

# Phage Overlay Assay for Quantification of Infectious Bacteriophage Particles

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The historical applications of plaque assays included isolation of virus and enumeration of viral particles in a solution. Plaque assays quantify viral particles by observing plaque forming units which are formed due to lytic viruses that lyse cells, and cells adjacent to them in a lytic life cycle within a confluent lawn of bacteria. We describe a method to prepare double agar overlay plaque assays, in an effort to quantify the viral particles in a solution.

## INTRODUCTION

The use of the plaque assay can be traced back to Felix d’Herelle in the early 1900s, who was credited with the “discovery” of bacteriophages (1). He used the plaque assay to observe the presence of these, then mysterious, particles that were able to pass through a filter, and caused clear spots on lawns of bacteria that were grown on agar in laboratory. He used this assay to isolate and study these particles further and to support his hypothesis for the existence of such an entity that was discredited by many at the time. However, the double overlay agar which in essence is the method used here, was originally devised by Andre Gratia who was the first to use the method for the quantification of phage particles (2). Beyond quantification and isolation, these assays can also be used to characterize plaque morphology and isolation of phage mutants.

One of the main uses of phage overlay assays is for the enumeration of the viral titer in a solution of virus (3, 4). The theory behind this method is that when a lytic virus attaches to the surface of a bacterium, injects its genome, replicates, processes its components to progeny viruses, it then lyses and releases these progeny viruses which causes the death of the host cell (5). These released viruses are then able to infect other cells and repeat the steps of replication and lysis. In the double overlay agar method, bacteria are mixed along with a dilution of phage in molten, low concentration agar which is then spread out over more solid, concentrated agar on a plate (3). The agar provides bacteria with nutrients and a growth medium which is fluid enough to allow them to

form a confluent lawn within. However, cells that become infected with the lytic virus before the solidification of agar allow replication of the virus within the cell. This virus then under goes its life cycle to produce progeny viruses that infect adjacent cells in the agar and continue to do this until there is a visible clearing that forms, called a plaque (5). In theory, each plaque is produced from one original viral particle that is able to infect one cell, which then contributes to the infection of adjacent cells to form what is known as a plaque forming unit (PFU) (4, 5). By counting the PFUs, one can determine the amount of infectious viral particles in the original dilution.

**Key words:** double, overlay, plaque, assay, protocol, method, bacteriophage, quantification

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## MATERIALS AND EQUIPMENT

### Materials:

- Isolated Pure Desired Phage Line
- Desired Cell Line
- M9 Media (or another desired media)
- Select™ Agar
- PBA and PTA

### Equipment:

- Bunsen Burner
- Biosafety Cabinet
- Autoclave
- Spectrophotometer (NanoDrop™ 2000)
- 60 °C Incubator
- 37 °C Incubator
- 4 °C Fridge
- Water Bath or Heating Block (Water bath preferably)
- Test-tubes (12 x 75 or larger)
- Eppendorf tubes (1.5 mL or 2 mL)
- Striker or BBQ lighter
- Pipetman™ P1000, P100, and P20

## PROTOCOL

All the steps below should be performed in sterile conditions using a Bunsen burner. Exercise precaution, as the phage can aerosolize and infect other cells easily and thus the tubes containing phage should be handled carefully. It is highly recommended that phage work is done in the Biosafety Cabinet (B2) to prevent the phage from aerosolizing and contaminating other laboratory equipment.

**A. Phage Bottom Agar (PBA) Plates:** PBA is used to make sterile plates on which Phage Top Agar (containing the cells and phage) is overlaid to observed plaque formation.

- ➔ Mix ingredients for the media of choice, add 1.5% Select™ agar and autoclave using the liquid cycle that is appropriate for the volume used. Let the agar cool to about 60°C (warm to the touch) and pour into sterile petri plates. Pour just enough volume to cover the bottom of the plate. Let it cool on the bench top until the agar solidifies and store the inverted plates at 4 °C.

**B. Preparation of cells for overlay assay:** The cells need to be in the right phase of growth for a sufficient bacterial lawn formation. This is required for the formation of concise round plaques.

- ➔ Set-up a 5 mL overnight culture of your cells in the media of interest. On the day of performing the phage overlay assay, seed the cells in a 1:100 dilution in the required volume of media. Grow the cells on a shaker at 37 °C at 200 rpm until the OD<sub>600</sub> is around 0.5. The OD<sub>600</sub> can be measured in a 1 mL cuvette on the nanodrop™ 2000.

**C. Preparation of Phage Top Agar (PTA):** PTA is used to plate the mixture of cells and phage to observe the plaques. It is usually of a lower agar concentration when compared to the PBA. This allows for the lateral diffusion of the growing cells inside the low concentration agar.

- ➔ Mix ingredients for the media of choice, add 0.75% Select™ agar and autoclave using the liquid cycle appropriate for the volume used. When the autoclave run is over, transfer the bottle containing PTA to the 60 °C incubator if it is not to be used right away. Approximately 30 minutes before the pouring of the plates using PTA, transfer the bottle from 60 °C

incubator to the 47 °C water bath. Aliquot 3 mL of PTA into sterile pre-warmed (47 °C) test tubes with caps and keep it in the 47 °C water bath until needed. This is to ensure that the PTA does not solidify prior to mixing in cells and phage.

**D. Phage dilution and infection:** Use multiple dilutions of the phage isolate to find a desired dilution at which between 20 and 200 plaques are observed. This can also be used to determine the viral titer of the phage solution, in plaque forming units (PFUs).

- Mix 1 ml of cells at OD<sub>600</sub> of 0.5 with 100 µL of the desired dilution of the phage. Incubate the cells for 8 to 10 minutes. Make sure the incubation step does not exceed 10 minutes as most phages have their first lytic cycle between 10 to 12 minutes post infection (6). Centrifuge the cells at 5000 × g for 2 minutes and discard the supernatant to remove phage un-adhered to the cells. Centrifugation and discarding of the supernatant is not necessary, but it is useful if the study is based on adherence or infectivity. If the cells were centrifuged and the supernatant was discarded, suspend the cells in 1 mL of appropriate media.

**E. PTA overlay with cells and phage:** Time is crucial in this step and phage infection and incubation times need to be staggered accordingly.

- When the cells are suspended in in 1 mL of media (Protocol Step D), immediately transfer the suspension of cells with phage to the test tube containing 3mL of liquid PTA at 47 °C. Replace the cap, mix by vortexing (press down on the vortex head once till you see the vortex inside the tube), remove cap, pour on to a labelled PBA plate and swirl until the top agar has evenly covered the top surface of the PBA. A platform shaker can be used to aid in the swirling process. Once the PTA has solidified, set the plate aside to cool for 10 minutes. Invert the plates and incubate at 37 °C for 6 to 12 hours. The top agar is less dense than the bottom agar and can slide off the plate when inverted; inverting quickly can prevent this from happening. Count the PFUs post incubation to determine the number of infectious virus particles present in the PTA. This data can be used to calculate the number of Plaque forming units (PFU) in the original phage preparation.

## ANTICIPATED RESULTS AND CONTROLS

1. PFUs should be clear and round patches within a confluent lawn of cells, which would happen if the cell lines are permissible to infection by the phage are used for the study.
2. Some cells might require a different concentration of agar in PTA, depending on their growth and diffusion rates to form confluent lawn of cells.
3. Round and concise plaques should be expected by 6 to 12 hours after plating the PTA. The times of incubation may vary based on the cells, the phage and the density of the plated cells.
4. A Control plate containing cells with no phage should contain a confluent lawn of cells.
5. A Control plate containing phage with no cells should show no growth.
6. A Control plate with just PTA without added cells nor phage should show no growth.

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## TROUBLESHOOTING

Observation/ Issue	Possible Explanation	Solution
<ul style="list-style-type: none"> <li>The control plate with only cells show confluent lawn, but my phage dilutions do not.</li> </ul>	<ul style="list-style-type: none"> <li>The amount of phage used in the assay is too high, thus all the cells have been lysed.</li> <li>The incubation time has exceeded the time for one lytic cycle, thus all the cells were infected during the incubation time.</li> </ul>	<ul style="list-style-type: none"> <li>Use a lower dilution of phage for the assay.</li> <li>Create a serial dilution of the phage stock and titer the contents thus you can choose how much phage to add.</li> </ul>
<ul style="list-style-type: none"> <li>Some plaques are bigger than others.</li> </ul>	<ul style="list-style-type: none"> <li>There might have been a lytic progeny of phage when the PTA was poured and was being swirled. Thus, that area where the lysis happened presents a bigger plaque than the smaller cycle.</li> </ul>	<ul style="list-style-type: none"> <li>Reduce the time of incubation for the phage to adhere to the cells.</li> <li>The assay can also be done without the incubation time by just adding the phage to the cells and plating instantly. This process does not remove any un-adhered phage.</li> </ul>
<ul style="list-style-type: none"> <li>None of my plates show a confluent lawn.</li> </ul>	<ul style="list-style-type: none"> <li>The cells might be in the stationary phase or death phase when used in the assay. This usually happened when the OD<sub>600</sub> of cells is above 0.8.</li> <li>The PTA was not allowed to cool to 47°C prior to adding the cell and phage solution and all the cells have died due to high temperatures.</li> </ul>	<ul style="list-style-type: none"> <li>Use a lower OD<sub>600</sub> of the cells ensuring they are in the growth phase so they continue to grow in the PTA.</li> <li>Turn the temperature of the water bath to 47°C. Before mixing PTA with the cell and phage solution, use one tube with 3mL of PTA to check the temperature of the agar solution using a thermometer. Discard tube used to check temperature. This can be an issue as the PTA takes a long time to cool to 47°C after being removed from the autoclave.</li> </ul>
<ul style="list-style-type: none"> <li>My plaques show circular patterns or irregular shapes.</li> </ul>	<ul style="list-style-type: none"> <li>The circular patterns may be due to the PTA solidifying too soon when they PTA is swirled to cover the surface of the PBA. This can produce plaques in the patterns where the agar solidified first.</li> <li>The irregular shapes of the plaques might also be due to overlaying plaques. This happens when the amount of phage added is high.</li> </ul>	<ul style="list-style-type: none"> <li>Make sure the temperature of the agar does not fall below 45°C as this will not provide enough time for the plating process. The agar will solidify too fast.</li> <li>This can be reduced by decreasing the amount of phage added to the assay.</li> </ul>
<ul style="list-style-type: none"> <li>I see spots on colony growth embedded on the agar</li> </ul>	<ul style="list-style-type: none"> <li>The cells couldn't diffuse in the concentration of agar in the PTA.</li> </ul>	<ul style="list-style-type: none"> <li>Use a lower agar concentration for the PTA and adjust temperature if necessary.</li> </ul>