

Gene Transfer into Neural Cells In Vitro Using Adenoviral Vectors

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ABSTRACT

Adenoviral vectors are excellent vehicles to transfer genes into the nervous system due to their ability to transduce dividing and nondividing cells, their ability to be grown to very high titers, and their relatively large insert capacity. Also, adenoviral vectors can sustain very long-term transgene expression in the CNS of rodents and in neurons and glial cells in culture. Successful gene transfer into the nervous system is dependent on the development, production, and quality control of vector preparations, which need to be of the highest quality. This unit provides protocols to clone, rescue, amplify, and purify first-generation adenoviral vectors. Detailed quality control assays are provided to ensure that vector preparations are devoid of contamination from replication-competent adenovirus and lipopolysaccharides. Also included are methodologies related to adenoviral-mediated gene transfer into neurons and glial cells in culture, and the analysis of transgene expression using immunocytochemistry, enzymatic assays, and fluorescence-activated cell sorting (FACS) analysis. *Curr. Protoc. Neurosci.* 45:4.23.1-4.23.43. © 2008 by John Wiley & Sons, Inc.

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INTRODUCTION

Adenovirus (Ad) vectors offer an extremely efficient system for delivery of transgenes or shRNA, allowing gene expression in a wide range of target cells, including neural cells. Important features of adenovirus vectors include: the ability to infect post-mitotic cells, including neurons; the capacity to be grown to high titers [up to 10^{13} virion particles per ml (vp/ml)]; an insert capacity of ~7.5 kb; high levels of transgene expression; long-term stable expression in the brain (described in rats and mice); and the ability to infect target cells with more than one vector. Moreover, adenoviruses are rarely associated causatively with brain pathology (Thomas et al., 2001; Barcia et al., 2006; Zirger et al., 2006). In addition to these features, recombinant adenovirus vectors have also been used to achieve targeted and regulated expression using tissue-specific or regulatable promoters, and controlled expression by gene dosage and promoter selection (Smith-Arica et al., 2000; Xiong et al., 2006; Candolfi et al., 2006, 2007).

Adenovirus vectors can be exploited to target expression to brain neocortical and glial cells in culture (Morelli et al., 1999; Smith-Arica et al., 2000). This unit describes protocols to generate adenoviruses, to assess whether they infect and express transgenes within identified brain cells in culture, and to transfer missing functions to cellular models of human diseases. Initially, the procedures for generation of recombinant replication-defective adenoviruses by homologous recombination of shuttle vectors within human embryonic kidney 293 cells, and their subsequent isolation by endpoint dilution, purification, and amplification, are discussed (see Basic Protocol 1). This protocol also presents steps for

cesium chloride gradient purification and assessment of vector titers. It is assumed from the outset that readers will be familiar with the molecular biology techniques required for production and analysis of shuttle vectors containing the desired transgenes under the control of selected promoters, and with the techniques and skills necessary for culture of mammalian cell lines *in vitro*. The procedures employed in the authors' laboratory for identification and characterization of recombinant adenovirus vectors is presented in the support protocols. These include techniques for extraction of viral DNA and Southern blotting (see Support Protocol 1), and PCR detection of transgenes (see Support Protocol 2), along with the essential quality control assays for detection of contamination by bacterial lipopolysaccharide and replication-competent adenovirus, and for the determination of the particle-to-infectious (plaque-forming) unit ratio (see Support Protocol 3), to which all recombinant vectors generated should be subjected.

After description of the methods for recombinant adenovirus vector generation, the techniques employed for infection of neurons and glia *in vitro* (see Basic Protocol 2) are presented, along with support protocols outlining the procedures for the establishment of primary cultures of neocortical neurons (see Support Protocol 4), ventral-mesencephalic neurons (see Support Protocol 5), and neocortical glial cells (see Support Protocol 6). Support Protocol 7 describes the detection of transgene expression in cultured cells by Xgal cytochemical assay, immunocytochemical means, and fluorescence-activated cell sorting (FACS).

Adenovirus vectors are not limited to gene transfer in mammalian neural systems. They are equally efficacious for gene transfer into cells of the gastrointestinal (including hepatic), cardiovascular, musculoskeletal, and pulmonary systems, and these protocols will be of value to researchers working in these fields as well.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a humidified 37°C, 5% CO₂ incubator unless otherwise stated.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

BASIC PROTOCOL 1

PREPARATION OF RECOMBINANT ADENOVIRUS VECTORS BY HOMOLOGOUS RECOMBINATION

Ad vectors for gene transfer and therapy have been constructed using derivatives of human adenovirus serotypes 2 or 5 (Ad2 or Ad5). First-generation recombinant adenovirus vectors (Ads) for gene transfer typically have deletions in the E1 region to render the vector replication-defective, thereby preventing virus spread and target cell lysis. These vectors can be propagated in the E1-complementing human embryonic kidney cell-derived 293 line (Graham et al., 1977). The largest viable E1 deletion reported for first-generation vectors extends from nucleotide 342 to 3523, removing nearly all the E1 sequences between the viral packaging domain and the coding sequence for adenoviral gene pIX.

The E3 region, which is dispensable for growth in culture, is also deleted in many Ad vectors to allow the cloning of larger inserts. The largest E3 deletion reported to date is 3.1 kb (from nucleotide 27,865 to 30,995 of Ad5), which prevents expression of all E3 genes (Bett et al., 1994). Since Ad is capable of packaging a genome that is 5% larger than the wild-type genome, the largest E1/E3 deletions provide a total cloning capacity of 8.3 kb. Most of the Ad vectors in use today are derived from the Ad5 strain *dl1309*

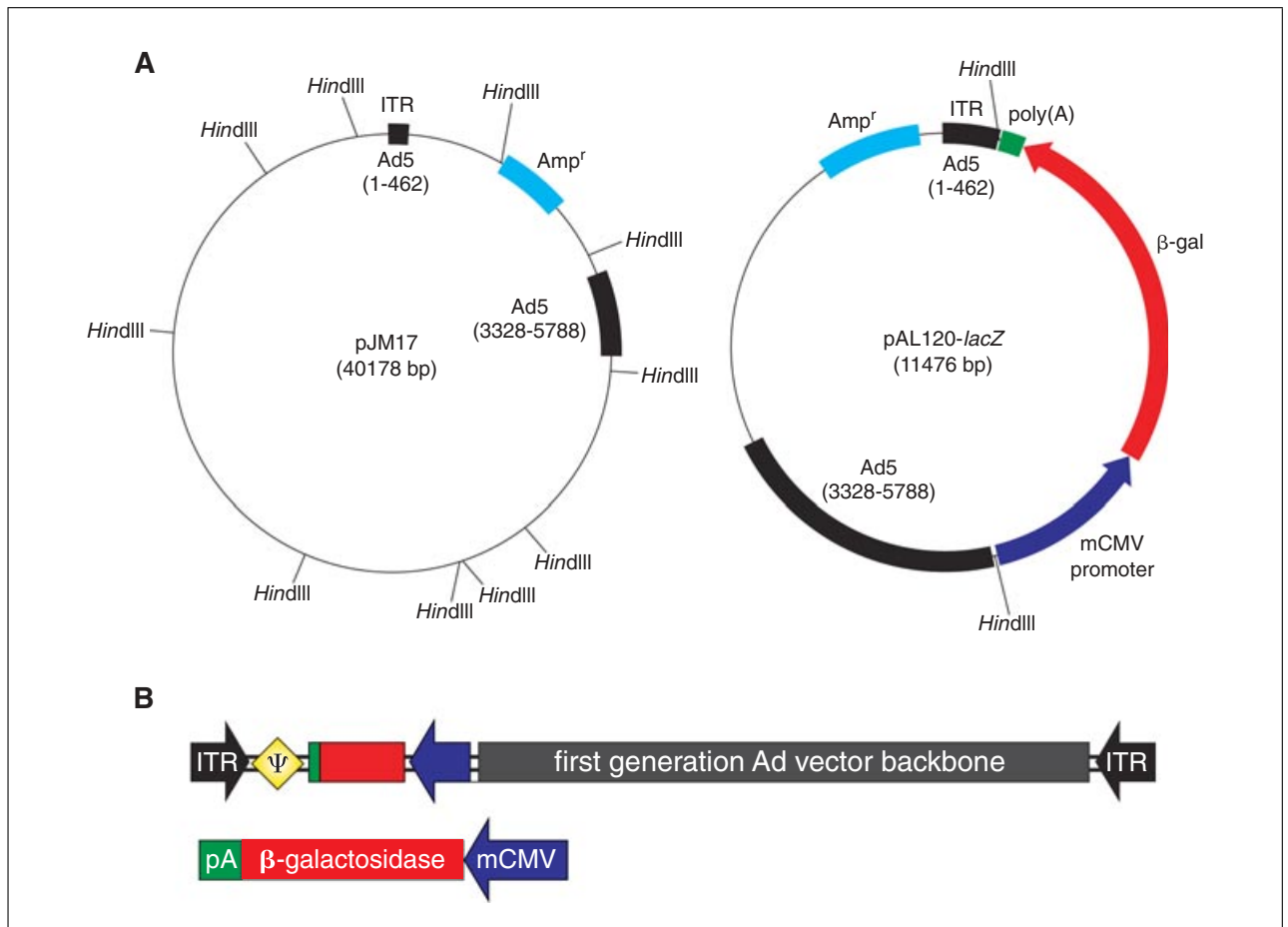


Figure 4.23.1 Schematic diagram of shuttle vectors used for construction of recombinant adenoviruses. **(A)** The transfer vector pAL120-βgal was constructed from plasmid pXCX2 (Spessot et al., 1989) with the addition of a linker (containing the *Hind*III cloning site) at the unique *Xba*I cleavage site. *lacZ* was cloned into pAL120 under mCMV promoter control and upstream of a polyadenylation signal. The transfer vector provides the expression cassette with flanking sequences from the Ad5 E1a gene region. The plasmid pJM17 contains the entire Ad5dl309 genome and the prokaryotic vector sequence pBRX inserted into the E1a gene at 3.7 map units. Insertion of the prokaryotic vector makes pJM17 too large to be packaged into Ad nucleocapsids. Cotransfection of both plasmids into 293 cells allows homologous recombination, resulting in the replacement of the entire E1a region and prokaryotic sequences with the *Hind*III expression cassette. The genome of the recombinant vector is reduced sufficiently in size to be packaged into nucleocapsids, allowing vector replication and resulting in plaque formation in the 293 cell monolayer. **(B)** A linear diagram of the Ad genome is shown, illustrating the position of the expression cassette.

or its derivative plasmid, pJM17, which carry a deletion/substitution in E3, but can still express the E3 19 kD and 11.6 kD proteins.

There are several approaches that can be used for the construction of first-generation recombinant Ad vectors. To insert a foreign gene or cDNA in place of the E1 region, the desired gene (usually together with exogenous promoter and polyadenylation sequences) is first inserted into a shuttle plasmid in which the insertion site replacing E1 is flanked by the remaining Ad sequences from the left end of the genome. The sequence is then rescued into the virus either by ligation of the expression cassette with DNA derived from the right end of the genome followed by transfection into 293 cells, or by homologous recombination following cotransfection of the shuttle plasmid with restricted viral DNA or with a second plasmid carrying Ad genomic sequences, e.g., pJM17 or pBHG10 (Fig. 4.23.1). A number of Ad genomic plasmids have been engineered to minimize the generation of replication-competent virus or viruses that do not contain the transcription unit. Other approaches have been developed to facilitate rescue of the insert into Ad genomic sequences prior to transfection of 293 cells, especially for cases

in which homologous recombination in 293 cells would not be efficient. Such strategies include homologous recombination in *Escherichia coli* between the insert and genomic sequences maintained as a plasmid (Chartier et al., 1996), homologous recombination in yeast between the insert and Ad genomic sequences maintained as a yeast artificial chromosome (Ketner et al., 1994), and construction of adenoviral vectors by lambda phage genetics (McVey et al., 2002). The following protocol is used to generate first generation replication-deficient adenoviruses by homologous recombination in 293 cells.

Materials

Adenoviral transfection plasmid, e.g., pJM17 or pBHG10 (Microbix Biosystems),
and shuttle plasmid pMV60 or pAL120 (constructed from plasmid pXCX2;
Spessot et al., 1989; Löwenstein et al., 1996)
Plasmid purification system (e.g., Maxi-prep, QIAGEN)
293 cells (ATCC #1573; ECACC #85120602)
293 cell medium (see recipe), prewarmed to 37°C
Low-Tris/EDTA buffer (LTE; see recipe)
2 M CaCl₂
2× HEPES-buffered saline (HBS; see recipe)
Dulbecco's phosphate-buffered saline (D-PBS; CellGro, VWR cat. no. 45000-082),
prewarmed to 37°C
Serum-free MEM, prewarmed
TransIT-293 transfection reagent (Mirus Bio, cat no. MIR2700)
1% Virkon solution (Anachem)
Liquid nitrogen
Tris-Cl, pH 8.0 (APPENDIX 2A)
5% (w/v) sodium deoxycholate
2 M MgCl₂
10 mg/ml RNase I
DNase I (see recipe)
1.33 and 1.45 g/ml cesium chloride (CsCl) solution (see recipe)
10% (v/v) glycerol
25-cm² and 175-cm² plastic tissue culture flasks (Corning)
15-ml and 50-ml sterile polypropylene conical tubes (Greiner)
Sterile flame-polished Pasteur pipets
Automatic pipettor
1.5-ml screw-cap microcentrifuge tubes
37°C water bath
24-well and 96-well tissue culture plates (Corning)
200-μl pipet tips
60-mm tissue culture dish
15-cm dish
500-ml sterile polypropylene conical bottle
Beckman 14-ml Ultra-Clear centrifuge tubes (cat. no. 344060)
Serological pipets
5-ml syringes
Wide-bore needles (18-G spinal needles; Sherwood Medical)
Black permanent marker
Beckman ultracentrifuge and SW-40 rotor
Slide-A-Lyzer Dialysis Cassette (extra strength; Pierce, cat. no. 66380)
Additional reagents and equipment for counting cells using a hemacytometer and
culture of mammalian cells (APPENDIX 3B), characterization of recombinant
adenovirus vectors (see Support Protocol 1), and dialysis (CPMB APPENDIX 3C and
APPENDIX 1A in this manual)

CAUTION: The following procedures should be carried out in a Class 2 (BCL-2) tissue culture suite in a Class 2 laminar flow cabinet. All media used should be discarded into a solution of 1% Virkon and all plasticware which has been in contact with the recombinant adenovirus should be washed in a solution of 1% Virkon prior to autoclaving and incineration.

Prepare shuttle plasmids

1. Prepare adenoviral transfection plasmids, such as pJM17 or pBHG10, and the shuttle plasmids, such as pMV60, pAL120 (mCMV promoter), pAL119 (hCMV promoter), or p Δ E1sp1A, using a system such as Maxi-prep and assay them for recombination as described in Support Protocol 1.

The assay for recombination should be performed prior to proceeding to the co-transfection stage. The vector system used most frequently by the authors is shown in Figure 4.23.1.

Cotransfect plasmid into 293 cells (Fig. 4.23.2A)

2. The day before cotransfection, thinly seed 293 cells in a fresh 25-cm² tissue culture flask in 5 ml of 293 cell medium (Löwenstein et al., 1996). Incubate overnight.

APPENDIX 3B describes general mammalian cell tissue culture techniques.

Aim to achieve ~60% confluency on the day of transfection for optimum transfection efficiencies.

3. The next day, immediately before transfection, aspirate the medium and add 5 ml of fresh, prewarmed 293 cell medium.

Calcium phosphate transfection

- 4a. Pipet a volume containing 5 μ g of the shuttle plasmid containing the transcription unit and a volume containing 5 μ g pJM17 (or pBHG10) into a sterile 15-ml polypropylene conical tube.

Maximum volume should be <15 μ l.

- 5a. Add LTE buffer to achieve a final volume of 210 μ l and mix gently by flicking the tube.
- 6a. Add 30 μ l of 2 M CaCl₂, flick tube to mix, and centrifuge gently (1 min at 200 \times g, room temperature) to ensure that all the solution is in the bottom of the tube.
- 7a. To a second tube add 240 μ l of 2 \times HBS. Using a sterile glass Pasteur pipet and an automatic pipettor, add the DNA-CaCl₂ mixture to the tube containing the HBS while very gently bubbling air into the mixture. Continuing bubbling for 1 min.
- 8a. Allow precipitate to form for 30 min at room temperature.
- 9a. Add the HBS-DNA-CaCl₂ mixture directly to 5 ml fresh, prewarmed 293 cell medium in a 25-cm² tissue culture flask containing the 293 cells, and tilt dish gently to ensure even distribution of the mixture across the cell layer.

Check that a fine precipitate has formed on the cells by viewing at high magnification.

- 10a. Leave DNA precipitate on the cells while incubating for 16 hr.
- 11a. Gently aspirate the medium and add 5 ml D-PBS to wash the cells. Aspirate the PBS, gently wash the cells once with fresh prewarmed 293 cell medium, then add 6 ml fresh prewarmed 293 cell medium and return to the incubator.

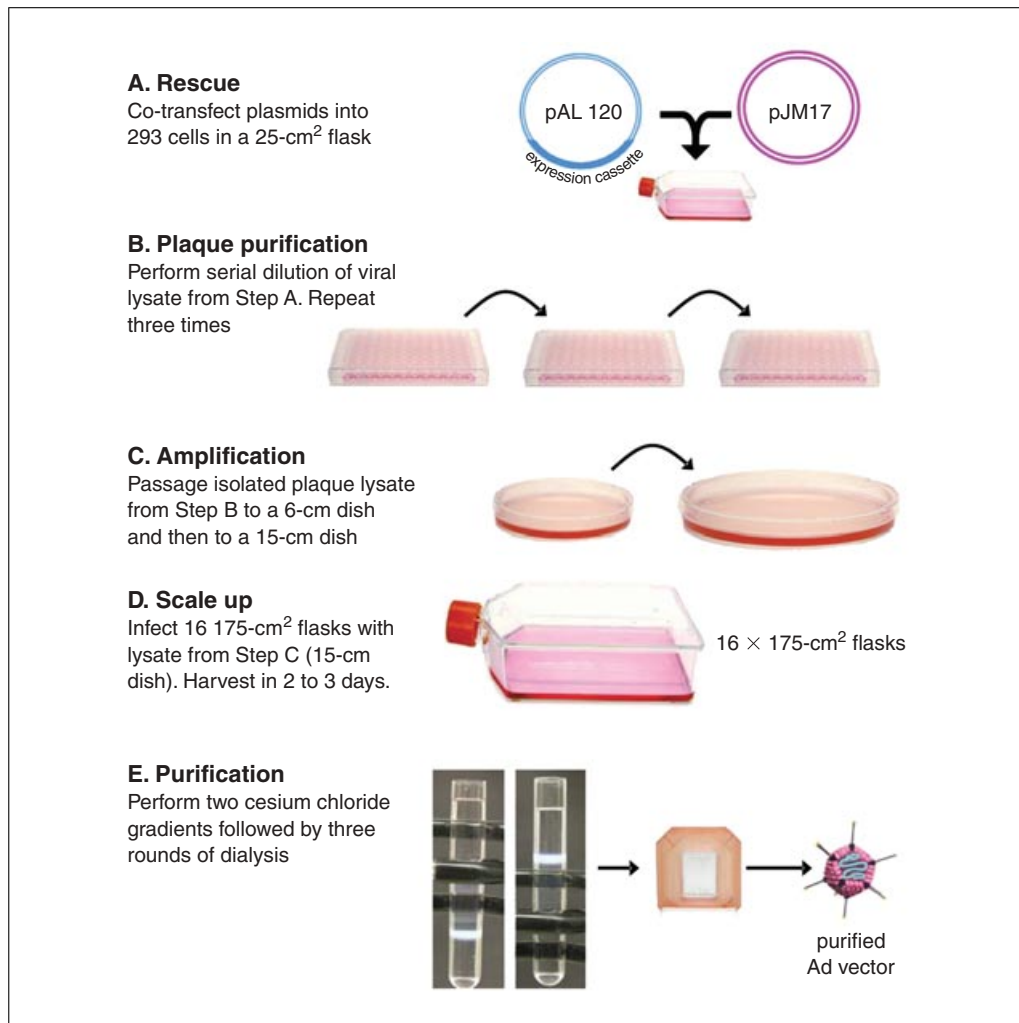


Figure 4.23.2 Schematic illustration of first generation vector rescue, amplification, and purification. **(A)** A shuttle plasmid (i.e., pAL120) and the vector genome plasmid are co-transfected onto 293 cells. Cells are incubated until CPE forms. **(B)** An individual adenovirus vector is isolated by three successive rounds of plaque purification. **(C)** An individual vector plaque is then amplified by two successive passages on 293 cells. **(D)** The vector is finally scaled-up in 16 to 20 175-cm² flasks of 293 cells. **(E)** The Ad vector is purified by two rounds of cesium chloride density gradients followed by three rounds of dialysis.

Alternative transfection method using TransIT-293 transfection reagent

This method uses a lipid-based transfection reagent that is designed specifically for the 293 cell line to provide high transfection efficiency and minimal cellular toxicity. Over 80% transfection efficiency can regularly be achieved using this reagent with the modified protocol below.

- 4b. Pipet 500 μ l of prewarmed serum-free MEM into a 1.5-ml microcentrifuge tube.
- 5b. Add 15 μ l of TransIT-293 transfection reagent. Vortex 10 sec.
- 6b. Incubate 10 min at room temperature.
- 7b. Dilute 5 μ g of the shuttle plasmid containing the transcription unit and 5 μ g pJM17 (or pBHG10) in 25 μ l of LTE.
- 8b. Add 25 μ l of DNA solution to medium-TransIT-293 transfection reagent mixture. Gently pipet up and down.

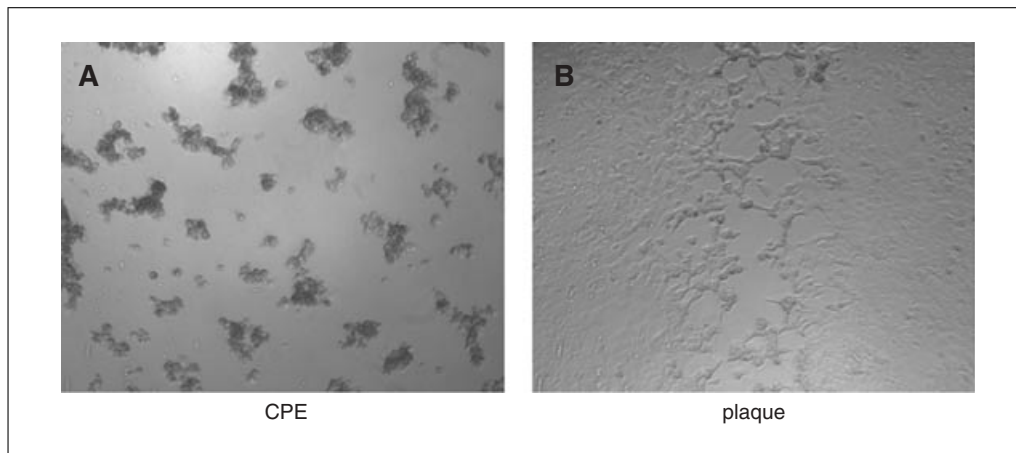


Figure 4.23.3 Recombinant adenovirus plaque formation with 293 cells. **(A)** Full cytopathological effect (CPE) is shown in the left panel. **(B)** A visible plaque can be seen spreading across the 293 cell monolayer.

- 9b. Incubate 20 min at room temperature.
- 10b. Add complex directly to 25-cm² tissue culture flask containing the 293 cells (~50% confluency), and tilt dish gently to ensure even distribution of the mixture across the cell layer.

A nonviral transfer shuttle plasmid containing the transgene lacZ, such as pAL120 (Fig. 4.23.1), can be used as a control to determine representative transfection efficiency. Incubate the cells for 2 days, then check the number of transfected cells using the method described for Xgal staining of infected cultures (Support Protocol 8). Aim for a transfection efficiency >30% for successful generation of a recombinant adenovirus. Alternatively, a shuttle plasmid containing the transgene green fluorescent protein (GFP) can be used in conjunction with a inverted fluorescent microscope to monitor transfection efficiency and vector rescue.

- 11b. Return to the incubator for 16 hr. Wash gently, as described in step 11a.
12. Replenish medium every 3 days, or more often if it becomes acidic (represented by change in the medium color from red to orange/yellow). Discard all media and wash all plastics used in a solution of 1% Virkon, then autoclave.

If recombination has occurred, plaques will become visible from 6 days after transfection (Fig. 4.23.3). Allow plaques to spread throughout the monolayer. Retain all cultures for up to 25 days before discarding as negative, feeding the cells every 3 to 4 days with fresh prewarmed 293 cell medium.

All the progeny generated using this system are likely to contain the desired insert.

13. Harvest the cells into a sterile 15-ml polypropylene conical tube (the cells should detach from the surface of the flask on gentle washing or tapping) and centrifuge 15 min at 300 × g, 4°C.
14. Aspirate the supernatant and resuspend the cells in 100 μl D-PBS.
15. Lyse the cells by rapidly freeze-thawing three times in liquid nitrogen/37°C water bath, pellet the cellular debris by centrifuging 15 min at 300 × g, 4°C, and transfer the virus-containing supernatant to a sterile screw-cap microcentrifuge tube. Store at -70°C.

The supernatant can be stored for several years at -70°C, but the number of infectious viral particles will decrease over time.

To ensure that the subsequent virus stock is derived from a single infectious viral particle, plaque purify the recombinant virus three times by endpoint dilution (steps 16 to 26).

Isolate recombinant adenovirus by endpoint dilution (Fig. 4.23.2B)

16. Seed each well of a 96-well tissue culture plate with 3×10^3 293 cells in 100 μ l prewarmed 293 cell medium. Incubate overnight.
17. On the next day, place 10 μ l of the recombinant adenovirus stock (from step 15) in 990 μ l of fresh, prewarmed 293 cell medium (dilution factor = 10^{-2}). Using this 10^{-2} dilution, prepare a series of further 10-fold dilutions in prewarmed 293 cell medium using sterile microcentrifuge tubes or a 24-well tissue culture plate. Continue until reaching a dilution factor of 10^{-11} .
18. Aspirate the medium from the 96-well plate (from step 16).
19. Add 100 μ l of each of the viral dilutions to triplicate wells, starting with the highest dilution (10^{-11}) and working down to the lowest (10^{-2}) dilution.

Since higher concentrations of virus are being transported at each step, it is not necessary to change tips on the pipet.

20. At 24 hr post infection, add an additional 100 μ l of fresh, prewarmed 293 cell medium to each well.
 21. Monitor the wells daily for evidence of plaque formation, changing the medium every 3 days or sooner if the medium becomes acidic (characterized by medium turning orange/yellow; Fig. 4.23.3).
 22. After 8 days, locate the endpoint wells (the wells containing the highest dilution of viral stock which contains a plaque).
 23. Harvest the cells from the triplicate endpoint wells by scraping with the end of a 200- μ l pipet tip.
 24. Place the cells from each well in separate sterile screw-cap microcentrifuge tubes, gently pellet cells in a microcentrifuge by centrifuging 15 min at $300 \times g$, room temperature, remove the supernatants, and resuspend the cell pellets in 100 μ l D-PBS.
 25. Lyse the cells by rapidly freeze-thawing three times in liquid nitrogen/ 37°C water bath. Pellet the cell debris by microcentrifuging 15 min at $300 \times g$, room temperature, and transfer the supernatant to a fresh sterile screw-cap microcentrifuge tube.
26. Repeat steps 16 to 25 two more times, selecting the cell lysate from one well at random from the three endpoint wells from step 24. Store the supernatants from the other two wells at -70°C as backup.

The triple purification takes a total of 24 days.

Viral stock: Purify recombinant adenovirus vectors (Figure 4.23.2C)

Production and purification of the viral stock is undertaken in two steps. The first step is the production of a master stock of the endpoint dilution-purified recombinant adenovirus. It is recommended that at this stage the viral preparation be thoroughly characterized for the presence of the transcription unit within the Ad genome (see Support Protocol 1). The second step, i.e., cesium chloride gradient centrifugation, entails the purification of the recombinant virus to a level suitable for in vivo gene delivery, and also concentrates the virus, allowing titers ranging from 10^{11} to 10^{12} pfu/ml to be obtained. For infections of neural cells in vitro, use cesium chloride-purified Ads, due to the cytotoxicity of less pure preparations. The investigator must also check the purity of the viral preparations as outlined in Support Protocol 3.

Produce master stock

27. Seed a 60-mm tissue culture dish with $\sim 2 \times 10^6$ 293 cells in 3 ml of prewarmed 293 cell medium to reach 70% to 80% confluency the following day.
28. In a sterile 15-ml polypropylene conical tube, gently mix 3 ml of fresh, prewarmed 293 cell medium and 100% of the recombinant virus stock produced after the third round of plaque-purification (from step 26).

After 2 to 3 days a plaque will form that will extend throughout the entire cell monolayer (this appearance is referred to as cytopathological effect, CPE; Fig. 4.23.3A).

29. Detach cells from the flask by tapping the side of the flask gently with the hand and then decant the cell suspension into sterile 1.5-ml tubes, each containing 300 μ l of cell suspension.
30. Lyse the cells by rapidly freeze-thawing three times in liquid nitrogen/37°C water bath.

At this point, the supernatant can be stored at -70°C for up to several years.

31. Infect 300 μ l of cell lysate onto a 15-cm dish of 293 cells at 80% to 90% confluency (seed $\sim 2 \times 10^7$ 293 cells per dish the day before).
32. Check culture 48 hr later; full CPE should be observed (Fig. 4.23.3). At this point harvest the cells by tapping the plate and pipetting the cell suspension up and down.
33. Transfer to 50-ml conical screw-cap tube and centrifuge 15 min at $300 \times g$, 4°C.
34. Remove supernatant and resuspend the cell pellet in 500 μ l D-PBS.
35. Lyse the cells by rapidly-freeze thawing three times in liquid nitrogen/37°C water bath.

36. Centrifuge the cell lysate 15 min at $300 \times g$, 4°C. Transfer supernatant into a new 50-ml conical tube. Store at -70°C until ready for scale-up.

The master viral stock will need to exhibit signs of full CPE by 48 hours. If this is not the case, repeat steps 31 to 36 using a larger virus inoculum.

Prepare large-scale recombinant adenovirus stock (Figure 4.23.2D)

37. Grow sixteen 175-cm² flasks of 293 cells to 80% to 90% confluency in 293 cell medium.
38. Prepare 400 ml of inoculation solution by adding 100% of cell lysate (from step 36) to 400 ml of 293 cell medium.
39. Aspirate the medium from 175-cm² flasks and inoculate each flask with 25 ml inoculation solution from step 38.
40. Return the flasks to the incubator and incubate until full CPE is observed (Fig. 4.23.3).

Optimal time for observation of CPE should occur between 48 and 72 hr after infection.

41. When CPE can be observed throughout the monolayer, detach the cells by tapping the side of each flasks gently with your hand.
42. Harvest the cell suspensions from each flask into a sterile 500-ml polypropylene conical bottle and pellet the cells by centrifuging 20 min at $300 \times g$, 4°C.
43. Discard supernatant and resuspend the cell pellet in 10 ml of 100 mM Tris-Cl, pH 8.0 in a 50-ml screw-cap conical tube.

At this point, 10% glycerol can be added and cells can be stored at -80°C .

Purify recombinant adenovirus by cesium chloride gradient centrifugation (Fig. 4.23.2E)

44. Add 1 ml of 5% sodium deoxycholate and thoroughly mix by vigorously shaking the tube. Incubate 30 min at room temperature with occasional mixing.

The lysate should become highly viscous.

45. Add 100 μ l of 2 M $MgCl_2$, 50 μ l of 10 mg/ml RNase A, and 50 μ l of 10 mg/ml DNase I (10 mg/ml). Incubate for 1 hr at 37°C with frequent mixing.

The solution should become much less viscous (Palmer and Ng, 2003).

46. Centrifuge 30 min at maximum speed, 4°C. Remove and save supernatant.
47. Pipet 0.5 ml of 1.50 g/ml CsCl solution into a Beckman 14-ml centrifuge tube. Then, using a serological pipet, slowly deliver 3.0 ml of 1.35 g/ml CsCl solution on top of the 1.50 g/ml layer. Mark the interface between the 1.35 g/ml and 1.50 g/ml density layers with a black permanent marker. Next, layer 3.0 ml of 1.25 g/ml CsCl so the less dense layer “floats” on top of the more dense layer (Revah et al., 1996; Tollefson et al., 1999).
48. Carefully layer up to 7 ml of the cell lysis supernatant containing Ad vector (from step 46) onto the gradient.
49. Carefully fill the remainder of the centrifuge tube with 100 mM Tris·Cl, pH 8.0, up to 2 mm from the top of the tube. Place the tube inside the rotor bucket prior to leaving the class 2 laminar flow cabinet. Weigh the bucket containing the tube and prepare a balance tube of identical weight (weighed to \pm 0.05 g).
50. Centrifuge 1 hr at 15,000 \times g, 10°C, in a Beckman ultracentrifuge with a swinging-bucket SW-40 rotor, using maximum acceleration and minimum deceleration.
51. Very carefully remove the tubes from the buckets in the class 2 laminar flow cabinet and remove the banded virus by side-wall puncture, \sim 1 cm below the level of the band, with a 5-ml syringe and wide-bore (18-G) needle (Fig. 4.23.4A through C).

Generally 2 to 3 ml of virus is extracted from the ultracentrifuge tube.

The virus band should be localized to the black mark on the tube at the location of the original interface between the 1.35 g/ml and 1.25 g/ml density layers.

52. Transfer 100% of the virus band isolated in step 51 into a new 14-ml ultracentrifuge tube. Fill the remainder of the tube with 1.35 g/ml CsCl solution up to 2 mm from the top of the tube.
53. Centrifuge in the SW40 rotor for \sim 18 hr at 15,000 \times g, 4°C.
54. Remove the viral band as in step 51 and inject the solution containing cesium chloride and Ad vector into the Slide-A-Lyzer Dialysis Cassette (Fig. 4.23.4D).
55. Dialyze the Slide-A-Lyzer Dialysis Cassette containing the virus against three changes of the buffers as follows at 4°C:
- 500 ml of 10 mM Tris·Cl pH 8.0 for \sim 6 hr,
 - 500 ml of 10 mM Tris·Cl pH 8.0 overnight,
 - 450 ml of 10 mM Tris·Cl pH 8.0 supplemented with 50 ml of 10% (v/v) glycerol for \sim 4 hr.
56. Divide the virus into 5- and 10- μ l aliquots and store at -70°C . Titer the recombinant virus by endpoint dilution (steps 57 to 64).

The virus can be stored frozen for several years, but the titer will decrease slowly over time. Once an aliquot is defrosted it should not be refrozen.

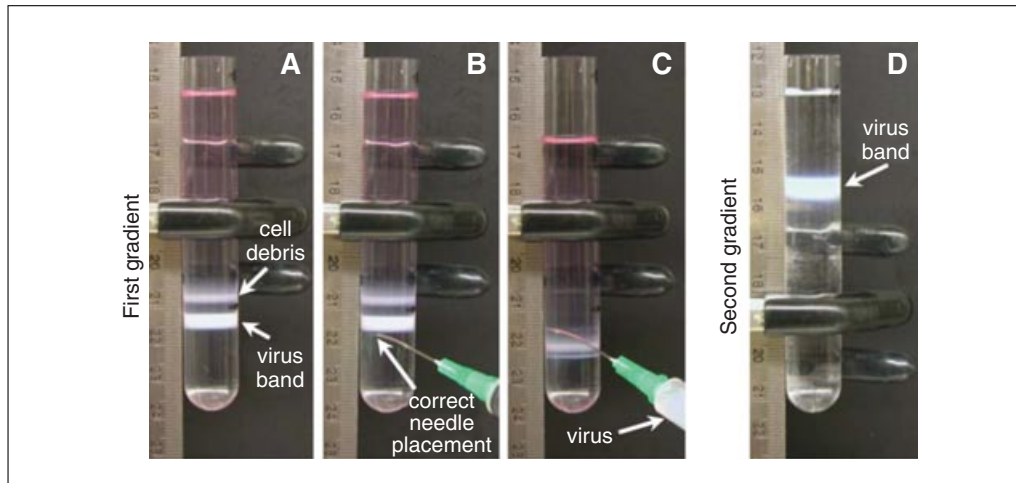


Figure 4.23.4 Vector banding on cesium chloride gradients. (A) The relative size and positions of the two major bands present after the first round of ultracentrifugation is indicated by white arrows. (B) The correct placement and angle of the needle is depicted. (C) An image of the density gradient following extraction of the viral band. Notice the level of cell debris band has dropped compared to the previous image following extraction of the vector band. Also, notice the white opaque color of the solution containing the vector with the syringe. (D) The vector band is shown following the second round of ultracentrifugation. Notice the absence of cellular debris.

Table 4.23.1 Dilution Scheme for Titering Recombinant Adenoviruses by Endpoint Dilution

Mixture	Dilution
10 μ l of recombinant adenovirus stock + 990 μ l of fresh prewarmed 293 cell medium	10^{-2}
100 μ l of 10^{-2} dilution + 900 μ l of fresh prewarmed 293 cell medium	10^{-3}
100 μ l of 10^{-3} dilution + 900 μ l of fresh prewarmed 293 cell medium	10^{-4}
100 μ l of 10^{-4} dilution + 900 μ l of fresh prewarmed 293 cell medium	10^{-5}
100 μ l of 10^{-5} dilution + 900 μ l of fresh prewarmed 293 cell medium	10^{-6}
500 μ l of 10^{-6} dilution + 500 μ l of fresh prewarmed 293 cell medium	5×10^{-7}
500 μ l of 5×10^{-7} dilution + 500 μ l of fresh prewarmed 293 cell medium	2.5×10^{-7}
etc. to a final end dilution of	7.63×10^{-12}

Titer recombinant adenoviruses by endpoint dilution

57. Seed each well of a 96-well tissue culture plate with 3×10^3 293 cells in 100 μ l 293 cell medium/well.
58. On the following day, prepare a series of 10- and 2-fold dilutions of the recombinant adenovirus (from step 56) in sterile microcentrifuge tubes or using a 24-well tissue culture plate as shown in Table 4.23.1.
59. Aspirate the medium from the 96-well plate.
60. Add 100 μ l of each of the viral dilutions to triplicate wells, starting with the highest dilution (7.63×10^{-12}) working down to the 10^{-2} dilution.

Performing the assay in triplicate offers greater assurance of the accuracy of the final titer. Since higher concentrations of virus are being transported at each step, it is not necessary to change tips on the pipet.

61. Return the plate to the incubator.

Table 4.23.2 Titer of Recombinant Adenovirus Preparations Corresponding to Serial Dilution Endpoint Wells

Well	1	2	3	4	5	6
Dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	5.0×10^{-7}
pfu/ml	1.0×10^3	1.0×10^4	1.0×10^5	1.0×10^6	1.0×10^7	2.0×10^7
Well	7	8	9	10	11	12
Dilution	2.5×10^{-7}	1.25×10^{-7}	6.25×10^{-8}	3.12×10^{-8}	1.56×10^{-8}	7.81×10^{-9}
pfu/ml	4.0×10^7	8.0×10^7	1.6×10^8	3.2×10^8	6.4×10^8	1.28×10^9
Well	13	14	15	16	17	18
Dilution	3.91×10^{-9}	1.95×10^{-9}	9.77×10^{-10}	4.88×10^{-10}	2.44×10^{-10}	1.22×10^{-10}
pfu/ml	2.56×10^9	5.12×10^9	1.02×10^{10}	2.05×10^{10}	4.10×10^{10}	8.19×10^{10}
Well	19	20	21	22	23	24
Dilution	6.10×10^{-11}	3.05×10^{-11}	1.53×10^{-11}	7.63×10^{-12}	Uninfected controls	
pfu/ml	1.64×10^{11}	3.28×10^{11}	6.55×10^{11}	1.32×10^{12}		

62. At a time point 24 hr post infection, add an additional 100 μ l of fresh, prewarmed 293 cell medium to each well.
63. Monitor the wells daily for evidence of plaque formation.
64. After 8 days, read the plate for the highest dilution in which all three wells contain plaques.

See Table 4.23.2 to determine the total number of plaque-forming units/ml. An alternative protocol for mathematically calculating pfu after titrating the recombinant adenovirus is illustrated in Löwenstein et al. (1996).

SUPPORT PROTOCOL 1

CHARACTERIZATION OF RECOMBINANT ADENOVIRUS VECTORS

Each recombinant vector should be thoroughly characterized to assess the presence of the transcription unit, i.e., promoter-transgene-poly(A), after cotransfection, after plaque purification, and after each subsequent CsCl purification procedure. This requires restriction digestion of viral DNA and Southern blotting using a specific probe for the transgene. PCR analysis of transgene and/or transcription unit that includes the promoter and poly(A) tail is also described (Support Protocol 2). This assessment is critical and should be performed on all viral preparations before use, as the transcription unit may be lost or modified due to recombination during viral purification and amplification in 293 cells. Replication-competent adenoviruses may arise during any stage of the procedure, with concomitant loss of the appropriate transcription unit.

Materials

- 293 cells (ATCC #1573, ECACC # 85120602)
- Recombinant adenovirus (e.g., Basic Protocol 1)
- 293 cell medium (see recipe)
- Dulbecco's phosphate-buffered saline (D-PBS; CellGro, cat. no. 21-031-CV), prewarmed to 37°C
- Virion lysis buffer (see recipe)
- 5 M NaCl
- 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (APPENDIX 2A)
- 100% and 70% ethanol
- Sterilized distilled water
- RNase Type I-A (Sigma)
- Dig DNA labeling and detection kit (Roche)

Restriction enzymes appropriate for digesting plasmids of interest
 Shuttle plasmid containing transgene
 1% agarose gel containing ethidium bromide (*APPENDIX 1N*)
 1-kb DNA step ladder (Promega)
 0.25 M HCl
 0.4 M NaOH
 0.5× SSC (prepare from 20× stock; *APPENDIX 2A*)
 Prehybridization solution (see recipe)
 Hybridization solution (prehybridization solution containing 5 μl denatured Dig DNA probe for every ml solution), prepare fresh just before use
 0.2× SSC (prepare from 20× stock; *APPENDIX 2A*) containing 0.1% (w/v) SDS
 0.02× SSC (prepare from 20× stock; *APPENDIX 2A*) containing 0.1% (w/v) SDS
 0.01× SSC (prepare from 20× stock; *APPENDIX 2A*) containing 0.1% (w/v) SDS
 Color buffers 1, 2, 3, and 4 (see recipes)
 25-cm² tissue culture flasks
 Screw-cap microcentrifuge tubes
 Styrofoam box
 Nytran nucleic acid and protein transfer membrane, pore size 0.45 μm (Schleicher & Schuell)
 Plastic bags for hybridization
 Hybridization oven
 95°C water bath or heating block
 Additional reagents and equipment for DNA extraction (*APPENDIX 1G*) and agarose gel electrophoresis (*APPENDIX 1N*)

NOTE: This protocol is a modified Hirt procedure (Gluzman and Van Doren, 1983). For steps 1 to 4, make sure that all solutions are disposed of in 1% Virkon and that all plasticware is autoclaved before disposal.

Extract DNA from recombinant adenovirus-infected cells

1. Culture a 25-cm² flask of 293 cells to 70% to 80% confluency and then inoculate with the recombinant adenovirus at a multiplicity of infection (MOI) of 3 in 5 ml of fresh 293 cell medium and return the flask to the incubator.
2. When the cells become rounded (after ~40 hr), collect them by gently tapping on the side of the flask and subsequently pipeting the cell/medium suspension up and down.
This is an earlier harvest than when growing up recombinant adenoviruses, as the quality of the DNA is better if the cells are harvested before the monolayer completely detaches.
3. Pellet the cells by centrifuging 10 min at 300 × g, 4°C, rinse the cells with D-PBS, and pellet the cells again by centrifuging 10 min at 300 × g, 4°C.
4. Decant the supernatant and resuspend the pellet in 400 μl virion lysis buffer. Transfer to a screw-cap microcentrifuge tube and incubate for 2 hr at 37°C.
5. Add 100 μl of 5 M NaCl and store the tube overnight in an ice/water mixture in a Styrofoam box (ideally this should be kept refrigerated).
6. Centrifuge 1 hr at 15,000 × g, 4°C, and harvest the supernatant.
7. Extract residual proteins in the supernatant with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol, then centrifuge 15 min at 15,000 × g, 4°C.

APPENDIX 1G provides additional details on DNA extraction.

8. Remove the aqueous layer. Precipitate the DNA by adding 2 vol of 100% ethanol and incubate for 1 hr at -70°C or overnight at -20°C.

9. Centrifuge 10 min at $15,000 \times g$, room temperature. Carefully remove the supernatant using a pipet and discard.
10. Wash the pellet in 1 ml 70% ethanol. Centrifuge 15 min at $15,000 \times g$, room temperature, and discard the supernatant. Allow the DNA pellet to air dry.
11. Resuspend the DNA pellet in 20 μ l sterilized distilled water containing 0.1 mg/ml RNase Type I-A and incubate for 30 min at 37°C. Store the resuspended DNA at -20°C for up to several years (Puntel et al., 2006).

Characterize recombinant adenovirus by Southern blot

12. Label ssDNA probe with digoxigenin-dUTP using the kit and instructions supplied by Roche.

Aim to make a probe no larger than 2000 bp which encompasses at least part of the promoter/transgene sequence that has been inserted into the recombinant adenovirus.

13. Using suitable restriction enzymes, digest the purified recombinant adenovirus DNA (from step 11), the original adenoviral vector, (i.e., pJM17 or pBHG10) as a negative control, and the shuttle plasmids containing the transgene to use as a positive control.
14. Electrophorese the three samples along with a DNA ladder on a 1% agarose gel containing ethidium bromide until all the bands have separated and are clearly visible (Fig. 4.23.5).

For information on agarose gel electrophoresis, see APPENDIX 1N.

15. Note the relative positions of the DNA ladder fragments and the fragments of the recombinant adenovirus and its controls by photographing the gel or by measuring the gel directly on an UV illuminator.
16. Rinse the gel with distilled water.

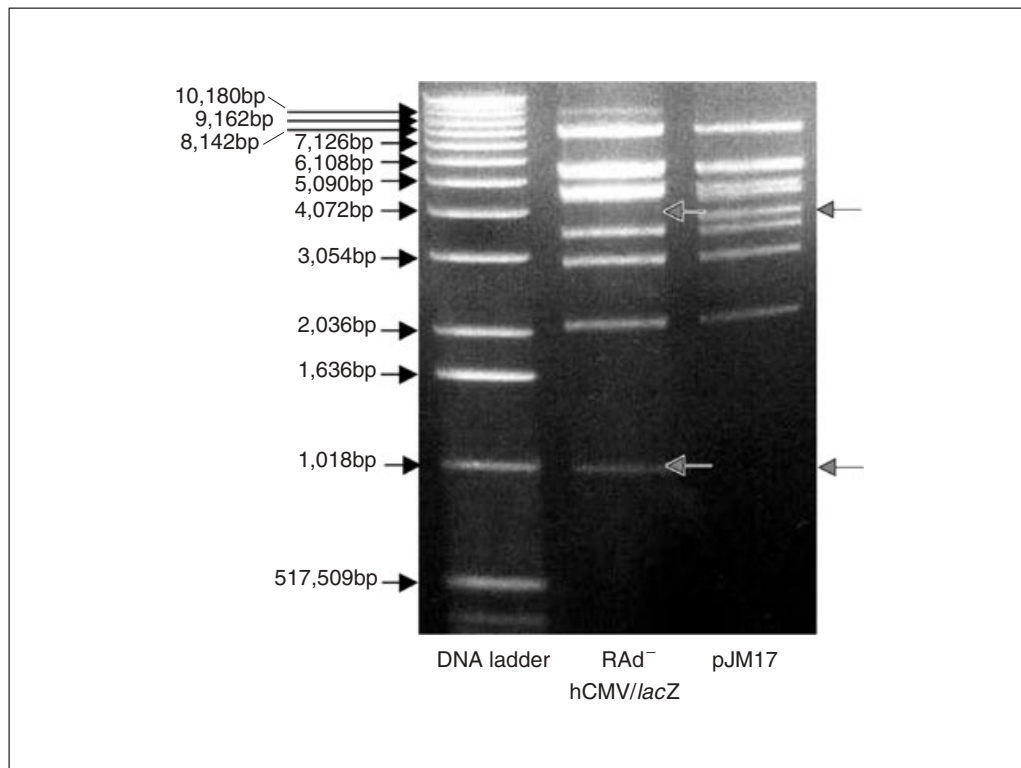


Figure 4.23.5 *Hind*III digest of pJM17 and Ad-hCMV/*lacZ*. After digestion with *Hind*III at 37°C, the samples were run on a 1% agarose gel containing ethidium bromide. Note the different band patterns between pJM17 and Ad-hCMV/*lacZ*, indicated by the shaded arrows.

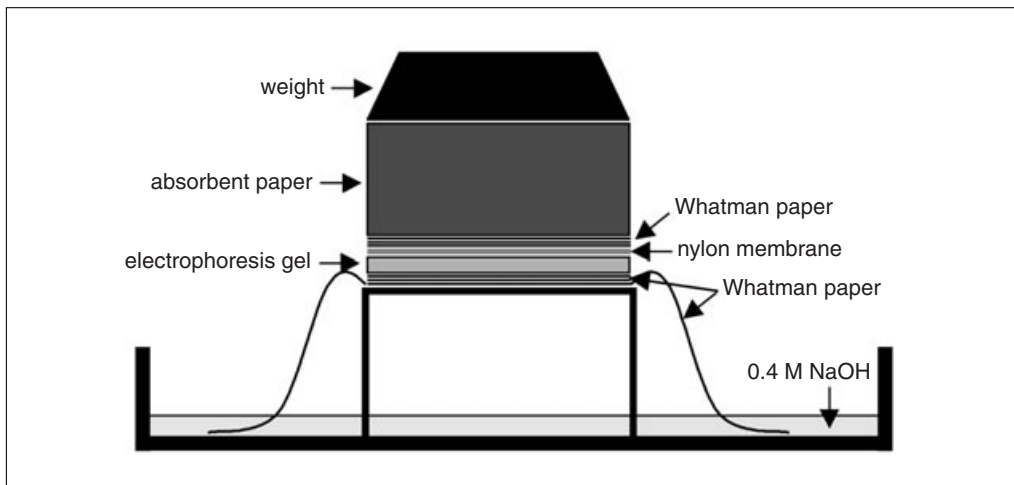


Figure 4.23.6 Transfer of DNA to nylon membrane.

17. Depurinate the DNA by soaking the gel for 30 min in 10 vol of 0.25 M HCl with gentle shaking.

Depurination causes nicking of the DNA, allowing the fragments to pass easily through the gel during transfer.

18. Rinse the gel once with distilled water for 2 min.
19. Denature the DNA by soaking the gel twice in 5 vol of 0.4 M NaOH for 10 min each time with gentle shaking.
20. Meanwhile, prepare the Nytran nucleic acid and protein transfer membrane (sterile filter pore size 0.45 μm), following the manufacturer's instructions.
21. Prepare the blotting stack as shown in Figure 4.23.6, being careful to ensure that no air bubbles are trapped between layers, as this can significantly affect the success of the DNA transfer. Allow transfer to proceed overnight.
22. Carefully remove the nylon membrane and wet it in $0.5\times$ SSC, then place it in a plastic bag containing prehybridization solution, making sure there is enough prehybridization solution to cover the membrane completely. Seal the bag so it is airtight, with no bubbles, then incubate for 1 hr at 68°C .
23. Heat denature the probe for 10 min at 95°C and then immediately place it on ice.
24. Open the bag containing the membrane and exchange the prehybridization solution for fresh hybridization solution containing 5 μl of probe/ml of hybridization solution. Seal the bag again and hybridize overnight at 68°C .

Hybridization solution and the probe can be reused up to four times over several months before discarding. Store at -20°C when not in use.

25. Remove the membrane from the bag and wash three times (5 min/wash) in $0.2\times$ SSC containing 0.1% SDS at room temperature.
26. Wash twice (5 min/wash) in $0.02\times$ SSC containing 0.1% SDS at room temperature.
27. Wash for 30 min in $0.01\times$ SSC containing 0.1% SDS at 65°C .
28. Wash membrane for 2 min in color buffer 1.
29. Block membrane by incubating for 30 min in color buffer 2 in a sealed plastic bag, with no bubbles.
30. Remove the membrane from the bag and wash for 2 min in color buffer 1.

31. Dilute AP-conjugated anti-digoxigenin antibody (from the Dig DNA kit) 1:5000 in color buffer 1 and incubate it with the membrane in a sealed plastic bag, with no bubbles, for 30 min at room temperature.
32. Remove membrane from bag and wash twice for 15 min with color buffer 1 to remove unbound antibody conjugate.
33. Equilibrate membrane 2 min in color buffer 3.
34. Incubate membrane in freshly prepared color substrate solution (from the Dig DNA kit) in a sealed bag in the dark, again with no bubbles. Do not shake or mix solution while it is developing.

A color precipitate should start to form within a few minutes and is likely to be complete in 12 hr. Occasionally it may be necessary to allow color development for as long as 24 hr.
35. When the desired level of color is reached, stop the reaction by removing the membrane from the bag and washing it in color buffer 4 for 5 min.
36. Photograph, scan, or photocopy the membrane while wet, as the color will fade slightly when dried.

**SUPPORT
PROTOCOL 2**

PCR ANALYSIS OF RECOMBINANT ADENOVIRAL DNA

This protocol requires two separate PCR reactions, amplifying first the expression cassette and then a region of the adenovirus type 5 genomic DNA. The design of primer pairs should encompass unique sections of the expression cassette and the adenoviral genome. Shown below are unique pairs of primers for the IVa2 transcription unit of the adenoviral type 5 genome present in both recombinant and wild-type adenovirus, located at 4071 to 4090 bp and 4738 to 4757 bp, which produce a PCR product of 0.69 kb, and the E1B transcription unit of the adenoviral type 5 genome present only in wild-type (replication competent) adenovirus, which produce a PCR product of 0.56 kb (Dewey et al., 1999).

Materials

Primers for IVa2:

Forward: 5'-AAGCAAGTGTCTTGCTGTCT-3'

Reverse: 5'-GGATGGAACCATTATAACCGC-3'

Primers for E1B:

Forward: 5'-CAAGAATCGCCTGCTACTGTTGTC-3'

Reverse: 5'-CCTATCCTCCGTATCTATCTCCACC-3'

2% agarose gel (APPENDIX 1N)

Additional reagents and equipment for PCR and agarose gel electrophoresis
(APPENDIX 1N)

NOTE: With all PCR experiments, it is essential that samples and reagents be kept as clean as possible to minimize contamination and DNA degradation. Laboratory coats and clean latex gloves should be worn routinely and procedures to prepare the PCR samples should be undertaken in a laminar flow cabinet.

1. Dilute the recombinant viral DNA, shuttle plasmid, and adenoviral genomic vector, i.e., pJM17 or pBHG10, to a concentration of 100 ng/ml with sterile distilled water in separate tubes.

The shuttle vector provides the positive control for the expression cassette. The vector containing the adenovirus genome provides a positive control for the adenovirus genomic region to be amplified and a negative control for the expression cassette. The recombinant viral DNA should be positive for the transcription unit and also the adenovirus genomic region (see Fig. 4.23.7).

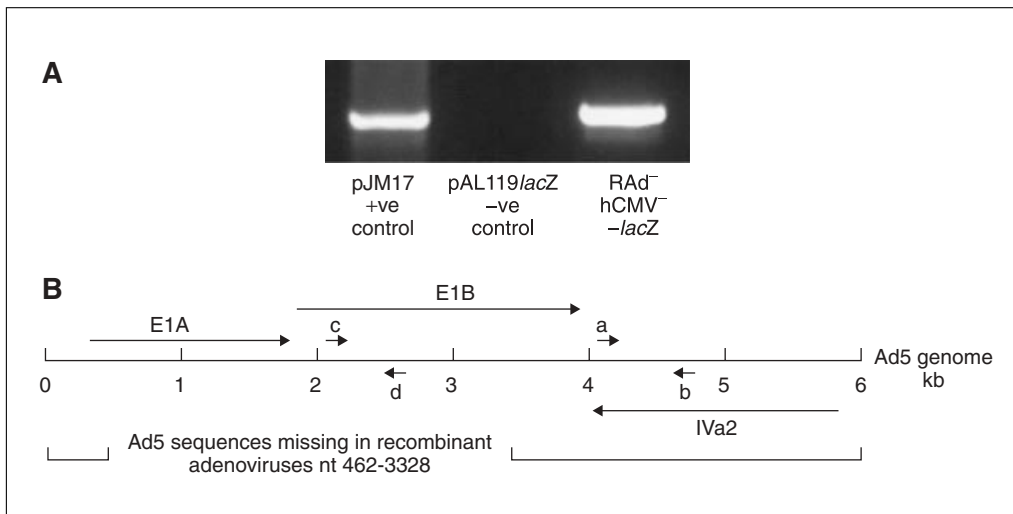


Figure 4.23.7 PCR detection of the IVa2 transcription unit from the Ad5 genomic sequence. **(A)** The extracted DNA from Ad-hCMV//lacZ was tested for the presence of the IVa2 transcription unit by PCR. **(B)** Schematic of first 6000 bp of the adenoviral type 5 genome showing the sites at which the primer pairs amplify their respective transcription units and the sequences removed by recombination. Primer pair (a,b) refer to the IVa2 transcription unit, while primer pair (c,d) refer to the E1B transcription unit.

2. In a sterile PCR tube add the following components (100 μ l final volume):

- 2 μ l 10 mM dNTPs
- 5 μ l 20 ng/ml first primer of pair (final concentration 1 ng/ml)
- 5 μ l 20 ng/ml second primer of pair (final concentration 1 ng/ml)
- 10 μ l 10 \times Taq DNA polymerase buffer
- 1 U Taq DNA polymerase
- 1 μ l 100 ng/ μ l template
- 4 μ l 25 mM MgCl₂ (1 mM final)
- 72 μ l sterile H₂O.

3. Overlay reaction mixture with 60 μ l mineral oil and then place tube in thermal cycler.

4. Carry out PCR amplification (the following example of a program is for the adenoviral type 5 genomic DNA primers under Materials, above):

1 cycle:	5 min	94°C	(initial denaturation)
	30 sec	56°C (IVa2) or 57°C (E1B)	(annealing)
	1 min	72°C	(extension)
35 cycles:	30 sec	94°C	(denaturation)
	30 sec	56°C (IVa2) or 57°C (E1B)	(annealing)
	1 min	72°C	(extension)
1 cycle:	10 min	72°C	(final extension)
	indefinite	4°C	(hold).

The PCR cycle will have to be optimized to the primers used.

5. Run 10 μ l of the PCR product on a 2% agarose electrophoresis gel.

The rest of the PCR product can be stored for several years at -20°C. An example of the PCR product generated by the above reaction is shown in Figure 4.23.7.

**QUALITY CONTROL OF RECOMBINANT ADENOVIRUS VECTORS:
ASSAYS FOR (1) LIPOPOLYSACCHARIDE CONTAMINATION,
(2) REPLICATION-COMPETENT VIRUS, AND (3) DETERMINATION
OF PURITY**

One of the main problems with the standard strategies for Ad vector generation and large-scale vector production is the inadvertent generation of E1-positive replication-competent adenoviruses (RCA). RCA contamination is detrimental for several reasons. RCA can direct high levels of expression of late viral genes that are sufficient to induce an immune response when administered *in vivo*. In addition, RCA can act as a “helper” for replication of E1-deleted vectors, potentially increasing the effective dose of the vector, as well as its mobilization throughout the target cells/tissue.

In culture, contaminating RCA can be generated by recombination between the vector and the E1 sequences, extending from nucleotide 1 to 4344 (Louis et al., 1997), carried by the complementing 293 cell line. The amount of RCA in a virus preparation can then be amplified, particularly if the RCA has a significant growth advantage over the recombinant vector during propagation (Lochmuller et al., 1994). To reduce RCA generation, alternative E1-complementing cell lines have been derived. Some of these are discussed in the Commentary (see Critical Parameters and Troubleshooting).

Another approach to reduce the occurrence of RCA involves the modification of the vector backbone by deletion or rearrangement of the pIX gene—which is colinear with E1B—thereby reducing the frequency of recombination between E1 sequences in 293 cells and those in the adenoviral vector (Krougliak and Graham, 1995; Hehir et al., 1996). An alternative method is the insertion of a DNA “stuffer” sequence into the E3 region of the vector, reducing the generation of RCA since recombination between the “stuffed” vector and the E1 sequences in 293 cells yields a virus too large to be packaged (Parks et al., 1996).

Another major source of concern is contamination of the viral preparation with lipopolysaccharide (LPS). The source of LPS could be any material/reagent used in the preparation of the viral stocks. In sufficient levels, LPS is able to elicit an immune response in its own right when administered *in vivo*, thereby altering the host response to the recombinant adenoviral vector. It is therefore crucial that all viral stocks to be used for *in vitro* and *in vivo* infection be checked for the presence of LPS and RCA.

Materials

- Recombinant adenovirus preparation to be tested
- Limulus Amebocyte lysate pyrogen kit (Cambrex, cat. no. 50-647U)
- HeLa cells (ATCC #CCL-2; ECACC #93021013)
- Maintenance medium (see recipe), prewarmed to 37°C
- Dulbecco’s phosphate-buffered saline (CellGro, VWR, cat. no. 45000-434), prewarmed to 37°C
- Virion lysis buffer (see recipe)
- Virus storage buffer (see recipe)
- 175-cm² flasks
- 15-cm tissue culture dishes
- 37°C incubator
- 24-well tissue culture plate
- 50-ml conical tube
- Screw-cap microcentrifuge tube
- 56°C water bath
- Spectrophotometer

To assess lipopolysaccharide (LPS) contamination

- 1a. Assay levels of LPS using the Limulus Amebocyte lysate test kit, following the manufacturer's instructions.

All vectors which have a LPS value of >2.0 endotoxin units per milliliter are considered positive for LPS contamination.

The Limulus Amebocyte lysate test is a semiquantitative test for Gram-negative bacterial endotoxin. Incubation in the presence of endotoxin causes gelation; in the absence of LPS gelation does not occur.

To assess levels of replication-competent adenovirus contamination in a recombinant adenovirus preparation

This protocol is a modified version of the technique of Dion et al. (1996).

- 1b. Maintain HeLa cells in 175-cm² flask. When cells approach confluency, split 1:3.
- 2b. *Day 1:* When cells approach confluency, seed 3×10^6 HeLa cells in eight 15-cm tissue culture dishes in 20 ml of maintenance medium.
- 3b. *Day 2:* At a time point 24 hr later, count the cells in one of the dishes. Multiply this number by six to obtain the total number of cells on six 15-cm tissue culture dishes. Calculate the amount of virus necessary to infect the total number of cells on six 15-cm tissue culture dishes with an MOI of 30 pfu/cell.
This represents a test dose of 1×10^9 pfu.
- 4b. Prepare 60 ml of inoculation solution by adding the amount calculated in the previous step to 60 ml maintenance medium.
- 5b. Aspirate the medium from six of the 15-cm tissue culture dishes and infect each dish with 10 ml of the inoculation solution prepared in the previous step. For the negative control, add 10 ml of fresh maintenance medium only. Incubate 6 hr at 37°C.
- 6b. At a time point 6 hr later, add an additional 10 ml maintenance medium to each dish and return to the incubator.
The total volume in each dish will be 20 ml.
- 7b. *Day 4:* At a time point 48 hr after infection, seed each well of a 24-well plate with 5×10^4 HeLa cells and incubate at 37°C in maintenance medium.
- 8b. *Day 6:* At a time point 72 hr after infection, harvest the supernatant from the six 15-cm dishes and pellet the detached cells by centrifuging 15 min at $300 \times g$, 4°C. Keep the supernatant from each dish separate in a 50-ml conical tube.
- 9b. Carefully remove the medium from the HeLa cells in the 24-well plate.
- 10b. Add 300 μ l of each of the seven supernatants harvested from step 8 to 24-well plate (from step 8b) in three separate wells (triplicate). This represents a total of 21 wells in the 24-well plate that will be infected. Overlay the remaining 3 wells of the 24-well plate with 300 μ l maintenance medium.
- 11b. Incubate 6 hr, then overlay with 700 μ l prewarmed maintenance medium to obtain a final volume of 1 ml medium per well. Resume incubation.
- 12b. *Day 9:* At a time point 72 hr later, add 1 ml fresh, prewarmed maintenance medium to each well.

Plaques will appear in replication-competent adenovirus contaminated cultures within 6 days post-medium replacement in the 24-well plate. Monitor daily. If medium becomes acidic, add additional medium.

To determine purity of recombinant adenovirus preparation

This approach is based on the well characterized absorption of pure adenovirus at 260 nm and 280 nm (Burlingham et al., 1974; Liebermann and Mentel, 1994; Ng and Graham, 2002). An A_{260} of 1.0 corresponds to a viral particle concentration of $1.1 \times 10^{12}/\text{ml}$, and the A_{260}/A_{280} ratio for pure virus is 1.3 (Burlingham et al., 1974). The A_{260}/A_{280} ratio is decreased for defective particles, which contain less DNA, and is increased by impurities in the preparation, notably cellular RNA.

- 1c. Thaw a 10- μl aliquot of the recombinant virus and dilute 1:20 to 1:50 in virion lysis buffer in a screw-cap microcentrifuge tube to make a final volume of 50 μl .
- 2c. In a second tube, make an equivalent dilution of virus storage buffer in a final volume of 50 μl of virion lysis buffer in a separate tube to serve as a negative control.
- 3c. Incubate for 10 min at 56°C.
- 4c. Turn on UV light in UV spectrophotometer during incubation.
- 5c. Blank the UV spectrophotometer with the entire 50 μl from step 2c.
- 6c. Read the entire 50 μl of each treated sample from step 1c at OD_{260} and OD_{280} nm.
- 7c. Calculate the virus particle concentration (see Equation 4.23.1) and the 260:280 ratio (see Equation 4.23.2).

$$260 : 280 = \text{OD}_{260} / \text{OD}_{280}$$

Equation 4.23.1

$$\text{vp/ml} = \text{OD}_{260} \times \text{dilution factor} \times 1.1 \times 10^{12}$$

Equation 4.23.2

- 8c. Calculate the ratio of defective to infectious viral particles (i.e., virus particles/plaque-forming unit as assayed by serial dilution endpoint; see Equation 4.23.3).

$$\text{vp:pfu} = \frac{\text{vp/ml}}{\text{pfu/ml}}$$

Equation 4.23.3

The particle/infectious unit ratio of wild-type Ad5 is 20. Packing of recombinant genomes is less efficient than wild-type.

BASIC PROTOCOL 2

INFECTION OF NEURONAL AND GLIAL CELLS IN PRIMARY CULTURE

At a low multiplicity of infection (i.e., low number of infectious viral particles per cell) replication-deficient adenoviruses can promote efficient transgene expression with no detectable cytotoxicity or breakthrough to early/late phase adenovirus gene expression. Promoters such as the major immediate early human cytomegalovirus promoter have been shown to induce extremely high levels of transgene expression (up to 35% of the total cell protein; Wilkinson and Akrigg, 1992). However, such overexpression of transgenes can in itself be toxic. For example, at $\text{MOI} > 100$, the recombinant adenovirus containing the herpes simplex virus gene thymidine kinase has been shown to have a toxic effect

in some cell types due to high level expression of its transgenic protein (Windeatt et al., 2000).

At even higher MOIs, i.e., 500 to 1000, toxicity can be associated with the virion penton-base protein. There may also be a breakthrough to adenovirus early/late gene expression. Breakthrough can result in the triggering of a cellular stress response, such as activation of NF κ B, which is associated with the induction of a cell-mediated response to adenovirus proteins.

An advantage of using recombinant adenovirus is that multiple infections within the same cell with different recombinant vectors are possible. For example, the authors currently use a two-recombinant-cell type-specific and regulatable vector system that requires both vectors to infect the same cell for the system to work (Smith-Arica et al., 2000). This may also be useful in evaluating interactions between transgenes (e.g., certain receptor subunits and associated G proteins).

Materials

Frozen virus (Basic Protocol 1)

1% Virkon

Plating medium (see recipe) or VM culture medium (see recipe), prewarmed to 37°C

Neocortical glial culture (see Support Protocol 6)

Neocortical neuronal culture (see Support Protocol 4)

Ventral-mesencephalic (VM) culture (see Support Protocol 5)

Dulbecco's phosphate-buffered saline (D-PBS; CellGro, VWR, cat. no. 45000-434), prewarmed to 37°C

37°C water bath

Autoclave

1. Remove the viral aliquot(s) from the freezer and quickly thaw in a 37°C water bath until the ice just disappears, and then place on ice.
2. Move reagents to a suitable laminar flow cabinet. Prepare a beaker of 1% Virkon for disposal of contaminated plastic and liquids.
3. Dilute the virus in a minimum volume of plating medium or VM culture medium to the appropriate virus dose (MOI). For a 35-mm well, do not exceed a volume of 0.7 to 1 ml.
4. Place the inoculum on ice. Just prior to infection (in step 5), gently warm it to 37°C.
5. Carefully remove all medium from the wells containing neuronal or glial cells in culture (prepared in Support Protocols 4, 5, or 6), and gently wash the cells with prewarmed D-PBS. Remove the wash and then gently add the warmed inoculum to the culture.
6. Return the plate to the incubator and gently rock the plates every 15 min for the next 2 hr to circulate the virus.
7. After 12 hr, adjust the volume of each well to 2 ml with prewarmed plating medium or VM culture medium.
8. Sterilize all items that have been in contact with the recombinant adenovirus by autoclaving.

Cultures can be processed to assess transgene expression or other physiological properties 48 to 72 hr post-infection (as described in Support Protocols 4 through 7).

**PREPARATION OF LOW-DENSITY PRIMARY NEOCORTICAL
NEURONAL CULTURES**

These cultures will survive for 5 to 9 days, but if long-term maintenance is required of the cultures, the neurons can be co-cultured with glia, as described in the Commentary (see Critical Parameters and Troubleshooting). The authors recommend that the investigator consult an atlas of the developing rat nervous system before attempting this protocol (Paxinos et al., 1994).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

Pregnant rat (E17-18)
70% ethanol
Hanks' balanced salt solution, calcium- and magnesium-free (CMF-HBSS; CellGro, VWR, cat. no. 45000-458), ice-cold (sterile)
1 M HEPES buffer solution (CellGro, VWR, cat. no. 45000-690)
0.05% (w/v) trypsin/0.02% (w/v) EDTA (CellGro, VWR, cat. no. 45000-082)
Plating medium (see recipe), prewarmed to 37°C

Sterile dissecting equipment
Petri dishes
Dissection microscope (e.g., Leica)
15- and 50-ml polypropylene conical tubes
37°C incubator
Sterile flame-polished glass Pasteur pipets
70- μ m nylon cell strainer
35-mm well diameter tissue culture plates
Poly-L-lysine coated 22-mm² glass coverslips (see recipe)

Additional reagents and equipment for counting cells using a hemacytometer and mammalian cell tissue culture (*APPENDIX 3B*)

Perform dissection

1. Sacrifice a pregnant rat (E17-18) using an accepted procedure and disinfect the animal's fur with 70% ethanol.
2. Using sterile dissection tools, make a large opening in the skin of the abdomen, leaving the abdominal wall intact.
3. Use fresh sterile tools to open a large area of the abdominal wall and reveal the embryos in the uterus.
4. Prepare ice-cold sterile CMF-HBSS containing 10 mM HEPES (add from 1 M HEPES stock). Remove both horns of the uterus, move to a laminar flow hood, and place each horn into a petri dish containing the ice-cold CMF-HBSS/10 mM HEPES.
5. Remove the embryos from the uterus, decapitate them, and place the heads in a fresh petri dish with ice-cold CMF-HBSS/10 mM HEPES.
6. With the aid of a dissecting microscope, use fine curved forceps to remove the embryo's nose, and then peel away the skin layer to reveal the top of the skull.
7. Using the point of the forceps, gently open the skull, remove the brain, and transfer it to a petri dish containing ice cold CMF-HBSS/10 mM HEPES; repeat this procedure for all the embryos.

This can be done in the same petri dish.

8. Remove the meninges from the brains and dissect out the cortex, cutting it into pieces of $\sim 1 \text{ mm}^3$.

Prepare tissue pieces

9. Transfer the tissue pieces to a sterile 15-ml polypropylene conical tube, add 150 μl of 0.05% trypsin/0.02% EDTA, and adjust volume to 15 ml with fresh CMF-HBSS/10 mM HEPES to aid dispersion and avoid formation of clumps among the cells.
10. Incubate for 15 min at 37°C. Mix by inversion every 5 min.
11. Allow the tissue to settle and remove as much of the medium as possible. Add fresh HBSS/10 mM HEPES to a total volume of 15 ml and leave for 5 min at room temperature. Repeat three times. Resuspend the last wash in 15 ml of plating medium, invert three times and allow tissue to settle.
12. Remove all but 5 ml of plating medium. Dissociate the tissue by triturating in the remaining medium using sterile glass flame-polished 5-ml Pasteur pipets. Once the suspension is homogenous, adjust volume to 15 ml with prewarmed plating medium.
13. Filter through a 70- μm nylon cell strainer into a 50-ml polypropylene conical tube.

Count cells

14. Count cells using a hemacytometer (*APPENDIX 3B*) and then adjust the density to 1×10^6 per ml with prewarmed plating medium.
15. Add 1 ml of the neuronal suspension to individual wells of a 35-mm well diameter tissue culture plate, each containing a poly-L-lysine-coated 22- mm^2 coverslip. Place in incubator overnight.
16. Remove cellular debris by replacing the medium with 1 ml of fresh prewarmed plating medium.

Twelve embryos should yield 36 wells plated at 1×10^6 cells/well. The cells will take a couple of days to establish a neuronal-like morphology and become suitable for further studies. Refrain from changing the medium too frequently, as this will promote the growth of contaminating non-neuronal cells such as glia and fibroblasts. The neuronal suspension may alternatively be plated onto 35-mm diameter tissue culture wells which have been coated in poly-L-lysine.

PREPARATION OF LOW-DENSITY PRIMARY VENTRAL-MESENCEPHALIC CULTURES

This procedure details the preparation of low-density primary ventral-mesencephalic cultures containing tyrosine hydroxylase immunoreactive neurons. The authors recommend that the investigator consult an atlas of the developing rat nervous system before attempting this protocol (Paxinos et al., 1994).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

- Pregnant rat (E14)
- 70% ethanol
- Hanks' balanced salt solution, calcium- and magnesium-free (CMF-HBSS; CellGro, VWR, cat. no. 45000-458), ice-cold (sterile)
- 1 M HEPES buffer solution (CellGro, VWR, cat. no. 45000-690)
- 0.05% (w/v) trypsin/0.02% (w/v) EDTA (CellGro, VWR, cat. no. 45000-082)
- VM culture medium (see recipe)

SUPPORT PROTOCOL 5

Gene Cloning, Expression, and Mutagenesis

4.23.23

Sterile dissecting equipment
Petri dishes
Dissection microscope (e.g., Leica)
29-G, 0.5-in. needles
15-ml conical polypropylene tube
Sterile flame-polished glass Pasteur pipets
70- μ m nylon cell strainer
22-mm well diameter tissue culture plates
Poly-L-ornithine and laminin coated 16-mm diameter glass coverslips (see recipe)
Additional reagents and equipment for counting cells using a hemacytometer and mammalian cell tissue culture (*APPENDIX 3B*)

Perform dissection

1. Sacrifice a pregnant rat (E14) using an accepted procedure and disinfect the animal's fur with 70% ethanol.
2. Using sterile dissection tools, make a large opening in the skin of the abdomen, leaving the abdominal wall intact.
3. Use fresh sterile tools to open a large area of the abdominal wall to reveal the embryos in the uterus.
4. Prepare ice-cold sterile CMF-HBSS containing 10 mM HEPES (add from 1 M HEPES stock). Remove both horns of the uterus, move to a laminar flow hood, and place each horn into a petri dish containing the ice-cold CMF-HBSS/10 mM HEPES.
5. Remove the embryos from the uterus.
6. With the aid of a dissecting microscope, remove the fetal brain, isolate the brainstem, and remove the meninges.

The mesencephalic flexure is deeply curved at this stage of development.

7. Make a cut at the junction of the diencephalon and mesencephalon using the prominent diencephalic protuberance as a point of reference.
8. Using 29-G, 0.5-in. needles as micro-knives, use one needle to steady the tissue, position the brainstem on its dorsal surface, and split the tectum medially through the ventricular opening.
9. Cut away the most rostral portions of the ventral brainstem and the tectum to leave the medial portion of the remaining rostral brainstem ($\sim 1 \text{ mm}^3$).
10. Dissect out the ventral mesencephalon from each embryo and place the dissected ventral-mesencephalon into a 15-ml polypropylene tube containing 5 ml of CMF-HBSS/10 mM HEPES on ice.
11. Incubate the tissue with 50 μ l 0.05% trypsin/0.02% EDTA for 15 min at 37°C, mixing by inversion every 5 min. Then, rinse the tissue three times for 5 min per wash with sterile CMF-HBSS/10 mM HEPES.
12. Remove the HBSS and add 5 ml of fresh cold VM culture medium.
13. Gently disperse the cells by triturating using sterile flame-polished glass Pasteur pipets, then filter through a sterile 70- μ m nylon cell strainer.

Count cells

14. Count cells using a hemacytometer (*APPENDIX 3B*), then adjust volume to 2×10^5 cells/500 μ l with fresh, 37°C VM culture medium.

15. Add 500 μ l of the cell suspension to individual wells of a 22-mm-well-diameter plate, each containing a previously prepared 16-mm diameter glass coverslips coated with poly-L-ornithine and laminin and place the plate in an incubator overnight.
16. Replace the medium with 500 μ l of fresh prewarmed VM culture medium to remove any cellular debris.

Twelve embryos should yield ~48 wells if plated at this density.

17. Replace the medium twice the following day, using fresh prewarmed VM culture medium to remove cellular debris.

Tyrosine hydroxylase-positive neurons can be identified by immunocytochemistry after 1 to 3 days in culture (see Support Protocol 7).

PREPARATION OF NEOCORTICAL GLIAL CULTURES

This protocol will produce a glial culture enriched in astrocytes and follows a protocol almost identical to the above procedure for preparing neocortical neuronal cultures (Support Protocol 4), except that 0- to 3-day postnatal rat pups are used. Low numbers of cortical oligodendrocytes do grow within the astrocytic cultures. Detailed protocols are available for obtaining pure oligodendrocyte cultures (Noble and Mayer-Proschel, 1998).

The authors recommend that the investigator consult an atlas of the developing rat nervous system before attempting this protocol (Paxinos et al., 1994).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

- 0- to 3-day-old rat pups
- 100% ethanol
- Hanks' balanced salt solution, calcium- and magnesium-free (CMF-HBSS; CellGro, VWR, cat. no. 45000-458), ice-cold (sterile)
- 1 M HEPES buffer solution (CellGro, VWR, cat. no. 45000-690)
- Plating medium (see recipe), prewarmed to 37°C
- Sterile dissecting equipment, including fine curved forceps
- Dissection microscope (e.g., Leica)
- 15- and 50-ml conical polypropylene tubes
- Sterile flame-polished glass Pasteur pipets
- 70- μ m nylon cell strainer
- Centrifuge
- 35-mm well diameter tissue culture plates
- Poly-L-ornithine/laminin-coated 22-mm² coverslips (see recipe)
- Additional reagents and equipment for counting cells using a hemacytometer and mammalian cell tissue culture (*APPENDIX 3B*)

Perform dissection

1. Sacrifice 0- to 3-day-old rat pups according to an accepted procedure, and then immerse them in 100% ethanol in a laminar flow cabinet and decapitate with a sterile scalpel blade.
2. Prepare ice-cold sterile CMF-HBSS containing 10 mM HEPES (add from 1 M HEPES stock). Place the heads in CMF-HBSS/10 mM HEPES.

SUPPORT PROTOCOL 6

3. Hold the head with curved forceps inserted into the eye sockets and using a fine pair of scissors make a cut into the skull at the base of the skull and open it to reveal the brain.
4. Remove the brain, carefully remove the meninges with the aid of a dissecting microscope, and then dissect out the cortex and cut it into 1-mm³ pieces using sterile curved forceps.
5. Place the tissue fragments in 5 ml of fresh ice-cold CMF-HBSS/10 mM HEPES in a 15-ml polypropylene conical tube.
6. Dissociate the tissue by triturating using flame-polished glass Pasteur pipets until no clumps are visible.
7. Adjust volume to 15 ml with CMF-HBSS/10 mM HEPES and pass through a 70- μ m nylon cell strainer into a 50-ml polypropylene conical tube.
8. Centrifuge for 15 min at $300 \times g$, 4°C, with no brake.

Count cells

9. Remove supernatant, resuspend in 10 ml plating medium, and count cells using a hemacytometer (*APPENDIX 3B*).
10. Adjust density to 1×10^6 cell/ml with plating medium and place 1 ml of the cell suspension in individual wells of a 35-mm well diameter tissue culture plate, each containing a poly-L-ornithine/laminin-coated 22 \times 22-mm coverslip.
11. Add an additional 1 ml of plating medium and transfer to incubator.
12. Continue incubation, feeding the cells every 3 to 4 days.

Cells should reach confluency after 1 to 2 weeks. Twelve pups should yield at least 36 wells when seeded at 1×10^6 cells/well. It is possible to passage the cells, but this will also increase the number of fibroblasts that contaminate the culture.

SUPPORT PROTOCOL 7

THREE ASSAYS TO DETECT TRANSGENE EXPRESSION WITHIN INFECTED NEURONAL AND GLIAL CELLS IN PRIMARY CULTURE: (1) Xgal STAINING, (2) FLUORESCENCE IMMUNOCYTOCHEMICAL STAINING, AND (3) FACS ANALYSIS

This protocol describes three methods for identifying transgenes expressed by recombinant adenoviruses. The Xgal histological staining of infected cultures is used only for virus expressing the marker transgene *E. coli* β -galactosidase. The fluorescence immunocytochemical and fluorescence-activated cell sorting (FACS) procedures can be used for all other transgenes, if suitable antibodies are available. Immunocytochemistry allows the investigator the opportunity to monitor the expression of the transgene morphologically within specific cell types. Fluorescence-activated cell sorting of cultures allows the investigator to combine the identification of individual cell types infected by the recombinant viruses with semiquantitative analysis of the level of transgene expression.

Materials

- Infected cells in primary culture
- Dulbecco's phosphate-buffered saline (D-PBS; CellGro, VWR, cat. no. 45000-434), prewarmed to 37°C
- 1% Virkon
- 4% paraformaldehyde solution (see recipe)
- Phosphate-buffered saline (PBS; see recipe)
- 0.3% (v/v) Triton X-100 in PBS

Xgal staining solution (see recipe) containing 1 mg/ml 5-bromo-4-chloro-3-indoyl β -D-galactopyranoside (Xgal; Sigma) in dimethyl sulfoxide (DMSO)
Tris-buffered saline (TBS; see recipe)
Mowiol 4-88 mounting solution (see recipe)
10 mM sodium citrate, pH 6.0
0.5 mg/ml sodium borohydride
50 mM ammonium chloride
10% and 1% (v/v) normal blocking serum (preferably from species in which secondary antibody was raised) in PBS
Primary antibody
Fluorescent secondary antibody labeled with Alexa-Fluor 488 or Alexa-Fluor 488 (Molecular Probes)
Pro-Long Gold Anti-Fade Reagent (Molecular Probes, cat. no. P36930)
0.05% (w/v) trypsin/ 0.02% (w/v) EDTA (CellGro, VWR, cat. no. 45000-082)
Plating medium (see recipe), ice-cold
FACS staining buffer (see recipe)
FACS permeabilizing buffer (see recipe)
Parafilm
Humidified chamber (e.g., sealable box containing a damp tissue)
Fine forceps
12-well plate
Glass slides
Epifluorescence microscope
35-mm tissue culture plates
Polypropylene centrifuge tubes suitable for fluorescence-activated cell sorting (different machines require different tubes so check with FACS manufacturer)
Centrifuge
Fluorescence-activated cell sorter (Becton Dickinson) 15-ml and 50-ml polypropylene conical tubes

Xgal staining of infected cultures

- 1a. Gently aspirate medium from the cells and wash with D-PBS. Dispose of medium and washes in 1% Virkon.
- 2a. Fix the cells for 15 min with 2 ml fresh 4% paraformaldehyde solution.
- 3a. Wash the cells three times, each time for 5 min with 5 ml PBS.
- 4a. Permeabilize cells for 10 min with 2 ml of 0.1% Triton X-100 in PBS.
- 5a. Wash the cells three times, each time for 5 min with 5 ml of PBS.
- 6a. Incubate cells for 3 hr in the dark at 37°C with 2 ml Xgal staining solution containing 1 mg/ml Xgal dissolved in DMSO.
Dissolve Xgal in DMSO at a ratio of 20 mg of Xgal per 500 μ l of DMSO. This stock solution can be stored in the dark at -20°C. However, it is best to always make the solution fresh.
- 7a. Wash cells three times, each time for 5 min with 5 ml PBS, then once for 5 min with 5 ml TBS. Allow to air dry or apply coverslip after addition of 100 to 200 μ l of the Mowiol 4-88 mounting solution.

Fluorescent immunocytochemistry of infected cultures

- 1b. At the appropriate time (usually 2 to 3 days) after infection, working in the laminar hood, wash the coverslips with the infected cell culture with 2 ml of D-PBS, disposing of each wash in 1% Virkon.

- 2b. Fix cells for 15 min with 2 ml fresh 4% paraformaldehyde solution.
- 3b. Wash cells gently three times, each time with 5 ml PBS.

At this point coverslips can be transferred to a 12-well plate for ease of manipulation and washing
- 4b. Permeabilize the cells by incubating for 10 min with 2 ml 0.3% Triton X-100 in PBS.
- 5b. Wash cells three times, each time with 2 ml PBS.
- 6b. Fill well with prewarmed 10 mM sodium citrate buffer (60°C). Allow to cool to room temp on bench (~20 min).
- 7b. Wash cells three times, each time with 2 ml PBS.
- 8b. Add 1 ml of 0.5 mg/ml sodium borohydride. Incubate 10 min.
- 9b. Wash cells three times, each time with 2 ml PBS.
- 10b. Add 1 ml of 50 mM ammonium chloride. Incubate 10 min.
- 11b. Wash cells three times, each time with 2 ml PBS.
- 12b. Incubate for 1 hr with 10% normal blocking serum in PBS.

Try to use the normal serum of the species in which the secondary antibody has been raised to minimize nonspecific staining.
- 13b. Wash briefly with 1% normal blocking serum in PBS.
- 14b. Place primary antibody diluted in 1% normal blocking serum (20 µl per coverslip) onto a strip of clean Parafilm in a humidified chamber and use fine forceps to invert the coverslips over the antibody dilutions.
- 15b. Incubate 4 hr at room temperature or overnight at 4°C.
- 16b. Replace coverslips into a 12-well plate and remove unbound primary antibody thoroughly with five 5-min washes each with 5 ml of 1% normal blocking serum in PBS.
- 17b. Incubate coverslips with 20 µl fluorescent-conjugated secondary antibodies in 1% blocking serum in PBS, using Parafilm as above in a dark humidified box (e.g., the box described in step 14 covered in foil), for 1 hr at room temperature, and perform all subsequent washes under dimmed ambient light.
- 18b. Replace coverslips into a 12-well plate and wash with PBS thoroughly six times, each time for 5 min.
- 19b. Wash briefly in TBS, then mount by inverting onto 20 µl of Pro-Long Gold Anti-Fade reagent on glass slides.
- 20b. Allow to harden at 4°C overnight before viewing under epifluorescence.

Figure 4.23.8 shows examples of neocortical neuronal and glial cultures stained with dye-conjugated antibodies and viewed under epifluorescence.

Fluorescence-activated cell sorting (FACS) analysis

- 1c. Aspirate medium from cells and wash cells gently with D-PBS. Dispose of the medium and wash solutions in 1% Virkon.

A single well from a 6-well plate will generally provide enough cells to run 2 to 3 test samples; however, it is best to use a single well per test.

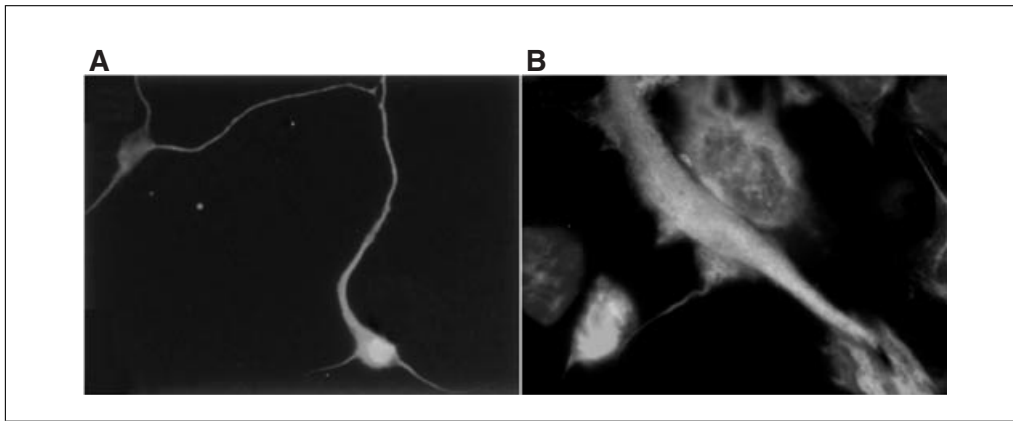


Figure 4.23.8 Neocortical neuronal and glial cultures viewed under epifluorescence. **(A)** Neocortical neuronal culture infected with a Ad encoding β -galactosidase at an MOI of 30. Cell type was identified using a monoclonal mouse anti-MAP-2 (1:1000; Sigma) and Texas red dye-AffiniPure F(ab')₂ fragment donkey anti-mouse IgG (1:100; Jackson). Transgene was identified using a rabbit anti- β -gal (kindly supplied by J. Price) and FITC donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch). **(B)** Neocortical glial culture infected with a Ad encoding HSV1-TK at an MOI of 30. Cell type was identified with a monoclonal mouse anti-GFAP (1:20; Boehringer Mannheim) and Texas red dye-AffiniPure F(ab')₂ fragment donkey anti-mouse IgG (1:100; Jackson ImmunoResearch), and the transgene was identified using a rabbit anti-TK (1:100; kindly supplied by D. Klatzmann), and FITC donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch).

- 2c. To each 35-mm well add 200 μ l of 0.05% (w/v) trypsin/0.02% (w/v) EDTA and incubate for 2 min.
- 3c. Lightly tap the tissue culture plate to dislodge the cells, then add 2 ml of ice-cold plating medium supplemented with 20% FBS to each well.
- 4c. Dispense the cell suspension into a polypropylene centrifuge tube suitable for use on a fluorescence-activated cell sorter and pellet the cells by centrifuging 15 min at $200 \times g$, 4°C.
- 5c. Aspirate all but 100 μ l of the medium from the tube, resuspend the pellet by brushing the bottom of the centrifuge tube along a rack, and add 5 ml of FACS staining buffer.
- 6c. Centrifuge 15 min at $200 \times g$ at 4°C, aspirate and resuspend (as in step 5c), then fix the cells on ice for 10 min in 1 ml 4% paraformaldehyde solution.

A quick vortex immediately after addition of the 4% paraformaldehyde solution to the cells prevents the cells from clumping together, which will cause problems later when the suspension is passed through the fluorescence activated cell sorter. If the cells do clump, however, pass the cell suspension through a 70- μ m nylon cell strainer to eliminate the clumps.
- 7c. Centrifuge the cells 10 min at $500 \times g$, room temperature, aspirate and resuspend (as in step 5c), and then wash cells with 5 ml FACS staining buffer.
- 8c. Centrifuge the cells 10 min at $500 \times g$, room temperature, then aspirate supernatant and resuspend pellet in 5 ml FACS permeabilizing buffer.
- 9c. Incubate 20 min at room temperature then centrifuge the cells 10 min at $500 \times g$, room temperature, and aspirate the supernatant.
- 10c. Resuspend the cells in 100 μ l FACS permeabilizing buffer, add the primary antibody (diluted in FACS permeabilizing buffer) at the appropriate dilution, and mix by gentle vortexing. Incubate 1 hr at room temperature.

- 11c. Add 5 ml FACS permeabilizing buffer, centrifuge 10 min at $500 \times g$, room temperature, decant supernatant and resuspend pellet in 100 μ l FACS permeabilizing buffer.
- 12c. Add fluorescent secondary antibody at the appropriate dilution. Mix by gently vortexing, and incubate for 45 min in the dark at room temperature.
- For fluorescein conjugates use a 1:50 dilution, i.e., 2 μ l neat antibody to 100 μ l resuspended cells; for R-phycoerythrin use 1:25, i.e., 4 μ l neat antibody to 100 μ l resuspended cells. Texas red conjugates are not suitable for FACS.*
- 13c. Add 5 ml FACS permeabilizing buffer, centrifuge as before (step 11c), and resuspend in 1 ml FACS staining buffer.
- 14c. Sort the cells with respect to their size (forward scatter) and granularity (side scatter). Set the level of fluorescent compensation using negative and single-color positive controls before reading the test samples (Morrelli et al., 1999; Cowsill et al., 2000).

Neurons are smaller and less granular than glial cells, and it is therefore easy to distinguish the two cell populations.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

293 cell medium

Minimum essential medium with Earle's salts (CellGro, VWR, cat. no. 45000-384)
10% fetal bovine serum (FBS; Omega Sci., cat. no. FB-01)
1 \times MEM nonessential amino acids (CellGro, VWR, cat. no. 45000-700)
2 mM L-glutamine (CellGro, VWR, cat. no. 45000-676)
1 U/ml penicillin/1 μ g/ml streptomycin (CellGro, VWR, cat. no. 45000-650)
Store up to 1 month at 4°C

All of the above ingredients are manufactured by CellGro/MediaTech and are distributed by VWR with the exception of the fetal bovine serum which is purchased directly from Omega Scientific.

Color buffer 1

100 mM Tris hydrochloride
150 mM sodium chloride
Adjust pH to 7.5 with 1 M NaOH
Store up to 6 months at room temperature

Color buffer 2

At least 1 hr prior to use, dissolve blocking reagent from the Dig DNA labeling and detection kit (Roche) in color buffer 1. Since the reagent does not dissolve rapidly, use mild heat to assist dissolution (50° to 70°C). Store up to 6 months at -20°C .

Color buffer 3

100 mM Tris hydrochloride
100 mM sodium chloride
50 mM magnesium chloride
Adjust pH to 9.5 with 1 M NaOH
Store up to 6 months at room temperature

Color buffer 4

10 mM Tris hydrochloride
1 mM EDTA (*APPENDIX 2A*)
Adjust pH to 8.0 with 1 M NaOH
Store up to 6 months at room temperature

Coverslips, poly-L-lysine or poly-L-ornithine treated

Clean coverslips: Prepare a solution of sulfo-chromic acid by dissolving 100 g potassium dichromate in 850 ml distilled water. Add 50 ml sulfuric acid; allow to cool for several minutes then add an additional 50 ml of sulfuric acid. Adjust volume to 1000 ml with distilled water. Place 22 × 22-mm or 16-mm diameter glass coverslips in a polytetrafluoroethylene (PTFE) rack and wash twice, each time for 10 min in distilled water. Place racks into sulfo-chromic acid for 18 to 36 hr. Wash off all traces of sulfo-chromic acid under running tap water and leave in distilled water for 2 hr. Wash again with distilled water for an additional 2 hr. Wrap the rack in aluminum foil and bake for 1 hr at 180°C, and then allow to cool to room temperature. In a laminar flow cabinet, place coverslips in 35-mm or 22-mm diameter tissue culture plates using sterile fine curved forceps.

Coat cleaned coverslips in 0.1 mg/ml poly-L-lysine solution for neocortical neuronal or glial cultures or 0.1 mg/ml poly-L-ornithine solution for ventral-mesencephalic cultures. If the coverslips are not to be used immediately, they should be stored dry after washing in distilled water. They can be stored up to 2 months in a sterile container (Löwenstein et al., 1996).

To prepare poly-L-lysine coated coverslips: Prepare a filter-sterilized stock solution of poly-L-lysine by dissolving 10 mg poly-L-lysine in 0.15 M sodium tetraborate (pH 8.5). Dilute the poly-L-lysine stock solution 10-fold with sterile distilled water to obtain a working solution of 0.1 mg/ml. Using a sterile Pasteur pipet, add 5 to 7 drops of the working solution of poly-L-lysine to each coverslip. If cleaned correctly, the poly-L-lysine will spread out to cover the entire coverslip. Allow to dry in laminar flow cabinet overnight. Wash with sterile distilled water twice for 2 hr. Add 1 ml of plating medium (see recipe) and incubate at 37°C in an atmosphere of 5% CO₂ until used.

To prepare poly-L-ornithine/laminin coated coverslips: Prepare a filter-sterilized stock solution of poly-L-ornithine by dissolving 10 mg poly-L-ornithine hydrobromide in 0.15 M sodium tetraborate (pH 8.5). Dilute the poly-L-ornithine stock solution 10-fold with sterile distilled water to obtain a working solution of 0.1 mg/ml. Add 1 ml/well of poly-L-ornithine working solution to 24-well plates or glass coverslips in 12-well plates, and allow to dry at room temperature for 1 to 4 hr in a laminar flow hood. After poly-L-ornithine coating, wash the plates twice, each time for 5 min, with distilled water. Add 0.5 ml of laminin (dissolved in sterile water to a final concentration of 10 mg/ml) to each well, and allow to stand for 2 to 24 hr at 37°C. Before seeding the cells, wash the plates three times, each time for 5 min, in Dulbecco's PBS (CellGro, VWR cat. no. 45000-434) and once in VM culture medium (see recipe).

CsCl solutions; 1.25, 1.35, and 1.5 g/ml

1.25 g/ml solution:
54.0 g CsCl
146.0 g of 10 mM Tris-Cl, pH 8.0 (*APPENDIX 2A*)

continued

**Gene Cloning,
Expression, and
Mutagenesis**

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1.35 g/ml CsCl solution:

70.4 g CsCl

129.6 g of 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

1.50 g/ml CsCl solution:

90.8 g CsCl

109.2 g of 10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)

Dissolve CsCl in the appropriate amount of 10 mM Tris·Cl buffer, pH 8.0 (by weight)

Filter sterilize

Prepare these solutions fresh just prior to use

DNase I

100 mg bovine pancreatic deoxyribonuclease I

10 ml of 20 mM Tris·Cl (pH 7.4; *APPENDIX 2A*)

50 mM NaCl

1 mM dithiothreitol

0.1 mg/ml bovine serum albumin

50% (v/v) glycerol

Store in 1-ml aliquots up to 3 months at -20°C

FACS permeabilizing buffer

Dulbecco's PBS (D-PBS; CellGro, VWR, cat. no. 45000-434) containing:

1% fetal bovine serum (FBS; Omega Sci., cat. no. FB-01) decomplexed by heating at 56°C

0.1% Triton X-100

Store up to 6 months at 4°C

FACS staining buffer

D-PBS containing 1% decomplexed fetal bovine serum (Omega Sci., cat. no. FB-01). Store up to 6 months at 4°C .

HEPES-buffered saline (HBS), 2×

50 mM HEPES, sodium salt

280 mM sodium chloride

1.5 mM disodium orthophosphate

Adjust pH to 7.12 with 1 M NaOH

Filter sterilize

Store up to 6 months at -20°C

Low-Tris/EDTA buffer

Prepare the following two solutions:

100 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2A*)

10 ml 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*)

Combine the two solutions

Filter sterilize

Store up to 6 months at -20°C

Maintenance medium

Dulbecco's Minimum Essential Medium with Earle's salts (CellGro, VWR, cat. no. 45000-312)

10% fetal bovine serum (FBS; Omega Sci., cat. no. FB-01)

1× MEM nonessential amino acids (CellGro, VWR, cat. no. 45000-700)

continued

2 mM L-glutamine (CellGro, VWR, cat. no. 45000-676)
1 U/ml penicillin/1 µg/ml streptomycin (CellGro, VWR, cat. no. 45000-650)
Store up to 1 month at 4°C

All of the above ingredients are manufactured by CellGro/MediaTech and are distributed by VWR with the exception of the fetal bovine serum which is purchased directly from Omega Scientific

Mowiol 4-88 mounting solution

Dispense 6.9 g of 99% to 100% glycerol in a 15-ml polypropylene conical tube. Add 2.4 g of Mowiol 4-88 (Calbiochem) and mix. Add 6 ml distilled water and leave for 2 hr at room temperature. Transfer to a 50-ml polypropylene conical tube and add 12 ml of 0.2 M Tris-Cl, pH 8.5 (*APPENDIX 2A*), and incubate for 10 min at 50°C with occasional stirring (a shaking water bath with occasional stirring may be useful because Mowiol can take a very long time to dissolve). Transfer the tube to a shaking platform and leave overnight. Centrifuge 15 min at 1000 × *g*, 4°C. Remove supernatant and divide into 1-ml aliquots into plastic pop-cap microcentrifuge tubes. Store up to 3 months at –20°C. Thaw just before use and do not refreeze.

Paraformaldehyde solution, 4%

Solution A: Mix 8 g paraformaldehyde with 80 ml distilled water. Heat to 50° to 60°C and add drops of 1 M NaOH until the paraformaldehyde dissolves. Cool and filter into 100 ml of solution B.

Solution B: Prepare 100 ml of 0.2 M PBS (see recipe) containing 0.12 M sucrose.

Adjust pH of the solution A/solution B mixture to 7.5 and bring volume to 200 ml. Store at 4°C and use within 24 hr.

Phosphate-buffered saline (PBS), 0.2 M

Solution A: Prepare 800 ml of 0.2 M disodium orthophosphate in 0.9% sodium chloride.

Solution B: Prepare 200 ml of 0.2 M sodium dihydrogen orthophosphate in 0.9% sodium chloride.

Add solution B to solution A with stirring until the pH is 7.5. Store up to 6 months at room temperature.

Plating medium

Minimum essential medium with Earle's salts (CellGro, VWR, cat. no. 45000-384)
10% fetal bovine serum (FBS; Omega Sci., cat. no. FB-01)

1 × MEM nonessential amino acids (CellGro, VWR, cat. no. 45000-700)

2 mM L-glutamine (CellGro, VWR, cat. no. 45000-676)

1 U/ml penicillin/1 µg/ml streptomycin (CellGro, VWR, cat. no. 45000-650)

Store up to 1 month at 4°C

Prehybridization solution

5 × SSC (*APPENDIX 2A*)

1% (w/v) blocking reagent (from Dig DNA labeling and detection kit)

0.1% (w/v) *N*-lauroylsarcosine

0.02% (w/v) SDS

Blocking reagent does not dissolve readily. Heat to 60°C for 30 min. Store up to 6 months at –20°C.

Tris-buffered saline (TBS)

50 mM Tris base
0.9% sodium chloride
Adjust pH to 7.4 with 1 M HCl
Store up to 6 months at room temperature

Virion lysis buffer

20 mM Tris·Cl, pH 7.8 (*APPENDIX 2A*)
10 mM EDTA, pH 7.8
1% (w/v) sodium dodecyl sulfate (SDS)
1 mg/ml proteinase K (Sigma)
Store up to 6 months at room temperature

Virus storage buffer

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2A*)
Add glycerol to 10% v/v
Use immediately

VM culture medium

1:1 mixture of Dulbecco's modified Eagle medium (CellGro, VWR, cat. no. 45000-312) and Ham's F12 medium (CellGro, VWR cat. no. 45000-354)
10% fetal bovine serum (FBS; Omega Sci., cat. no. FB-01)
4.0 mM L-glutamine (CellGro, VWR, cat. no. 45000-676)
1 U/ml penicillin/1 µg/ml streptomycin (CellGro, VWR, cat. no. 45000-650)
Store up to 1 month at 4°C

All of the above ingredients are manufactured by CellGro/MediaTech and are distributed by VWR with the exception of the fetal bovine serum which is purchased directly from Omega Scientific.

Xgal staining solution

5 mM potassium ferrocyanide
5 mM potassium ferricyanide
2 mM magnesium chloride
20 mg Xgal dissolved in 500 µl DMSO
Add ddH₂O to 20 ml
Keep solution in the dark at room temperature and use immediately

COMMENTARY

Background Information

A brief introduction to adenoviruses

Adenoviruses (Ads) were first described in 1953 and were soon utilized for the study of a vast number of molecular and cell biology phenomena, e.g., oncogenesis, DNA replication, transcriptional regulation, and protein synthesis. Although Ads have been isolated from many species, including birds and mammals, the human Ads have been the most studied. There are fifty known serotypes of human Ads that have been divided into six subgroups. Most of the recombinant adenovirus vectors used for gene transfer are based on serotype 2 and 5 of subgroup C. All human Ads studied

to date show many similarities. Their genome consists of 30 to 40 kbp of double-stranded DNA, which is flanked by 100- to 140-bp inverted terminal repeats (ITRs) containing an origin of DNA replication. The packaging region, which is needed for encapsulation of the genome, is located at the left-hand side of the genome near the ITR. DNA replication occurs through two phases after infection, early and late. Early-region transcription units are, E1A, E1B, E2, E3, and E4. The late transcription units are initiated from the major late promoter (MLP) and include L1 through L5. Ads do not integrate into the host-cell chromosome, but instead form a nuclear-located episome from

which their gene transcription is initiated. As a consequence, gene expression has a tendency to be transient, and there is a need for repeated administration if prolonged gene expression is required.

Adenoviral gene deletions in recombinant vectors

The first-generation recombinant adenovirus vectors (Ads) that were developed for gene transfer contained deletions of the entire E1A and E1B transcription units, rendering them replication incompetent (Berkner and Sharp, 1983, 1984). These early transcription units are essential for regulation of adenoviral transcription. The human embryonic kidney cell line 293 has the genes inserted in *trans*, thereby allowing the growth and propagation of Ads. The deletion of the E1 transcription units was initially assumed to be sufficient to ablate the expression of other early and late viral genes; however, it is now known that at high MOIs the deletion of E1 can be overcome, probably through the action of cellular *trans* activators with E1-like activity, overriding the absence of transcription from the Ad. The Ads most commonly used for gene transfer, described in this unit, cannot progress through the lytic cycle in normal cells. Nevertheless, the presence of viral gene expression due to high MOIs can lead to changes in cell proliferation and can cause apoptosis in vitro.

Cellular responses to adenoviral infection

It has been established that infection of cells by adenovirus can lead to the induction of tissue necrosis factor α (TNF- α), which can trigger apoptosis (Cartmell et al., 1999). The wild-type adenovirus gene products E1B (19 kD), E3 (14.7 kD), and an E3 (10.4 kD)/E3 (14.5 kD) protein complex can protect adenovirus-infected cells from lysis by TNF- α . Furthermore, the adenovirus E1A proteins are also known to induce apoptosis, which is inhibited by the E1B 19 kD protein or Bcl-2 (Mahr and Gooding, 1999). As some current Ads are deleted with respect to both the E1 and E3 regions, it was postulated that these vectors may have a diminished effect on cell lysis. However, data from two separate in vitro cell systems have indicated that E1-deleted Ads can still induce apoptosis in a targeted cell population despite the deletion of the early regions known to encode proteins that regulate apoptosis (Easton et al., 1998). An alternative strategy was the deletion of the E4 region alongside the deletion of E1 in order to alter the phenotype for apoptosis. It was demonstrated that the E4orf6 protein can bind to the p53

cellular tumor suppressor protein, blocking its ability to activate transcription. This resulted in the prevention of p53-mediated apoptosis. However, as an affirmation of the complexities inherent in this vector system, absence of the E4 gene led to down-regulation of the promoter within the inserted transcriptional cassette, causing a reduction in persistence of expression of the transgene (Lavoie et al., 2000).

Adenovirus vector-induced target cell cytotoxicity

Target cell toxicity may arise as a consequence of cellular proteins contaminating the virus preparation or from defective viral particles within the Ad preparation. An Ad purification and large-scale preparation protocol involving ultracentrifugation using two cesium chloride density gradients is described in this unit (see Basic Protocol 1). This minimizes cytotoxic contaminants and optimizes the ratio of viral vectors with respect to defective and/or empty capsids. The cytotoxicity of Ads prepared using these protocols is minimal (Löwenstein et al., 1996; Shering et al., 1997) and vector/defective vector ratios are usually between 25:1 and 40:1. The titers that can be routinely achieved starting from twenty 175-cm² flasks of 293 cells are $\sim 10^{12}$ plaque-forming units/ml, with a band volume of 700 μ l to 1 ml. For in vitro experiments, neuronal cells and glial cells should not be infected with MOIs >500. If higher MOIs are used, cell rounding and detachment will occur, with concomitant loss of cell viability. The neuronal and glial cell culture protocols in this unit are optimized for Ad infection experiments, maximizing cell attachment and good morphology for cell biology/physiology assessment.

Promoter selection: Cell-type specificity

A further challenge in the use of Ads involves the search for promoters that can not only provide optimal gene expression, but also cell-type-specific expression. Many Ads have utilized very strong constitutive promoters that are most often of viral origin, such as the Rous sarcoma virus long terminal repeat, the SV40 early promoter, or the human cytomegalovirus major immediate early promoter. These lack cell-type specificity and therefore raise safety issues such as those related to the expression of the transcription cassette within non-target cells. In order to establish specificity at a cellular level, investigators are attempting the alteration of the virus capsid proteins so that they bind to cell-specific receptors.

However, the most common approach is the utilization of weaker cell-type specific promoters, such as the glial fibrillary acidic protein (GFAP) promoter, specific to astrocytes, or the neuron-specific enolase (NSE) promoter, specific to neurons. These allegedly cell-type-specific promoters have met with mixed results. The GFAP promoter has been shown to induce specific expression of a reporter gene in an Ad both in vitro and in vivo, whereas the NSE promoter did not confer cell-type specificity in vitro, despite being predominantly neuron-specific in vivo (Smith-Arica et al., 2000).

Promoter selection: Regulation of gene expression

Strategies to regulate expression of the transcription cassette are currently being developed. The insertion of a drug-dependent regulatory element within the Ad genome would confer control of levels of expression from the transcription cassette. An example of such a regulatory element is the tetracycline response element (TRE) described by Gossen and Bujard (1992). This inducible system requires, first, the insertion of the TRE before the reporter gene or gene of interest and, second, the insertion of the transactivator (tTa) with its expression driven by a promoter which can be cell-type specific. Absence of the drug doxycycline allows the binding of the tTa to the TRE, driving the expression of the gene of interest. The presence of doxycycline inhibits the binding of tTa to TRE, thereby preventing the expression of the transgene (Candolfi et al., 2006; Xiong et al., 2006; Smith-Arica et al., 2000).

Critical Parameters and Troubleshooting

Cell lines for vector propagation

The protocols described in this unit employ the human embryonic kidney (HEK) 293 cell line for generation and propagation of recombinant adenoviruses. The HEK 293 cell line has been transformed by genomic integration of nucleotides (nt) 1-4344 of the adenovirus type 5 (Ad5) genome. Virtually all adenovirus vectors for in vivo and clinical studies have been produced in 293 cells, but other cell lines may also be used for this purpose. Human embryonic retinal (HER) 911 cells contain nt 79-5789 of the Ad5 genome (Fallaux et al., 1996), PER.C6 HER cells contain nt 459-3510 (Fallaux et al., 1998), and NCL A549 (a human lung carcinoma cell line)-derived cells have been generated that contain nt 505-4034 (Imler

et al., 1996). These alternative cell lines each offer some advantages over 293 cells. Plaques have been observed to form in 911 cell monolayers after 3 to 4 days, compared to the 4 to 10 days required for 293 cells, and vector yields of up to three times those observed from vector propagation in 293 cells have been reported (Fallaux et al., 1996). 911 cells do not, however, circumvent the risk of generation of replication-competent adenovirus (RCA, i.e., wild-type Ad5) by homologous recombination between shuttle plasmids containing the right-hand sequence of the adenovirus genome (e.g., pJM17, pBHG10) and the left-hand fragment of the Ad5 genome integrated into the cell genome. RCA may also be generated by recombination between the genome of recombinant adenoviruses derived from shuttle vectors which contain sequences homologous to the genomically integrated sequence (e.g., pAL120, which contains nt 1-341 and 3524-5790). In contrast, vector propagation in PER.C6 cells—in which no overlap occurs between the integrated sequence and that present within shuttle plasmids—has been reported to prevent generation of RCA while allowing vector yields comparable to 293 cells (Fallaux et al., 1998). PER.C6 cells are difficult to obtain, however, and are currently available only to large-scale producers of recombinant vectors for clinical purposes. The risk of RCA generation is substantially reduced in NCL A549-derived cells because the left-hand ITR is absent from the integrated Ad5 sequence, thereby necessitating two recombination events for RCA to arise; however, vector yields are lower than in 293 cells (Imler et al., 1996).

Optimization of transfection efficiency: Factors related to 293 cell culture

One of the most critical factors, if not the most critical factor in determining the efficiency of vector generation by cotransfection into 293 cells, is the condition of the 293 cells at the time of cotransfection. The cells are grown in an incubator at 37°C in the presence of 5% CO₂ and 100% humidity. The efficiency of transfection decreases with increasing passage number and, ideally, cells employed for the purpose of cotransfection should have been passaged no more than 50 times. Cells at higher passage can be used, but the decreased efficiency of transfection is accompanied by a prolongation of the time required for plaque generation, which increases the risk of generation of RCA at this very early stage of vector production. It is also sensible

to check the morphology of the 293 cells immediately prior to cotransfection. The cells should look as normal as possible at 24 hr post-passage. Some minor degree of cell death is almost inevitable during calcium phosphate precipitation, and this is likely to be substantially greater if the cells have not resumed a normal morphology after passaging. Confluence of ~40% ensures that the cells are growing rapidly at the time of cotransfection, which optimizes efficiency. At later stages of virus propagation, it is more appropriate to infect flasks of 293 cells when the cells are 70% to 80% confluent. The addition of fresh medium to the cells at the time of infection ensures that the majority of cells are actively growing, thereby ensuring optimal expression of the cellular apparatus for protein expression and subsequently maximal vector production. Cells that are completely confluent should not be used for vector propagation.

Optimization of transfection efficiency:

Physical factors

The quality of the DNA used for cotransfection is another important determinant of the efficiency of the procedure. DNA should be purified on a QIAGEN column or equivalent, or by cesium chloride gradient centrifugation. The large plasmids employed for cotransfection (pJM17 is in excess of 40,000 bp) require that care be taken to avoid shearing of the plasmids during mixing of the cotransfection reagents. It is possible to mix the reagents by vortexing rather than by bubbling the mixture, but this typically reduces transfection efficiency by >50%. It goes without saying that the plasmids to be cotransfected must be characterized prior to use. The integrity of the shuttle plasmids that bear the transgenic sequences to be inserted into the recombinant vector must be unequivocal. The orientation of promoter, transgene, and poly(A) are obviously important with respect to subsequent transgene expression, although this is not a critical influence on transfection efficiency. More significantly, the large shuttle plasmids containing the majority of the Ad5 genome (e.g., pJM17 or pBHG10) are prone to rearrangement after transformation. It is important that the correct *Hind*III digest pattern be confirmed before the purified plasmid is used.

The pH of the transfection solutions (discussed in Basic Protocol 1) is also critical in ensuring optimal calcium phosphate precipitation. Once prepared, the solutions are best stored at -20°C or lower until required.

Assessment of cotransfection efficiency

The efficiency of cotransfection can be assessed by transfection of a plasmid expressing a marker gene, such as *lacZ* (*E. coli* β -galactosidase), under the control of a promoter appropriate to 293 cells into a 25-cm² flask of 293 cells at the time of cotransfection. The flask of 293 cells can then be assessed for marker gene expression 48 to 72 hr post-transfection. Under optimal conditions in the authors' laboratory, transfection of >60% of cells has been achieved. It is of course possible to use other methods of transfection than calcium phosphate, such as cationic liposomes (e.g., LipofectAMINE or DOTAP), but these methods offer little practical benefit over carefully optimized calcium phosphate precipitation.

Successful generation of recombinant vectors typically manifests as the formation of visible plaques within 14 days of cotransfection. Occasionally, however—and particularly if cotransfection efficiency has been suboptimal—a longer period may be required before plaques appear. Flasks of 293 cells may be retained for as long as 25 days post-transfection, and it is not unheard of for successful homologous recombination to give rise to plaque formation as late as day 21. At this point after cotransfection, however, the 293 cells will be markedly overconfluent and it may be difficult to determine whether cell death is due to virus formation resulting in cytopathological effect (CPE) or simply to overconfluence. The morphology of 293 cells that have died in a very confluent culture is very similar to the morphology of cells that have died as a consequence of virus infection. It is thus vital that unequivocal plaque formation be observed before a cotransfection is deemed to have been successful.

Identification of successful recombinant vectors

Prior to virus isolation and purification it is essential to confirm that the recombinant vector contains the correct transgene and promoter elements. To this end, it is necessary to infect a 25-cm² flask of 293 cells at 70% to 80% confluency with ~30 μl of the cell suspension obtained after harvesting of the cotransfection culture. Once CPE has spread throughout the cell monolayer, the cells can be harvested and the recombinant viral DNA extracted as described in Support Protocol 1.

The first step in virus identification is verification of the restriction exonuclease digest

pattern of the viral DNA. Any restriction exonuclease may be used for this purpose and the choice of enzyme depends, at least in part, upon the inserted transgene. As a rule, however, the authors use *HindIII*, as this gives a characteristic and easily interpreted pattern with recombinant viruses derived from Ad5. The exact band pattern obtained with any restriction enzyme depends upon the inserted transgene and the shuttle vectors employed for vector generation. In this respect, it is probably most expedient to have access to the band pattern obtained by digestion of a virus containing a marker transgene that has been generated using the same initial shuttle vectors (e.g., pAL120 and pJM17) as the vectors to be identified. The advantage of this approach is that transgene function—and therefore integrity of the transgene-promoter construct—can be readily identified in the marker virus by demonstration of marker gene expression. The digest pattern of the marker virus DNA may then act as a template for comparison with other recombinant vectors.

Identification of successful recombinant vectors: Southern blotting versus PCR

Once the predicted restriction exonuclease digest pattern has been confirmed, it is necessary to confirm the identity of the transgene by Southern blotting or PCR as described in Support Protocols 1 and 2. Both methods allow the identification of a length of DNA corresponding to the transgene \pm promoter sequence within the recombinant vector. Each method has advantages and disadvantages. PCR is more rapid and less cumbersome, but merely confirms that there is an insert within the recombinant virus of the size appropriate for the inserted transgene. Southern blotting is more laborious, but confirms that the transgene shares the identity (to some degree at least) of the gene from which the probes were generated, as well as confirming its size. Realistically, either method provides sufficient circumstantial evidence, if positive, to justify proceeding to virus isolation by endpoint dilution. Once three rounds of endpoint dilution have been successfully performed, verification of virus identity is necessary again before further purification and amplification.

Determination of transgene expression in vitro

Before proceeding to cesium chloride gradient purification of recombinant adenovirus stocks, it is essential to confirm transgene expression in vitro. In the case of vectors bearing marker transgenes, this is a relatively straight-

forward matter, but other recombinant viruses will require immunocytochemical verification of recombinant protein expression in vitro. The choice of cell line for immunocytochemical investigation in vitro depends upon, among other things, the transgene concerned and the use to which it will be put in vivo. HeLa or COS-7 cells are generally used for this purpose, but detailed consideration of the choice of cell line is beyond the scope of this unit.

When recombinant vector identity and expression of transgene have been confirmed, it is safe to proceed to cesium chloride gradient purification of the vectors for subsequent in vitro or in vivo use.

Points of note for cesium chloride gradient purification

The result of cesium chloride gradient purification is a recombinant virus preparation which is free of the majority of defective particles (virus capsids containing an incomplete genome) and cellular debris. During the process of retrieval of the intact virions it is important to ensure that no contamination arises from accidental mixing of gradient bands. The simplest way to achieve this is to harvest the band by side puncture of the centrifuge tube. The needle employed in this step should be 19-G to 21-G. It is possible to remove the upper layer of cellular debris after the first centrifugation step using a sterile Pasteur pipet inserted into the centrifuge tube from above (not by side puncture). This diminishes the risk of contamination prior to removing the virus band. If this is performed, care must be taken to ensure that the band of intact virions is not disturbed.

Storage and maintenance of recombinant virus stocks

Adenoviruses are very sensitive to low pH and high temperature. It is vital at all stages of preparation that care be taken to adhere to the pH recommended for each reagent. This is particularly true for the solutions against which the viruses are dialyzed after cesium chloride gradient purification. A drop in the pH of the dialysates by 1.0 is likely to lead to a loss of $\sim 90\%$ of infectious particles.

Once aliquotted, the virus should be stored at -70°C until used. Thawing of the virus stock will result in a significant loss of infectious particles, such that an aliquot that has been thawed once cannot reliably be used for further infections. Bearing this in mind, it is important to freeze any aliquot that is to be used for titration before assessing the viral titer; otherwise any titer obtained is likely to

be a significant overestimate of the number of infectious units/ml of the aliquots stored at -70°C . The virus stocks are stable at -70°C for several years, although it is wise to repeat the titration of a stock that has been stored for any prolonged period of time (arbitrarily, >1 year).

Transport of adenovirus vectors

Transport of adenovirus vectors represents a particular problem. The most practical method of transporting viruses over long distances is on dry ice. However, many of the cryotubes widely used for storage of viral vectors are permeable to CO_2 . As a consequence, relatively brief (24 hr) storage of viruses on dry ice can lead to a loss of $>99\%$ of infectious particles as the pH of the storage buffer drops in the presence of CO_2 . A variety of commercially available containers are impermeable to CO_2 , and it is absolutely essential that any cryotubes that are to be transported on dry ice should be carefully wrapped in Parafilm and sealed in a CO_2 -impermeable container.

With care, it is possible to achieve viral titers in excess of 10^{12} infectious units/ml and to maintain these titers in storage for periods of years.

Assessment of levels of replication-competent adenovirus contamination within recombinant adenovirus preparation

As discussed earlier, one of the principal difficulties in generation of recombinant adenoviruses in 293 cells is the potential for contamination of vector stocks by RCA. In vitro, RCA are capable of direct toxic effects on cells in addition to affecting intracellular second messengers and cytokine release. The presence of RCA in vivo is likely to give rise to exaggerated inflammatory responses. All of these effects may have a critical influence on any experimental outcomes. Two basic methods of detection of RCA in vector stocks exist. These are the supernatant rescue assay (SRA, described in Support Protocol 3) or PCR for E1 region genes, which are absent from recombinant viruses but present in RCA. Both methods have their relative merits and disadvantages, as discussed below.

Supernatant rescue assay versus PCR

SRA is more sensitive than PCR for detection of RCAs (Dion et al., 1996). It relies upon the fact that HeLa cells will allow replication of RCA, but not recombinant viruses. The limit of the sensitivity of this assay is essentially how many HeLa cells can be grown to be infected (see Support Protocol 3). A 10-cm

diameter tissue culture dish contains ~ 5 to 6×10^6 HeLa cells at 90% confluency. Infecting each dish at an MOI of 30 allows $\sim 1.6 \times 10^8$ infectious units to be examined. If six such dishes are infected, 1×10^9 infectious units can be examined, and so on. The appearance of plaques (identical to those seen in infected 293 cells) in the HeLa cells cultured in 24-well plates indicates the presence of RCA in the recombinant vector stocks. It is possible to calculate the degree of contamination from the number of wells on the 24-well plate in which CPE is seen. It is probably more useful to repeat the assay with a ten-fold decrease in the initial infecting dose, and to continue to do so until no CPE is seen in the 24-well plate cultures after 6 days. In practice, it is of little value to use an initial dose in excess of 10^9 infectious units, as few gene transfer protocols will require a vector dose in excess of this.

The PCR methods are less time-consuming than SRA, but are not as sensitive. In one comparison, the most sensitive PCR method employed was able to detect the equivalent of 5000 RCA genomes in the presence of 10^9 recombinant vectors—i.e., 1 RCA against a background of 200,000 recombinant viruses (Dion et al., 1996).

The presence of RCA at a concentration in excess of 1 per 10^9 infectious units of vector stock will require that the virus be prepared again from the master stock of the third round of purification by endpoint dilution.

Primary cortical neuronal, ventral mesencephalic neuronal, and cortical glial cultures

A critical parameter when setting up primary cultures is the timed nature of the pregnancies. It is important for the investigator to establish the breeding schedule of the animal house and when they consider a pregnancy as day 0. The protocols described take day 0 as the time of mating. For the neocortical glial culture, pups can be taken on postnatal days 0 to 3; for neocortical neuronal cultures embryonic (E) days 18 or 19 are used. However, it is critical that, for the ventral mesencephalic primary neuronal culture, the embryos be taken on E14.

With all three protocols for the growth of primary cultures of brain cell types, the investigator can expect to find substantial cell death 24 hr post plating. Gently removing these cells by changing the medium at this point will “clean up” the culture and promote cell growth. Medium changes in the neuronal cultures should be kept to a minimum, as

changing the medium can promote growth of glia and fibroblasts. The astrocytic glial cultures can be expected to survive for 2 to 3 weeks after plating, while the morphology and overall quality of neuronal cultures tend to deteriorate after 5 days unless the neurons are cocultured with glial cells as described in the paragraph below. The investigator may also find low levels of bacterial contamination within the cultures due to the nature of the culture production. It may therefore be wise to isolate these cultures in their own incubator and continually monitor them. With the concentration of antibiotics indicated in the protocol, occasional bacterial contamination can be contained without jeopardizing the culture or further experiments.

Low-density primary cultures of neocortical neurons can be cocultured with glial cultures for long-term maintenance. To do this, sterilized paraffin wax beads should be placed at the corners of the coverslip in a laminar flow cabinet. This is done by first heating the sterilized wax gently on a hot plate. Allow a small amount of molten wax to enter a sterile pipet tip by capillary action. The tip should then be placed on the corners of the coverslip and a small quantity of wax expelled to form small beads ~2 mm in height. Prepare the coverslips with poly-L-lysine or poly-L-ornithine/laminin as described (see Reagents and Solutions), then 3 hr after plating the neuronal culture (to allow the neuronal cells to attach to the coverslip), invert the coverslip onto a well containing confluent neocortical glial cells which have been "conditioned" for the neurons by switching to neuronal plating medium 3 days previously.

Xgal staining of infected cultures

It should be noted that some cell types have high endogenous levels of β -galactosidase activity. This can be eliminated by adding 5 mM ethyleneglycol-bis-(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) to the phosphate-buffered saline, which will block the endogenous activity but allow detection of the bacterial enzyme.

An enzymatic assay is a useful tool for the investigator to examine the levels of transgene expression within cells. However, it is important to note that it becomes difficult to differentiate activity from background levels when there are few cells infected or when using a weak promoter. Therefore, Xgal staining is a particularly useful tool to determine infectability of cell types with an Ad encoding *lacZ* by

examining the percentage of cells expressing the transgene.

Fluorescent immunocytochemistry of infected cultures

When selecting antibodies for fluorescent immunocytochemistry, attention should be paid to the normal blocking serum to be used. It is best to use the normal blocking serum of the same species in which the secondary antibody fluorescent conjugate has been raised (for example if the secondary antibody is a goat anti-rabbit fluorescein conjugate, then normal goat serum should be used to block any background staining). This should avoid any possible cross-reactions between the different antibodies used. This, however, is not always possible, and in this case the investigator should select an alternative that is not of the same species as the primary antibody. High levels of background can be eliminated by quenching the remaining reactive aldehyde groups by incubating with 50 mM ammonium chloride in PBS for 15 min (after the primary antibody incubation). The investigator should refer to the supplier's guidelines for the working dilutions of the antibodies, but it is usually necessary to optimize the exact dilutions for each particular application.

Fluorescence-activated cell sorting of infected cultures

For fluorescence-activated cell sorting, the investigator will usually have to determine whether the antibodies are suitable for this purpose. It does not necessarily follow that an antibody that works perfectly for immunocytochemistry will work for FACScan. Secondary antibody choice is also very important. For example, in dual-labeling experiments, the combination of secondary antibodies labeled with fluorescein and with R-phycoerythrin proved to give the easiest interpretation of results, as they are clearly separated into two distinct fluorescent channels.

Anticipated Results and Time Considerations

Generation of recombinant vectors

The generation of a cesium chloride gradient-purified vector ready for experimental use is likely to take ~3 months after all shuttle vectors have been prepared.

If cotransfection is performed carefully and in quadruplicate, it is probable that a recombinant virus will be generated at the first attempt, although this can never be

guaranteed. Successful homologous recombination is likely to give rise to flask-wide CPE ~14 days after co-transfection. The first viral DNA purification, restriction exonuclease digestion and Southern blot (if this is preferred to PCR) will take ~7 days after infection of a flask of 293 cells with the crude cell lysate from the cotransfection flask. Each round of plaque purification by serial dilution requires 8 days (24 in total), and an additional 7 days will be required to verify the identity of the plaque-purified vector by restriction exonuclease digestion and Southern blotting. Hence, the production of a plaque-purified vector suitable for amplification and cesium chloride gradient purification will take at least 50 days.

The generation of a master stock of recombinant adenovirus with a titer in excess of 10^8 infectious units/ml will require at least 2 days for full CPE to be achieved in the infected flask(s) and a further 8 days for titration by serial dilution endpoint assay. If a titer of 10^8 is not achieved at the first attempt, each further amplification/assay step will require an additional 10 days.

Once a master stock of sufficiently high titer has been obtained, preparation of a cesium chloride gradient-purified vector stock is relatively quick. After infection at an MOI of 3, CPE will develop throughout each infected flask in 48 to 72 hr.

Prior to use, this vector stock preparation must be titrated again by serial dilution endpoint assay, which will require 8 days. Once the titer of the preparation is known, the stock can be assayed for the presence of RCA, which will take a further 9 days. Once the titer of the preparation is known and it has been confirmed to be free of RCA, it may be used for experimental purposes. This will have required a minimum of 84 days of continuous labor. However, researchers can reasonably generate three or four different Ads at any one time. At this stage, it is likely that the purified vector will have a titer between 1×10^{10} and 1×10^{12} . Occasionally titers in excess of 10^{12} may be achieved. This usually requires great care to minimize the volume in which the recombinant vector band is removed from the second-round cesium chloride gradient. It is unusual to obtain titers of $<10^{10}$ from a cesium chloride preparation. Such a result would be likely to imply a failure at some stage of production and should prompt a careful reappraisal of the implementation of the techniques described.

Preparation of primary cultures

The main temporal consideration in preparation of primary cultures is the dissection of

the embryonic and postnatal tissue. The investigator will find that, at first attempt, the dissections will take up to 3 hr for a whole litter. It is essential that this time be reduced to 1 to 1.5 hr, as this yields a drastic increase in the viability of the culture and a reduction in time for the culture to stabilize and become experimentally useful. It must also be noted that neocortical glial cultures take a long period of time (up to 2 weeks) to establish a monolayer, as they grow very slowly. Nevertheless, they can be used for experimental purposes once they reach 50% confluency. It is not advisable to use a confluent culture for viral infection, as the infectability of the cells will be reduced.

Incubation times with primary antibodies for use in immunocytochemistry and fluorescence-activated cell sorting will determine the length of time needed for these protocols. The authors have found that most primary antibodies work best with overnight incubations at 4°C for immunocytochemistry, but this can be reduced to 4 hr if incubation is done at room temperature. Likewise, for the fluorescence-activated cell sorting procedure, an incubation time of 1 hr at room temperature has been described; this can be increased to overnight at 4°C.

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Key References

- Graham et al., 1977. See above.
This paper demonstrates the transformation of the human embryonic kidney cell line with adenoviral DNA, as described in this unit.
- Berkner and Sharp, 1983. See above.
Paper in which Berkner first discussed the use of adenoviruses as "cloning vehicles" for gene transfer, and her system for generating adenoviruses by cotransfection of plasmids.
- Berkner and Sharp, 1984. See above.
The first generation of a recombinant adenovirus expressing a heterologous gene.