Isolation of cyanophages by liquid bioassays

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Abstract

Multiwell plates are convenient for isolating cyanophages from environmental samples using the liquid bioassay approach (Table 1). This protocol describes a typical procedure (96-well assay) used to detect and isolate cyanophages from marine samples that lyses *Synechococcus* sp. strain DC2 (also known as CCMP1334 or WH7803). Using this method, greater than 10^5 lytic phages per milliliter of seawater have been detected in the Gulf of Mexico (Suttle and Chan 1994) that lyses this permissive target host.

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Guidelines

As mentioned earlier, there are some basic similarities between the isolation procedures for bacteriophages and cyanophages. However, the specific protocols for isolation of cyanophages differ substantially from the procedures presented in the previous section and are described in detail in the following sections. Figure 2 shows possible strategies one could follow, depending on the suspected titer of cyanophages in the sample of interest. These assays assume that the target cells are unialgal and clonal. If cultures are not unialgal or clonal, complete lysis of the culture may not occur or plaques could be obscured by contaminating bacteria. Axenic cultures of the host are preferred for the plaque assay, but not necessary for liquid assays. Many strains of marine cyanobacteria can be purchased from culture collections. Alternatively, new hosts can be isolated from the natural environment of interest. However, it can take a lot of time and effort to produce clonal cultures. As well, many cyanobacteria do not grow well on solid substrate. Unless the target host of interest is already cultured on solid substrate, the simplest method for isolation of novel cyanophages would be via the liquid bioassay. It is simple, inexpensive, and the host need not be axenic.

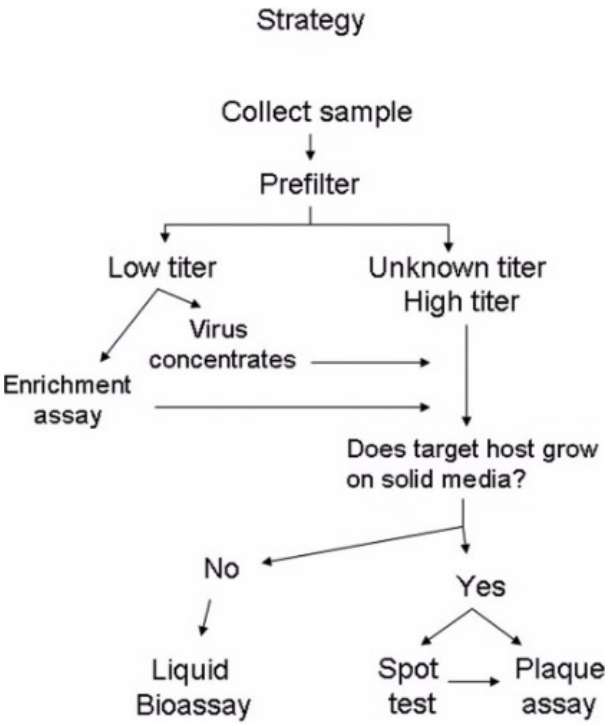
In principle, a small volume of a water sample is added to the host culture and monitored over time for signs of infection. The treated cultures are compared with control cultures by eye for obvious signs of viral infection such as total lysis (clearing) of the culture, decrease or change in overall pigmentation of the culture, or clumping and settling of cells to the bottom of the culture vessel. This approach has been used to isolate and detect cyanophages from seawater as well as marine sediment samples (Suttle and Chan 1993; Waterbury and Valois 1993; Wilson et al. 1993; Suttle 2000). Multiwell plates (e.g., Corning brand 24- or 96-well polystyrene plates with lids) are the culture vessel of choice. They are conducive to the screening of many samples, require minimal culture volumes, and take up little incubator space. Glass culture tubes with screw caps (e.g., 13-mm or 25-mm diameter) may be substituted and are useful for screening larger sample volumes. These glass tubes allow nondestructive monitoring of the in vivo chlorophyll fluorescence of the cultures using a fluorometer (e.g., Turner Designs TD700) or similar. Any lysis of the cells would result in a decrease in relative fluorescence (rf) compared with the control cultures.

Liquid assays can be used for all aquatic cyanobacteria and for screening all types of samples, including sediments, and host cells need not be axenic. Samples to be tested are not subjected to the possibility of elevated temperatures encountered when using plaque assays.

Table 1. Suggested volumes of target cells and sample to use for the liquid bioassay.

Culture vessel	Cell volume	Sample volume	Max. total volume
Plate, 96-wells	200-250 µL	50-100 µL	300 µL
Plate, 24-wells	2.5-3 mL	0.2-0.5 mL	3.5 mL
Tube, 13 100 mm	3.5-4 mL	0.5-1 mL	4.5 mL
Tube, 25 150 mm	30-35 mL	2-10 mL	40 mL

Figure 2. A flowchart suggesting various strategies of cyanophage isolation depending on type of sample and characteristics.



Tips and Tricks:

1. Use neutral density screens (gray or black window screening) to attenuate the light. Low light levels enhance development of pigments, which allows for easier discrimination of lysed versus unlysed cultures in the wells.

2. Condensation forming on lids can occur due to temperature changes in drafty incubators. Excessive condensation can make it difficult to visualize the wells. Sandwich the full plates between a layer of empty plates to insulate the cultures from temperature shifts.

3. Sealing the lid to the bottom of the plate helps to slow down evaporation of well contents, particularly the ones located at the plate perimeter.

Protocol

Step 1.
Collect ca. 50 mL seawater sample in a clean, acidwashed plastic (HDPE, PP, or PC) container or sterile Falcon tube.

Step 2.
Rinse container 3 times with the sample before filling.

NOTES
Amy Chan 01 Sep 2015
If not filtering right away, keep sample cold and in the dark.

Step 3.
Remove phytoplankton and bacteria from the water sample by filtration.

PROTOCOL

. Phytoplankton and bacteria filtration

CONTACT: Amy Chan
Step 3.1.
Filter sample using glass fiber filter (e.g., GC50, Advantec)

NOTES
Amy Chan 01 Sep 2015
This step may help to reduce premature clogging of the next filter.

Step 3.2.
Then filter with 0.2 µm or 0.45 µm PVDF filter (e.g., Millipore Durapore filters).

NOTES
Amy Chan 01 Sep 2015
As for the bacteriophage isolation, this filtration step may also remove some of the larger viral particles.

Step 4.
Store filtered seawater sample in the dark at 4°C (or on ice) until use.

Step 5.
Have ready, a culture of host cells in exponential growth (approximately 10^6 cells/mL).

Step 6.
Dilute cells ca. 10-fold with sterile media such as F/2 media (Guillard 1975)

NOTES
Amy Chan 01 Sep 2015
To about 1×10^5 cells/mL.

Step 7.
Allow a minimum of 30 mL host cells for every 96-well plate.

Step 8.
Using a multichannel pipette, aliquot cells into wells, cover plate with the lid and set aside in the incubator.

NOTES

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See table 1 for suggested volumes.

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Warning: make sure that the total volume of host and sample does not exceed the maximum capacity of the wells. There should be ca. 1–2 mm clearance from the top; excessive volume would cause overflow of the contents and subsequent cross-contamination of the wells.

Step 9.

Prepare 10-fold serial dilutions of the seawater sample (e.g., 0.5 mL sample added to 4.5 mL media in a 15-mL Falcon tube, up to 5 dilution levels) using sterile media as the diluents.

NOTES

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Note: triplicate dilutions series are recommended for determining the titer of lytic cyanophages in the sample.

Step 10.

Add diluted samples to wells: for example, 50 µL to 16 wells (2 rows of 8 wells each) for each dilution level.

NOTES

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With 5 dilution levels, that would leave 2 rows for the negative controls if using 96-well plates.

Step 11.

Replace the seawater sample with same volume of media for negative controls.

Step 12.

Cover plate with lid.

Step 13.

Carefully seal the lid to the plate using either parafilm or thin strips of plastic film.

Step 14.

Incubate plates at ca. 25°C, between 10 to 25 µmol quanta $m^{-2}s^{-1}$

Step 15.

Compare color development in the wells with the control wells.

NOTES

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Clear wells can be discerned from pigmented wells in 4 to 7 d.

Step 16.

Monitor wells daily for signs of lysis for 10 to 14 d.

NOTES

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Could be longer for slow growing host cells.

Step 17.

Choose a clear well from the highest dilution.

Step 18.

Transfer the lysate to a microtube.

Step 19.

Centrifuge the lysate for 5 min ca. 12,000g to pellet cell debris.

DURATION

00:05:00

Step 20.

Store the supernatant (about 250 µL) at 4°C and use it for further rounds of purification (via liquid or plaque assay).