Plaque Assay Protocols

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Information

History

Since the discovery of bacteriophage (phage, for short) early in the twentieth century, there have been two principal methods of growing virulent phage, both based on the ability of phage to kill the host bacterial cell. One method introduces phage into a fluid bacterial culture medium. After a period of incubation, the phage lyse the bacteria in the broth culture resulting in a clearing of the fluid medium. The other method, known as the plaque assay, introduces phage into a few milliliters of soft agar along with some bacterial host cells. This soft agar mixture is laid over a hard agar base (seeded-agar overlay). After a period of incubation, the phage lyse the bacterial cells in their vicinity resulting in zones of clearing on the plate known as plaques. Each plaque represents a single phage particle in the original sample. The former method is typically used for the growth of significant amounts of phage, while the latter method is typically used for the isolation and enumeration of phage particles. It is the latter method that will be described in this protocol.

F.W. Twort, an English physician, and Félix d'Herelle, a French-Canadian microbiologist, are both credited with the discovery of bacteriophage, Twort in 1915 and d'Herelle in 1917 (Duckworth, 1976). However, it is d'Herelle that first described the plaque assay method of isolating bacteriophage (d'Herelle, 1917). This technique was modified during the 1920's and 1930's as bacteriologists worked to grow bacteriophage, while wrestling with the question of whether bacteriophage were living entities or simply proteins that could self-multiply (Dreyer and Campbell-Renton, 1933; Ellis and Delbrück, 1939). (In this pre-Watson and Crick era, the prevailing hypothesis was that protein was the molecule of inheritance.) The protocol described here is the soft-agar overlay method as described by Gratia (1936), and adapted by Hershey, et al. (1943). This procedure is the one most frequently used today, and the one that is taught in most microbiology teaching laboratories.

Purpose

The purpose of the plaque assay is to grow isolated plaques of phage particles within a lawn of bacteria. Students can visualize the clearing of bacterial growth on the agar media, demonstrating the effect of phage on bacteria. This method also requires that students perform serial dilutions, and allows them the opportunity to apply a concept that most find

difficult to grasp.

Theory

Aliquots of diluted bacteriophage are mixed with host bacterial cells in several milliliters of soft agar, which are then spread onto agar plates containing media. The use of soft agar allows the phage to easily diffuse through the medium giving more consistent plaque formation. It also eliminates the problem of uneven absorption of the bacterial-phage solution into the hard agar that often caused uneven plaque formation on the plate (Ellis and Delbrück, 1939).

The bacteriophage adsorb onto the host bacterial cells, infect and lyse the cells, and then begin the process anew with other bacterial cells in the vicinity. After 6 – 24 hours, zones of clearing, plaques, are observable within the lawn of bacterial growth on the plate. Plaque characteristics are related to the type of bacteriophage as well as other physical and chemical characteristics of the system in which the bacteriophage are grown (Jones and Krueger, 1951).

RECIPE

Phage stock solution

Bacterial broth culture that is actively growing (ideally, early log phase, about 10^8 cells/ml, which, for a number of culture types, is indicated by light turbidity of the broth). It is essential to choose bacteria that are sensitive to (recognized by) the phage. A commonly used system is *E. coli* B with any of the T-even phages.

3 ml of 0.7% Soft (melted) agar with nutrients, in 50°C water bath to maintain molten state (LB is most commonly used, but trypticase-soy and nutrient media have been used successfully. Selection should be based on the media that is most effective for growth of the bacteria.)

1.5% Hard agar base plates with nutrients, kept warm in 35° C incubator (typically, 100 mm x 15 mm plates are used, but smaller ones can be used as well)

Sterile 0.85% saline or broth for dilutions

Sterile empty tubes for dilutions

Micropipetters

Sterile transfer pipettes

Sterile microfuge tubes

Chloroform, optional

PROTOCOL

This protocol is optimized for plaque formation with *E. coli* phage T4

A. Dilution of phage stock

Phage stock should be serially diluted to achieve a plaque count on plates of 100-250 pfu (plaque forming units). Generally, if 0.1 ml is plated, this will be a titer of about 10^3 to 10^4 pfu/ml. Dilutions can be done in sterile water, saline, or broth. (Be sure that students mix the dilutions before making the next dilution. This can be done by pipetting in and out of the meniscus.) Students should plate a range of phage dilutions, for example 10^{-3} to 10^{-6} dilutions. (For instructions on serial dilutions, see Serial Dilution Protocols).

B. Plating of phage

1. Removing one soft agar tube at a time from the 50o C water bath, add 0.3 ml of bacteria to the 3.0 ml soft agar, followed by 0.1 ml of diluted phage. *(Some individuals prefer to include a pre-adsorption step prior to adding the bacterial-phage suspension to the agar. Diluted phage are added to 0.3 ml bacterial culture and allowed to adsorb to the bacteria for 15 minutes or so. The bacterial-phage suspension is then added to the soft agar and poured onto base plate. This results in more uniform plaque size and appearance (Pecota and Wood, 1996), but is not necessary, especially in the teaching laboratory.)*
2. Roll agar tube between palms to mix for 2 or 3 seconds, and quickly pour onto agar surface of warm base plate. (Be sure hard agar is dry with no condensation on it. Pre-warming the plates in a 37° incubator will reduce condensation.)
3. Do quick, but gentle figure eight patterns with plate to disperse soft agar over the surface of the base plate agar.
4. Allow soft agar to harden. Incubate inverted at 35° C. Appearance of plaques will depend on the phage, but should be visible in about 6 – 24 hours. If over-incubated, plaques can continue to enlarge. Given sufficient numbers of plaques, this may result in confluent lysis.

C. Observation

Observe and count the number of plaques on those plates that have between 30 and 300 plaques. Students can work through the dilution calculations to determine the number of pfu's/ml in the original sample of phage. * Note: plaques are not necessarily round. Although many are, many others take a variety of shapes and sizes.

D. Phage elution

Using a sterile transfer pipette, insert tip of pipette into center of a discrete plaque and remove a plug of soft agar. Place plug in 1 ml of sterile saline in microfuge tube, and use pipette to break up agar. Let tube to sit for 15 – 30 minutes to allow phage to diffuse out of agar. Do a rapid spin in minifuge to collect agar in bottom of tube. Remove supernatant to fresh, sterile microfuge tube. A couple of drops of chloroform can be added, if desired, but, we have not found it essential, and it must be removed before the phage can be used again. Phage can be stored indefinitely in refrigerator.

SAFETY

The ASM advocates that students must successfully demonstrate the

ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

TIPS AND COMMENTS

An interesting variation on this protocol is to obtain phage from an environmental source, e.g. waste water, a local stream, an insect (flies), etc. Filter sample through 0.45 micron membrane filter. Bacteria will be retained and phage will collect in filtrate. Add several milliliters of phage filtrate to young, actively growing bacterial broth culture. (Different bacteria can be tried, if so desired.) This step increases the titer of the phage. Grow cultures with shaking overnight at appropriate bacterial temperature. Pour some of broth culture into centrifuge tubes and spin down bacterial cells. Phage will be in supernatant and can be titered. The titer is typically low. Students can plate out phage with appropriate bacteria and observe the different types of plaques that form with different bacteria. This, also, gives students an appreciation of the ubiquity and diversity of bacteriophage in the environment.

Note that phage are highly host-specific. If you purchase phage, be sure you are using the correct species and strain of bacteria for the phage you have bought. If you use a different bacterial strain, you are unlikely to see any plaques.

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