 5 g, potassium phosphate-dibasic 16 g, in 200 ml distilled water)
- Warmed nutrient agar plates (6, 100 × 15 mm plates) (12–15 g agar/liter nutrient medium)
- Tubes containing 3 ml each of warm, top agarose (one per plate) (7.5 g agarose/liter nutrient broth, molten, cooled to 45°C)

Reagents
- Phosphate-buffered saline (PBS) (sodium chloride 1.6 g, potassium chloride 0.04 g, sodium phosphate-dibasic 0.22 g, potassium phosphate-monobasic 0.04 g in 100 ml)

Equipment
- 37°C incubator with shaker platform
- Water bath at 37°C
- Water bath at 45°C

Miscellaneous supplies
- 5 ml pipettes/pipettor
- 15 ml conical centrifuge tube
- Tube for collection and storage of phage filtrate
- Sterile 0.45 µm syringe tip filter
- 10 ml syringe without needle
- 1.5 ml microfuge tubes for preparing dilutions
- 1.0 ml serological pipettes/pipettor or micropipettor/tips (100–1,000 µl)
- Laboratory marker

## Procedure

Prior to today's lab, raw sewage was collected from a local sewage treatment plant. Yesterday, 50 ml of 1× nutrient broth was inoculated with *E. coli* B for overnight growth at 37°C with shaking.

### First Session: Amplification of Bacterial Viruses

1. Pipette 5 ml of 10× nutrient broth into the flask containing 40 ml of raw sewage.
2. Inoculate the sewage in the flask with 5 ml of an overnight culture of *E. coli* B.
3. Inoculate a separate flask containing 45 ml of 1× nutrient broth with 5 ml of an overnight culture of *E. coli* B (one per class).
4. Incubate both cultures at 37°C, shaking for 24 hours.

## Second Session: Bacteriophage Isolation and Plating:

Prior to today's lab, 2 ml of 1× nutrient broth was inoculated with *E. coli* B for overnight growth at 37°C with shaking. Earlier today, 100 ml of 1× nutrient broth was inoculated with a small volume of the overnight. This was done to obtain a culture in log growth by class time. *Note:* The instructor may choose to inoculate today's culture with the day-old "overnight" stored in the refrigerator.

1. Transfer 10 ml of the sewage-bacteria-bacteriophage culture into a centrifuge tube, and centrifuge the sample at 2,000 RPM for 5 minutes. Most of the remaining cells will be pelleted. The supernatant contains bacteriophage.
2. Prepare a 10 ml storage tube for the collection of bacteriophage supernatant as it is filtered. Then pipette the supernatant into a 10 ml syringe barrel fitted with a 0.45 micron filter. Gently slide the plunger, allowing the flow-through to drip into the storage tube. This step removes any remaining bacteria from the phage sample. The storage tube contains bacteriophage. It can be stored at 4°C and is stable for several months.
3. Prepare a series of microfuge tubes for making serial 10-fold dilutions of the bacteriophage suspension (performing the same dilution repeatedly in series is called serial dilution; see figure 37.4). Label six tubes 1–6. Into each tube, pipette 0.9 ml of sterile PBS.
4. ***Perform serial dilutions:*** Transfer 0.1 ml of phage suspension (that has been mixed well) into tube 1, and mix. Using the same pipette, transfer 0.1 ml of the sample from tube 1 into tube 2, and mix. Repeat this process, transferring 0.1 ml from tube 2 to tube 3, and so on, mixing each time, as shown in figure 37.4. Store the remaining phage suspension in the refrigerator.
5. Distribute 0.5 ml of log-phase *E. coli* into each of six microfuge tubes, labeled 1–6.

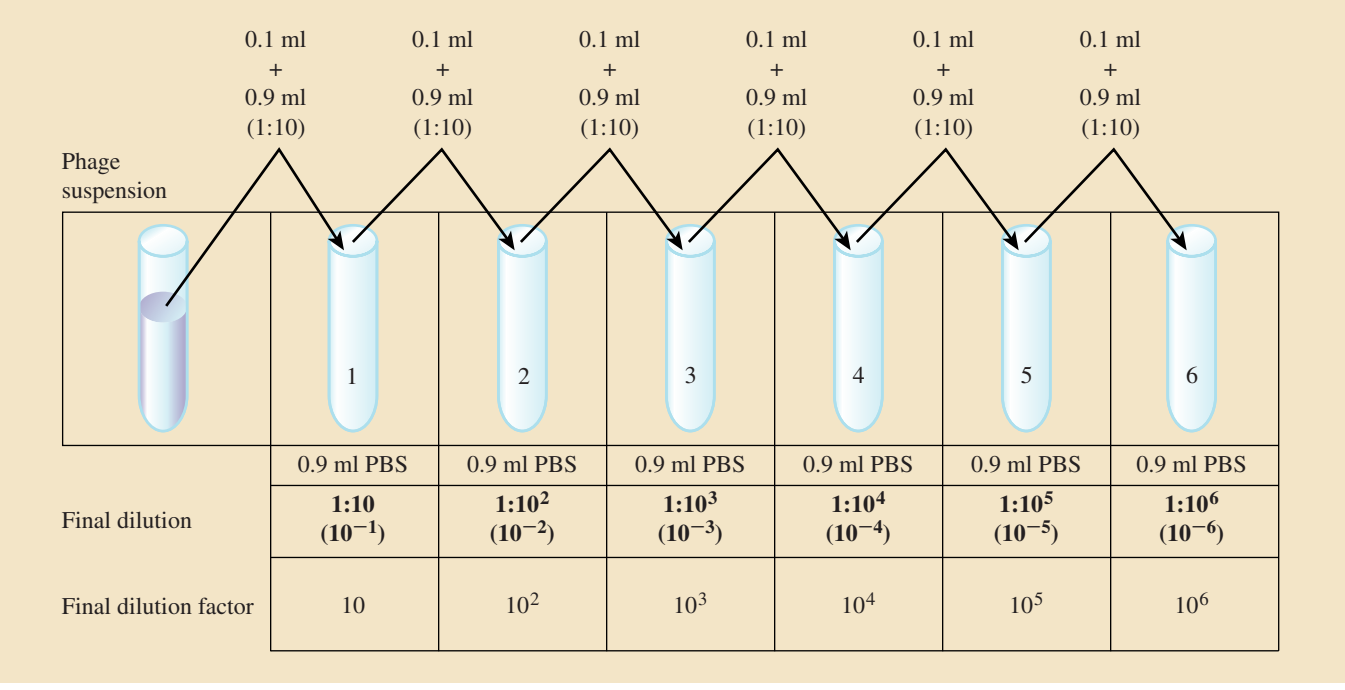


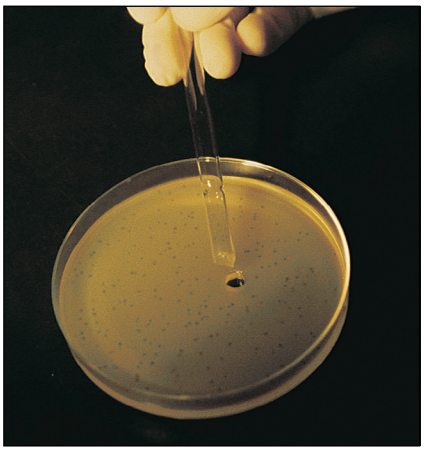

**Figure 37.4** Serial dilutions of bacteriophage suspension. First, pipette 0.9 ml of PBS (diluent) into each dilution tube (numbered 1–6. Then transfer 0.1 ml of phage suspension in series, mixing each time.

6. To each tube of bacteria, add 0.1 ml of the corresponding phage dilution (0.1 ml of dilution 6 to cell tube 6, and so forth). *Note:* If you work from the most dilute to the least dilute, you can use the same pipette. Cap the tubes, and mix gently by inverting them.
7. Incubate at 37°C for 10 minutes to allow the phage to adsorb (attach) to the bacteria. This is your cell-phage mix.
8. In the meantime, label six warm, dry, nutrient agar plates 1–6 (one for each infection). Write on the bottom plate along the plate edge. Keep the plates in the 37°C incubator until you are ready to use them.
9. When you are ready to plate cell-phage mixes, collect your warmed, labeled plates from the incubator. Add the contents of cell-phage tube 1 to a vial containing 3 ml of top agarose (molten, at 45°C). Quickly cap the tube, and mix it by gently inverting it three times. Quickly pour the mixture onto warmed plate 1 (figure 37.5). You can tip the plate slightly to spread the top agarose. Push the plate aside, but do not pick it up until the agarose solidifies.
10. Repeat step 9 for each of the remaining five samples, 2–6.
11. Allow the plates to cool without being disturbed for approximately 10 minutes. When the top agarose has solidified, incubate the plates, inverted, at 37°C for 24 hours.

**Figure 37.5** Plating phage. Once you have gently mixed the cell-phage–top agarose suspension by inverting it a few times, quickly pour the mixture onto a warmed agar plate.

## Third Session: Examination of Bacteriophage Plates, Phage Storage

1. Record the number of plaques on each plate in your laboratory report.
2. Using one of the least-crowded plates, pick an isolated plaque for long-term storage: Pipette 1 ml of PBS into a microfuge tube, and add 1 drop of chloroform. Then, using either the large or small end of a Pasteur pipette (depending on the size of the plaque and the space around it), pierce the agar surrounding the plaque, and pick out the agar "plug" containing the plaque (figure 37.6). Place the "plug," agar and all, into the 1 ml of PBS. The phage will diffuse into the PBS over time, and the chloroform will kill any remaining bacteria. Store the plaque in the refrigerator (4–10°C).



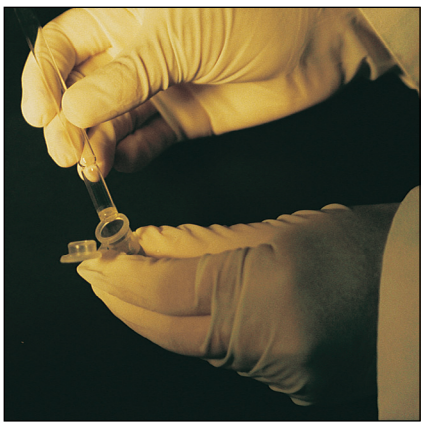


**Figure 37.6** Picking a phage plaque for storage. Pierce the agar surrounding the plaque, and pick out the agar "plug" containing the plaque. Transfer the plug into a microfuge tube containing 1 ml of PBS and a drop of chloroform.

EXERCISE

# LABORATORY REPORT 37

NAME _____ DATE _____

LAB SECTION _____

## Isolation of Bacteriophage from Sewage and Determination of Phage Titer

1. Count the plaques on each plate. *Note:* If the plate is very crowded, it may be easier to count if you divide the plate in quarters or eighths and then multiply the count by 4 or 8, respectively. Then complete the following table.

| Plate no. | Plaques per plate | Dilution factor | Volume of phage plated (ml) | Titer calculation $\frac{\text{(number of plaques) (DF)}}{\text{volume plated (ml)}}$ | Titer: plaque-forming units (PFU) per ml |
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2. Do the results in the far right-hand column agree? Should they agree? What is the average titer of the amplified, filtered phage suspension?

3. Approximately how many bacteriophages are in the phage filtrate you collected?

4. A protocol calls for $10^9$ phage particles as starting material. How much of your phage suspension would you need to have $10^9$ phages?

5. Why is bacteriophage titer expressed as PFU/ml and not bacteriophages/ml?

6. Take a look at one of the phage plates, and comment on the plaques you see with respect to their appearance and dimensions. Do they all look alike? If two plaques differ in size or shape, what might that indicate about the bacteriophages in the two plaques?

7. What is the purpose of the amplification step?

8. You used a 0.45 μm filter to separate bacteriophages from any whole bacteria that remained after centrifugation. Why was this a proper choice of filter pore size? How big is an *E. coli* cell? How big is a typical bacteriophage? What else might be present in the bacteriophage filtrate?

9. You picked a single plaque from a phage plate for long-term storage. It is expected that all of the phages in the storage tube are identical. Why?

10. Describe and diagram how a bacteriophage plaque arises on a bacterial lawn.

11. Bacteriophage $\lambda$ is a temperate phage. When $\lambda$ is plated with susceptible *E. coli*, the plaques are visible but they are cloudy, not clear. Why are the plaques cloudy?