Extinction dilution cloning for isolation of viruses infecting protists

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Abstract

This is a detailed outline of the extinction dilution method from:

Nagasaki, K., and G. Bratbak. 2010. Isolation of viruses infecting photosynthetic and nonphotosynthetic protists, p. 92–101. In S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

Please see the published manuscript for additional information.

Citation: Keizo Nagasaki and Gunnar Bratbak Extinction dilution cloning for isolation of viruses infecting protists. **protocols.io**
dx.doi.org/10.17504/protocols.io.dpx5pm
Published: 02 Dec 2015

Guidelines

List of materials and reagents:

- Bucket or water sampler (e.g., Van Dorn water sampler)
- Sediment sampler (e.g., Ekman bottom grab sampler)
- Centrifuge and centrifuge tubes (e.g., 15- or 50-mL Falcon tubes)
- Sterilized filter holder and membrane filters (0.2, 0.22, 0.45 or 0.8µm)
- Vacuum pump
- Sterile medium for algal culture (e.g., SWM-III, f/2)
- Sterile test tubes, pipette with tips and vortexer (for serial dilution)
- 24-well and 96-well cell culture plates (e.g., Falcon)
- 8-channel pipette and sterile reservoir tray for filling (for extinction dilution procedure)
- Plastic tape (for sealing the culture plates to avoid drying)
- Incubator (with light and temperature control)
- Inverted microscope
- Refrigerator, freezer, deep freezer, or liquid nitrogen container

Cloning and maintenance of microalgal viruses

When decay (i.e., bleaching, decrease in chlorophyll *a* fluorescence, clearing, etc.) of the tested host algal culture is detected in the screening procedure, the lytic factor should be cloned as soon as possible. In many cases clones have been obtained by using an extinction dilution procedure (e.g., Suttle 1993; Tomaru et al. 2004a,b). Briefly, the culture lysate is diluted with an adequate liquid medium in a series of 10-fold dilution steps. Aliquots (100 µL) of each dilution are added to 8 wells in cell-culture plates with 96 round-bottom wells, mixed with 150 µL of exponentially growing host culture, and incubated under the conditions suitable for the host's growth. Lysed cultures are removed from the most diluted wells in which lysis occurred, and the entire procedure is repeated.

The lysate in the most diluted wells of the second assay is sterilized by filtration through 0.1-µm (for ssRNA, ssDNA, or dsRNA viruses) or 0.2-µm pore size polycarbonate membrane filters (phycodnaviruses or large dsDNA viruses) and transferred into an exponentially growing host culture; certification of the lytic activity of the lysate is essential. After cell debris is removed by low-speed centrifugation, the supernatant is used as the clonal pathogen suspension.

Microalgal viruses are diverse in terms of stability, and a suitable protocol for maintaining infective viruses must be set up in each case. *Chlorella* viruses (PBCV-1) are so durable that significant decreases in titer are rarely seen as long as the viruses are kept refrigerated. In contrast, the titers of HaV, HcV, and PpV gradually decrease even when stored at 4°C in the dark. Isolated viruses may also be maintained in culture by routine transfer of the viral lysate to fresh host cultures. Loss of infectivity, however, caused for example by defective interfering particles (Bratbak et al. 1996), is a possible risk that should be taken seriously. Cryopreservation may, at least in some cases, be an alternative, and HaV and PpV have for example been stored at –196°C in 10–20% dimethyl sulfoxide and at –70°C in 10–20 % sucrose, respectively (Nagasaki and Yamaguchi 1999; Nagasaki 2001).

Comments

Filtration of the inoculum used for isolating viruses through 0.2–0.45-µm filters and later of the obtained lysates through 0.1–0.2-µm filters is a common procedure (see Table 1). Several workers have noted that some viruses do not pass or lose infectivity when filtered through certain types of filters and through filers with small pore size (i.e., 0.2 µm) (Van Etten et al. 1981; 1983; Suttle et al. 1991; Bratbak unpubl. data). Exceptionally large viruses such as the Mimivirus (~750 nm; La Scora et al. 2003; Xiao et al. 2005) will also be lost during filtration.

With use of the extinction dilution method for cloning, it should be noted that only the most abundant virus showing lytic activity to the host culture will be isolated. In other words, the less dominant viruses (if there are any) will be lost.

In most cases, lysis of the host culture will be a key criterion for detection of virus infection. Recovery and regrowth of the host in lysed cultures appears to be a common occurrence, however, and if this phenomenon occurs lysis may pass unnoticed if cultures are infected with a too high virus dose that results in weak lysis and rapid regrowth, or if cultures are left for too long before being inspected for lysis (Thyrhaug et al. 2003). Moreover, Mizumoto et al. (2008) have recently demonstrated that microscopic observation alone may be inadequate for detecting viral lysis, and infected cultures may be erroneously disposed of if the symptoms of lysis are weak.

Another issue that should be considered when preparing host cultures for isolation of virus is that viral susceptibility may change between life cycle stages with different ploidy levels in unicellular eukaryotes. Frada et al. (2008) has recently demonstrated that the haploid phase of *E. huxleyi* is resistant to EhVs that kill the diploid phase and that exposure of diploid *E. huxleyi* to EhVs induces transition to the haploid phase. The ensuing hypothesis, that the ploidy level of the host cultures may explain earlier unsuccessful attempts to isolate viruses and that viral induced transition between host ploidy levels may result in an apparent loss of infectivity during isolation, should be tested.

The lytic activity differs between various host–virus systems on the species level and also between various clonal combinations of host and virus within the same species (Nagasaki and Yamaguchi 1998; Mizumoto et al. 2008). The isolation procedures used so far may have selected for viruses having strong lytic activity because researchers inadvertently may prefer host–virus systems showing massive cell lysis. Viruses that are slow, latent, cause chronic infections, or are produced and released without killing or lysing the host may be hard to isolate but have an important ecological impact. The conception of viruses in natural ecosystems may thus be miscalculated if based only on the properties of the possibly “extreme” host–virus systems available in culture.

Brown algal phaeoviruses infect only the host spores or gametes and are reproduced in the

sporangia. Virus particles are not present in vegetative cells, but the genome of the FsV virus infecting *Feldmannia* sp. has recently been found integrated into the host genome in vegetative cells (Meints et al. 2008). Protocols for isolation and culturing of viruses infecting these macroalgae are described by Lanka et al. (1992) and Müller (1996).

Protocol

Preparation of dilution series

Step 1.
Prepare 9 tubes (numbered #1–#9) with 4.5 mL medium.

Preparation of dilution series

Step 2.
Add 500 µL of the virus filtrate (virus size fraction) to tube #1 and vortex.

Preparation of dilution series

Step 3.
Change the pipette tip and transfer 500 µL of suspension #1 to tube #2 and vortex.

Preparation of dilution series

Step 4.
Repeat the procedure to tube #8.

Preparation of cell culture plates

Step 5.
Pour vigorously growing algal host culture into the reservoir tray, fill the pipetter and add 150 µL culture to each well in lines 1–9 of a 96-well cell culture plate.

Preparation of cell culture plates

Step 6.
Fill the pipetter and add 150 µL culture to each well in lines 1–9 of a 96-well cell culture plate.

ANNOTATIONS

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Use an 8-channel pipetter filled from a reservoir tray (an ordinary pipette may be used but makes the work more tedious). When starting with the most diluted samples it is not necessary to change tips or reservoir tray while working with the same virus

Preparation of cell culture plates

Step 7.
Empty the tray.

Preparation of cell culture plates

Step 8.
Pour dilution tube #9 (control medium, no virus) into the reservoir tray.

Preparation of cell culture plates

Step 9.
Fill the pipetter and add 100 µL of to each well in line 9.

Preparation of cell culture plates

Step 10.
Empty the tray.

Preparation of cell culture plates

Step 11.

Repeat steps 7 and 8 for dilution tube #8–#1 and fill the respective well lines in the culture plate.

Preparation of cell culture plates

Step 12.

Put on the lid and seal tightly with plastic tape to avoid drying.

Preparation of cell culture plates

Step 13.

Incubate under appropriate conditions.

Inspection

Step 14.

Use an inverted microscope and inspect the culture plates for signs of cell lysis at regular intervals.

Inspection

Step 15.

Mark wells where lysis is observed and continue the incubation with daily inspections until no more lysis occurs.

Inspection

Step 16.

Prepare a second extinction dilution with virus from the most-diluted well.

Inspection

Step 17.

Propagate virus clone from the most diluted well in a larger volume and store appropriately.

Warnings

All work should be done in a clean bench.