Isolation of cyanophages by plaque assays

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Abstract

More than 40 years ago, Safferman and Morris (1963) used the plaque assay method to isolate the first cyanophage that infects a freshwater filamentous cyanobacterium, *Plectonema boryanum*. Since then, this approach has been used successfully to detect and isolate a number of different phages infecting marine *Synechoccocus* and *Prochlorococcus* (e.g., Suttle and Chan 1993; Waterbury and Valois 1993; Wilson et al. 1993; Sullivan et al. 2003).

The advantages of this method are that results are easily interpreted as plaques formed on pigmented lawns can be easily identified. Since a plaque is the result of a single infection event, the virus can be easily purified and cloned. The first part of the procedures described as follows: (1) preparation of base plates and (2) preparation of top agar/agarose are applicable also for bacteriophage plaque assays.

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Guidelines

For example, purified agar or agarose (1% w/v) is added to your media of choice and autoclaved. This will provide a support base for the top agar/agarose overlay as well as nutrients for the host cells. For best results, use plates within 1 week of pouring.

Tip: plates can be fast-tracked: dry plates at 37°C; leave lids slightly ajar; monitor closely to prevent over drying.

Considerations: Depending on the composition of the media used, the addition of solidification agents (in particular the combination of high salinity seawater-based media and common agar such as Bacto Agar) can often result in the formation of precipitates when autoclaved together. These □flocks can sometimes interfere with interpretation of the plaque assay. Moreover, impurities in common agar can negatively affect the growth of the host cells. Here are some suggestions on how to reduce the formation of these precipitates. Some testing may be required to determine the best combination to use for your particular situation.

a. Do not use common agar; rule of thumb□the whiter the agar, the □cleaner□ it is.
b. Use commercially available purified agar or agarose; or clean common agar using a washing procedure such as the one outlined in Waterbury and Willey (1989).
c. Reduce the salinity of seawater media with purified water; e.g., to 20-□25 psu.
d. Add purified agar or agarose to autoclaved media aseptically and then melt the agar/agarose in the microwave (bring to a short boil 2-□3 times to completely dissolve the agar/agarose).
e. For cells that will grow in artificial media, prepare media and gelling agent at 2x□ concentration and

autoclave separately. When cooled to ca. 60°C, gently mix the gelling agent into the media and dispense immediately.
f. In the case of artificial media, add agar/agarose to filter-sterilized media and melt the gelling agent in the microwave

Protocol

Step 1.
Prepare base plates

PROTOCOL

. Base agar plates

CONTACT: Amy Chan

NOTES
Amy Chan 30 Sep 2015
For example, purified agar or agarose (1% w/v) is added to your media of choice and autoclaved. This will provide a support base for the top agar/agarose overlay as well as nutrients for the host cells. For best results, use plates within 1 week of pouring.

Prepare base plates
Step 1.1.
Add 5 g purified agar or agarose to 500 mL culture media in a 1-L Erlenmeyer or media bottle.

Prepare base plates
Step 1.2.
Gently stir to disperse the agar/agarose.

Prepare base plates
Step 1.3.
Autoclave for 20 to 25 min to sterilize.

DURATION
00:25:00

Prepare base plates
Step 1.4.
When cooled to about 60°C, dispense 15 to 20 mL per plate.

NOTES
Amy Chan 02 Feb 2016
the suggested volume is suitable for 15 x 100 mm diameter petri plates; adjust accordingly for smaller or larger plates

Prepare base plates
Step 1.5.
To reduce condensation forming on the insides of the lids, leave lids slightly ajar to allow escape of steam or stack the plates immediately after pouring.

Prepare base plates
Step 1.6.
Invert plates once the agar has solidified to prevent condensation from dripping onto the surface of the agar.

Prepare base plates

Step 1.7.
Plates can be used about 12 h after pouring if the agar surface is not wet; a longer time is needed if conditions are humid.

DURATION
12:00:00

NOTES
Amy Chan 30 Sep 2015
If the surface of the bottom agar is too moist, the top agar/agarose will not stick to the bottom plate and will slide off when the plate is inverted.

Step 2.
Prepare top agar/agarose.

PROTOCOL

. Top agarose

CONTACT: Amy Chan

NOTES
Amy Chan 30 Sep 2015
Prepare 100 mL portions of 0.4 to 0.5% (w/v) of purified agar, agarose or low-melting point (LMP) agarose (i.e., Invitrogen #15517-022) in your media of choice. Although LMP agarose can be quite expensive, it is recommended for temperature sensitive samples and cells, since it solidifies at ca. 25°C. Purified agars, as well as low-melting point agars and agaroses are available for a range of lower temperatures (consult the following websites for more details: www.sigmaaldrich.com and www.invitrogen.com)

Step 2.1.
Autoclave or microwave sterilize on the day of the assay.

Step 2.2.
Dispense 2.5 to 3 mL into 13-×-100–mm glass disposable culture tubes (Fisher Scientific #1496127).

Step 2.3.
Transfer tubes to a water bath or dry heat block set at the appropriate temperature.

NOTES
Amy Chan 02 Sep 2015
(e.g., ca. 30 to 32°C for LMP agarose, ca. 40–42°C for purified agar or agarose)

Step 2.4.
Cover tubes with foil or cap, allow for temperature to equilibrate.

Step 2.5.
For each water sample, prepare triplicate tubes.

NOTES
Amy Chan 02 Sep 2015
Control tubes containing cells are only used to monitor lawn growth.
Amy Chan 02 Sep 2015
For best results (smooth lump-free top agar), use freshly prepared top agar/agarose since repeated re-melting of solidified agar/agarose can give inferior results.

Step 3.
Prepare target indicator cells.

PROTOCOL

. Preparing target indicator cells

CONTACT: Amy Chan

Step 3.1.
Grow the cyanobacteria in liquid media, harvest in exponential growth and adjust cell density to about 10^7 to 10^8 cells/mL.

NOTES

Amy Chan 30 Sep 2015
If necessary, cells can be concentrated by gentle centrifugation and resuspended in media. Preliminary testing may be required to determine the best cell density to use for your particular host organism. The objective is to start with a lawn of cells that will have the capacity for additional growth during the length of the assay. Depending on the growth rate of the target cells, one can expect plaques to appear on the lawn as early as 3 to 4 d to weeks after infection. The initial lawn of cells will be very faint in color. However, the lawn will develop into an evenly distributed dense layer of cells within 7 to 10 d. If the lawn is too thin, plaques will go undetected. If the lawn is too thick, the cells could run out of nutrients prematurely which may result in poorly developed plaques.

Step 4.
Prepare the sample: Environmental samples should be prefiltered as described earlier.

NOTES

Amy Chan 30 Sep 2015
If high titers are expected, serial dilutions of the sample may need to be performed.

Assay

Step 5.
Adsorb 50 to 100 µL sample (as is, and 10-fold serial dilutions, up to ca. 3 levels) to 0.5 mL target cells under the usual culturing conditions (e.g., for *Synechococcus* sp. strain DC2, constant 5–25 µmol quanta $m^{-2}s^{-1}$, at 25°C), agitate occasionally to encourage adsorption of phage to host.

Assay

Step 6.
After 1 h, transfer virus: host mixture to 2.5 mL soft agar.

DURATION
01:00:00

Assay

Step 7.
Quickly and gently vortex the mixture and pour the entire tube contents onto the surface of the agar plate.

Assay

Step 8.
Working rapidly, gently rock and swirl the plate to spread the mixture evenly onto the plate surface before the agar starts to gel.

Assay

Step 9.

Set aside on a flat surface to harden (about 1 h).

DURATION
01:00:00

NOTES
Amy Chan 30 Sep 2015
For best results, the total volume of cells + virus + soft agar is between 3 to 4 mL. Larger volumes would make it easier to pour, but is not recommended as the top layer would be too thick, and plaques could form on top of one another.

Assay

Step 10.
Prepare a control plate containing only cells; this plate will allow you to monitor cell growth.

Assay

Step 11.
Seal plate with parafilm, flip plates upside down.

Assay

Step 12.
Incubation of plates under constant low light conditions (5 to 25 µmol quanta $m^{-2}s^{-1}$) will produce darker lawns thus allowing for easier detection of plaques.

NOTES
Amy Chan 30 Sep 2015
Plaques will appear within 1 to 2 weeks, depending on the growth rate of the host cells.

Assay

Step 13.
Note the number of plaque forming units (PFUs), plaque size, and morphology.

Assay

Step 14.
Choose a well-isolated plaque on a plate that contains less than 100 PFUs.

Assay

Step 15.
Harvest the plaque using a Pasteur pipette: gently press the tip of the pipette into the plaque to the bottom agar; using gentle suction, remove the plug.

Assay

Step 16.
Transfer the plug to 1 mL media and vortex briefly to break it up.

Assay

Step 17.
Place the tube at 4°C and allow the phages particle to elute from the plug overnight to form a plaque lysate.

DURATION
18:00:00

Assay

Step 18.
Vortex and centrifuge the sample (ca. 12,000g for 10 min) to pellet cyanobacteria and agar.

DURATION
00:10:00

Assay

Step 19.

Transfer the supernatant to a new tube; typical titer of the plaque lysate can be 10^4 to 10^5 PFU mL^{-1}.

Assay

Step 20.

Repeat steps 14 to 19 for a minimum of 3 plaques. Choosing plaques with different morphologies may result in the isolation of different phages.