Plaque Assay: A Method to Determine Viral Titer as Plaque Forming Units (PFU)

Overview

Viruses that infect procaryotic organisms, called bacteriophages or simply phages, were identified in the early 20th century by Twort (1) and d'Hérelle (2) independently. Phages have since been widely recognized for their therapeutic value (3) and their influence on human (4), as well as global, ecosystems (5). Current concerns have fueled a renewed interest in the use of phages as an alternative to modern antibiotics in treatment of infectious disease (6). Essentially all phage research relies on the ability to purify and quantify viruses, also known as a viral titer. Initially described in 1952, this was the purpose of the plaque assay (7). Decades and multiple technological advancements later, the plaque assay remains one of the most reliable methods for determination of viral titer (8).

Bacteriophages subsist by injecting their genetic material into host cells, hijacking the machineries for production of new phage particles, and eventually causing the host to release numerous progeny virions through cell lysis. Because of their minute size, bacteriophages cannot be observed using solely light microscopy; therefore, scanning electron microscopy is required (Figure 1). Additionally, phages cannot be cultivated on nutritional agar plates like bacteria, as they need host cells to prey on.

Figure 1: The morphology of a bacteriophage, here exemplified by an E. coli phage, can be studied using scanning electron microscopy. Most bacteriophages belong to Caudovirales (tailed bacteriophages). This particular phage has a very short tail structure and an icosahedral head, placing it in the family of Podoviruses.

The plaque assay (Figure 2) is based on incorporation of host cells, preferentially in log-phase growth, into the medium. This creates a dense, turbid layer of bacteria able to sustain viral growth. An isolated phage can subsequently infect, replicate within, and lyse one cell. With each lysed cell, multiple adjacent ones become immediately infected. Several cycles in, a clear zone (a plaque) can be observed in the otherwise turbid plate (Figure 2B/Figure 3A), indicating the presence of what was initially a single bacteriophage particle. The number of plaque forming units per volume (i.e. PFU/mL) of a sample, can thus be determined from the number of plaques generated.
Figure 2: Testing for plaque forming units (PFU) is a common method for determining the number of bacteriophages in a sample. (A) The base of a sterile Petri dish is covered with an appropriate solid nutrient medium, followed by a mixture of soft media, susceptible host cells and a dilution of the original bacteriophage sample. Note that the phage-suspension could, in some cases, also be evenly spread-out across the surface of already solidified soft agar. (B) Growth of the host bacteria forms a lawn of cells in the top agar layer. Bacteriophage replication generates clear zones, or plaques, caused by host cell lysis.

Figure 3: Results from PFU-testing show multiple plaques generated by bacteriophages. Due to lysis of susceptible host cells, plaques can be seen as clearing zones in the bacterial lawn, either with (A) full clearance, or (B) partial re-growth caused by generation of resistant bacteria (or possibly by temperate phages in the lysogenic cycle).

Certain temperate phages can adopt what is referred to as a lysogenic lifecycle, in addition to the formerly described lytic growth. In lysogeny, the virus assumes a latent state through incorporation of its genetic material into the genome of the host cell (9), often conferring resistance to further phage infections. This is sometimes revealed through a slight clouding of the plaque (Figure 3B). It is worth noting however, that plaques can also appear blurred due to re-growth of bacteria that have evolved resistance to the phage independent of previous phage infections.

Viruses can attach, or adsorb, to only a limited range of host bacteria (10). Host ranges are further limited by intracellular anti-viral strategies such as the CRISPR-Cas system (11). The resistance/sensitivity towards specific phages displayed by bacterial subgroups has historically been used to categorize bacterial strains into different phage types (Figure 4). Although the effectiveness of this method has now been surpassed by novel sequencing techniques, phage typing can still provide valuable information about bacteria-phage interactions, for instance, facilitating the design of a phage cocktail for clinical usage.
Figure 4: Phage sensitivity of different bacterial strains. Soft agar plates with *Cutibacterium acnes* strain (A) AD27 and (B) AD35, were spotted with 21 different *C. acnes* bacteriophages. Only phage 11 were able to infect and kill AD27 while strain AD35 showed sensitivity towards all phages. This technique, termed phage typing, can be used to divide bacterial species and strains into different subgroups based on phage susceptibility.

**Procedure**

1. **Set-up**
   1. Before commencing any work involving microbes, make sure that the work space is sterilized (e.g. wiped with 70% ethanol). Always wear a lab coat and gloves, keep long hair tied back, and ensure that any wounds are particularly well protected.
   2. When finished, sterilize all surfaces and thoroughly wash/sterilize hands and wrists.

2. **Protocol**
   1. **LB Media preparation**
      **Note:** Depending on the host bacterial strain and the bacteriophage, a different liquid medium may be more suitable for the initial culturing of the host bacterial strain or a different solid medium may be more suitable for subsequent growth. Lysogeny broth (LB) is used in this protocol for the broth and the agar.
      1. Mix 4 g LB in 200 mL distilled water, in triplicate, for the LB broth, the solid bottom agar, and the soft top agar. All solutions should be prepared in containers able to hold twice the final volume to prevent overflow while autoclaving.
      **Note:** If performing the assay in triplicate, prepare double the amount of LB bottom agar.
      2. Adjust the pH of all three solutions to 7.4 using NaOH or HCl as appropriate.
      3. Add 3 g of agar power to the bottom agar bottle to make a 1.5% agar solution for the solid agar.
      4. Add 1.2 g of agar power to the top agar bottle to make a 0.6% agar solution for soft agar.
      5. Place the bottles, with semi-tightened caps, in an autoclave set to 121°C for 20 min to sterilize the solutions. Close the caps as soon the run is finished to prevent contamination.
      6. When the LB media has reached a temperature of approximately 45-50°C, add 450 μL of sterile 1 M CaCl$_2$ to all three of the 200 mL LB solutions to make a final concentration of 2.25 mM.
      7. Pour 15 mL aliquots of the solid agar medium into sterile Petri dishes (avoid shaking to prevent foaming) and allow the agar to solidify for a few hours or overnight at room temperature (Figure 4A). Plates can be stored upside down at 4°C for several days.
   2. **Culturing of host cells**
      1. One day prior to the assay, add 10 μL of *E. coli* culture to 10 mL of LB broth.
      2. Incubate the bacteria at 37°C overnight at 160 rpm in a shaking incubator.
      3. The morning of the assay, add 0.5 mL of the overnight culture to 10 mL of fresh LB.
      **Note:** If you are performing the assay in triplicate, prepare double the amount of this culture.
      4. Incubate the at 37°C at 160 rpm in a shaking incubator until the bacterial culture is in log-phase growth. This can be determined spectrophotometrically by an OD600 of 0.5-0.7.
      5. Keep the culture at room temperature until the bacteria are to be added to the top LB agar.
   3. **10-fold Serial dilution of bacteriophage**
      1. Add 180μL of LB broth to seven wells in the first row of a to a 96-well plate.
      **Note:** It is suggested to perform the dilution in triplicate in order to increase its statistical reliability. To do this, prepare additional dilutions of the bacteriophage in the second and third rows of the plate.
      2. Carefully vortex the original bacteriophage sample to ensure homogeneity and transfer 20 μL into the first well.
      3. Mix the sample well by pipetting and down.
      4. Transfer 20 μL of the resulting suspension into the second well.
      5. Continue the serial dilution by transferring 20 μL of the solution in the second well into the third well, and so on, until sixth well, leaving the seventh well as a negative control to which no phage-suspension will be added. This will create a dilution range of $10^{-1}$-$10^{-6}$.
   4. **Plating**
1. Label the base of the Petri dishes (previously prepared in step 2.1.7) with name, date and a short sample description (including the phage sample dilution factor). Pre-heat the Petri dishes in 37°C incubator one hour before the assay.

2. Melt the solidified soft agar-medium (typically done using a microwave; solidified agar melts at 85°C), and let it come to around 45°C. The heated media can be placed in a 45°C water bath for ~1h to reach an appropriate temperature. It should be hot enough to remain in liquid form, but cool enough to not kill added bacteria.

3. Mix 4 mL bacterial culture (from step 2.2) with 35 mL LB soft agar (at 45°C). Swirl to evenly distribute the cells but avoid shaking to prevent foaming (Figure 4A).

4. Label one sterile test tube for each of the serial dilution steps and one for the control sample, for a total of 7 labeled test tubes. Place 5 mL aliquots of the bacterial culture/soft agar mixture from step 2.4.3 into the 7 tubes. Work quickly through step 2.4.6 because such small volumes of agar-based media will quickly solidify at room temperature.

5. Add 100 μL of the control sample (from step 2.3) to the control test-tube and swirl carefully. Discard the used pipette tip and transfer the same volume from each of the serially diluted bacteriophage samples (step 2.3) to their respective test tubes, swirling to mix.

6. Immediately transfer the 5 mL mixtures onto the labelled, pre-heated, solid agar plates (Figure 4A). Gently swirl the plates to even spread the mixtures.

Note: If you are performing the assay in triplicate, repeat steps 2.4.3-6 two more times.

7. Seal each plate with laboratory film, and allow both layers to solidify properly at room temperature (approximately 15 minutes) before placing them up-side-down a 37°C incubator, stimulating growth of both the bacteria and the phage, for 24 hours or until plaques develop. It typically takes about 1-5 days for plaques to appear (Figure 4B), but the timing varies considerably depending on incubation conditions, medium, and the bacterial species.

### 3. Data Analysis and Results

1. **Counting plaques**

   1. Ensure that no plaques are visible in the plates marked “control”, as this would indicate viral contamination.

   2. Begin with the plates labelled 10⁻¹, containing the most diluted bacteriophage sample. Count the plaques without removing the lid, marking them with a pen as you go to indicate which plaques have already been counted.

   3. Count the remaining plates. Some plates may have too few or too many plaques to count. Use the plates with 10-150 plaques for further analysis.

2. **Calculating PFU**

   1. Divide the number of plaques by the dilution factor, (ex. 10⁻⁶ for the most diluted sample) to obtain the number of Plaque Forming Units (PFU) in 100 μL of phage mixture. 

   Note: If performing the assay in triplicate, use the average number of plaques from the three plates.

   2. To determine the concentration (in PFU/mL) of the original sample, multiply by an additional dilution factor of 10, since only 100 μL of sample was plated. (ie \( \frac{1000 \mu L}{100 \mu L} \times 10 = 100 \))

   3. Calculate the mean value of the PFU/mL for all dilutions that had between 10 and 150 plaques to attain a more reliable result.

### Applications and Summary

Despite multiple technological advances, plaque assays remain the gold standard for determination of viral titer (as PFU) and essential for isolation of pure bacteriophage populations. Susceptible host cells are cultivated in the top coat of a two layered agar-plate, forming a homogenous bed enabling viral replication. The initial event where an isolated bacteriophage in lytic lifecycle infects a cell, replicates within it, for isolation of pure bacteriophage populations. Susceptible host cells are cultivated in the top coat of a two layered agar-plate, forming a homogenous bed enabling viral replication. The initial event where an isolated bacteriophage in lytic lifecycle infects a cell, replicates within it, preventing foaming (Figure 4A). Gently swirl the plates to even spread the mixtures.

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### References