A Simplified System for Rapid Generation of Recombinant Adenoviruses

A Practical Guide for using the AdEasy System

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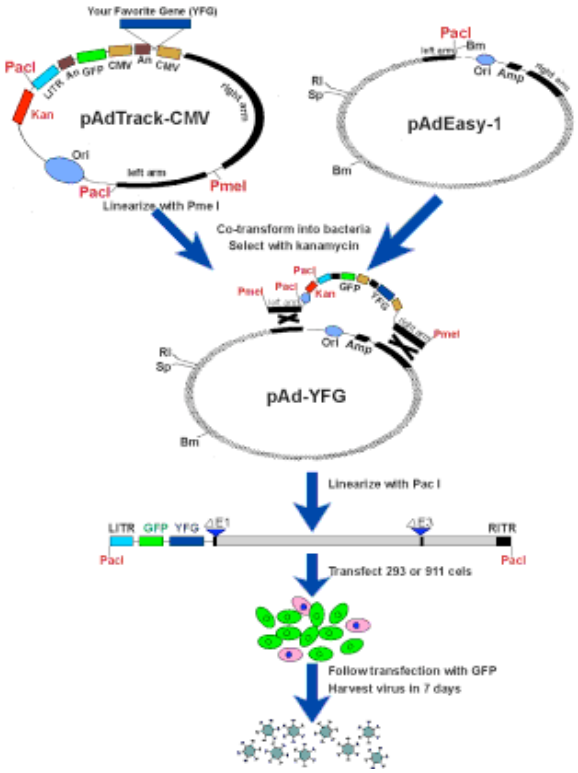
I. Introduction

Summary

Recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications. We here report a strategy which simplifies the generation and production of such viruses. A recombinant adenoviral plasmid is generated with a minimum of enzymatic manipulations, employing homologous recombination in bacteria rather than in eucaryotic cells. Following transfections of such plasmids into a mammalian packaging cell line, viral production is conveniently followed with the aid of green fluorescent protein, encoded by a gene incorporated into the viral backbone. Homogeneous viruses can be obtained from this procedure without plaque purification. This system should expedite the process of generating and testing recombinant adenoviruses for a variety of purposes.

Outline of the AdEasy system



Schematic Outline of the AdEasy System. The gene of interest is first cloned into a shuttle vector, e.g. pAdTrack-CMV. The resultant plasmid is linearized by digesting with restriction endonuclease Pme I, and subsequently cotransformed into E. coli. BJ5183 cells with an adenoviral backbone plasmid, e.g. pAdEasy-1,. Recombinants are selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analyses. Finally, the linearized recombinant plasmid is transfected into adenovirus packaging cell lines, e.g. 293 cells. Recombinant adenoviruses are typically generated within 7 to 12 days. The "left arm" and "right arm" represent the regions mediating homologous recombination between the shuttle vector and the adenoviral backbone vector. An: polyadenylation site; Bm: BamHI, RI: EcoRI; LITR: left-hand ITR and packaging signal; RITR: right-hand ITR; Sp: SpeI.

II. General Considerations

1. Because PmeI and PacI sites are designed to linearize the final constructs for

transformation and transfections, avoid using these sites in you inserts. (If you absolutely cannot avoid Pme1 and Pac1 sites, you can still use these vectors, but with more difficulty (employing partial digestions or digestion with EcoRI and recA-assisted restriction endonuclease (RARE) cleavage).

2. Avoid cloning elements that are present more than once in the vector (e.g., CMV promoters) in head-to-head orientations.

3. Except for pAdEasy-1 and pAdEasy-2, all other constructs (including recombinant adenoviral plasmids) confer resistance to kanamycin (*NOT ampicilin*).

4. High competence of BJ5183 cells is important to obtain homologous recombinants because these cells have relative low transformation efficiency. Therefore it is important to carefully follow the protocol for preparation of these cells.

5. Purification of plasmid DNAs: Standard CsCl gradient purification for all plasmid DNAs used in the experiments (especially pAdEasy-1 and pAdEasy-2) is recommended, though miniprep DNAs made by commercial kits (e.g. Nucleobond) may also be acceptable (see FAQ's section).

6. References:

T.-C. He, et al (1998) PNAS 95: 2509-2514.

H. Hermeking, et al (1997) Molecular Cell 1:3-11.

T.-C. He, et al (1998) Science 281(5382):1509-12

T.-C. He, et al (1999) Cell 99:1-20.

III. Generation of Recombinants in Bacterial Cells

1. Prepare electrocompetent BJ5183 cells in 20 micro**liters/tube aliquots (see Appendex A and B**).

2. Linearize the shuttle plasmids with Pme I. Usually one-fifth of a miniprep (typically 100-500 ng) is sufficient. After digestion, DNAs are phenol-chloroform extracted, ethanol precipitated, and resuspended in 6.0 microliters of ddH_2O.

3. Co-transform Pme I-digested shuttle plasmid with 1.0 microliter of **adenoviral backbone vector (100 ng/ul). Twenty microliters of electrocompetent *E. coli* BJ5183 cells were added and electroporation was performed in 2.0 mm cuvettes at 2,500V, 200 Ohms, and 25 micro-FD in a Bio-Rad Gene Pulser electroporator. Resuspend transformation mix in 500** microliters of **L-broth (incubation at 37°C for 10-20 min is optional, depending on background, see FAQ's). Plate on 3 to 5 LB/Kan plates, and grow at 37°C overnight (16-20 hrs).**

4. Pick up 10 to 20 smallest colonies, and grow them in 2 ml L-broth containing 25 microgram/ml kanamycin for 10-15 hours.

5. Perform minipreps using the conventional alkaline lysis method, and check the sizes of supercoiled plasmids by running one-fifth of a miniprep on 0.8% agarose gel.

6. Restriction digest DNA from clones with Pac I. Candidate clones usually yield a large fragment (near 30 kb), plus a smaller fragment of 3.0kb or 4.5kb.

7. Re-transform two microliters of the correct recombinant miniprep DNA into DH10B (or your favorite plasmid propagation strain). Plasmids are purified by CsCl-banding (see **Appendix D**) or by using commercially available purification kits for transfection of 293 cells.

NOTE: **A more efficient approach to generating adenoviral recombinants has recently been described in the section "AdEasy Got Easier".**

IV. Viral Production in 293 or 911 Cells

1. 293 cells (E1-transformed human embryonic kidney cells) or 911 cells (E1-transformed human embryonic retinal cells) in one or two T-25 flasks at 2 x 10^6 cells per flask ~24 hours prior to transfection. The confluency should be about 50% to 70% at the time of transfection.

2. On the day of transfection, digest recommbinant adenovial plasmids with PacI (usually 4 µg DNA is needed to transfect one T-25 flask). Ethanol precipitate the plasmids and resuspend in 20 µl of sterile H_2O.

3. Perform a standard Lipofectamine transfection according to manufacture's manual. Mix 4 µg of PacI-digested plasmid and 20 µl of Lipofectamine (GIBCO BRL) for each T-25 in 500 µl of OptiMem I medium, and incubate at room temperature for 15-30 min.

4. While waiting, remove growth medium from recipient cells and wash them once with 4 ml serum-free medium (e.g. plain DMEM, or Hank's). Add 2.5 ml OptiMem I per T-25 flask. Return to 37°C CO_2 incubator for ~10 min.

5. Add Lipofectamine-DNA mix to the flasks, and return to 37°C CO_2 incubator.

6. Remove medium containing Lipofectamin-DNA mix four hours later, and add 6 ml fresh complete DMEM (10% FBS, 1% Pen/Strep). Do not change the lipo/DNA medium if a significant amount of floating cells are observed; sometimes this happens with 293 cells and doesn't necessarily indicate a problem. If lots of floating cells are seen, add 6.0 ml complete DMEM to each flask and incubate at 37°C overnight. Change medium next morning.

7. Transfections and viral productions can minotored by GFP expression if pAdTrack-based vectors are used. No obvious plaques or CPE are easily observed by standard microscopy up to 2 weeks post transfection in most cases. However, plaques are always observed under fluorescence using the GFP marker.

8. Scrape cells off flasks with a rubber policeman (not trypsin) at 7 to 10 days post-transfection and transfer to 50 ml conical tubes. Spin cells in a benchtop centrifuge and resuspend pellet in 2.0 ml HBSS or sterile PBS. Freeze cells in dry ice/methanol bath, and thaw in a 37^{o}C water bath. Vortex vigorously. Repeat freeze/thaw/vortex for 3 more cycles (four cycles total). Do not let virus supernatants warm up. Spin samples briefly anc store supernatant at -20^{o}C.

9. Infect two 50% to 70% confleunt T-25 flasks of 911 or 293 cells using 30-50% of the above viral supernatants for each flask. CPE or cell lysis should become evident at 2 to 3 days post infection. Productive infections easily observed with the AdTrack vectors.

10. Collect viruses when a third to half of the cells are detached, usually 3 to 5 days post infection. Presence of the recombinant adenoviruses can be confirmed by Western blot and/or PCR (for PCR, take 5 µl virus sup plus 10 µl PCR-grade Protease K at 55^{o}C for 1 hour, then boil samples for 5 min. spin briefly, use 1 to 2 µl for PCR).

11. Scrape cells off and prepare viral supernatants as described in **Step 8** above. You should have at least 10^7 infectious particles/ml at this stage, and often much more. Each round of amplification should give at least 10-fold more virus than present in the previous round.

12. To amplify further, repeat the infection of cells using 30-50% of the viral supernatant from **Step 11**, using T75 flasks instead of T25 flasks. Titers can be measured at any time, which is particularly easy with AD-Track vectors. Simply infect 293 cells with various dilutions of viral supernatant and see how many are green 18 hours later. Without AdTrack, viruses can be plaque titered or titered by limiting dilution using standard methods; we find these methods much less simple and quantitative than that employing the GFP marker, but these have to be used if GFP is not present.

V. Preparation of High Titer Viral Stocks

1. Plate 911 or 293 cells in T-75 flasks to be 90% confluent at time of infection (about $1x10^7$ cells/T-75). Usually, fiften to twenty T-75 flasks are sufficient to make a high titer stock.

2. Infect cells with virus sup at a multiplicity of infection (MOI) of 5 to 10 PFU per cell. When all cells have rounded up and about half of the cells are detatched (usually at 3 to 4 days post infection), harvest and combine all flasks. Spin 5 min in a benchtop centrifuge (~500

g), and remove the supernatant.

3. Resupend pellet in 8.0 ml sterile PBS. Perform four cycles of freeze/thaw/vortex. Centrifuge lysate in Sorvall HS4 rotor at 6000 rpm (7000 g) 4°C for 5 min.

4. Weigh 4.4 grams of CsCl in a 50-ml conical tube, transfer 8.0 mls of clear virus sup to the tube, and mix well by vortexing. Transfer the CsCl solution (about 10 ml, density of 1.35 g/ml) to a 12 ml polyallomer tube for SW41 rotor. Overlay with 2 ml mineral oil. Prepare a balance tube. Spin the gradient in SW41 rotor at 32,000 rpm, 10°C, for 18 to 24 hours.

5. Collect virus fraction (about 0.5 to 1.0 ml) with a 3cc syringe and an 18g needle. Mix with equal volume 2X Storage Buffer (2X Storage Buffer = 10 mM Tris, pH 8.0, 100 mM NaCl, 0.1% BSA, and 50% glycerol, filter sterilized). Store virus stocks at -20°C.

6. Check viral titer by GFP (preferred) of by plaque assays (**see below**) or by immunohistochemical staining, or simply read OD at 260 nm. To read OD, add 15 µl virus to 15 µl blank solution (Blank Solution = 1.35g/ml CsCl mixed with equal vol 2X Storage Buffer) plus 100 µl TE/0.1% SDS; vortex 30 seconds, centrifuge 5 min. measure A260. One A260 unit contains ~1012 viral particles (particles:infectious particles =~20:1).

VI. APPENDICES

A. Protocol for making electrocompetent bacteria cells

Note: This is a generic protocol for making electrocompetent bacterial cells.

1) Use a fresh colony or frozen stock of DH10B cells to inoculate 10 ml of LB medium in a 50 ml tube (for BJ5183 cells, to inoculate 10ml LB containing 30 ug/ml streptomycin). Grow cells in a shaker overnight at 37°C.

2) Dilute 1 ml of cells into 1000 ml of LB medium (for BJ5183, use streptomycin-containing LB medium) in eight 1 liter flasks (125 ml each). Grow for 4 to 5 hour with vigorous aeration at 37°C, until A_{550} is ~0.8.

3) Collect cells in four 250 ml conical centrifuge tubes and incubate on ice for 10 minutes

to 1 hour. (The longer the cells are incubated the higher the competency.)

4) Pellet cells by centrifuging at 3,000 rpm (2,600 g) at 4°C for 10 min.

5) Wash the cell pellet by resuspending in 1000 ml of sterilized, ice-cold WB (WB = 10% ultra pure glycerol, 90% distilled water, v/v)

6) Centrifuge the cell suspension at 3,000 rpm (2500 g) for 30 min.

7) Repeat steps 5 and 6.

8) Pour off the supernate roughly, gently pipet most of supernate off, leaving about 20 ml, transfer the cell suspension to a 50 ml tube. Spin at 3,000 rpm x 10 min, and pipet all but 5 ml of the supernate out (**for BJ5183 cells, the final total volume should be limited to 2-3 mls**).

9) Resuspend cell pellet in the WB remaining in the tube. Aliquot 20 ul per tube (**the tubes should be pre-chilled at -80°C**) and store the aliquots at -80°C.

B. Checking Competency of Electrocompetent Cells

Purified plasmid DNA (10 ng/ul) 1 µl
DH10B competent cells 20 µl

1) Transform cells with Bio-Rad gene pulser (200 Ohms/25µF/ 2.5 KV).

2) Add 1 ml of L-Broth at room temperature.

3) Shake cells at 37°C for 1 hour in 50 ml tube.

4) Make 100-fold and 1000-fold serial dilutions of the cells in L-Broth.

5) Plate 100 ul of diluted cells on L-agar with 100 ug/ml ampiciliin.

6) Incubate overnight @ 37°C. Titer should be $>10^8$ colonies/ug, and we often achieve 10^9 colonies/ug.

C. Plaque Assay for Purification and Titration of rAdenoviruses

Note: this only needs to be done for adenoviruses without GFP marker. In those which

include GFP marker, titration of infectious units (i.e., those resulting in expression of GFP) is simply determined by fluorescence microscopy of your favorite cell type.

1. Remove all but 2 ml per well of medium from 6-well plates containing 80% to 90% confluent 911 or 293 cells. Infect with appropriately diluted virus for 3 to 6 hours. Infect cells with 6 different dilution titers (e.g. 10^{-3} to 10^{-8}ml/well).

2. Prepare the overlay agar as following: autoclave 100 ml of 2.8% Bacto-Agar (Difco) and keep warm at 45°C water bath. For 100 ml overlay:

	Stock Conc.	Final Conc.	ml/100ml overlay
2 X BME (GIBCO)	2 X	1X	50.0
HEPES	1.0M	20.0mM	2.0
MgCl2	1.0M	12.5mM	1.25
FBS	100.0%	10.0%	10.0
Pen/Strep	100 X	1 X	1.0
	mix well and incubate at 37°C bath.		
Bacto-Agar	2.8%	1.0%	36.0

Mix well and swirl at 37°C water bath, add 4 ml/well for 6-well plate.

Note: If you want to melt 2.8% agar stock, microwave or heat it in boiling water bath. Prepare 50 ml overlay at a time. To prevent the pre-drying of agar before plaque formation, it is important to add sterile PBS or Hank's medium in the space between wells, and wrap the plates with SaraWrap.

3. Return the plates to 37°C CO_2 incubator. On days 5 - 7, overlay 2 to 3 ml agar containing nuetral red (from 100x stock, available from GIBCO-BRL) to each well. Plaques should be visible 16-30hrs after the neutral red overlay. (In general, plaques should form in 911 cells in 4 or 5 days and in 293 cells after 5-7 days).

4. Pick 5 to 10 well-isolated plaques for each recombinant by punching out agar plugs with a sterile pasteur pipet. Store agar plugs in 200µl HBSS medium. Perform freeze/thaw/vortex cycle 4 times. Spin samples briefly.

5. Infect one well of 6-well plate of 911 or 293 cells (about 80-90% confluency at time of infection) with 50-70% virus sup of each plaque for 3 to 5 days (depending on the titer of individual plaque). Presence of recombinant virus can be confirmed by Western blot or PCR.

6. Collect viral supernatant using four cycles of freezing/thawing, as described earlier in this guide. The virus sup can be used to further infect T-25, and then T-75 flasks, for large scale preparation.

D. CsCl Banding of Plasmid DNA

1. Collect 400 ml cultures (from one 1L flask) in 500 ml centrifuge containers in Sorvall GS3 rotor (holds up to six 500 ml containers). Spin @ 6k (for 10 min @ 4^{o}C in Sorvall centrifuge.

Alternative: use 250 ml conical tubes, cf. 3000 RPM (2500 g) in IEC at 4 degrees for 15 minutes and follow "Wizard Protocol" for alkaline lysis and isopropanol precipitation steps..

2. Resuspend pellets in 7.5 ml BDI (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA stored @ 4 oC; made as follows: 4.5 gm glucose, 12.5 ml 1 M Tris pH 8.5, 25 ml 0.2M EDTA, 460 ml H2O, pH 8.05).

3. Transfer resuspended pellets to 50ml polypropylene tubes. Add 2.5 ml 20 mg/ml lysozyme in BDI. Vortex and sit @ RT for 5 min.

4.Add 20 ml BDII (made fresh) (= 0.125N NaOH/1% SDS; make this by combining 1 ml 50% NaOH, 10 ml 10%SDS, and 89 ml H_2O). Invert tubes gently and sit on ice for 10 min.

5. Add 15 ml BDIII (3M K+, 5M Acetate pH 4.8; made as follows: 147.3 g K.Acetate, 57.5 ml Acetic Acid, H_2O to 500 ml, pH should be ~4.8). Invert sharply several times and incubate on ice for 10 min. Spin in HS4 for 45 min at 7000 rpm.

6. Transfer 21 ml of supernatant to each of two 50 ml polypropylene tubes containing 14 ml isopropanol. Mix and let sit @RT for 5 min. Spin in HS4 rotor for 15 min @ 4.2 K rpm (4800 g) at 4°C.

7. Wash pellet with 70% ethanol. Remove last dregs of ethanol with pipet tip. Dissolve pellets in a total of 8.3 ml LoTE (4 ml per pellet).

8. Weigh 8.8 gm CsCl (Ultrapure) in weigh boat and put into a centrifuge tube. Add exactly 8 ml DNA solution. Density should be 1.57 - 1.60, but you generally do not need to measure this if you have weighed and pipetted out DNA carefully.

9. Add 800 ul 1 mg/ml ethidium bromide in TE.

10. Transfer solution to Ti 70 or Ti 50 ultracentrifuge tubes (both SETON 6041). screw on collars, put tops on. Tighten with screwdriver.

11. Opposite tubes should weigh within 100 mg of each other.

12. Place in Ti 70 rotor and spin @ least 16 (--> 20 hr) @ 50.000 rpm (brake @ 800rpm) @ 20^{o}C.

Alternatively, you can use a Ti 50 rotor and spin at 48 K for the same amount of time.

13. Prepare 2 syringes (3 cc) with 1" 22 gauge needles for each prep. Label syringes so you won't mix them up.

14. Carefully remove tubes from rotor with hemostat.Remove inner nut from centrifuge tubes.

15. Put on face shield. Set up tubes in ring stand above UV transilluminator. Insert needle

just above supercoiled band and bevel down to aspirate the band.

16. Pour remaining CsCl in chemical waste and wash centrifuge tube cones and nuts. The plastic centrifuge tubes are disposable but the nuts and cones are not.

17. Extract with an equal volume of H_2O saturated 1-butanol in 15 ml polypropylene tube. Saturated butanol = 40 ml butanol + 10 ml H_2O; the butanol layer stays on top.

18. Vortex ---> sit 10-60 sec @ RT. Discard top (red) layer

19. Repeat three times (total of 4 extractions). Add equal volume of H_2O-saturated butanol each time. (If begin with 1.5 ml, end with ~ 2.5 ml of solution).

20. Add 5 volumes LoTE to final volume of DNA in a 50 ml tube. Vortex, and add 2 volumes of ethanol (e.g., if 2.5 ml DNA, add 12.5 ml LoTE, mix, then add 30 ml ethanol).

21. Centrifuge 60 min @ 4200 rpm (=4800 g).

22. Wash with 75% ethanol twice. Re-spin and remove last drop of 75% ethanol. Do not speedvac.

23. Dissolve in 0.5 ml LoTE.

E. Alkaline Lysis Protocol for Plasmid Minipreps.

1. Pellet 2 ml overnight coli culture in 2-ml eppendorf tubes, and spin for 1 min.

2. Discard the supernatants,

3. Add 200 ul Resuspension Buffer (50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA pH8.0), and vortex briefly.

4. Add 200 ul Lysis Solution (0.2N NaOH and 1% SDS), gently mix by inverting the tubes several times.

5. Add 200 ul Precipitation Solution (5M potassium acetate 60 ml, glacial acetic acid 11.5 ml, and water 28.5ml), mix well by inverting the tubes several times,

6. Spin the tubes at top speed for 3 min.

7. Pour the supernatant to a new set of 1.5ml tubes. Add 500ul 2-propanol and mix well.