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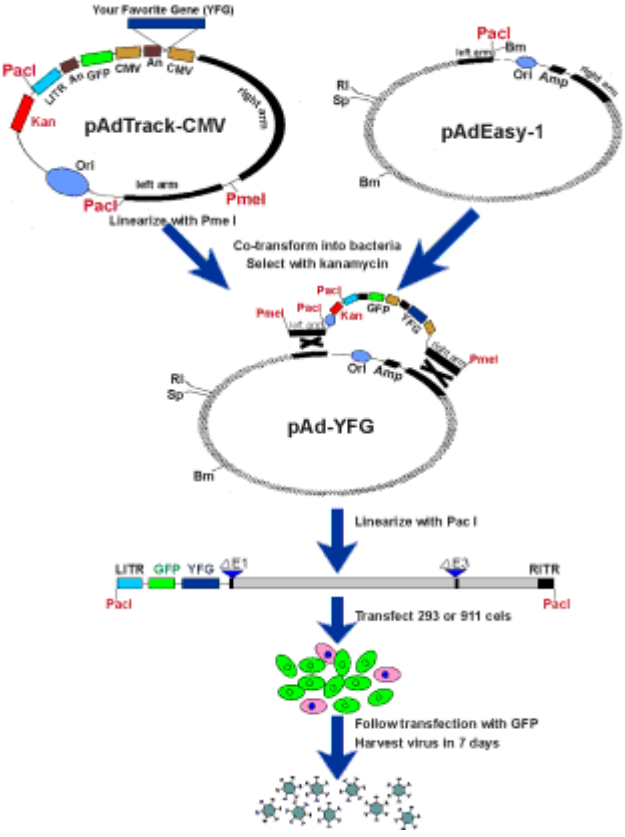
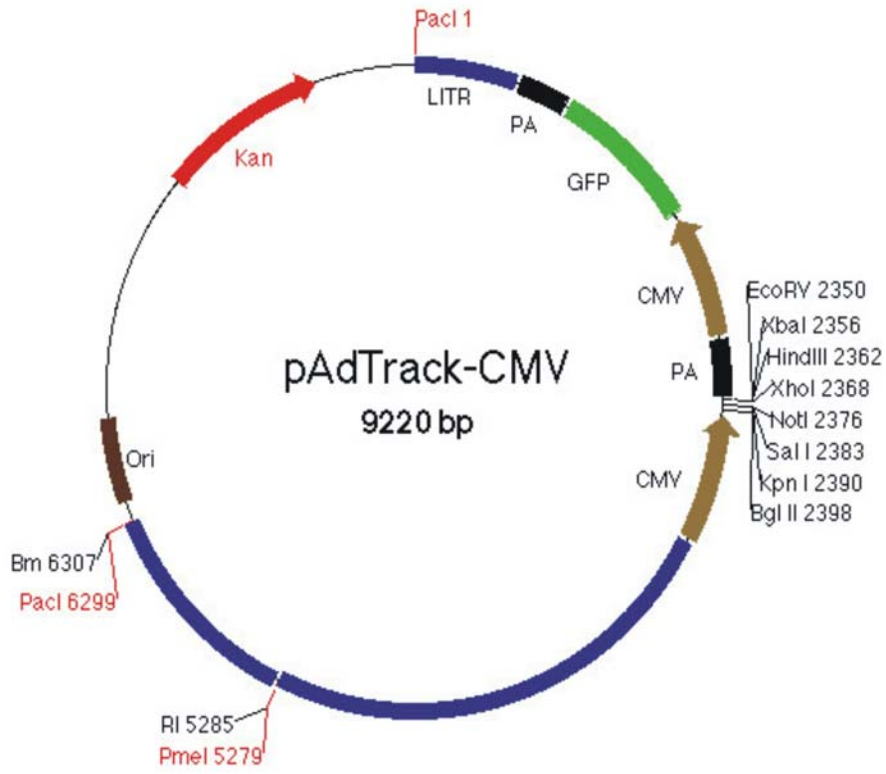
## Generation of Adenovirus

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-AD-1 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.



## Cloning / General Considerations

1. Clone cDNA of interest into pAd-Track CMV using the multiple cloning site. 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***). (Use Ad-Easy-2 for inserts greater than ~7kb.)

4. High competence of BJ5183-AD-1 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.

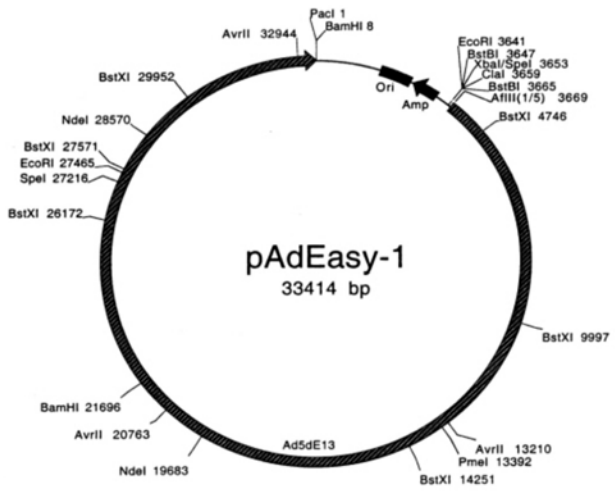
5. References:


*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.*

## Generation of Recombinants in Bacterial Cells

1. Purchase electrocompetent BJ5183-Ad-1 cells (Stratagene).

2. Linearize 1ug of the shuttle plasmid (Ad-Track CMV with your cDNA of interest) with PmeI. After digestion, DNAs are phenol-chloroform extracted, ethanol precipitated, and resuspended in $H_2O$ to 0.1ug/ul. Can store at -20.

Recombination:

1. Chill the required number of DNase-free microcentrifuge tubes and electroporation cuvettes (0.2 cm gap) on ice. (0.1cm will also work)

2. Referring to the instructions provided with the electroporator, set the following parameters on the instrument: 200 Ω, 2.5 kV, 25 μF.
(**Note:** Electroporator needs to be warmed up before use-20 minutes)

3. Remove the BJ5183-AD-1 electroporation competent cells from –80°C storage and thaw on ice.
(**Note:** keep the cells on ice all the times)

4. Gently pipet 40 μl of the competent cells into each of the chilled microcentrifuge tubes.

5. Into one tube of cells, pipet 1 μl (0.05 to 0.1 μg) of linearized shuttle vector.

6. Into a second tube of cells, pipet 1 μl of the provided transformation control DNA. (Optional)

7. Transfer the shuttle vector transformation mixture (from step 5) into a chilled electroporation cuvette and tap the cuvette gently to settle the mixture to the bottom. (Pipet the cells using a fine pipet tip, and make sure the cells spread in the BOTTOM of the cuvette. *Avoid bubbles!)*

8. Slide the cuvette into the chilled electroporation chamber until the cuvette connects with the electrical contacts.
(**Note:** make sure the orientation of the cuvette is correct otherwise it will not fit into the chamber. Keep the lid of cuvette closed during the electroporation. Make sure the cuvette connects with the contacts)

10. Pre-measure 1 ml LB in a separated bacterial culture tube for each sample.

11. Pulse the sample once by continuously pushing the button on the left- (Automatic Charge & Pulse), until you hear the 'pop' being careful of the spark, then quickly remove the cuvette. **Immediately** add (by pouring) 1 ml of sterile LB broth and pipet up and down to resuspend the cells using the plastic pipet in the bag of cuvette.
*You can also use SOC medium.

12. Transfer the cell suspension to a sterile 15-ml Falcon 2059 polypropylene tube.

13. Incubate the cell suspensions at 37°C for 1 hour with shaking at 225–250 rpm.

14. For the linearized shuttle vector transformations, plate the entire volume of cell suspension onto LB agar plates containing kanamycin; Spread different volumes of cells on three plates (50 µl, 100 µl, and 850 µl respectively) such that the entire volume is plated.
(**Note:** Separate the cell culture in the different plates, and no more than 200 µl/plates. Make sure spread all of the cultures because you only get very few colonies eventually. The electorporation process will kill 99% cells!)

15. For the transformation control, plate 10 µl and 100 µl of the cells on LB-kanamycin agar. When plating less than 100 µl, first place a 100-µl pool of LB broth on the plate, pipet the cells into the broth, and then spread the mixture.

16. Incubate the plates overnight at 37°C.

Screening for Positive Recombinants

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

2. 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.

3. Restriction digest DNA from clones with PacI. Candidate clones usually yield a large fragment (near 23-30 kb), plus a smaller fragment of 3.0kb or 4.5kb. ***** **It is very important to do extensive restriction mapping**

**at this step to be sure the recombination event did not alter your cDNA in any way.**

4. Re-transform (don't forget the kanamycin) one microliter of the correct recombinant plasmid minipreps into XL10-Gold bacterial cells and do a Qiagen maxi prep.

5. Verify that your recombinant is expressing by transiently transfecting your positive clone into 293 cells and harvesting protein (6-well plate) or RNA (10cm dish) for a western or northern to check for overexpression. Use the Lipofectamine protocol for transfection. Do not linearize plasmid for this transient transfection.

## Viral Production in 293 Cells

1. 293 cells (E1-transformed human embryonic kidney 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. One day prior to the transfection, change the culture media, to Pen/Strep FREE!

2. On the day of transfection, digest recombinant adenoviral plasmids with PacI . For 2 T-25s, digest 9ug in 50ul total volume. Ethanol precipitate the plasmids by adding 50ul of water, 50ul 7.5M ammonium acetate pH=7.5, and 200ul ethanol to the 50ul digest. Resuspend pellet in 45 µl of sterile $H_2O$. Run 5ul of DNA on gel to check digest.

3. Perform a standard Lipofectamine transfection according to manufacture's manual. Mix 4 µg of PacI-digested plasmid and 20 µl of Lipofectamine 2000 (GIBCO BRL) for each T-25 in 500 µl of OptiMem I medium (serm free), 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 HBSS). Add 2.5ml OptiMem I per T-25 flask. Return to 37°C $CO_2$ incubator for ~10 min.

5. Add Lipofectamine-DNA mix to the flasks, mix gently, 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 do not aspirate off DNA mix, just 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 be monitored by GFP expression if pAdTrack-based vectors are used. However, plaques are always observed under fluorescence using the GFP marker.

Harvest

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 IEC at 1000rpm for 5 minutes at 4C and resuspend pellet in 2.0 ml HBSS.

Freeze cells in dry ice/methanol bath (or 95% ethanol is fine-make sure ethanol coever the dry ice), 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 (5 minutes) and store supernatant at -20$^{o}$C.

9. Infect two 50% to 70% confleunt T-25 flasks of 293 cells using 30-50% of the above viral supernatants for each flask. 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 supernatant 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.

## Titering Virus

1. Plate cells of interest into a 6 well plate.

2. The next day, infect with different amounts of virus by washing off media and adding back media containing either 0, 0.5, 1, 1.5, 2, 4, 6, 8, 10ul virus/ml of media. Incubate 24-48 hours.

3. After 24 hours, GFP signal should begin to be apparent. Ideally, you want to pick the concentration of virus that infects approx 80-90% of cells without much cell death after 48 hours.

4. In order to confirm that you are infecting similar amounts of Ad-GFP and Ad-YFG (your favorite gene), you should perform a western blot and look for GFP. Alternatively, RT-PCR or northern blot can be performed. **********THIS SHOULD BE PERFORMED FIRST BEFORE MOVING ON TO YOUR EXPERIMENTAL ENDPOINTS TO BE SURE WHAT YOU ARE COMPARING IS ACCURATE.

5. To perform western, harvest protein using the whole cell extract protocol for cells. In brief, lyse cells with RIPA buffer, collect, incubate on ice 20minutes, spin down cells, pull supernatant through an 18g syringe. Spin and save supernatant for BCA and western blot.

6. If doing a northern, will have to start in larger wells such 10cm dishes.