SUPPLEMENTARY MATERIAL FOR:

Use of herpes virus amplicon vectors to study brain disorders

Rachael L. Neve¹, Kim A. Neve², Eric J. Nestler³, and William A. Carlezon, Jr.¹

BioTechniques 39:__-__ (September 2005)

Protocol for Packaging Amplicon DNA Vector into Virus

Methods for titering the amplicon virus and for generating and titering helper stocks are detailed by Lim and Neve (1). Methods for in vivo use of herpes simplex virus (HSV) vectors are described by Carlezon and Neve (2). The timeline for the following protocol for packaging amplicon DNA vector into virus is shown in Supplementary Protocol S1. This protocol will yield virus stocks with a ratio of amplicon to helper of approximately 2:1.

Transfect 2-2 Cells

1. Maintain 2-2 cells [VERO cells containing the IE2 (ICP27) gene and promoter (3)] at 37°C in a humidified, 10% CO₂ incubator in Dulbecco’s modified Eagle’s medium (DMEM) + 10% defined fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Do not try to substitute any other brand of FBS. When a fresh aliquot of cells is thawed, it should be passaged at least twice before the cells are plated for the transfection. It is important to keep the cells subconfluent prior to and during the transfection; if they are allowed to become confluent at any point, they will not transfect well even if they are split to make them subconfluent. Cells may reach confluence for the subsequent infections.

2. Two days before the transfection, plate 2-2 cells at 3 × 10⁵ per 60-mm dish in 5 mL DMEM + 10% FBS.

3. On the day of the transfection, dilute 2 μg DNA [purified by a Qiagen (Valencia, CA, USA) column and resuspended in 1 mm Tris, pH 7.5, 0.1 mm EDTA] with 250 μL OptiMEM™ (Invitrogen, Carlsbad, CA, USA) in a sterile 1.5-mL microcentrifuge tube at room temperature. Add 8 μL of Lipofectamine Plus™ reagent (Invitrogen). Incubate at room temperature for 15 min.

4. Dilute 12 μL of Lipofectamine 2000 into 250 μL of OptiMEM in a second tube; add it to the 250-μL DNA mixture in the first tube. Leave the DNA-Lipofectamine mixture at room temperature for 15 min, for liposomes to form.

5. Remove the medium from the plates, wash them once with 2 mL OptiMEM (prewarmed to 37°C), and replace with 2 mL OptiMEM. Add the DNA/Lipofectamine mixture to the cells dropwise evenly over the whole plate. Incubate at 37°C for 3–5 h.

6. Rinse the plates 3 times with Dulbecco’s phosphate-buffered saline (D-PBS) and replace the medium with 5 mL DMEM + 10% FBS. Incubate the cells overnight at 37°C.

Superinfact Transfected Cells - P0

7. Sixteen hours after the time of the medium change following the transfection, prewarm DMEM + 5% FBS to 37°C.

8. Remove the medium from the plates and add 5 mL DMEM + 5% FBS. Add approximately 10⁶ plaque-forming units (pfu) of helper virus 5dl1.2 per plate and incubate at 37°C until 95% of the cells show cytopathic effects (CPE). The cells should round up but remain attached to the plate. This will take about 30 h.

Harvest P0 Cells by Hypotonic Lysis

9. Remove the medium from each plate and transfer it to a 15-mL tube. Add 1 mL of 0.1x D-PBS + Ca²⁺, Mg²⁺ to the cells on the plate and leave it on the cells at room temperature for at least 2 min for the cells to lyse completely. Use a disposable fine-tipped transfer pipet to collect the lysate and transfer it to the tube containing the medium. Add 100 μL 10x D-PBS to restore the ionic strength of the medium. Store the virus overnight (or longer) at -70°C or lower.

Amplify Virus Stock - P1

10. Two days before the P1 infection, plate fresh 2-2 cells: 4 × 10⁵ cells per 60-mm dish in 5 mL DMEM + 10% FBS. Incubate them at 37°C for 2 days.

¹Harvard Medical School and McLean Hospital, Belmont, MA, ²VA Medical Center and Oregon Health & Science University, Portland, OR, and ³The University of Texas Southwestern Medical Center, Dallas, TX, USA
11. Thaw the P0 virus stock quickly in a 37°C waterbath. Centrifuge it at 1350× g for 5 min to pellet cell debris but not virus particles.
12. Replace the medium on the 2-2 cells with 4 mL DMEM + 5% FBS and add 4 mL of the P0 supernatant. Incubate at 37°C overnight.
13. Harvest the P1 viruses as described in step 10 when they show 95% CPE (21–24 h). The P1 virus stocks can be frozen at this point, but it is preferable to proceed immediately to the P2 infection, so as not to disrupt the flow of the packaging procedure.

**Amplify Virus Stock - P2**

14. Two days before the P2 infection, plate fresh 2-2 cells: 1 × 10⁶ per 100-mm dish in 10 mL DMEM + 10% FBS (2 dishes per sample). Incubate them at 37°C for 2 days.
15. Centrifuge the P1 virus stock at 1350× g for 5 min to pellet cell debris but not virus particles.
16. Replace the medium on the 2-2 cells with 6.0 mL DMEM + 5% FBS per dish and add 4.0 mL of P1 supernatant per dish. Incubate the cells at 37°C overnight.
17. Harvest the P2 viruses when they show 95% CPE (21–24 h). Remove the medium from each pair of plates to a 50-mL tube. Add 2 mL of 0.1× D-PBS to the cells on each plate and leave it on the cells at room temperature for at least 2 min. Use a disposable fine-tipped transfer pipet to collect the lysate and transfer it to the tube containing the medium. Add 400 μL 10× D-PBS to restore the ionic strength of the medium. The P2 virus stocks can be frozen at this point, but it is preferable to proceed immediately to the P3 infection, so as not to disrupt the flow of the packaging procedure.

**Amplify Virus Stock - P3**

18. Two days before the P3 infection, plate fresh 2-2 cells: 1.2 × 10⁶ per 100-mm dish in 10 mL DMEM + 10% FBS (6 dishes per sample). Incubate the cells at 37°C for 2 days.
19. Centrifuge the P2 virus stock at 1350× g for 5 min to pellet cell debris but not virus particles.
20. Replace the medium on the 2-2 cells with 6 mL DMEM + 5% FBS per dish and add 4.0 mL of P2 supernatant per dish. Incubate the cells at 37°C overnight.
21. Harvest the P3 viruses when they show 95% CPE (21–24 h). Scrape the cells with a cell lifter. Transfer the cells and medium to 50-mL polypropylene conical tubes.
22. Freeze/thaw the cells three times using a dry ice/ethanol bath and a 37°C water bath. Minimize the amount of time that they are thawed in the 37°C bath.
23. Following the third freeze of the cells, the crude cell lysates may be stored at -70°C or lower until they are ready to be processed by sucrose gradient centrifugation.

**Purification of Virus from the Crude Cell Lysate**

24. Transfer the crude cell lysates to 50-mL polystyrene tubes and sonicate them for 2 min in a cup sonicator (power setting 6, 50% duty cycle, 1 second cycle). Centrifuge the lysates at 1350× g for 10 min to pellet cell debris but not virus particles.
25. Prepare sucrose step gradients (Figure S1) in 40-mL (25 × 89 mm) Ultra-Clear Beckman SW28 ultracentrifuge tubes (Beckman Coulter, Fullerton, CA, USA) or their equivalent at room temperature by layering the following sucrose solutions into the tube: 7 mL 60% sucrose in D-PBS + Ca²⁺, Mg²⁺, 6 mL 30% sucrose in D-PBS + Ca²⁺, Mg²⁺, and 3 mL 10% sucrose in D-PBS + Ca²⁺, Mg²⁺. Three tubes are needed for each virus preparation.
26. Load 20 mL crude virus onto the gradient in each tube and centrifuge it for 1 h at 112,000× g (SW28 rotor, 25K rpm) at 18°C.
27. Immobilize each tube in a clamp on a ring stand and place it in front of a black background. The virus will appear as a sharp, very thin band at the 30%/60% interface, while debris will collect in a diffuse band close to the 10%/30% interface.
28. Using a 3-mL syringe with an 18-g needle attached, pierce the tube underneath the band, with the beveled edge of the needle pointing upwards. Slowly pull the band into the syringe in a volume of 2 mL. Transfer this to an 11.5-mL (14 × 89 mm) Ultra-Clear Beckman SW41 tube and discard the remainder.
29. Dilute the virus in each tube with 9.5 mL D-PBS + Ca²⁺, Mg²⁺ and gently mix the contents of each tube by pipetting it up and down.
30. Centrifuge at 125,000× g (SW41 or SW40, 26K rpm, 18°C) for 1 h 15 min. Carefully aspirate the supernatant.
31. Add 200 μL 10% sucrose in D-PBS + Ca²⁺, Mg²⁺ to the opalescent pellet in each tube and resuspend the pellet by shaking it slowly in a rack.

Figures S1. Diagram of the protocol for purifying and concentrating the virus from the crude cell lysate. See steps 25–32 of the packaging protocol for details.
Protocol S1. Timetable for Packaging Amplicon DNA into Virus

| Day 1 | Plate P0 (5 × 10⁵ cells in a 60-mm dish). |
| Day 2 | Transfect DNA into P0 cells. |
| Day 3 | Superinfect P0 with ~10⁶ plaque-forming units (pfu) helper virus. Plate P1 (4 × 10⁵ cells in a 60 mm dish). |
| Day 4 | Harvest and freeze P0. Plate P2 (1 × 10⁶ cells in each of two 100-mm dishes). |
| Day 5 | Infect P1 with P0 lysate. Plate P3 (1.2 × 10⁶ cells in each of six 100-mm dishes). |
| Day 6 | Harvest P1. Infect P2 with P1 lysate. |
| Day 7 | Harvest P2. Infect P3 with P2 lysate. |
| Day 8 | Harvest P3. Do three freeze-thaws and store at -70°C or lower. |
| Day 9 and following | Do sucrose gradients. |

Notes

1. Large numbers of cells are needed throughout the packaging procedure, particularly if a large number of amplicon vectors are being packaged in parallel. To minimize the number of dishes used, it is helpful to maintain the cells in 150-mm dishes.

2. The freeze-thaws of the P3 lysates must be done in polypropylene tubes. Polystyrene tubes will crack. However, the lysates must be moved to polystyrene tubes for the sonication, following the freeze-thaws.

3. It is essential to use HyClone Defined FBS rather than any other type or brand of FBS. FBS from other suppliers causes the cells to divide on a different schedule, completely changing the timing of the packaging procedure.

4. The entire virus packaging procedure must be carried out under sterile conditions.

5. At the final step, if the virus is resuspended immediately by trituration, without an overnight incubation in the buffer, it is very difficult to get it into suspension. Moreover, the mechanical disruption necessary to resuspend the virus under these conditions will greatly decrease the viability of the virus. We therefore recommend slow resuspension overnight at 4°C.

6. The 2-2 cells are passaged by trypsinization. Remove the medium in which the cells are maintained (DMEM + 10% FBS), wash the plates 2× (very important) with Ca²⁺/Mg²⁺-free D-PBS, and incubate the plates at 37°C with a thin layer of trypsin-EDTA for 10 min.

REFERENCES

