Chapter 7

Viral Packaging and Transduction of Adult Hippocampal Neural Progenitors

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Abstract

Genetic manipulation of adult hippocampal neural progenitor cells is a useful technique for exploring gene function through gain of function and loss of function mutations or RNAi. Furthermore, the introduction of new genes can "re-program" progenitor cell behavior to force a desired lineage in signaling environments that are not normally permissive for that cell fate. Additionally, by using a systems biology approach, neural progenitors can even be taught new behaviors and responses to signaling. In this chapter, we describe protocols for retroviral and lentiviral packaging and transduction of progenitors. These techniques are important for studying the role of various genes in progenitor fate choice.

Key words: Neural, Hippocampus, Progenitors, Rat, *In vitro*, Retrovirus, Lentivirus, Viral transduction, Viral production/purification, Infection

1. Introduction

Genetic manipulation of adult hippocampal neural progenitor cells (AHNPCs) is crucial to understanding the roles various genes play in progenitor fate choice (e.g., self-renewal vs. differentiation). Expressing the constitutively active or dominant negative mutant of an endogenous gene, overexpressing the wild-type gene, or utilizing shRNA expression cassettes to induce RNAi can provide insight into that gene's role in the cellular process of interest. Alternatively, one may wish to transduce cells with reporter constructs (e.g., luciferase or GFP) to readily monitor gene expression.

In this chapter, we describe the methods for packaging retroviral and lentiviral vectors and the subsequent protocols for transduction of AHNPCs *in vitro*. Virus produced in this manner can also be used to transduce AHNPCs *in vivo*. To inject virus into the brain follow the protocol describing AHNPC engraftment in Chapter 6.

Viral production begins when "helper plasmids" expressing viral structural and enzymatic proteins are cotransfected into human embryonic kidney (HEK) 293T cells along with a "vector plasmid" or "transfer vector" carrying the genetic cargo of interest flanked by two lentiviral or retroviral long terminal repeats (LTR). The deletion of all viral genes between the two LTRs renders the virus replication incompetent. The transfected cells package the vector plasmid cargo into viral particles, which can then be purified and concentrated by ultracentrifugation in a sucrose step gradient. After determining the concentration of infectious units in this concentrate, the virus is ready to transduce cells.

The production and purification processes are identical for both retrovirus and lentivirus; however, the plasmids used for transfection differ. Retroviral production requires structural and enzymatic genes provided by the pCMV *gag-pol* and pcDNA3 IVS VSV-G plasmids (1) in addition to a transfer vector containing the gene of interest flanked by retroviral LTRs. One such transfer vector is pCLPIT (1), which confers puromycin resistance and places the transgene under the control of a tetracycline-controlled transactivator (tTA) resulting in "tet-off" gene regulation (2). Transfer vectors with other selection markers (e.g., GFP, neomycin, or a GFP-puromycin fusion) and/or promoters (e.g., ubiquitin promoter) may be used depending on the intended application.

Lentiviral production requires pMDLg/pRRE encoding the *gag* and *pol* genes (3), pcDNA3 IVS VSV-G (same as retrovirus), and pRSV Rev (3). In addition to the structural genes provided by the above plasmids, lentiviral production also requires a transfer vector containing the gene of interest between lentiviral LTRs, such as pHIV CS (4). Similar to the retroviral vectors discussed above, the lentiviral transfer vector can be modified to express desired selection markers or promoters, depending on the application.

Both retrovirus and lentivirus are capable of efficiently transducing AHNPCs with an insert of up to 9,000 base pairs (5); however, retrovirus will only infect cells that are actively replicating, whereas lentivirus will infect all cells. This makes retrovirus ideal for transducing an AHNPC culture or progenitor cells within a live animal brain, since it will only target the actively dividing progenitors and leave terminally differentiated cells uninfected.

2. Materials

2.1. Viral Packaging

2.1.1. Calcium Phosphate Transfection of 293T Cells

1. 293T HEK cells (ATCC, Manassas, VA). See vendor's instructions for recommended subculturing protocol.

2. 10 cm tissue culture plates.

3. Iscove's Modified Dulbecco's Medium (IMDM, MediaTech, Herndon, VA) with L-glutamine, HEPES, and phenol red;

without α-thioglycerol, β-mercaptoethanol, and sodium biocarbonate.

4. Fetal bovine serum (FBS, Invitrogen, Bethesda, MD). Aliquot and store at –20°C. Avoid freeze/thaw.

5. Penicillin/streptomycin, liquid (PS, Invitrogen). Contains 10,000 U/ml penicillin and 10,000 μg/ml streptomycin. Store at –20°C.

6. Working media (IMDM + FBS/PS). Dissolve 8.84 g IMDM and 1.86 g $NaHCO_3$ in 450 ml deionized water. Sterilize by 0.22 μm bottle-top filtration. Supplement with 50 ml FBS (10% v/v) and 5 ml PS (1% v/v). Store supplemented medium at 4°C for up to 1 month. Warm to 37°C before use.

7. 5 ml round bottom culture tubes (commonly used for flow cytometry) (BD Falcon, Bedford, MA).

8. Sterile tissue culture water.

9. 2.5 M $CaCl_2$ in tissue culture water. Sterilize by 0.22 μm syringe filtration. Store at room temperature.

10. 2× HEPES buffered saline (HeBS). Dissolve 1.64 g NaCl and 1.19 g HEPES in 80 ml tissue culture water. Add 933 μl of 150 mM Na_2HPO_4 solution (tissue culture grade Na_2HPO_4 in tissue culture grade water). pH to 7.05 with NaOH. Sterilize by 0.22 μm syringe filtration. Store at room temperature. Do not refrigerate because refrigeration will adversely affect the pH.

11. For retroviral production, the following high-quality plasmids at 1 mg/ml: pCMV *gag-pol*, pcDNA3 IVS VSV-G, and transfer vector of interest (see Note 1). Plasmids should be purified by Maxiprep purification using a kit from one of many manufacturers (i.e., BioRad, Invitrogen, Qiagen, etc.). For purification instructions, we refer the reader to the manufacturer's Maxiprep kit instructions.

12. For lentiviral production, the following high-quality plasmids at 1 mg/ml: pMDLg/pRRE, pcDNA3 IVS VSV-G, pRSV Rev, and transfer vector of interest (see Note 1). See item 11 for plasmid purification.

2.1.2. Viral Purification by Ultracentrifugation

1. Optima™ L-Series Preparative Ultracentrifuge (Beckman Coulter, Fullerton, CA).

2. SW-41 swinging bucket ultracentrifuge rotor (Beckman Coulter) (see Note 2).

3. Ultracentrifuge tubes (Beckman Coulter). 9/16 × 3½ in Ultra Clear Tubes. Although the manufacturer does not claim sterility, we have found that sterility is maintained if tubes are opened and handled in a biosafety cabinet with good aseptic technique (see Note 3).

4. Working media (IMDM + FBS/PS) (see Subheading 2.1.1, item 6).

5. Flathead screwdriver.

6. Tweezers.

7. 0.45 μm syringe filters.

8. Sterile phosphate buffered saline (PBS), pH 7.4 (Invitrogen). Store at 4°C.

9. 20% w/v sucrose in PBS. Sterilize by 0.22 μm syringe filtration and store at 4°C.

2.2. Quantification of Viral Titer

2.2.1. Titering by Fluorescent Marker Expression

1. 6-well poly-L-ornithine/laminin coated tissue culture plates (see Chapter 5, Subheading 2.1.1 for preparation).

2. AHNPCs (see Chapter 5, Subheading 3.1 for subculturing protocol).

3. Dulbecco's Modified Eagle Medium/Nutrient Mix F-12 (DMEM/F-12) with HEPES and L-Glutamine but WITHOUT phenol red (Invitrogen) supplemented with 1% (v/v) N-2 Supplement (Invitrogen). Store supplemented medium at 4°C for up to 1 month.

4. Accutase (Innovative Cell Technologies, San Diego, CA).

5. Basic fibroblast growth factor (FGF-2, Peprotech, Rocky Hill, NJ). FGF-2 stock solution: Dissolve in DMEM/F-12 + N-2 at 0.1 mg/ml, aliquot, and store at –20°C. Once thawed, aliquot can be stored at 4°C for up to 1 week.

6. Hemacytometer.

7. Sterile PBS, pH 7.4 (Invitrogen). Store at 4°C.

8. 5 ml round bottom culture tubes (commonly used for flow cytometry) (BD Falcon).

9. Flow cytometer (Beckman Coulter FC-500 or equivalent).

2.2.2. Titering by Drug Resistance Selection

1. All reagents in Subheading 2.2.1, items 1–7.

2. 96-well tissue culture plate.

3. Puromycin, cell culture tested (Sigma, St. Louis, MO). Puromycin stock solution: Dissolve in tissue culture water at 1 mg/ml and sterilize by 0.22 μm syringe filtration. Aliquot and store at –20°C. Working aliquots can be stored at 4°C for up to 1 month.

4. Cell Proliferation Reagent WST-1 (Roche, Indianapolis, IN). Upon receipt, thoroughly thaw at 37°C and swirl/pipette to ensure all aggregates dissolve. Then aliquot and store at –20°C. Protect from light. Aliquots can be frozen/thawed if necessary. Before use, warm again to 37°C to dissolve aggregates.

5. Multichannel pipette appropriate for 10 μl volume.

6. 96-well microplate reader.

2.3. Transduction of Adult Neural Progenitors

1. All reagents in Subheading 2.2.1, items 2–6.

2. Poly-L-ornithine/laminin coated culture dishes: 3.5, 6, and 10 cm (see Chapter 5, Subheading 2.1.1 for preparation instructions).

3. Puromycin, cell culture tested (Sigma). See Subheading 2.2.2, item 3 for preparation of puromycin stock solution.

3. Methods

3.1. Viral Packaging

Production of retroviral and lentiviral vectors is accomplished in two steps: (1) Calcium phosphate transfection of 293T cells with all necessary plasmids and (2) concentration and purification by ultracentrifugation through a sucrose step gradient.

Although transfection may be accomplished by other means (e.g., lipofectamine), we have found that the calcium phosphate method gives high transfection efficiencies in 293T cells for very low cost. Approximately 24 h after transfection, virus will begin to be secreted into the culture supernatant, and we recommend harvesting and purifying the supernatant at 48 and 72 h post infection to obtain optimal viral yields.

3.1.1. Calcium Phosphate Transfection of 293T Cells

1. Inoculate 293T cells onto 2×10 cm culture dishes in 10 ml IMDM + FBS/PS per plate (see Note 4). Plates should be seeded with approximately 3×10^6 cells/plate (~30% confluent). Incubate overnight at 37°C in 5% CO_2. Cells are ready for transfection once they are attached to the plate, actively dividing, and ~50–70% confluent (see Note 5).

2. For retroviral production: In a 5 ml culture tube, mix 1.72 ml sterile water, 20 μl transfer vector, 12 μl pCMV *gag-pol*, 8 μl pcDNA3 IVS VSV-G, and 2 ml 2× HeBS.

   For lentiviral production: In a 5 ml culture tube, mix 1.72 ml sterile water, 20 μl transfer vector, 10 μl pMDLg/pRRE, 7 μl pcDNA3 IVS VSV-G, 3 μl pRSV Rev, and 2 ml 2× HeBS.

3. Dropwise, add 240 μl of 2.5M $CaCl_2$. Pipette up/down 2–3 times to mix. Incubate at room temperature for 2 min (see Note 6).

4. Dropwise, add 2 ml of the reaction mixture to each 10 cm plate. Incubate at 37°C in 5% CO_2 overnight. Retroviral vectors carrying biohazardous cargo (e.g., oncogenes) and lentiviral constructs in general are considered Biosafety Level 2 biohazards; therefore, proper safety and waste

handling protocols should be in place before proceeding. Consult with your institutional safety committee for approval of all viral vector work prior to initiating studies. All subsequent steps, unless otherwise noted, should be performed at Biosafety Level 2.

5. Replace the media on both plates with 12 ml warm IMDM + FBS/PS 8–14 h post-transfection (see Note 7).

6. Approximately 48 h post-transfection, there is sufficient virus in the supernatant for purification. Proceed to Subheading 3.1.2 for purification by ultracentrifugation (see Note 7).

3.1.2. Purification by Ultracentrifugation

1. Clean and sanitize centrifuge buckets with 70% ethanol. In a biosafety cabinet, spray ethanol into each bucket and aspirate. Then, dry by wrapping lint-free wipes around tweezers, inserting into the bucket, and rotating. Use care to avoid scraping the tweezers on the bucket as they will score the metal. Leave buckets in the biosafety cabinet with the caps off to dry. Ensure that buckets are dry before inserting the centrifuge tubes, because residual ethanol will cause cracking in the centrifuge tubes.

2. Approximately 48 h post-transfection, pool supernatant from the culture plates into a 50 ml centrifuge tube. Replace the media with 12 ml warm IMDM + FBS/PS, and incubate the plates overnight at 37°C in 5% CO_2. This will be used for a second purification the following day.

3. Centrifuge the collected viral supernatant at 1400 RCF for 3 min to remove cellular debris.

4. Filter through a 0.45 μm syringe filter to remove smaller particulates.

5. Pipette 9 ml of filtrate into each of two ultracentrifuge tubes (if using the larger SW-28 rotor, see Note 2).

6. Using 1 ml serological pipettes, add 1 ml of 20% sucrose to the bottom of each tube. Add the sucrose slowly such that a well-defined, clear layer is seen below the viral supernatant. Use care in subsequent steps to prevent disturbing this sucrose layer.

7. Add another 1 ml of filtrate to fill each tube.

8. Seal the tubes in centrifuge buckets and weigh them to ensure that they are properly balanced. Add leftover viral supernatant as necessary to balance (see Note 8).

9. Once the buckets are balanced, re-seal and place them in the rotor. Also seal any unused buckets and place them in the rotor. Place the rotor in the ultracentrifuge, and pull a vacuum in the chamber.

10. Spin at 25,000 rpm (77,000 average RCF) for 1 h 30 min at 4°C (if using the larger SW-28 rotor, see Note 2). To prevent disruption of the sucrose layer during acceleration, set the ultracentrifuge to slowly accelerate to 170 rpm during the first 3 min of the spin. Similarly, set the ultracentrifuge to slowly decelerate from 170 rpm to 0 rpm over 3 min at the end of the spin.

11. Remove the buckets from the ultracentrifuge and bring into a biosafety cabinet.

12. Using tweezers sanitized with 70% ethanol, remove the tubes from the buckets. The virus will be in an invisible pellet at the bottom of the tube.

13. Vacuum aspirate the media layer from each tube by running the aspirating pipette around the wall of the tube in a circular motion as the liquid level is decreasing. Aspirate into the sucrose layer, but do not remove the sucrose layer.

14. Allow residual media to run down the side of the tube into the sucrose layer.

15. Aspirate the remaining liquid with a fresh aspirating pipette by tilting the tube and allowing the liquid to run into the pipette. This prevents accidental aspiration of the viral pellet.

16. Add 100 µl of cold PBS to the centrifuge tube. Keep the virus cold for all subsequent steps. It inactivates relatively rapidly at higher temperatures.

17. Seal with Parafilm® and store at 4°C for at least 2 h to allow the pellet to loosen, but do not incubate longer than 24 h.

18. Repeat step 1 to sanitize the centrifuge buckets.

19. Repeat steps 1–17 the following day to collect more virus.

20. Pipette all viral concentrates up/down several times to ensure that the viral pellet is completely dissociated. Pool viral concentrates in a sterile 1.5 ml tube.

21. Optional: purification for *in vivo* infection (see Note 9). In a fresh ultracentrifuge tube, dilute the concentrated viral stock in PBS to 10 ml total volume. Prepare a balance with 10 ml PBS and follow steps 9–18 and 20 to spin the virus.

22. Aliquot and store at –80°C. Avoid freeze/thaw. Viral stocks are stable at –80°C for approximately 3 months, after which they should be re-titered. Follow Subheading 3.2 to determine viral concentration.

3.2. Quantification of Viral Titer

Accurate knowledge of infectious viral concentration is crucial for a well-controlled transduction. Too much virus will result in multiple infectious events per cell, causing cells to express multiple copies, resulting in poor experimental control and repeatability.

However, insufficient virus will give very few infectious events, potentially making selection extremely difficult.

Depending on the selection marker that is included with the transgene of interest, infectious titer can be determined in one of two ways: fluorescent marker expression or drug resistance. To titer by fluorescent marker expression (e.g., GFP), cells are infected with a dilution series of freshly prepared virus, grown for 2 days, and analyzed by flow cytometry to determine the fraction of fluorescent (infected) cells. Similarly, when titering by drug selection, cells are infected with a dilution series, and then a drug is added (e.g., puromycin) to which infected cells are now resistant. After the uninfected cells have died, the number of remaining cells is quantified and compared to an uninfected control to determine the fraction of infected cells. If using drug resistance, we recommend using puromycin, because it kills all uninfected cells within 3 days, while other drugs can take much longer. For this reason, this protocol is designed for cells expressing puromycin resistance.

It is important to note that the infectious titer is not the same across all cell types, as different cells are differentially susceptible to retroviral and lentiviral transduction. Therefore, we recommend titering virus on the cell type that will be transduced to avoid possible differences in infectivity from one cell type to the next.

3.2.1. Titering by Fluorescent Marker Expression

1. Thaw a 6-well laminin coated culture plate.

2. Prepare AHNPCs. Follow Chapter 5, Subheading 3.1.1, steps 1–11. Determine the cell concentration of the cell suspension by hemacytometer. This solution is generally quite concentrated, so a 1:10 dilution in DMEM/F-12 + N-2 prior to counting is usually necessary. Keep cell suspensions well-mixed throughout the counting and diluting process.

3. Dilute cells to 1.4×10^6 cells in 14 ml (100,000 cell/ml) in DMEM/F12 + N-2. Supplement with 2.8 μl of 0.1 mg/ml FGF-2 stock solution (20 ng/ml final concentration).

4. Aspirate PBS from the culture plate and add 2 ml of cell suspension to each well. Rock the plate several times to mix, but do not swirl. Incubate at 37°C in 5% CO_2 for approximately 4 h.

5. Thaw a viral aliquot in a 37°C water bath for approximately 30 s. If titering retrovirus, we recommend using 16 μl. If titering lentivirus, which is generally produced at a higher concentration, we recommend using 4 μl. Other amounts may be used as necessary.

6. Prepare a 5-point, two-fold dilution series of virus in PBS. For example, if starting with 16 μl of virus, the resulting dilution series will have 16, 8, 4, 2, and 1 μl of virus diluted in PBS such that the final volume of each dilution is 16 μl.

7. Add the dilutions to each well on the 6-well plate. Leave one well as an uninfected control. Gently rock the plate to mix. Incubate for approximately 48 h at 37°C in 5% CO_2.

8. Aspirate media from each well and add 100 μl Accutase. Incubate at 37°C for approximately 2 min. Rock the plate to dislodge the cells.

9. Add 900 μl DMEM/F-2 + N-2 to each well and pipette up/down several times to dissociate cells. Transfer cell suspension into a 5 ml culture tube.

10. Analyze by flow cytometry to determine the fraction of GFP expressing cells (infected).

11. By assuming that infection events follow a Poisson distribution, calculate the multiplicity of infection (MOI, infectious units/cell) at each dilution point (see Note 10). Multiply the MOI by the initial cell number (200,000) to calculate the number of infectious units (IU) in each dilution. Determine viral concentration by calculating the slope of a plot of IU (*y*-axis) vs. volume of virus sample used (*x*-axis) (see Note 11).

3.2.2. Titering by Drug Resistance Selection

1. Follow Subheading 3.2.1, steps 1–5.

2. Follow Chapter 5, Subheading 3.1.1, steps 12–15 to continue culturing leftover AHNPCs. They will be used in 3 days to generate a standard curve.

3. Prepare a 4-point, twofold dilution series of virus in PBS. For example, if starting with 16 μl of virus, the resulting dilution series will have 16, 8, 4, and 2 μl of virus diluted in PBS such that the final volume of each dilution is 16 μl.

4. Add the dilutions to each well on the 6-well plate. Leave two wells as uninfected positive and negative controls. Gently rock the plate to mix. Incubate overnight (12–18 h) at 37°C in 5% CO_2.

5. Add 1.2 μl puromycin stock solution to each well (0.6 μg/ml final concentration) except for one of the uninfected controls. This becomes the positive control. Incubate 48 h at 37°C in 5% CO_2.

6. To generate a standard curve, dissociate, dilute, and count the leftover cells maintained in parallel by following Chapter 5, Subheading 3.1.1, steps 1–11.

7. Prepare standard curve and plate in triplicate 100 μl per well of each cell concentration on a 96-well plate. The cell concentrations can be varied based on typical titers for your specific virus, but we recommend 40,000, 20,000, 10,000, 5,000, 2,500, 1,250, and 0 (DMEM/F-12 + N-2 only) cells per well.

8. Aspirate media from each well of the 6-well plate and add 100 μl Accutase. Incubate at 37°C for approximately 2 min. Rock/shake the plate to dislodge the cells.

9. Add 900 μl DMEM/F-2 + N-2 to each well and pipette up/down several times to dissociate cells.

10. Transfer 100 μl of cell suspension from each well to 3 wells on the 96-well plate. Ensure that the cell suspensions stay well mixed.

11. Thaw WST-1 at 37°C for at least 5 min. Briefly vortex to ensure all aggregates dissolve.

12. Using a multichannel pipette, add 10 μl WST-1 to each well. Gently rock plate to mix. Incubate at 37°C for 2 h.

13. Rock on shaking platform for approximately 1 min.

14. Measure samples against blank (media only) on microplate reader. Measure absorbance at 440 and 700 nm. Subtract A_{700} from A_{440}. This is the experimental readout. For the infected cells, subtract the experimental readout of the uninfected negative control from the readout for the infected wells.

15. Plates can then be returned to the incubator and reanalyzed up to 4 h after WST-1 addition. Use the standard curve to determine the best read. This will depend on the cell concentrations of the standard curve, but we have generally found that 2–3 h post WST-1 addition provides the best results.

16. Use the standard curve to interpolate cell numbers. Divide the number of cells in the experimental wells by the number of cells in the uninfected positive control to determine the fraction of puromycin resistant (infected) cells. Follow Subheading 3.2.1 step 11 to complete the calculations (see Note 11).

3.3. In Vitro Transduction of Adult Neural Progenitors

Here, we describe the transduction and selection of AHNPCs based on puromycin selection. Alternatively, cells can be selected by fluorescence activated cell sorting (FACS) for expression of a fluorescent marker (e.g., GFP); however, we have found FACS to be a harsh procedure for AHNPCs. Approximately 50% of the cells survive the procedure, and those that do survive have long recovery times. This cell death and slow recovery will select for a cell population that is capable of surviving the harsh conditions of FACS. Unfortunately, the characteristics of this population are poorly understood, and furthermore they may not be representative of the typical AHNPC.

1. Thaw a 6-cm and a 3.5-cm laminin coated culture dish.

2. Prepare AHNPCs. Follow Chapter 5, Subheading 3.1.1, steps 1–11. Determine the cell concentration of the cell suspension by hemacytometer. This solution is generally quite

concentrated, so a 1:10 dilution in DMEM/F-12 + N-2 prior to counting is usually necessary. Keep cell suspensions well-mixed throughout the counting and diluting process.

3. Inoculate 750,000 cells into the 6 cm dish in 4 ml of DMEM/F-12 + N-2. Supplement with 0.8 μl of 0.1 mg/ml FGF-2 stock solution (20 ng/ml final concentration).

4. As a negative control, inoculate 200,000 cells into the 3.5 cm dish in 2 ml DMEM/F-12 + N-2. Supplement with 0.4 μl of 0.1 mg/ml FGF-2 stock solution (20 ng/ml final concentration). Dilution of the FGF-2 stock may be required, depending on the accuracy of available pipettes at such a low volume.

5. Incubate 4–8 h at 37°C in 5% CO_2.

6. Add 750,000 IU of virus to the 6 cm plate, resulting in an MOI of 1 IU/cell (see Note 12). Gently rock the plate to mix and incubate 1–2 h at 37°C in 5% CO_2 (see Note 13).

7. Aspirate and replace the medium with 4 ml of fresh, warm DMEM/F-12 + N-2. Supplement with 20 ng/ml FGF-2. Incubate overnight (12–18 h) at 37°C in 5% CO_2.

8. Add 2.4 μl of puromycin stock solution to the 6 cm plate and 1.2 μl to the 3.5 cm plate (final concentration 0.6 μg/ml). Gently rock the plates to mix and incubate at 37°C in 5% CO_2.

9. Depending on the transgene, cellular "behavior" (morphology, proliferation, multipotency, etc.) will vary greatly between viruses. Therefore, monitor the plates daily and take appropriate action as necessary (nothing, replating the cells, passing to a larger/smaller plate, or media change). Some general guidelines:

   (a) Some cell death is normal, depending on the MOI. If the viral titer was correct and infection events follow a Poisson distribution, then at an MOI of 1 IU/cell approximately 37% of cells will remain uninfected and will die upon puromycin selection.

   (b) All cells on the negative control plate should be dead and floating 72 h post puromycin addition.

   (c) Regardless of cell growth/death, replace the media with fresh, warm DMEM/F-12 + N-2 + 20 ng/ml FGF-2 + 0.6 μg/ml puromycin every 48 h.

   (d) If cells are growing in clumps, follow Chapter 5, Subheading 3.1.1 to remove them from the plate, dissociate, and replace on a fresh plate of the same size.

   (e) At very low cell density proliferation will slow significantly. Therefore, if more cell death than expected occurs, it may become necessary to pass the cells down to a

smaller plate (i.e., 3.5 cm dish or even a 12-well plate). Follow Chapter 5, Subheading 3.1.1 for subculturing procedure.

   (f) If the cells are >70% confluent, follow Chapter 5, Subheading 3.1.1 to pass all cells to a larger plate.

10. Culture the cells under puromycin for 4 days. At that point, if cell growth has stabilized, they are ready for experimentation. We also recommend cryo-preserving several aliquots. See Chapter 5, Subheading 3.1.2 for cryo-preservation procedure.

4. Notes

1. In addition to encoding the gene of interest, we also recommend including a selection marker into the transfer vector. This marker is crucial for both eliminating uninfected cells and for viral titering. A fluorescent marker, such as GFP, will allow for selection and titering by flow cytometry, cell sorting, or microscopy. However, a drug resistance marker, such as puromycin or neomycin, will allow for selection by cell survival. We have found that a GFP-puromycin fusion protein (6) is an excellent marker, because it combines the ease of titering by flow cytometry with the ease of drug selection.

2. The SW-41 rotor has a working volume of 11 ml (10 ml viral supernatant + 1 ml 20% sucrose). For larger-scale purifications, we recommend the SW-28 rotor, which has a working volume of 35 ml (32 ml viral supernatant + 3 ml 20% sucrose). The centrifugation protocol is similar for the two rotors; however, the SW-28 should be spun at 24,000 rpm, while the SW-41 should be spun at 25,000 rpm.

3. If using the SW-28 rotor, use $1 \times 3\frac{1}{2}$ in UltraClear tubes (Beckman Coulter).

4. The number and size of plates may be varied depending on the amount virus required, the application, and the size of the centrifuge rotor available. Each 10 cm plate will yield approximately 10^5–10^8 infectious units (IU); therefore, 2×10 cm plates generally provide adequate viral amounts for most *in vitro* applications with a typical MOI of 1 IU/cell. However, most *in vivo* experiments require ~3 μl per animal of viral stock at approximately 10^9 IU/ml; therefore, the number and/or size of plates must be scaled accordingly.

5. Although antibiotics are included in the 293T cultures, good aseptic technique in a laminar flow biosafety cabinet is still required for all viral production and purification steps.

The virus will be used to infect AHNPCs, which are cultured without antibiotics; therefore, great care should be taken to prevent contamination of viral stocks.

6. Although we have generally found that 2 min incubation is ideal, optimal incubation times may vary from 1 to 3 min depending on the batch of HeBS. To check the optimal incubation time, transfect 293T cells with a GFP-expressing vector on a 6-well plate at various time points. Qualitatively assess transfection efficiency the following day by fluorescent microscopy.

7. At 24 h post-transfection cells should look normal. At 48 h post-transfection some cell death and detachment may occur, but the culture should still look mostly healthy. At 72 h post-transfection widespread cell death and detachment may occur. The amount of cell death will depend on the size and type of viral insert.

8. Tubes must be completely full (11 ml for SW-41, 35 ml for SW-28); otherwise, they will collapse during centrifugation. Make up any unused volume with fresh working media.

9. *In vivo* transduction of AHNPCs requires very pure virus to prevent inflammation of the injection site. A second ultracentrifuge spin removes the residual contaminants from the 293T culture media that can cause this inflammation.

10. Assuming a Poisson distribution, the fraction of infected cells is given by

$$x = 1 - e^{-\lambda}$$

where x is the fraction of infected cells, and λ is the MOI (IU/cell). Solving the above equation for the MOI gives

$$\lambda = -\ln(1-x).$$

11. The production/purification protocol written here will typically generate 10^5–10^8 retroviral infectious units for the pCLPIT vector plasmid. Lentiviral amounts are typically 3–4 orders of magnitude higher for a pHIV CS vector plasmid. One of the most significant factors affecting production is the size of the DNA sequence being packaged. Large inserts result in low titers and vice versa.

12. Depending on the application, other MOIs may be used. Note that infectious events follow a Poisson distribution, so at an MOI of 1 IU/cell, approximately 37% of cells will be uninfected, while approximately 26% of cells are multi-infected.

13. Do not leave virus on the cells for more than 2 h. Residual serum in the virus from the 293T culture will induce AHNPC differentiation after prolonged incubation.

Acknowledgment

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