ABSTRACT

Genetically modified adenoviruses (Ads) make attractive vectors for the delivery of exogenous DNA to mammalian cells for basic science and gene therapy applications. Ad vector production consists of (1) cloning a transgene into an infectious plasmid by in vivo recombination in bacteria, (2) rescuing and propagating the vector in complementing cells, and (3) purifying the vector. All of this can be accomplished using commercially available reagents, plasmids, and cell lines. Helper-independent Ads have a large cloning capacity (5–14 kbp) and transduce efficiently in a wide range of both quiescent and proliferating cell types. They propagate readily and produce high-titer stocks ($10^{11}$ to $10^{13}$ vector particles from a 3-liter culture). Furthermore, Ads rarely integrate into the host genome and are relatively safe. However, Ad vector production typically takes 4–6 weeks, and promiscuous host-cell transduction can occur in vivo. Furthermore, immune responses against viral proteins encoded by the vector backbone can occur, and transgene expression by Ad vectors is relatively short. Here, we discuss the current methods for the generation, propagation, purification, and characterization of helper-independent Ad vectors.

INTRODUCTION, 

PROTOCOL, 

Construction and Characterization of Ad Vectors, 

MATERIALS, 

Reagents, 

Equipment, 

METHODS, 

Generation of Infectious Ad Plasmids, 

Cell Preparation, 

Transfection for Rescue of Ad Vectors, 

Analysis and Expansion of Plaque-isolated Ad Vector, 

Large-scale Preparation of Ad Vectors, 

Vector Purification, 

Characterization of Purified Ad Vectors, 

ACKNOWLEDGMENTS, 

REFERENCES,
Genetically modified Ads make attractive vectors for the delivery of exogenous DNA to mammalian cells for basic science and medical applications. Ad vectors are relatively easy for nonspecialists to produce, because current methods for Ad vector generation require only basic laboratory skills in molecular biology and cell culture. These vectors have a relatively large cloning capacity and are easily propagated and purified to high titer. Furthermore, Ad vectors do not integrate, but they are capable of long-term gene expression. Although Ad vectors transduce a wide range of mammalian cell types in a cell-cycle-independent manner, this promiscuity makes delivery to specific tissues in vivo difficult.

Most Ad vectors are based on the well-characterized human Ad serotypes 2 or 5. The 36-kbp linear, double-stranded DNA genome encodes genes that are classified based on the timing of their expression (Fig. 1). Early genes (E1, E2, E3, and E4) are expressed before the onset of DNA replication. Proteins encoded by the early genes function to activate other Ad genes, replicate the viral DNA, interfere with immune recognition of infected cells, and modify the host-cell environment to make it more conducive to viral replication. The late genes (the major late transcription unit, pIX, and IVa2) are expressed after DNA replication and primarily encode proteins involved in capsid production and packaging of the Ad genome. The viral DNA also contains the origins of replication (the inverted terminal repeats [ITR], ~100 bp located at both the left and right end) and the packaging sequence (~150 bp located immediately adjacent to the left ITR).

The most commonly used type of Ad vector is the E1-deleted or first-generation Ad (fgAd) vector, which has a cloning capacity of approximately 5 kbp (Danthinne and Imperiale 2000). Typically, the transgene is inserted in place of the E1 region (Fig. 1). Since E1 is essential for virus replication, these vectors must be propagated in E1-complementing cell lines such as 293 (Graham et al. 1977), 293N3S (Graham 1987), 911 (Fallaux et al. 1996), or PER.C6 (Murakami et al. 2002). The E3 region is unnecessary for replication in vitro, and its removal increases vector cloning capacity to 8 kbp. Currently, the most efficient method for producing fgAd vectors is by construction of “infectious” Ad plasmids in bacteria (Chartier et al. 1996; He et al. 1998). This method uses recombination-proficient (RecA+) bacteria to transfer a transgene cassette into an Ad genomic plasmid, generating a DNA molecule that is essentially identical to the final virus construct. Transfection of the infectious plasmid into an E1-complementing cell line results in recovery of the desired recombinant Ad vector at a very high frequency (He et al. 1998).

The commercially available AdEasy system is widely used for recombination-mediated construction of Ad vectors. This consists of several variations of two plasmids (Table 1 and Fig. 2) (He

**FIGURE 1.** Simplified transcription map of the Ad5 genome. See text for details.
et al. 1998; He 2001). The shuttle plasmid (Table 2) contains a kanamycin (kan) resistance cassette flanked by the left and right ends of an E1-deleted Ad vector genome, a multiple cloning site located in the E1 locus, and several kilobase pairs of Ad5 DNA downstream from the E1 region (called the right arm of homology). The backbone plasmid encodes the majority of the Ad5 genome and an ampicillin resistance cassette. In vivo recombination between homologous sequences contained in both plasmids transfers the kan resistance cassette, left ITR and packaging sequence, and the transgene from the shuttle into the backbone plasmid, generating an infectious recombinant Ad vector (Fig. 2). The choices of shuttle and vector depend on the needs of the investigator, with the primary considerations being the promoter to be used, the size of the insert, and whether vector tracking is desired (Table 2). Two variations of pAdEasy (the Ad genomic plasmid) are available: pAdEasy-1 has an intact E4 region, whereas pAdEasy-2 is deleted of E4. Deletion of E4 increases the cloning capacity of resultant vectors, but this requires the use of E4-expressing 911 or 293 cells for vector propagation (Amalfitano and Parks 2002).

**FIGURE 2.** Schematic representation of the AdEasy system for Ad vector production. See text for details. (Modified, with permission, from He et al. 1998 [©National Academy of Sciences].)
<table>
<thead>
<tr>
<th>Supplier</th>
<th>Kit name</th>
<th>Catalog no.</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>AdEasy starter kit</td>
<td>JHU-23</td>
<td>AdEasier-1 cells (stably transformed with pAdEasy-1)</td>
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<td></td>
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<td>pShuttle</td>
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<td>BJ5183 cells</td>
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<td></td>
<td>pAdEasy-2</td>
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<tr>
<td></td>
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<td></td>
<td>pAdEasy1-GFP + β-gal (amplification control)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>pAdEasy2-GFP + β-gal</td>
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<td>Stratagene</td>
<td>AdEasy adenoviral vector kit</td>
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<td>pAdEasy-1</td>
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<td>pShuttle-CMV-lacZ</td>
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<td>BJ5183 electrocompetent cells</td>
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<td>XL10-Gold ultracompetent cells</td>
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<td>pUC18 (control DNA)</td>
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<td></td>
<td>AdEasy XL adenoviral vector kit</td>
<td>240010</td>
<td>BJ5183-Ad1 cells (similar to AdEasier-1 cells)</td>
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<td>pShuttle</td>
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<td>pShuttle-CMV</td>
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<td>pShuttle-CMV-lacZ (amplification control)</td>
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<td>AD-293 cells (Stratagene 293 cell derivative)</td>
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<td>AdEasy basic kit</td>
<td>AES1001</td>
<td>BJ5183 cells</td>
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<td>QBI-Infect+ (purified E1/E3-deleted Ad vector encoding lacZ)</td>
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<td>QBI-293A cells (Qbiogene 293 cell derivative)</td>
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<td>Basic kit components</td>
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<td>pShuttle-CMV</td>
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TABLE 2. Shuttle Vectors Available for Use with the AdEasy System

<table>
<thead>
<tr>
<th>Shuttle vector</th>
<th>Size (kbp)</th>
<th>Maximum insert size (kbp)</th>
<th>Supplier</th>
<th>GFP-tracer</th>
<th>Notes</th>
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<tr>
<td>pShuttle</td>
<td>6.6</td>
<td>1–7.5, 2–10.2</td>
<td>A, S, Q</td>
<td>no</td>
<td>Maximal cloning capacity; entire expression cassette ligated into MCS</td>
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<tr>
<td>pShuttle-CMV</td>
<td>7.5</td>
<td>1–6.6, 2–9.1</td>
<td>A, S, Q</td>
<td>no</td>
<td>Transgene ligated into MCS between CMV promoter and poly(A)</td>
</tr>
<tr>
<td>pAdTrack</td>
<td>8.3</td>
<td>1–5.9, 2–8.6</td>
<td>A</td>
<td>yes</td>
<td>GFP expression regulated by CMV promoter; entire expression cassette ligated into MCS</td>
</tr>
<tr>
<td>pAdTrack-CMV</td>
<td>9.2</td>
<td>1–5.0, 2–7.7</td>
<td>A</td>
<td>yes</td>
<td>GFP and transgene each regulated by CMV promoters in adjacent (head-to-tail) expression cassettes; transgene ligated into MCS between CMV promoter and poly(A)</td>
</tr>
<tr>
<td>pShuttle-IRES- hrGFP-1</td>
<td>8.9</td>
<td>1–5.3, 2–8.0</td>
<td>S</td>
<td>yes</td>
<td>One promoter regulates transgene and GFP expression in a dicistronic expression cassette; transgene cloned in-frame with carboxy-terminal FLAG epitope tag in MCS, between CMV promoter and poly(A).</td>
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<tr>
<td>pShuttle-IRES- hrGFP-2</td>
<td>8.9</td>
<td>1–5.3, 2–8.0</td>
<td>S</td>
<td>yes</td>
<td>One promoter regulates transgene and GFP expression in dicistronic expression cassette; transgene cloned in-frame next to carboxy-terminal HA epitope tag in MCS, between CMV promoter and poly(A).</td>
</tr>
</tbody>
</table>

*The maximum size insert will differ depending on whether the shuttle vector is recombined with pAdEasy-1 (intact E4) or pAdEasy-2 (E4-deleted), as indicated.

Abbreviations: (A) American Type Culture Collection; (S) Stratagene; (Q) Qbiogene.

GFP Tracer: Vector with a green fluorescent protein (GFP) expression cassette can be tracked visually during propagation, obviating the need for plaque purification.

Abbreviations: (MCS) Multiple cloning site; (poly[A]) polyadenylation signal; (CMV) cytomegalovirus immediate-early promoter/enhancer.
Protocol

Construction and Characterization of Ad Vectors

The shuttle, containing the transgene of interest, is produced using standard cloning procedures. Once obtained, the shuttle vector is linearized by digestion with Pmel and cotransformed into BJ5183 cells with the AdEasy backbone vector. After small-scale purification of DNA from BJ5183 cells (which do not maintain high copy numbers of plasmids), the DNA is transformed into a general-purpose cloning strain, such as DH5α. Once the structure of the vector is verified by restriction endonuclease mapping, clones are subjected to large-scale purification in CsCl gradients, and the resulting purified Ad vectors are characterized. For Troubleshooting guide, see Table 3.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Agarose (1% w/v)
Dissolve 1 g of agarose (UltraPure, Invitrogen, 15510-027) in 100 ml of sterile distilled H₂O. Sterilize by autoclaving for 20 minutes at 121°C. Store at room temperature and melt in a microwave oven before use.

Bacteria
BJ5183 cells (RecA-proficient)
These cells are supplied in a number of commercially available AdEasy kits. For details, see Table 1.
DH5α (RecA-deficient, Invitrogen)
Transformation-competent cells are prepared with rubidium chloride using a protocol similar to that of Sambrook et al. (1989) and stored at –80°C in 0.2-ml aliquots.

Buffer-saturated phenol <!>

Cell lines
Low-passage 293 cells (Microbix, Toronto, Canada)
A549 cells (ATCC, Manassas, Virginia)
Grow 293 and A549 cells in 150-mm dishes in a 37°C, 5% CO₂ incubator and split 1 to 2 or 1 to 3 when they reach about 90% confluence.

293N3S cells (Microbix)
Maintain 293N3S cells in 150-mm dishes as described above or in suspension. Grow the cells in suspension in maintenance medium in suspension flasks, agitated at 70 rpm. When they reach a density of 5 x 10⁵ cells/ml, dilute 1 to 2 or 1 to 3 with maintenance medium.

Cesium chloride gradient solutions (1.25 and 1.35 g/ml)
Dissolve 54.0 and 70.4 g of solid CsCl (Fisher Scientific) in 146.0 and 129.6 ml of dialysis buffer, respectively. Sterilize by filtration through a 0.2-μm filter.

Chloroform:isoamyl alcohol (24:1 v/v) <!>

2x Citric saline
270 mM KCl
30 mM sodium citrate
Sterilize by autoclaving for 45 minutes at 121°C.
### TABLE 3. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible cause(s)</th>
<th>Suggestion(s)</th>
</tr>
</thead>
</table>
| Few or no colonies after cotransformation of BJ5183 cells | Cotransformation conditions suboptimal | Try another, more efficient, transformation method, such as electroporation  
Correct antibiotic, or too much kan used | Plate the cells on 1.5% agarose LB plates supplemented with 25 μg/ml kan |
| Wrong strain of competent cells used | Ensure that BJ5183 cells are used |
| Competence of BJ5183 cells too low | Check the competence of the cells and generate new competent cells if necessary  
Use a different transformation method (e.g., electroporation) |
| Too many colonies after cotransformation of BJ5183 cells | Incomplete digestion of shuttle by Pmel | Use less DNA or more Pmel (also, ensure that Pmel is active)  
Check the digestion efficiency by agarose gel electrophoresis |
| Failure to generate plaques on 293 cells after initial transfection | Incomplete digestion with PvuI | Ensure that PvuI is active  
Examine digestion efficiency by agarose gel electrophoresis |
| Transfection efficiency too low | Optimize the transfection protocol by trying different amounts of DNA and transfection reagent  
Try another transfection reagent or method |
| DNA preparation not appropriate | Prepare DNA by CsCl gradient centrifugation  
Verify DNA concentration |
| 293 cell passages too high | Thaw a new aliquot of 293 cells |
| Defect in Ad vector backbone | Analyze the plasmid structure by digestion with several restriction enzymes; if a defect is detected, generate a new clone |
| Insert size exceeds upper limit of Ad packaging | Consult Table 2 |
| No virus band visible on CsCl gradients | DNA preparation not appropriate | Prepare DNA by CsCl gradient centrifugation  
Verify DNA concentration |
| Transgene product cytotoxic | Use a weaker or inducible promoter |
| Density of CsCl solutions incorrect | Verify densities by weighing 1 ml of each solution |
| Overlay the 1.35 g/ml CsCl with the 1.25 g/ml CsCl very carefully; ensure that a continuous, slow stream of 1.25 g/ml CsCl is ejected and that the phases do not mix |
| No transgene expression detected | The transgene or promoter is mutated | Analyze purified capsid DNA by restriction analysis and sequencing  
If an error is detected, screen other plaque isolates for transgene expression |
| The transgene not efficiently expressed | Verify that the promoter is active in the cell type being used  
Ensure that a Kozak sequence and polyadenylation sequence have been included in the construct |
| Purified virus is RCA | Examine replication on noncomplementing cells, such as A549  
If virus is RCA, or if RCA levels are high, purify the vector again, starting from the plaque purification step |
Complete medium
500 ml of minimum essential medium (MEM; Sigma-Aldrich)
500 ml of 10% fetal bovine serum (FBS; Sigma-Aldrich)
2 mM GlutaMAX (Invitrogen)
1X antibiotic-antimycotic (Invitrogen)
Dialysis buffer (10 mM Tris-HCl at pH 8.0)
   Sterilize by filtration through a 0.2-μm filter.
DNase I (Sigma-Aldrich)
   Prepare 10 mg/ml of DNase I in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM dithiothreitol,
   and 50% (v/v) glycerol.
Ethidium bromide
Isopropanol
Kanamycin (25 μg/ml)
Luria broth (LB) medium
MgCl2 (2 M)
   Sterilize by autoclaving for 45 minutes at 121°C.
Maintenance medium
500 ml of MEM (Sigma-Aldrich)
500 ml of 5% FBS (Sigma-Aldrich)
2 mM GlutaMAX (Invitrogen)
1X antibiotic-antimycotic (Invitrogen)
2X Maintenance medium
2X MEM (Invitrogen)
10% FBS (Sigma-Aldrich)
2X antibiotic/antimycotic (Invitrogen)
Phosphate-buffered saline (PBS)
137 mM NaCl
8.2 mM Na₂HPO₄
1.5 mM KH₂PO₄
2.7 mM KCl
   Sterilize by autoclaving for 45 minutes at 121°C.
Plasmids: Shuttle and backbone plasmids (see Tables 1 and 2)
Restriction endonucleases
   PacI restriction endonuclease (New England BioLabs)
Pmel (New England BioLabs)
   Other enzymes may be needed for cloning the gene of interest into the shuttle vector.
RNase A (10 mg/ml; Sigma-Aldrich)
NaCl (5 M)
   Sterilize by autoclaving for 45 minutes at 121°C.
Sodium deoxycholate (5% w/v)
   Sterilize by filtration through a 0.2-μm filter.
SDS–proteinase K solution
10 mM Tris-HCl (pH 7.4)
10 mM EDTA
1% SDS (w/v)
1 mg/ml proteinase K
SDS (0.1% w/v)-TE
   Dissolve 0.1 g of SDS in 100 ml of TE.
Sucrose (40% w/v)
   Dissolve 40 g of sucrose in 100 ml of PBS. Sterilize by filtration through a 0.2-μm filter.
SuperFect transfection reagent (QIAGEN)
Tris-HCl (10 mM)
1x TE
  10 mM Tris-HCl (pH 7.5)
  1 mM EDTA
Sterilize by autoclaving for 45 minutes at 121°C. For 0.1x TE, dilute the 1x stock solution with H2O and then sterilize by autoclaving for 45 minutes at 121°C.
1x Trypsin-EDTA
Dilute a 10x solution (Invitrogen) with PBS.

Equipment
Centrifuge (Avanti J-25 I, Beckman) equipped with a JLA 10.500 rotor (Beckman)
Heat sealer (e.g., Beckman Model 7700 Cordless Tube Topper)
Hemacytometer (VWR International, 15170-172)
Incubator (37°C, 5% CO2)
Laminar flow hood
Magnetic stirrer (five position, Bellco Glass, 7785-D2005)
Microcentrifuge tubes (1.5 ml)
Pasteur pipettes, sterile cotton-plugged
Petri dishes containing 1.5% agar (granulated agar; Fisher Scientific, BP1423-500) in LB medium supplemented with kanamycin (25 μg/ml)
Quick-Seal (16 x 76-mm ultracentrifuge tubes; Beckman, 344322)
Slide-A-Lyzer dialysis cassettes (10,000-kD m.w. cutoff, 0.5–3.0 ml) (Pierce, Rockford, Illinois)
Polypropylene tubes (13 ml capped)
Polystyrene tubes (5 ml)
Spinner flasks with impeller assembly: 250 ml (Bellco Glass, 1965-61002) and 3000 ml (Bellco Glass, 1965-61030)
Syringe (3 cc) equipped with a 22-gauge needle
Ultracentrifuge (e.g., Beckman Optima XL-100K ultracentrifuge)
  70.1 Ti rotor (Beckman)
  SW 41 Ti swinging bucket rotor (Beckman)
Ultra-Clear (14 x 89 mm) ultracentrifuge tubes (Beckman)

METHOD
Ad manipulations must be performed in a laboratory operating at biosafety level 2. A laminar flow hood and incubator should be dedicated to Ad work. Care should be taken to use only sterile equipment, reagents, and technique. Decontaminate and dispose of liquid and solid waste and disinfect contaminated surfaces.

Generation of Infectious Ad Plasmids

1. Generate a shuttle vector containing the transgene of interest using standard cloning procedures.
2. Digest 2 μg of the shuttle vector with 1 μl of of Pmel in a total volume of 20 μl overnight at 37°C.
3. Add 3 μl of Pmel-digested shuttle and 3.3 μl of 0.1 μg/μl supercoiled pAdEasy to a 13-ml capped polypropylene tube. Add 3 μl of Pmel-digested shuttle without pAdEasy to another tube as a negative control. Chill on ice.
4. Thaw two 0.2-ml aliquots of BJ5183 competent cells on ice.
5. Add 0.2 ml of BJ5183 cells to the DNA and incubate for 25 minutes on ice.
6. Incubate the tubes for 90 seconds at 42°C and then for 2 minutes on ice.
7. To each tube, add 1 ml of LB and incubate with shaking at 225 rpm for 25 minutes at 37°C.
8. Transfer the bacteria to 1.5-ml microcentrifuge tubes and centrifuge at 8500g for 1 minute at room temperature.
9. Aspirate 1 ml of LB and resuspend the pellet in a total volume of 0.2 ml.
10. Spread 0.1 ml of bacterial suspension on the surface of two petri dishes containing 1.5% solid agar in LB supplemented with 25 μg/ml kanamycin. Use one plate for the control suspension.
    No colonies should form on the negative control plate. However, if “background” colonies do appear, recombinant colonies will be noticeably smaller than those produced by bacteria transformed with undigested shuttle plasmid.
11. Choose four to eight small colonies and purify the plasmid DNA by small-scale alkali lysis. Suspend the DNA in a total volume of 25 μl of 0.1x TE.
12. Repeat Steps 5–10 with 5 μl of DNA and 100 μl of DH5α. Pick two colonies from each plate.
13. Purify the DNA by small-scale alkali lysis.
    Digest the DNA with several different restriction enzymes to verify the structure of the plasmid. Correct clones should be subjected to large-scale purification by CsCl buoyant density centrifugation and then used for generation of a recombinant Ad vector (see Step 15).

Cell Preparation

14. Prepare the cultured cells for transfection.

To prepare adherent 293 and A549 cells

a. Remove medium from 150-mm dishes of 293 or A549 cells.
b. Rinse monolayer twice with 5 ml of 1x citric saline (for 293 cells) or 2 ml of trypsin-EDTA (for A549 cells).
c. Remove all but 0.5 ml of the citric saline or trypsin-EDTA after the second rinse and leave the dishes for approximately 5–10 minutes at 22°C until cells begin to detach.
d. Tap the sides of the dishes to detach all cells.
e. Resuspend the cells in 11.5 ml of complete medium and distribute into new dishes.
   Incubate the plates so that the cells are about 90% confluent on the day of transfection.

To prepare of 293N3S cells in suspension

a. Transfer six confluent 150-mm plates of 293N3S cells to a 3-liter spinner flask. Bring the total volume in the flask to 1 liter with maintenance medium.
b. Every 1–2 days, remove 2 ml of suspension cells to a 15-ml polystyrene conical tube.
c. Add 2 ml of 2x citric saline and vortex vigorously for 10 seconds.
d. Incubate the cells for 15 minutes at 37°C.
e. Vortex vigorously for 10 seconds.
f. Count cells using a hemacytometer.
If cells are in clusters too large to count, continue to incubate at 37°C and vortex until clusters are broken up.

g. If the cell density is greater than $4 \times 10^5$ cells/ml, add 1 liter of maintenance medium to obtain 3 liters of cells with a final density of $3 \times 10^5$ to $5 \times 10^5$.

Transfection for Rescue of Ad Vectors

15. Digest 10 µg of the plasmid encoding the recombinant Ad genome (obtained from Step 13) with 2 µl of PacI in a total volume of 50 µl overnight at 37°C.

16. Mix 40 µl of PacI-digested DNA with 360 µl of MEM and 16 µl of SuperFect reagent in a round-bottom 5-ml polystyrene tube.

Other transfection reagents may be used in place of the SuperFect reagent.

17. Vortex vigorously for 10 seconds. Allow complexes to form for 15 minutes at room temperature.

18. Add 2.4 ml of MEM to each tube containing the DNA-SuperFect complexes. Mix well by titration.

19. Rinse four 35-mm plates seeded with 293 cells (as described above) twice with 2 ml of PBS.

20. Transfer 0.7 ml of the plasmid complex suspension to each 35-mm dish of 293 cells.

21. Incubate the cells with 5% CO₂ for 3 hours at 37°C.

22. After approximately 2.5 hours, melt 1% (w/v) agarose solution (minimum 1.5 ml per 35-mm dish) in a microwave oven. Equilibrate to 42°C in a water bath.

23. Equilibrate 2× maintenance medium (1.5 ml per 35-mm dish) to 37°C.

24. After 3 hours, remove the transfectant from the cells. Rinse the monolayer with 1 ml of PBS.

25. Mix equal volumes of the agarose solution and 2× maintenance medium. Add 3 ml to each 35-mm dish of 293 cells.

This must be done quickly to avoid solidification of the agarose, but gently to avoid disturbing the monolayer.

26. Allow the overlay to solidify (~15 minutes at 22°C) and return the cells to the incubator.

27. Incubate until plaques form (~7–12 days).

28. Choose several well-isolated plaques. Use a sterile cotton-plugged pasteur pipette to remove a plug of agarose over each plaque. Place each plug in a vial containing 1 ml of 4% sucrose PBS. Vortex briefly and store plaques at −80°C.

Initial Ad plaque isolates should be plaque-purified a second time (Steps 10–28) to ensure that the resulting virus is from a single clone.

Analysis and Expansion of Plaque-isolated Ad Vector

29. For each sample of plaque-purified vector obtained above, add 100 µl to two 35-mm plates of 293 cells (~90% confluence). Return cells to the incubator.

30. Allow the virus to adsorb for 1 hour, rocking the dishes every 10–15 minutes.

31. After the adsorption period, add 2 ml of maintenance medium to each plate and return them to the incubator.

32. Examine plates for cytopathic effect (CPE).

The cells should have a rounded morphology or be detached from the plate. Use one plate for analysis of the recombinant Ad structure. Reserve the contents of the other plate for vector expansion.


**To analyze the plaque-isolated Ad vector**

a. Once complete CPE is achieved, leave plates undisturbed for about 10 minutes in a laminar flow hood so that detached cells will come to rest on the bottom of the plate.

b. Carefully remove the medium. Resuspend the remaining cells in 0.2 ml of SDS–proteinase K solution. Transfer the suspension to a microcentrifuge tube.

c. Incubate the lysate overnight at 37°C.

d. Add 0.3 ml of TE to the lysate. Extract with 0.5 ml of buffer-saturated phenol, followed by 0.5 ml of chloroform:isoamyl alcohol.

e. Add 0.1 ml of 5 M NaCl and 0.5 ml of isopropanol.

f. Pellet the DNA by centrifugation at 20,000g for 10 minutes at 4°C.

g. Resuspend the DNA in 20–50 μl of TE and use 5–10 μl for digestion with appropriate restriction enzymes.

h. Examine the resulting banding pattern by electrophoresis on a 0.8% agarose gel, followed by staining with ethidium bromide.

**To expand plaque-isolated Ad vector**

a. Once complete CPE is achieved (Step 32), scrape the cells from the dish into the medium and remove to a 4-ml cryovial.

b. Add 40% sucrose PBS to a final concentration of 4%. Vortex briefly. Use immediately or store at –80°C until use.

c. Infect a 150-mm dish of 293 cells with 1 ml of inoculum. Return cells to the incubator.

d. Allow the virus to adsorb for 1 hour, rocking the dishes every 10–15 minutes.

e. After the adsorption period, add 2 ml of maintenance medium to each plate and return them to the incubator.

f. Examine plates for CPE. Once complete CPE is evident, remove the cells and medium to a 50-ml conical tube. Add 1/10 volume of 40% sucrose PBS to a final concentration of 4% sucrose and store inoculum at –80°C.

**Large-scale Preparation of Ad Vectors**

33. Thaw the inoculum in a 22°C water bath. If necessary, increase the volume of the inoculum to 30 ml using MEM.

**For large-scale preparation of Ad vectors using adherent 293 cells**

a. Remove medium from 30 150-mm dishes of 293 cells (~90% confluent at the time of infection), ten at a time, and replace with 1 ml of inoculum.

b. Allow the virus to adsorb 1 hour in a 37°C, 5% CO₂ incubator, rocking the plates every 10–15 minutes.

c. Add approximately 20 ml of maintenance medium to each dish and return the cells to the incubator.

d. Examine cells daily until complete CPE is evident (~2–3 days).
e. Remove medium and cells to two 500-ml polypropylene bottles. Most of the cells should be detached in the medium; however, any remaining cells are usually loosely attached and can be removed by tapping the sides of the dish.

f. Use the same pipette to rinse groups of ten dishes twice with 10 ml of PBS.

g. Centrifuge cells at 650g for 20 minutes at 4°C. Decant the medium and retain the cell pellets.

h. Resuspend the cells in 3 ml of 4% sucrose PBS and transfer to a 50-ml conical tube.

i. Use the same pipette to rinse the bottles once with 2 ml of 4% sucrose PBS and once with 4–5 ml of 4% sucrose PBS.

The total volume of the cell pellet should be about 15 ml. The cells can be processed immediately for vector purification or they can be stored at –80°C.

For large-scale preparation of Ad vectors using suspension-adapted 293N3S cells

a. Distribute a 3-liter culture of 293N3S cells into eight 500-ml centrifuge bottles.

b. Centrifuge at 650g for 20 minutes at room temperature. Decant the medium into sterile 1-liter bottles.

Retain 1 liter of spent medium and add it to the 3-liter flask. Return the flask to the incubator.

c. Use the spent medium to resuspend the cell pellets to a final volume of approximately 40 ml. Transfer the suspension to a 250-ml spinner flask. Rinse the bottles twice with 10 ml of spent medium and transfer to the spinner flask.

d. Add the thawed inoculum to the spinner flask.

The total volume in the flask should be about 100 ml.

e. Transfer the flask to the incubator and agitate the cells at 70 rpm for 2 hours.

f. Transfer the cells to the 3-liter suspension flask containing 1 liter of spent medium. Rinse the 250-ml spinner flask twice with approximately 250 ml of fresh maintenance medium and transfer to the 3-liter spinner flask. Add 500 ml of fresh maintenance medium to a final volume of 2 liters.

g. Remove 2 ml of the cell suspension to a 35-mm plate and place in a 37°C, 5% CO₂ incubator.

The cells should reattach to the plate.

h. Return the suspension flask to the incubator.

i. When complete CPE is evident on the 35-mm plate (~2–3 days), decant the suspension culture into 500-ml centrifuge bottles.

j. Centrifuge cells at 650g for 20 minutes at 4°C. Decant the medium and retain the cell pellets.

k. Resuspend the cells in 3 ml of 4% sucrose PBS and transfer to a 50-ml conical tube.

l. Use the same pipette to rinse the bottles once with 2 ml of 4% sucrose PBS and once with 4–5 ml of 4% sucrose PBS.

The total volume of the cell pellet should be about 15 ml. The cells can be processed immediately for vector purification or they can be stored at –80°C.
Vector Purification

34. Thaw pellets obtained by either method for large-scale preparation of Ad vectors in a 22°C water bath.

All volumes stated below are for a cell pellet with a total volume of 15 ml. Scale volumes accordingly.

35. Add 1.5 ml of 5% deoxycholate to the pellet. Incubate with frequent inversion for 30 minutes at 22°C.

The lysate should have a thick, highly viscous consistency.

36. Add 0.3 ml of 2 M MgCl₂, 0.15 ml of 10 mg/ml RNase A, and 0.15 ml of 10 mg/ml DNase I. Incubate with occasional inversion for 30–60 minutes at 37°C.

37. Once the viscosity of the lysate is near that of water, centrifuge at 1000 g for 10 minutes at 22°C.

38. Prepare CsCl step gradients in Ultra-Clear ultracentrifuge tubes (two tubes per virus).

a. Add 2 ml of 1.35 g/ml CsCl to each tube.

b. Carefully (i.e., with a steady stream, at a rate of ~30 sec/ml) overlay with 3 ml of 1.25 g/ml CsCl.

c. Carefully add equal volumes of cleared lysate (~6.5–7 ml) to each tube.

d. Balance the tubes and transfer to the buckets of a SW 41 rotor.

39. Use slow acceleration and deceleration profiles (500 rpm over ~5 minutes) to centrifuge the samples at 35,000 rpm for 1 hour at 10°C.

The viral band is the lowest band visible on the gradient and will be found at the interface between the 1.25- and 1.35-g/ml layers of the gradient.

40. Use a 3-cc syringe and a 22-gauge needle to pierce the tube approximately 1 cm below the virus. Turn the bevel so that it is parallel to the band and slowly remove, lowering the needle as the band lowers.

Virus from both step gradient tubes can be combined in a single Quick-Seal ultracentrifuge tube.

41. Fill the Quick-Seal tube containing the virus to the base of the neck with 1.35 g/ml CsCl. Use a heat sealer to seal the Quick-Seal tubes.

A balance tube can be prepared by filling another Quick-Seal tube to the base of the neck with 1.35 g/ml CsCl.

42. Centrifuge at 35,000 rpm with maximal acceleration and deceleration in a 70.1 Ti rotor overnight at 10°C.

43. Pierce the top of the sealed 70.1 Ti tube to form an air inlet. Use a 3-cc syringe and a 22-gauge needle to pierce the tube about 1 cm below the virus. Turn the bevel so that it is parallel to the band and slowly remove, lowering the needle as the band lowers.

Take care to minimize the volume extracted.

44. Inject the Ad into a prepared dialysis cassette. Remove the air bubble with the syringe.

45. Dialyze the Ad vector for 24 hours at 4°C against two 500-ml volumes of dialysis buffer.

46. Remove the vector from the dialysis cassette. Retain the syringe and 0.9 ml of dialysis buffer.

47. Add 40% sucrose PBS to the vector and the dialysis buffer to a final concentration of 4%. Store purified vector in small aliquots (~100–200 μl) at −80°C and the buffer at −20°C.

Ad vector stocks are stable for years at −80°C.
Characterization of Purified Ad Vectors

48. Further characterize the Ad vectors by assessing the genetic structure, determining titer, and examining for contamination with replication-competent adenovirus (RCA).

To confirm the genomic structure of the purified Ad vector

a. Rinse the syringe used to remove the vector from the dialysis cassette with 0.2 ml of SDS–proteinase K. Transfer the liquid into a 1.5-ml microcentrifuge tube.
b. Incubate overnight at 37°C.

c. Add 0.3 ml of TE to the lysate. Extract with 0.5 ml of buffer-saturated phenol, followed by 0.5 ml of chloroform:isoamyl alcohol.
d. Add 0.1 ml of 5 M NaCl and 0.5 ml of isopropanol.
e. Pellet the DNA by centrifugation at 20,000g for 10 minutes at 4°C.
f. Resuspend the DNA in 20–50 μl of TE and use 5–10 μl for digestion with appropriate restriction enzymes.
g. Examine the resulting banding pattern by electrophoresis on a 0.8% agarose gel, followed by staining with ethidium bromide.

To determine titer in infectious units by plaque-forming unit (pfu) assay

a. Prepare serial dilutions (10⁻⁴ to 10⁻⁹) of Ad vector in MEM.
b. Infect 293 cells (~90% confluent) in the wells of a six-well dish with 0.1-ml aliquots of each dilution. Return cells to the incubator.
c. Allow the virus to adsorb for 1 hour, rocking the dishes every 10–15 minutes.
d. After the adsorption period, overlay with agarose as described previously (Steps 22–26).
e. Incubate for 10–12 days. Count the number of plaques.
   Multiply the number of plaques in a well by the dilution factor to determine the vector titer in pfu/ml.

To determine vector titer in particles/milliliter

a. Dilute 20–50 μl of purified vector to a final volume of 1 ml in 0.1% SDS-TE.
   Use the dialysis buffer (from Step 46) as a blank control.
b. Incubate for 10 minutes at 56°C, vortex briefly, and centrifuge briefly.
c. Determine OD_{260}.
d. Calculate the number of particles/milliliter, based on the extinction coefficient of 1.1 x 10¹² for wild-type Ad (Maizel et al. 1968):

\[(\text{OD}_{260})(\text{dilution factor})(1.1 \times 10^{12})\]

A typical Ad vector preparation examined as described previously should have a ratio of particle to pfu of about 10 (Mittereder et al. 1996).
To detect presence of RCA in purified vector preparations

Recombination between vector DNA and Ad5 DNA present in 293 or 911 cells can result in transfer of E1 to the vector (Lochmuller et al. 1994), generating RCA. A549 cells do not express E1 and cannot support efficient replication of E1-deleted vectors. Therefore, after infection with purified Ad, only contaminating RCA will induce CPE in A549 cells.

a. Infect one 60-mm dish of 293 cells (~90% confluence) with $10^6$ pfu in 250 μl of MEM. Infect a second 60-mm dish with $10^7$ pfu in 250 μl of MEM. Infect a 150-mm dish with $10^8$ pfu in 1 ml of MEM. Return cells to the incubator.

b. Allow the virus to adsorb for 1 hour, rocking the dishes every 10–15 minutes.

c. After the adsorption period, add 2 ml of maintenance medium to each plate and return them to the incubator.

d. Once complete CPE is evident or 7 days pass, harvest the monolayer by scraping the cells into the medium, add 40% sucrose PBS to a final concentration of 4% sucrose, and store at −80°C.

e. Thaw the viruses obtained above and use 1 ml of each culture to infect an individual 150-mm dish of A549 cells. Add 1 ml of MEM to a fourth plate as a negative control.

f. Allow the virus to adsorb for 1 hour, rocking the dishes every 10–15 minutes.

g. After the adsorption period, add 2 ml of maintenance medium to each plate and return them to the incubator.

h. Compare the infected cells with the uninfected control daily for signs of CPE. Change the medium every 5 days, if necessary.

If CPE is evident (usually apparent by ~14 days postinfection), RCA is present in the purified stock. The relative amount of RCA to pfu can be inferred by comparing CPE on the three infected dishes.

i. Extract DNA from dishes showing signs of CPE. Analyze by restriction enzyme digestion and agarose gel electrophoresis.

A structure identical to that of wild-type Ad (due to the presence of E1) indicates the left end of the RCA genome, confirming contamination.

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