Viral and bacterial isolates, propagation and preparation of stocks

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Abstract

The following procedure is an adaptation of the original FLVP protocol from Hennes et al. (1995) for use with SYBR Green I stain. Normally, this protocol will take 2 days given that the softening of the viral pellet is an overnight step. However, if the viral pellet redissolves quickly, it can be accomplished in 1 day.

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Guidelines

The virus–host system used for the FLVP experiments was *Vibrio alginolyticus* strain PWH3a, a marine heterotrophic bacterium, and its phage PWH3a-P1, a species-specific dsDNA virus of the *Myoviridae* family. Both strains were originally isolated from the coastal waters of the Gulf of Mexico (Suttle and Chen 1992). The host was maintained as a –80°C glycerol stock to minimize any long-term culturing effects and was cultivated at 30°C with agitation (120–200 rpm) using Marine Luria-Bertani broth (MLB) (0.5 g L^{-1} each of casamino acids, peptone, and yeast extract, 0.3% vol/vol glycerol, in 25 psu ultrafiltrate base). The virus was amplified using the plate lysate/liquid elution method (Suttle 1993), substituting ultrafiltered (virus-free) seawater for sterile media as the eluting agent. The eluant from multiple plates was pooled into a 50-mL centrifuge tube and spun at ~4000g for 20 min to remove large debris. The supernatant was then collected and filtered through a 47-mm–diameter, 0.22-µm–pore-size Durapore (Millipore) membrane to remove any remaining host cells. The final viral stock was titered by plaque assay (Suttle 1993) and kept at 4°C in the dark until needed.

Materials and Equipment:

• amplified, concentrated virus stock (preferably ≥10^{10} viruses mL^{-1}); calculate amount of stock needed using the following guide: 1 tube (1.7 mL) of stock = 50 µL of FLVPs → 1 µL FLVPs/slide = 50 slides
• Screw cap 1.7-mL microcentrifuge tubes (often listed as 1.5 mL)
• 0.02-µm filtered water or appropriate filtered seawater medium for resuspensions; use the latter if the virus is destroyed by freshwater
• SYBR Green I dye working stock (Molecular Probes)
• RC80 Beckmann (or similar) ultracentrifuge with an SW40 (or similar) swinging-bucket rotor; alternatively, use a rotor capable of directly accepting microcentrifuge tubes in a centrifuge capable of reaching >50,000g
• epifluorescence microscope equipped with a 100× oilimmersion objective and a blue-light excitation filter (such as the Olympus U-MWB2/U-MWIB2 filters)

Protocol

Step 1.
Pipette 1.7 mL amplified 0.22-µm filtered virus stock into 1.7-mL screw cap tubes.

Step 2.
Float the microcentrifuge tubes in long ultracentrifuge tubes (14 × 95 mm) using water until they are just flush with the top of the ultracentrifuge tubes; balance them to within 1 g.

NOTES
Amy Chan 14 Oct 2015
Most standard brands of O-ring screw cap microcentrifuge tubes (10-mm–diameter bodies, 12-mm–diameter screw caps) fit snugly into the larger ultracentrifuge tubes.
Amy Chan 14 Oct 2015
Alternatively, use a rotor capable of directly accepting microcentrifuge tubes in a centrifuge capable of reaching >50,000g to pellet the viruses.

Step 3.
Load the ultracentrifuge tubes into the SW40 rotor and spin them at 133,000g for 1 h.

DURATION
01:00:00

NOTES
Amy Chan 14 Oct 2015
Alternatively, spin at lower speeds in a microcentrifuge-accepting rotor for an equivalent duration (i.e., 2.5 h at 50,000g).
Amy Chan 14 Oct 2015
A small, whitish pellet should be visible in the microcentrifuge tubes after centrifugation.

Step 4.
Recover the microcentrifuge tubes.

NOTES
Amy Chan 08 Feb 2016
Use a squirt bottle to add more water to 'float' out the microcentrifuge tube - recover microcentrifuge tube with tweezers or fingers. Pellet is not disturbed.

Step 5.
Remove the supernatant and resuspend the pellet as follows:

PROTOCOL

. Pellet resuspension

CONTACT: Amy Chan
Step 5.1.
Pipette off 1.5 mL using a P1000.

Step 5.2.
Switch to a P100 and gently remove nearly all of the supernatant.

NOTES
Amy Chan 14 Oct 2015
Remainder usually totals ~10 µL.

Step 5.3.
Add 40 µL water or seawater medium to bring the volume to 50 µL.

NOTES
Amy Chan 14 Oct 2015
Remember freshwater versus seawater choice for viral isolate.

Step 5.4.
Gently vortex the tubes to disrupt the pellets and place them at 4°C overnight to soften.

DURATION
18:00:00

Step 6.
Perform all subsequent steps under subdued light since the stain will fade if exposed.

Step 7.
Thaw the SYBR Green I, then add 1 µL stain to each concentrated virus tube and incubate for 15 min in the dark.

DURATION
00:15:00

Step 8.
Verify the staining (and monodispersal) of the viruses by pipetting 1 µL of the suspension onto a microscope slide.

Step 9.
Add an 18 × 18 mm coverslip and observe the slide under the epifluorescence microscope.

NOTES
Amy Chan 14 Oct 2015
A veritable “sea” of FLVPs should be visible, which will fade nearly instantaneously as you scan from field to field due to the lack of antifade. If you wish to observe the FLVPs for longer periods, add 1 µL of antifade (0.1% p-phenylenediamine) to the FLVPs before adding the coverslip.

Step 10.
Add 1.65 mL water or seawater medium to each tube and respin as above.

NOTES
Amy Chan 14 Oct 2015
The resulting pellet should be slightly orange in the case of SYBR Green I.

Step 11.
Remove the supernatant and resuspend as above (completes first wash out of stain).

Step 12.
Repeat steps 10 and 11.

NOTES
Amy Chan 14 Oct 2015
Completes second wash out of stain.

Step 13.
Repeat steps 10 and 11 again.

NOTES
Amy Chan 14 Oct 2015
Completes third wash out of stain and ends protocol.
Amy Chan 14 Oct 2015

Ensure that the resulting FLVPs are monodispersed after the three centrifugation wash steps. If not, gently vortex the tubes to disrupt the pellets and place them at 4°C overnight to soften. The FLVPs can then be used the following day (day 3) or stored at 4°C for a significant amount of time (see "Assessment").