Preface

Heart disease is the leading cause of death in developed countries. Recent experimental advances featuring cellular, molecular, and genetic tools and technologies offer the potential for new therapeutic strategies directed toward remediation of inherited and acquired heart diseases. Whether these recent basic science advances will ultimately translate to clinical efficacy for patients with heart disease is unknown and is important to ascertain. *Cardiac Cell and Gene Transfer: Principles, Protocols, and Applications* is designed to provide the reader with up-to-date coverage of a myriad of specific methodologies and protocols for gene and cell transfer to the myocardium. Each chapter features a "Notes" section that provides useful "how to" problem-solving insights that are often left unstated in standard published protocols.

Cardiac Cell and Gene Transfer: Principles, Protocols, and Applications addresses principles and applications of cell and gene transfer to the heart, including protocols for vector production and purification. Detailed step-by-step methods and applications for first/second-generation adenoviral vectors, adeno-associated vectors, gutted adenoviral vectors, and lentiviral vectors are included. Additionally, detailed methods for cardiac cell grafting and transplantation are provided, and these chapters highlight the prospects of cell-based therapies for cardiac repair. The book also covers specific in vivo techniques for cardiac gene transfer, and specifies subsequent cellular and organ-level physiological assessment techniques and protocols. Accordingly, this book is designed for basic science and clinical researchers in the academic, pharmaceutical, and biotechnology sectors of the cardiovascular community.

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Gutted Adenoviral Vectors for Gene Transfer to Muscle

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1. Introduction

Adenoviral vectors are a popular choice for gene transfer protocols because they are well characterized, have a relatively large cloning capacity (up to 36 kB), and can be grown to high titers (10^{13} particles/mL) ***(1)***. Despite these attributes, first-generation adenoviral vectors retain many viral genes that can elicit a strong immune response, severely limiting their utility for studies in vivo ***(2)***. Our laboratory and others have been developing "gutted" or helper-dependent adenoviruses, which lack all viral coding sequences and therefore should greatly enhance the persistence of the vector in vivo ***(3,4)***. We have used this technology to deliver to muscle full-length cDNAs of the largest known gene, dystrophin, under control of the mouse muscle creatine kinase enhancer plus promoter ***(4–6)***.

By design, gutted adenoviral vectors must be grown in the presence of a helper virus to supply *in trans* all the viral proteins required for growth and replication of the gutted genome. Consequently, the gutted vector must then be purified away from the helper virus that is simultaneously produced. Specific packaging cell lines may be very useful for limiting the production of infectious helper virus while promoting the growth of the gutted virus. This chapter describes the methods used by our laboratory for generating, expanding, and titering gutted adenoviral vectors for gene transfer to muscle.

1.1. General Features of Gutted Adenoviral Vectors

The structure of a gutted adenovirus is a double stranded DNA genome that has at its termini the adenoviral inverted terminal repeat (ITR) sequences. These sequences, along with the covalently attached adenoviral terminal pro-

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tein, serve as the natural origin of replication ***(4,7,8)***. Adjacent to the left ITR is the viral packaging sequence, which is made up of seven A/T-rich pseudo-repeats normally located between 240 and 375 bp from the left end of the adenovirus 5 (Ad5) genome ***(9)***. The remaining length of the sequence is comprised of the desired expression construct(s) including regulatory elements, reporter genes, and "stuffer" sequences. Although the maximum length of an adenoviral genome can be 37.6 kB ***(10)***, for the purpose of purification from helper virus (see below), we recommend that the total length of the gutted virus genome be 27–30 kB. Smaller genome sizes have been observed to rearrange, necessitating the inclusion of a noncoding stuffer DNA fragment if the expression cassette being studied is too small ***(11)***.

1.2. Role of the Helper Virus

The ideal helper virus provides robust adenoviral gene expression yet does not compete with or interfere with packaging of the gutted virus. We use helper viruses that are replication-deficient owing to deletion of viral sequences in the Ad early region 1 (E1A and B genes). These gene products are supplied by the packaging cell line. This strategy ensures that any helper virus that escapes negative selection and is copurified with the gutted virus cannot replicate in a nonpermissive cell. To restrict amplification of the helper virus, we use helper viruses that contain "floxed" packaging signals, i.e., the packaging signal is flanked by *lox*P sites. One of our packaging cell lines, C7-cre, constitutively expresses cre recombinase, which recognizes the *lox*P sites and excises sequences between them. This cell line is capable of selecting against expansion of the helper virus by removing the packaging signal from >99% of the helper virus genomes ***(12)***, conferring to the gutted virus a competitive advantage for packaging proteins and ultimately producing higher yields.

1.3. Construction of Viral Genomes in Plasmid Backbones

Although construction of the large plasmids that contain the gutted or helper virus genomes can be problematic, we have had good success using a method of homologous recombination in *E. coli*. With this method, expression cassettes and/or stuffer fragments are inserted into the gutted virus shuttle vector that contains the adenoviral ITRs and packaging signals. Unique restriction sites are inserted just outside of the ITRs and are used for template preparation prior to viral rescue (*see* **Subheading 3.1.**). This digestion releases the viral genome from the bacterial origin of replication and antibiotic resistance gene of the plasmid backbone.

1.4. Rescue, Amplification, and Purification of Gutted Viruses

To initiate the production of a gutted virus, linear templates of both the gutted and helper viruses are cotransfected into an E1A/E1B-complimenting cell

line, such as 293 cells ***(13)***. We use the C7 cell line, which was derived from 293 cells and stably expresses the adenoviral polymerase and preterminal proteins ***(14,15)***. These proteins improve the conversion of DNA templates to viral genomes, a process we refer to as viral "rescue" ***(16)***. If a plasmid-embedded helper virus is not available, one can also initiate gutted virus production using protease-digested viral DNA in the cotransfection, or simply by adding purified helper virus 16–20 h following transfection of the gutted virus template ***(3,4)***. When viral cytopathic effects (CPEs) are observed in all cells, the cells are harvested with their growth medium and the viral titer is amplified on larger monolayers of cells through three to six passages until the desired titer is achieved, usually 10^{11} particles per 150-mm dish. The virus from the final cell lysate is purified through two CsCl gradients: the first gradient separates the viruses from cellular components and debris, whereas the second gradient separates the gutted virus from the helper virus. The virus is then dialyzed, titered, and stored in aliquots at –70°C.

2. Materials

1. 293 Cells (or derivatives such as C7 or C7-cre cells).
2. Tissue culture dishes (60, 100, and 150 mm) and 24-well plates.
3. DMEM + FBS: Dulbecco's modified Eagle's medium with L-glutamine supplemented with 10% Fetal bovine serum (FBS) and 100 µg/mL each penicillin G and streptomycin (all from GibcoBRL, Rockville, MD).
4. Phenol/chloroform (1:1) mixture.
5. Ethanol.
6. 0.1X TE: 1 m*M* Tris-HCl, 0.1 m*M* EDTA, adjust pH to 8 with 1 *M* HCl.
7. 2X HEPES-buffered saline (HBS): 20 m*M* HEPES, 150 m*M* NaCl, pH 7.03, 0.22-µm filter sterilized.
8. $CaCl_2$, 2 *M*.
9. Chloroquine, 100 m*M*.
10. Phosphate-buffered saline (PBS), pH 7.4 (Gibco-BRL).
11. 30% Glycerol in water, 0.45-µm filter sterilized.
12. 10% NP-40 in sterile water.
13. 250-mL Centrifuge bottles (Kendro Laboratory Products, Newtown, CT).
14. 20% (w/v) polyethylene glycol (PEG) 8000, 2.5 *M* NaCl (0.45 µm filtered).
15. Cell scraper (Sarstedt, Newton, NC).
16. DNase I (10 mg/mL).
17. RNase A (10 mg/mL).
18. 20 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$.
19. CsCl (density 1.3 g/mL) in 20 m*M* Tris-HCl, pH 8.0 (0.45 µm filtered).
20. CsCl (density 1.34 g/mL) in 20 m*M* Tris-HCl, pH 8.0 (0.45 µm filtered).
21. CsCl (density 1.4 g/mL) in 20 m*M* Tris-HCl, pH 8.0 (0.45 µm filtered).
22. CsCl (powder).
23. Dialysis membrane or cassettes (10,000 mw cutoff; Slide-a-Lyzer, Pierce, Rockford, IL).

24. 20 m*M* HEPES, pH 7.4.
25. 20 m*M* HEPES, pH 7.4 with 5% sucrose.
26. Virion lysis solution: 0.1% sodium dodecyl sulfate (SDS), 10 m*M* Tris-HCl, pH 7.4, and 1 m*M* EDTA.
27. EcR-293 cells (Invitrogen, Carlsbad, CA) .
28. Ponasterone A (Invitrogen) in 100% ethanol.
29. 24-Well culture dish coated with poly-L-lysine (Becton Dickinson, Bedford, MA).
30. PBS with 0.5% glutaraldehyde.
31. NBT/BCIP tablets (Sigma, St. Louis, MO).
32. X-gal substrate solution: 1 mg/mL X-gal, 41 mg/mL $K_3Fe(CN)_6$, 52 mg/mL $K_4Fe(CN)_6 \cdot 3H_2O$, 1 m*M* $MgCl_2$ in PBS.
33. Taqman Universal PCR Master mix (Applied Biosystems, Foster City, CA).
34. PCR primers.
35. Taqman probe (Applied Biosystems).
36. Real-time polymerase chain reaction (PCR) thermocycler/detector.

3. Methods

3.1. Rescue of Gutted Viruses by Cotransfection

To initiate a gutted virus preparation, linear gutted and helper virus templates are cotransfected into C7 cells. After 6–10 d, when all the cells have been infected, the titer of the gutted virus will be between 10^5 and 10^7 transducing units (tu)/mL, and the helper virus titers will be between 10^8 and 10^9 tu/mL. Ideally, the gutted virus titer following rescue will be $>10^6$ tu/mL, allowing for a multiplicity of infection (MOI) of 1 for the gutted virus during the next passage. It has been reported that optimal titers of gutted virus are obtained when the termini of the gutted and helper input DNA are identical ***(17)***. When one uses purified viral DNA as the source for helper virus rescue, the termini are covalently linked to terminal protein, making this an ideal substrate for replication. In this case, the gutted virus template is an inferior competitor for replication by the C7 cells and will ultimately be produced at much lower titers.

1. Seed a 60-mm tissue culture dish with approx 10^6 C7 cells in 5 mL of DMEM + FBS. Incubate until these cells reach approx 80% confluency, usually overnight (*see* **Note 1**).
2. Digest 5 µg each of the gutted and helper plasmid DNAs to release the viral genome templates completely from the plasmid backbone (*see* **Note 2**).
3. Extract the digested DNA with phenol/chloroform 1 time and then ethanol-precipitate, using caution to avoid shearing the long DNA fragments during these steps. Resuspend the DNA pellet in 220 µL 0.1X TE, pH 8.0.
4. Add 250 µL of 2X HBS and mix (*see* **Note 3**).
5. Precipitate the DNA by slowly adding 31 µL of 2 *M* $CaCl_2$ with gentle and constant mixing. Incubate the solution for 20 min at 22°C.

6. Add 5.5 µL of 100 m*M* chloroquine to the culture medium, gently rock the plate, and then add the DNA dropwise to the cells. Incubate at 37°C for 4.5 h in a tissue culture incubator (*see* **Note 4**).
7. Glycerol shock the cells by aspirating the medium from the cells and gently washing the monolayer with prewarmed PBS. Aspirate the PBS and then add 1.5 mL of 15% glycerol/1X HBS solution to the cells. After 40 s, remove the glycerol solution and rinse the cells twice with PBS.
8. Re-feed the plates with fresh DMEM + FBS. Incubate at 37°C until viral CPE reaches 100%, usually 8–12 d (*see* **Note 5**).
9. Collect the cells and medium from the plate and freeze/thaw 3 times in a dry ice-ethanol bath and a 37°C water bath. Store at –70°C. This is referred to as P0.

3.2. Amplification and Purification of Gutted Adenovirus

The titer and absolute amount of gutted virus is increased through several rounds of infection (passages). Below is a general outline of how the gutted virus can be expanded on C7-cre cells. This procedure may be modified based on empiric data for each unique gutted virus.

3.2.1. Amplification Through Serial Passages

1. Prepare a 100-mm tissue culture dish with an 80% confluent monolayer of C7-cre cells.
2. Inoculate the cells with 1.3 mL of infected cell lysate (P0) obtained from the co-transfection procedure (*see* **Subheading 3.1.** and **Note 6**). Incubate until CPE is complete, usually 2–4 d. Harvest the lysate and freeze/thaw as described in **Subheading 3.1.**, **step 9**. Store at –70°C.
3. Titer the gutted and helper viruses in this lysate (P1) using one of the procedures described in **Subheading 3.3.**
4. Prepare a 150-mm tissue culture dish with an 80% confluent monolayer of C7-cre cells.
5. Inoculate the plate with 2 mL of infected cell lysate (P1) supplemented with the appropriate amount of purified helper virus. The additional amount required (if any) should be based on the titer of the helper virus in P1. The final helper virus MOI should be 5–10 tu per cell. Incubate the cells until CPE is complete, usually 2–4 d. Harvest the lysate and freeze/thaw as above. Store at –70°C.
6. Titer the helper and gutted viruses produced in this passage (P2) as described in **Subheading 3.3.**
7. The final 2 rounds of amplification are carried out as in **steps 5–6**, using 10 and, then 50–100 × 150-mm dishes of C7–cre cells (*see* **Note 7**).
8. When CPE is complete in the final round of infection, harvest the cells and medium by adding 1 mL of 10% NP–40 to dissolve all cell membranes and transfer the lysate into 250 mL centrifuge bottles. Freeze the lysate in a dry ice–ethanol bath and store at –70°C, or begin the purification procedure (*see* **Subheading 3.2.2.**).

3.2.2. Purification of Gutted Adenoviral Vector

Gutted adenoviruses can be purified using protocols available for conventional adenovirus vectors, except that additional centrifugation steps are required to separate the gutted from the helper virus. We have found that the methods of Graham and Prevec ***(1)*** and Gerard and Meidell ***(18)*** both work well. A modified version of the latter is presented below.

1. Centrifuge the virus-containing lysate at 12,000*g* for 10 min at 4°C to remove cellular debris.
2. Transfer the supernatant to fresh, sterile 250-mL bottles, 160 mL per bottle, and add 80 mL PEG/NaCl solution. Mix well and place bottles in ice for 1 h to precipitate the virus particles.
3. Collect the virus particles by centrifugation at 12,000*g* for 20 min at 4°C. Promptly pour off the supernatant and keep the bottles inverted to allow the liquid to drain. Using a tissue, carefully wipe out the neck of the bottle to remove all traces of solution (*see* **Note 8**).
4. Resuspend the virus in a small volume (usually 5 mL per 2 pellets) of 20 m*M* Tris-HCl, pH 8.0, 1 m*M* $MgCl_2$. This procedure is most easily accomplished using a flexible cell scraper to ensure that all the virus is retrieved. Transfer the virus solution to a 50-mL conical tube.
5. Add DNase I and RNase A to a final concentration of 50 µg/mL each. Incubate at 37°C for 30 min to remove any genomic or unpackaged nucleic acids that were coprecipitated with the virus particles.
6. To the virus solution, add CsCl to a final density of 1.1 g/mL (0.135 g CsCl per mL). When completely dissolved, pellet any residual debris by centrifuging at 8000*g* for 5 min at 4°C. Collect the supernatant and note the volume (*x*).
7. Prepare CsCl gradients in Beckman Ultra-Clear SW28 centrifuge tubes as follows: First, pipet (31-*x*) mL 1.3 g/mL CsCl solution into the empty tube. Second, slowly pipet 7 mL 1.4 g/mL solution *under* the 1.3 g/mL solution. Mark the interface of the CsCl solutions. Finally, overlay the virus-containing solution on the gradient.
8. Centrifuge at 53,000*g* for 4–16 h at 5°C.
9. After centrifugation, the viruses will appear in the gradient as a double opalescent band near the interface of the 1.4 and 1.3 g/mL solutions. Using an 18-gauge needle attached to a 5-mL syringe, pierce the side of the tube just below these bands and slowly collect this region of the gradient, usually 0.5–0.9 mL.
10. Transfer this solution directly into a quick-seal ultracentrifuge tube. Fill the tube with 1.34 g/mL CsCl, seal and centrifuge at 320,000*g* for 12 h followed by 73,000*g* for an additional 12 h at 5°C in a Beckman NVT65 rotor (or equivalent).
11. The band of gutted virus will be 4–5 mm above the helper virus. Use a dark background and strong, direct light to visualize the bands clearly. Pierce the top of the tube with a 16-gauge needle to prevent formation of a vacuum, then carefully insert an 18-gauge needle between the two bands, and slowly pull the gutted virus band. Repeat **steps 10–11** if desired, keeping in mind that, although addi-

tional gradients will increase the purity of the gutted virus prep, there will be a decrease in the overall yield.

12. Dialyze the gutted virus in 20 m*M* HEPES, pH 7.4, with three changes of buffer. For the last change, add 5% sucrose to the buffer.
13. Aliquot in small tubes and freeze in a dry ice/ethanol bath. Store at –70°C (*see* **Note 9**).

3.3. Assessing Gutted and Helper Virus Titers During Expansion and Following Purification

If the gutted and helper viruses each contain reporter genes, analysis of the titer of these viruses can be accomplished by a simple transduction experiment in a permissive cell line, followed by an assay for the reporter gene product. We typically include a β-galactosidase gene in our gutted viruses and a human alkaline phosphatase gene in the helper viruses, both under the control of an inducible promoter. If one or both of the viruses lack reporter genes, it will be necessary to estimate the viral titers according to genome copy number by Southern analysis or real-time PCR. Both these methods involve comparing dilutions of the virus preparation with a standard curve of known quantity of reference material, i.e., plasmid DNA. We routinely use real-time PCR to estimate the viral genome copy number in infected cell lysates. Finally, to assay the amount of replication competent helper virus, one can perform an adenovirus plaque assay in a complementing cell line, according to standard protocols ***(1)***, although this assay requires up to 14 d to complete. These assays are described below.

3.3.1. Colorimetric Assay for Transducing Units

The reporter genes in our viruses are driven by the ecdysone-responsive promoter from pIND (Invitrogen), which is induced when EcR-293 cells are treated with ponasterone A, an analog of ecdysone.

1. Plate approx 10^6 EcR-293 cells in 1 mL of complete medium per well of a 24-well cell culture plate. Incubate overnight to produce a monolayer of 100% confluence.
2. Dilute infected cell lysates in medium containing 5 µL/mL Ponasterone A. Typical dilutions are 10^{-4} – 10^{-2} for titering virus in a C7–cre cell lysate, and 10^{-8} – 10^{-6} for purified virus.
3. Replace culture medium on cells with 300 µL diluted virus solution. Incubate for 16 h at 37°C.
4. Remove medium, wash cells once with PBS, and then fix cells with 0.5% glutaraldehyde in PBS for 10 min at room temperature. Wash twice with PBS.
5. For alkaline phosphatase assays, inactivate the endogenous enyzme by incubating the cells in PBS at 65°C for 1 h.
6. Add 0.5 mL substrate solution (NBT/BCIP for alkaline phosphatase or X-gal for β-galactosidase) and incubate overnight at 37°C.

7. Count positively stained cells and calculate the number of transducing units per mL of lysate.

3.3.2. Real-Time PCR Assay for Genome Copy Number

Primer pairs and probe to detect gutted or helper viruses must not amplify endogenous sequences from the packaging cell line. (C7-cre cells contain adenovirus sequences from the left end of the genome, as well as the polymerase and terminal protein genes). For helper virus detection, we use a sequence found in the L2 region of the virus defined by the following primer sequences: forward, 5'-CGCAACGAAGCTATGTCCAA-3'; reverse, 5'-GCTTGTAA TCCTGCTCTTCCTTCTT-3'; and probe, 5'- VIC-CAGGTCATCGCGCC GGAGATCTA-TAMRA-3'. The gutted virus is detected using a primer/probe set made from a region of the murine MCK promoter.

1. Dilute the reference plasmid in PCR-grade water such that the samples contain decreasing copies of the target sequence, e.g. 10,000, 1000, 100, 10 copies/μL, etc.
2. Dilute infected cell lysate 10^{-3} in PCR-grade water (*see* **Note 10**).
3. To assay purified virus stocks, dilute the virus fivefold in virion lysis solution and incubate at 56°C for 10 min. Make additional 10-fold dilutions in water before performing the PCR assay.
4. Using the standard curve, calculate the genome copy number per mL (*see* **Note 11**).

4. Notes

1. It is helpful to achieve a very even distribution of cells over the entire surface of the plate. Uneven plating will lead to insufficient lysis in some areas and total CPE in others.
2. The plasmids can be digested together if the same enzyme is being used.
3. Prepare and test the efficiency of 2X HBS solutions as described ***(19)***, as commercial stocks often perform poorly. Store the 2X HBS at –20°C for up to 6 mo.
4. During this incubation time, dilute the 30% glycerol solution with an equal volume of 2X HBS and equilibrate it, the PBS, and the culture medium to 37°C.
5. Viral CPE is considered 100% when all cells are round and mostly detached from the tissue culture dish.
6. Because this lysate was prepared in C7 cells, in which the helper virus growth is unrestricted, it contains ample amounts of helper virus to support a second round of gutted virus growth. Later passages in the amplification procedure, which are prepared in C7-cre cells, will not contain sufficient amounts of helper virus and will need to be supplemented to support production of the gutted virus.
7. Following the infection of 10 × 150-mm dishes, it is prudent to determine the titer of the gutted virus. If the genome copy number is $>10^9$ copies/mL, proceed with the large-scale expansion of 50–100 dishes; otherwise, repeat the 10-plate infection until the titer reaches its maximum, and then inoculate the final set of plates.
8. The pellet will be a widely dispersed, opaque region that covers most of the side of the bottle. There may also be a small pellet of debris at the bottom of the bottle.
9. Prepare aliquots according to the volume required for the planned experimental

procedure, as freeze/thawing purified virus will cause a decrease in the infectious titer. Estimate the particle count prior to aliquoting by incubating 5 μL of virus solution in virion lysis solution at 56°C for 10 min. Particle number per mL is equivalent to $[(A_{260} \times 21)/0.909] \times 10^{12}$ P/mL ***(20)***.

10. Crude lysates must be diluted at least 1000-fold to eliminate quenching of fluorescence by components of the culture medium.
11. The calculated genome number is used to evaluate only the relative expansion of the virus in each passage since all genomes, packaged and unpackaged, are detected with this assay. For purified virus, however, we have found that the copy number correlates with the particle number as determined by A_{260} spectrophotometry.

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