Adenoviral Vectors

The AdEasy system was developed as a simple and efficient method for generating recombinant adenoviruses. Compared with traditional methods, the AdEasy system incorporates two unusual features. First, the viral backbone containing most of the adenoviral genome is supplied as a supercoiled plasmid rather than as linear viral DNA, which makes backbone amplification of the adenovirus much easier and also provides the possibility of generating stable recombinants. Second, the recombination step is performed in *E. coli* rather than in mammalian cells to take advantage of the high efficiency of homologous recombination in bacteria.

As outlined in Figure 12.4.1, the AdEasy system consists of three steps. The first is to subclone the genes of interest into a shuttle vector (e.g., pAdTrackCMV) containing two "arms" of viral sequence for homologous recombination with the adenoviral backbone vectors (i.e., pAdEasy-1 or pAdEasy-2). The second is to generate recombinant adenoviral plasmids between the *Pme*I-linearized shuttle vector and the supercoiled backbone vector in BJ5183 bacterial cells, using antibiotic selection with kanamycin. Unlike more than a dozen tested *E. coli* strains that harbored mutations in *recA, recBCD, recJ,* or *recF*, BJ5183 cells are not *recA* mutants but deficient in other enzymes that mediate recombination in bacteria (genotype: *endA, sbcB*$^{-}$*, recBC*$^{-}$*, str*^{R}; Hanahan and Gluzman, 1984), and have been chosen because of their ability to efficiently generate stable homologous recombinants. This process has proven most efficient when linearized donor molecules and circular recipient molecules were used. The final step is to generate recombinant adenoviruses by transfecting the recombinant adenoviral DNA into 293 cells. To efficiently generate adenoviruses, it is critical to liberate both ends (ITRs) of the recombinant adenoviral genome for the effective initiation of viral DNA replication through linearization with *Pac*I. In general, the production of adenoviruses is observed in 7 to 10 days after transfection.

The primary method (see Basic Protocol) provides detailed instructions for generating recombinant adenoviruses using the standard AdEasy system. An alternative which offers a simpler and more efficient approach to the production of recombinant adenovirus plasmids using the AdEasier cells is also provided (see Alternate Protocol; Fig. 12.4.2). The first supporting technique (see Support Protocol 1) describes preparation and purification of high-titer adenoviruses by equilibrium density gradient centrifugation on CsCl gradients. The second supporting method (see Support Protocol 2) describes the conventional plaque assay for titering adenoviruses. This protocol can also be used to plaque purify the adenoviruses. Also provided are detailed instructions for the preparation of electrocompetent BJ5183 *E. coli* cells (see Support Protocol 3). Preparation of high-quality, competent BJ5183 cells is one of the most important steps for using the AdEasy system. A description of the conventional alkaline lysis procedure for plasmid DNA minipreps is also given (see Support Protocol 4). This procedure is strongly recommended for the purification of shuttle plasmids that are used for homologous recombination in BJ5183 cells. The penultimate protocol (see Support Protocol 5) provides a brief procedure for adenoviral DNA preparation. Finally, the last protocol (see Support Protocol 6) describes a simple and efficient procedure for quick agarose-tube dialysis immediately prior to the use of adenoviruses.

The AdEasy system is freely available for academic researchers. For more detailed information, please visit the AdEasy website at *http://www.coloncancer.org/adeasy.htm*.

Contributed by Tong-Chuan He

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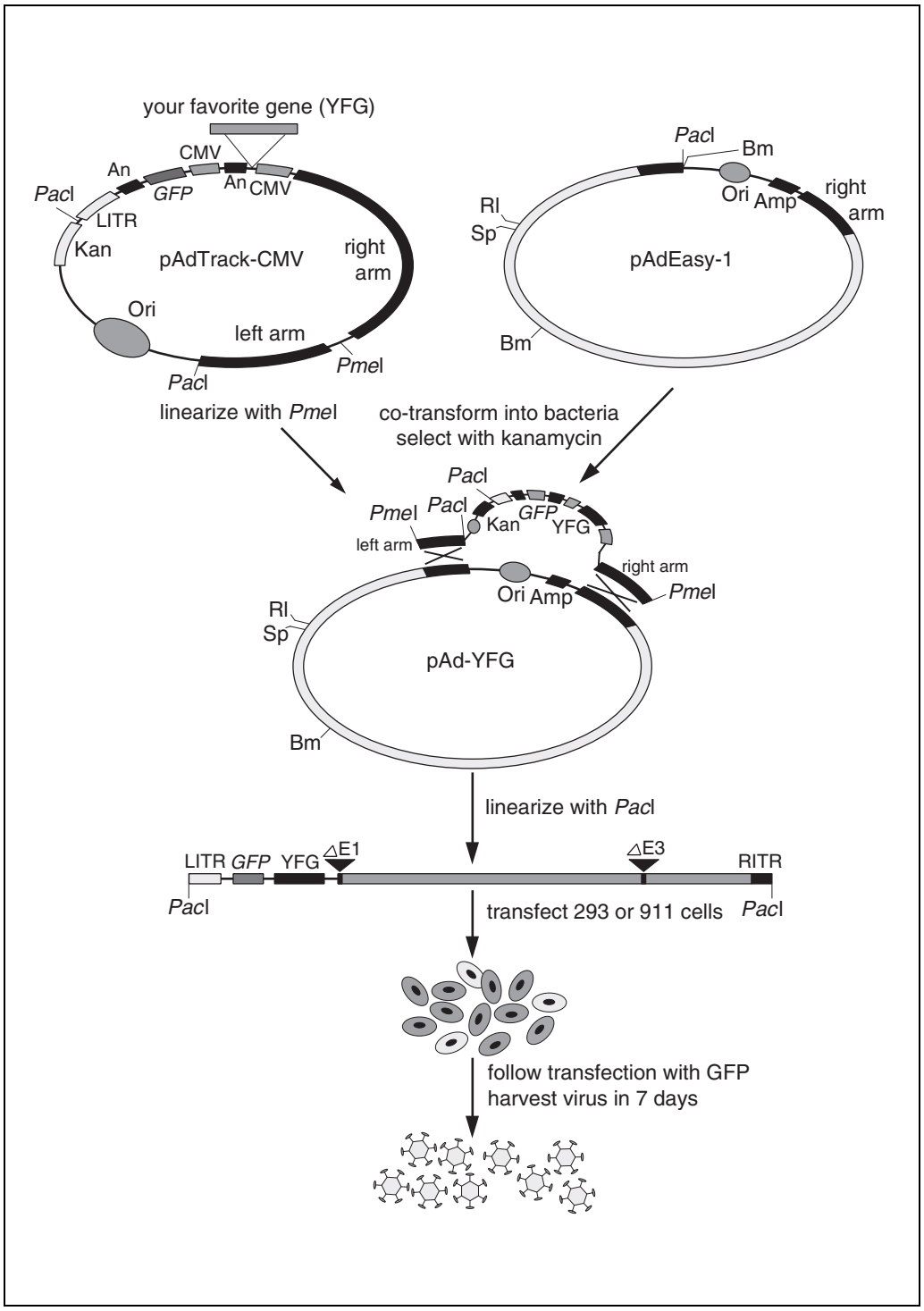

Figure 12.4.1 Schematic overview of the AdEasy technology. The gene of interest is first cloned into a shuttle vector (e.g., pAdTrack-CMV). The resultant plasmid is linearized by digesting with restriction endonuclease *Pme*I, and subsequently cotransformed into *E. coli*. BJ5183 cells with an adenoviral 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. Abbreviations: An, polyadenylation site; Bm, *Bam*HI, RI, *Eco*RI; LITR, left-hand inverted terminal repeat (ITR) and packaging signal; RITR, right-hand ITR; Sp, *Spe*I. Reproduced from He et al. (1998) with permission. Copyright (1998) National Academy of Sciences, U.S.A.

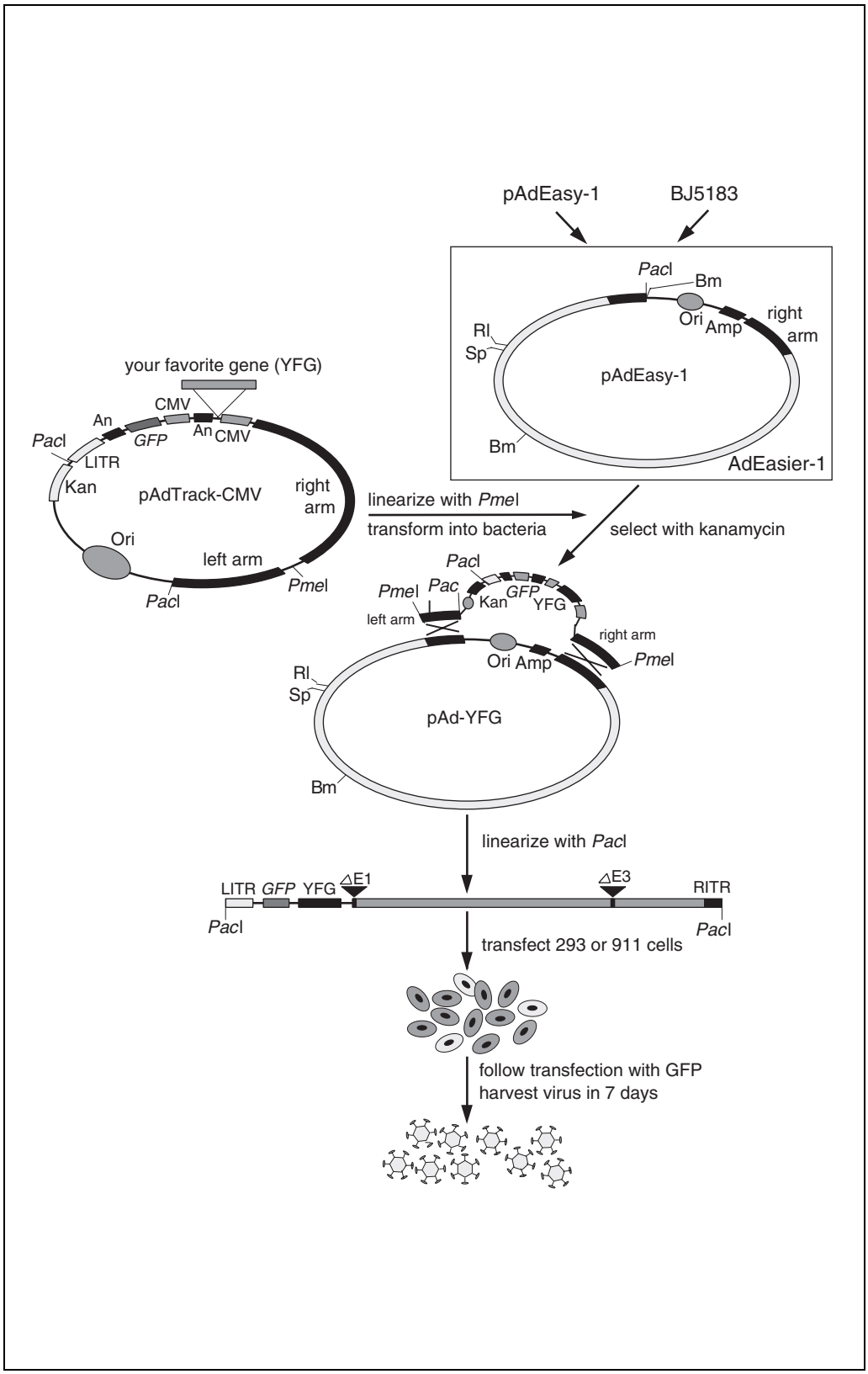

Figure 12.4.2 Schematic representation of the modified AdEasy technology. The revised methodology involves using the AdEasier cells that are BJ5183 derivatives containing pAdEasy backbone vectors (pAdEasy-1 shown) in order to increase the efficiency of generating recombinant adenovirus plasmids. The rest of methodology is virtually the same as the standard AdEasy system described in Figure 12.4.1. Adapted from He et al. (1998) with permission. Copyright (1998). National Academy of Sciences, U.S.A.

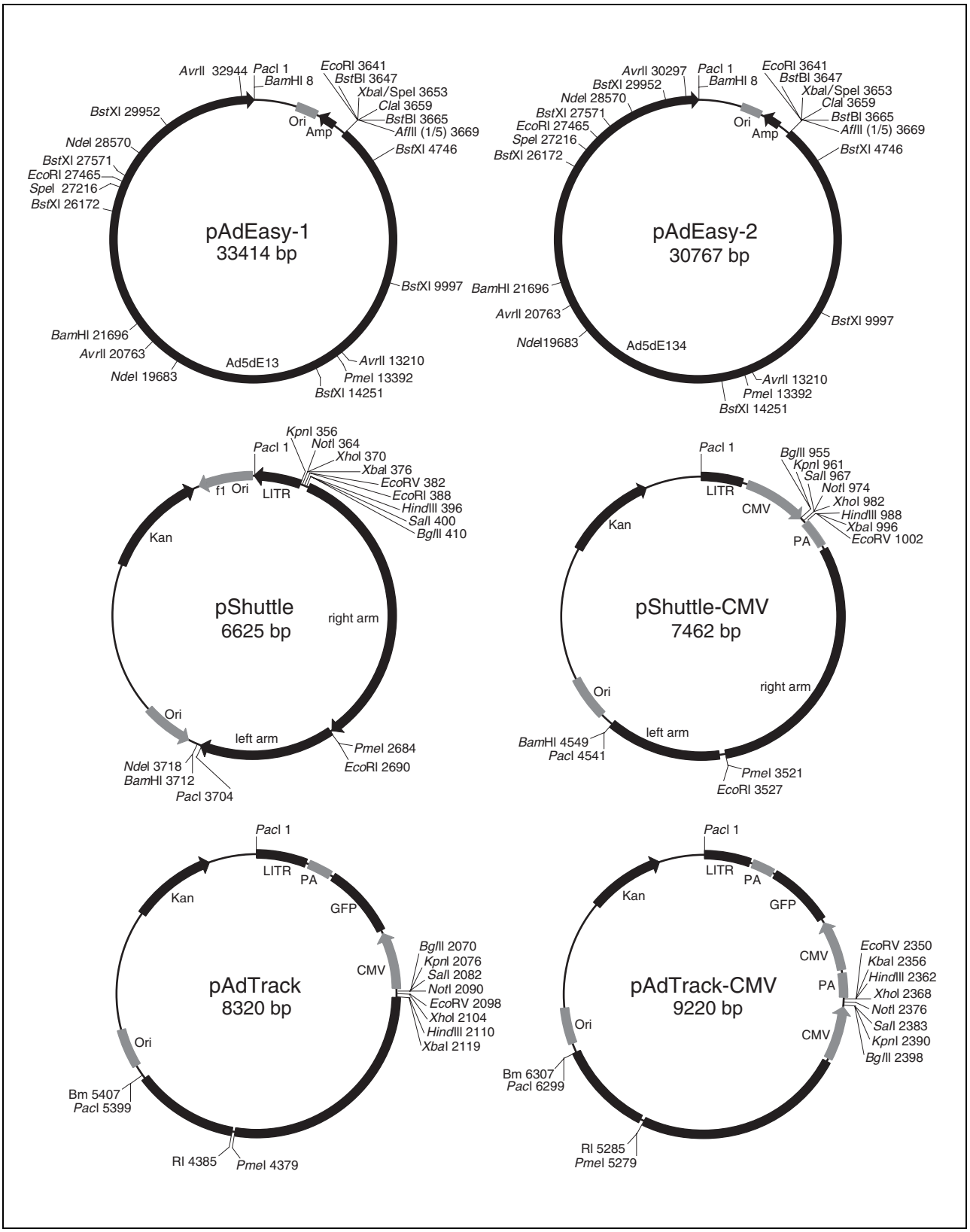

Figure 12.4.3 Shuttle vectors and adenoviral backbone plasmids used in the AdEasy technology. Abbreviations are defined in the legend to Figure 12.4.1. See text for details. Reprinted from He et al. (1998) with permission. Copyright (1998) National Academy of Sciences, U.S.A.

Table 12.4.1 Selection of the AdEasy Vectors

Shuttle plasmid	Adenoviral backbone	Packaging cells	Maximum insert size	GFP tracer	Use
pAdTrack-CMV	pAdEasy-1	293 or 911	5.0 kb	Yes	Expression of gene of choice under CMV promoter with a built-in GFP tracer
pAdTrack-CMV	pAdEasy-2	911-E4	7.7 kb	Yes	Expression of gene of choice under CMV promoter with a built-in GFP tracer
pAdTrack	pAdEasy-1	293 or 911	5.9 kb	Yes	Expression of gene(s) of choice under your favorite promoter with a built-in GFP tracer
pAdTrack	pAdEasy-2	911-E4	8.6 kb	Yes	Expression of your favorite gene(s) under promoter of choice with a built-in GFP tracer
pShuttle-CMV	pAdEasy-1	293 or 911	6.6 kb	No	Expression of genes in the absence of GFP tracer
pShuttle-CMV	pAdEasy-2	911-E4	9.1 kb	No	Expression of genes in the absence of GFP tracer
pShuttle	pAdEasy-1	293 or 911	7.5 kb	No	Expression of large or multiple genes in the absence of GFP tracer
pShuttle	pAdEasy-2	911-E4	10.2 kb	No	Expression of large or multiple genes in the absence of GFP tracer

BIOSAFETY

According to the National Institutes of Health biosafety guidelines based on risk assessment, manipulations on human adenoviruses should be performed in a laboratory operating at Biosafety Level 2 (BL2) as approved by the user's Institutional Biosafety Committee. The requirements include the use of laminar flow hoods, the establishment of proper procedures for decontamination and disposal of liquid and solid waste, and the disinfection of contaminated surfaces and equipment. Also see *UNIT 12.1* for a discussion of safety issues related to gene therapy.

STRATEGIC PLANNING

Choice of Vectors

The AdEasy system provides four different shuttle vectors. As illustrated in Figure 12.4.3, the pShuttle vector is the basic shuttle with the maximal capacity for accommodating foreign genes and the flexibility of desired promoter for transgene expression. The pShuttle-CMV vector contains a built-in CMV promoter–driven expression cassette. The pAdTrack and pAdTrack-CMV vectors incorporate independent expression cassettes carrying the green fluorescent protein (GFP) marker to facilitate tracking of transgene expression. However, both vectors have significantly reduced capacity for accommodating foreign genes.

Two adenoviral backbone vectors are offered by the AdEasy system. The pAdEasy-1 is an E1 and E3 double-deletion vector. Hence, the AdEasy-1 derived recombinant adenoviruses can be propagated in E1-expressing packaging cells, such as 293 or 911 cells. pAdEasy-2 is an E1, E3, and E4 triple deletion vector. Thus, propagation of AdEasy-2-derived vectors requires the use of packaging cell lines expressing both E1 and E4. Because the E4 gene product is highly toxic to mammalian cells, its expression is usually controlled by inducible promoters. There are several such E1/E4 packaging lines currently available; however, because of the leakiness of most inducible systems, the E4 expressing cells are often lost after serial passages and it is difficult to generate high-titer AdEasy-2-derived viruses. In this unit, the focus is on the procedures for generating recombinant

adenoviruses with the AdEasy-1 system. The features and utility of different vectors are listed in Table 12.4.1 to help researchers choose an appropriate combination of vectors.

Cloning Genes of Interest into Shuttle Vectors

General guidelines for cloning genes of interest into shuttle vectors are provided below; however, researchers are also advised to consult with general molecular biology manuals for full details on cloning techniques and protocols.

If the gene of interest and the shuttle vector do not have correctly positioned restriction sites, it may be necessary to blunt-end one or both restriction sites with T4 DNA polymerase. In some cases, it may be more convenient to introduce new restriction sites at one or both ends by linker ligations or by PCR amplification. Introduction of restriction sites by PCR is quick and efficient, but the amplified genes must be verified by DNA sequencing.

If the pShuttle or pAdTrack vector is chosen, users have to provide a promoter and a polyadenylation signal for the transgene expression cassette. For all shuttle vectors, it is absolutely critical to include a consensus Kozak sequence in front of the coding sequences.

Because *Pme*I and *Pac*I sites are designed to linearize the final constructs for transformation and transfections, these sites should be avoided in the inserts. If they cannot be avoided, these vectors can still be used, but with more difficulty, by employing partial digestions, digestion with *Eco*RI and recA-assisted restriction endonuclease (RARE) cleavage, or site-directed mutagenesis to eliminate the restriction sites of interest.

If multiple gene expression cassettes are desired, it is critical to avoid cloning the same elements (e.g., CMV promoters) in head-to-head orientations. Deletion of the sequence between the two elements may occur if a homologous recombination event takes place. However, this type of unwanted recombination can be avoided by placing the repetitive elements in a head-to-tail orientation.

BASIC PROTOCOL

GENERATION OF RECOMBINANT ADENOVIRAL VECTORS USING THE AdEasy METHOD

To generate recombinants, linearized shuttle vector DNA is cotransformed with supercoiled pAdEasy-1 plasmid into electrocompetent BJ5183 cells. Homologous recombinants are selected on the basis of kanamycin resistance, size, and restriction mapping. Recombinant plasmids are propagated in a bacterial strain less prone to recombination than BJ5183, such as DH10B cells. Finally, the purified recombinant adenovirus plasmids are digested with *Pac*I to liberate both ITRs. The *Pac*I-digested DNAs are introduced into the packaging cell line 293 by lipofection. Viral production should be observed 7 to 10 days after transfection.

Materials

Gene of interest
LB medium with kanamycin (*APPENDIX 2D*)
Restriction endonucleases (AdEasy specific, *Pac*I, and *Pme*I or *Eco*RI)
Shuttle vector DNA (Quantum Biotechnologies or Stratagene)
7.5 M ammonium acetate (*APPENDIX 2D*)
seeDNA (Amersham Pharmacia Biotech)
20 mg/ml glycogen (Roche Molecular)
25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (*APPENDIX 3C*)
70% and 100% ethanol

Electrocompetent BJ5183 cells (see Support Protocol 3, or Quantum Biotechnologies or Stratagene)
pAdEasy-1 supercoiled adenoviral backbone vector (Quantum Biotechnologies or Stratagene), CsCl purified
LB/kanamycin plates (*APPENDIX 2D*)
0.8% (w/v) agarose gel
Competent DH10B cells or other cells not prone to recombination
293 cells (E1-transformed human embryonic kidney cells)
LipofectAMINE reagent (Life Technologies)
Opti-MEM I medium (Life Technologies)
Dulbecco's modified Eagle medium (DMEM; *APPENDIX 3G* or Life Technologies)
Complete DMEM: DMEM with 10% FBS, 1% penicillin/streptomycin
HBSS or sterile PBS (Life Technologies)

15-ml conical tubes
37°C orbital shaker
2-mm electroporation cuvettes, ice cold
Bio-Rad Gene Pulser electroporator (or similar apparatus)
37°C bacteria incubator
25-cm^2 tissue culture flasks
37°C, 5% CO_2 incubator
Cell scrapers (rubber policeman)
50-ml conical centrifuge tubes
Dry ice/methanol bath

Additional reagents and equipment for alkaline lysis (see Support Protocol 4), phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation of DNA (*APPENDIX 3C*), preparation of electrocompetent BJ5183 cells (see Support Protocol 3), agarose gel electrophoresis (*UNIT 2.7*), and CsCl purification of plasmids (e.g., *UNIT 5.3*)

NOTE: All cell culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All solutions, reagents, and equipment coming into contact with cells must be sterile, and proper sterile and antiseptic techniques should be used accordingly. Biohazard wastes containing adenoviruses should be disinfected with chlorine bleach.

NOTE: Skip steps 6 to 10 if the AdEasier cells are used to generate recombinants as described (see Alternate Protocol).

Clone gene into shuttle vector

Clone the gene of interest directly into the chosen shuttle vector, taking into consideration the issues described above (see Strategic Planning). It is a good practice to confirm transgene expression in the shuttle vectors by transient transfection assays before starting adenovirus construction. Integrity of the transgenes in final recombinant adenoviral plasmids should also be analyzed by diagnostic restriction endonuclease digestions or PCR amplification.

1. Grow candidate gene of interest clones in 2 ml LB/kanamycin in a 15-ml conical tube and culture by shaking overnight in a 37°C orbital shaker.

2. Purify plasmid DNA using the alkaline lysis procedure (see Support Protocol 4). Confirm the presence and orientation of the transgene by restriction analysis and/or PCR amplification.

   All shuttle vectors and recombinant adenoviral plasmids confer resistance to kanamycin, except pAdEasy-1 and -2, which are ampicillin resistant. Vector maps and sequences can be found at the AdEasy website (http://www.coloncancer.org/adeasy.htm).

3. Linearize the confirmed shuttle vector with *Pme*I or *Eco*RI restriction enzyme. To ensure a complete digestion, use a 100-µl restriction reaction with 5 µl enzyme. Ensure that the digestion is complete to minimize background, and verify on an agarose gel.

   One-fifth to one-quarter of each miniprep (~0.1 to 0.5 µg DNA) is usually sufficient for one cotransformation in BJ5183 cells.

4. To the 100-µl DNA restriction solution, add 100 µl distilled, deionized water, 100 µl of 7.5 M ammonium acetate, and 2 µl seeDNA (or substitute with 2 µl of 20 mg/ml glycogen); extract with 300 µl of 25:24:1 phenol/chloroform/isoamyl alcohol, pH 8.0.

   The phenol/chloroform/isoamyl alcohol extraction and subsequent ethanol precipitation are described in APPENDIX 3C.

5. Transfer the top layer of DNA solution to a clean tube. Precipitate with 600 µl of 100% ethanol by centrifuging 5 min at 16,000 × *g*, room temperature. Wash the pellet two times with 70% ethanol to eliminate residual salts. Resuspend DNA in 8 µl distilled, deionized water.

   It is not necessary to gel purify the linearized vector, because the purification process may reduce the transformation efficiency, and, more importantly, may generate undesired nicks in the DNA. Also, dephosphorylation of the linearized vector is not recommended to help reduce background.

Generate recombinant adenovirus plasmids in BJ5183 cells

6. Prepare electrocompetent BJ5183 cells in 20 µl/tube aliquots kept at –80°C (see Support Protocol 3). When ready for transformation, thaw aliquots and keep competent cells on ice.

7. To 20 µl competent cells, add 8.0 µl *Pme*I-digested shuttle vector (from step 5) and 1.0 µl pAdEasy-1 supercoiled adenoviral backbone vector (stock 100 ng/µl). Limit the final volume to ≤30 µl.

8. Carefully transfer the bacteria/DNA mix to an ice-cold 2-mm electroporation cuvette, avoiding formation of bubbles, and keeping the cuvette on wet ice. Deliver the pulse at 2500 V, 200 Ω, and 25 µFD in a Bio-Rad Gene Pulser electroporator.

9. Resuspend transformation mix in 500 µl LB medium. Plate on 3 to 4 LB/kanamycin plates, and grow overnight (16 to 20 hr) at 37°C.

   Optionally, the transformed cells can be incubated 10 to 20 min at 37°C prior to plating.

   Although the conventional chemical transformation method can be used for the cotransformation experiments, it is not recommended because of its significantly lower efficiency of transformation.

10. Pick 10 to 20 of the smallest colonies, and grow each in 2 ml LB medium containing 25 µg/ml kanamycin for 10 to 15 hr in a 37°C orbital shaker.

    It can be challenging to pick up smaller colonies if the bacterial cells are not evenly spread during plating. Try to pick up the small colonies in well-isolated and evenly plated areas.

The author has attempted to screen for potential recombinants by PCR using the primers across the recombination junctions, and found that it is not very helpful because of the high false-positive rate.

In order to obtain recombinant adenovirus plasmids more efficiently, users are encouraged to follow the alternative method listed at the end of this section (see Alternate Protocol).

11. Perform minipreps using the conventional alkaline lysis method as described (see Support Protocol 4). Check the size of supercoiled plasmids by running 1/5 of each miniprep on a 0.8% agarose gel (e.g., *UNIT 2.7*).

    Potential recombinants usually run slower than the 12-kb band of a 1-kb ladder (Life Technologies) and often the yield is much lower than that of the shuttle vector background clones.

12. Perform *Pac*I restriction digestion on candidate clones. Correct recombinants usually yield a large fragment (~30 kb) and a smaller fragment of 3.0 or 4.5 kb.

    When digesting the recombinants with PacI, the smaller fragment can be either 3.0 or 4.5 kb. Both types of clones are correct because the homologous recombination can also occur between the two ori regions. In this case, PacI digestion will yield 30+ and 4.5 kb fragments. However, both types of recombinants are equally efficient in generating adenoviruses.

13. Retransform 1 µl correct recombinant plasmids into DH10B (or other plasmid propagation strain not prone to recombination). Perform further restriction analysis on the clones to confirm their primary structure. Finally, purify plasmids by CsCl-banding (e.g., *UNIT 5.3*) or using commercial purification kits in preparation for transfection of 293 cells.

    If the background is high, consider one of the following modifications. (1) Do not incubate the bacteria mix after electroporation but directly plate them on LB/kanamycin plates; (2) try to reduce the amount of shuttle vector DNA used in the PmeI digestion; and (3) try to minimize the possibility of introducing nicks into the shuttle vector DNA (e.g., use the alkaline lysis procedure to prepare the shuttle plasmids).

    Because of the higher frequency of recombination and rearrangement of plasmids in BJ5183 cells, one should not attempt to regrow the BJ5183 culture for the candidate recombinant clones. Instead, potential recombinant plasmids should be recovered from BJ5183 cells as early as possible (no later than 20 hr) and, once confirmed, should be retransformed into DH10B or other common strains used for plasmid propagation.

Generate recombinant adenoviruses in 293 cells

14. Plate 293 cells (or 911 cells) in one or two 25-cm^2 tissue culture flask(s) at 2×10^6 cells per flask, ~12 to 20 hr prior to transfection.

    The confluency should be ~50% to 70% at the time of transfection.

    Transfection of one flask is usually sufficient to generate viruses for further amplification. However, multiple flasks can be used for transfection if high initial titers and quicker amplifications are desired.

15. On the day of transfection, digest recombinant adenoviral plasmids with *Pac*I. To ensure complete digestion, carry out restriction reactions in 100-µl volumes. Precipitate digested plasmids with ethanol and resuspend in 20 µl sterile water.

    Usually, 4 µg DNA is needed to transfect one 25-cm^2 tissue culture flask.

16. Perform a standard LipofectAMINE reagent transfection according to manufacturer's manual. Mix 4 µg of *Pac*I-digested plasmid and 20 µl LipofectAMINE reagent for each 25-cm^2 tissue culture flask in 500 µl of Opti-MEM I medium, and incubate DNA/LipofectAMINE reagent mix for 15 to 30 min, room temperature.

17. While waiting for the incubation, remove growth medium from 25-cm^2 tissue culture flasks plated with 293 cells. Gently add 4 ml serum-free medium (e.g., plain DMEM or HBSS medium) to wash residual serum-containing medium. Remove DMEM and add 2.5 ml Opti-MEM I per 25-cm^2 tissue culture flask. Return for ~10 min to 37°C, 5% CO_2 incubator.

    Special precautions are needed when washing the 293 cells because 293 cells are usually less adherent to the flasks. In most cases, one wash is sufficient. Furthermore, if more than ten flasks are used for transfections, wash no more than five flasks at a time to minimize detachment of 293 cells.

18. Add DNA/LipofectAMINE mix dropwise to the 25-cm^2 tissue culture flasks, and return them to 37°C, 5% CO_2 humidified incubator.

19. Remove medium containing DNA/LipofectAMINE mix 4 to 6 hr later, and add 7 ml fresh complete DMEM.

    Do not change the DNA/LipofectAMINE medium if a significant number of floating cells are observed. Sometimes this happens with 293 cells but does not necessarily indicate a problem. If a large number of floating cells are observed, add 6.0 ml complete DMEM to each flask and incubate 10 to 12 hr at 37°C. Remove the medium and add 7 ml fresh medium to each 25-cm^2 tissue culture flask.

20. If pAdTrack-based vectors are used, monitor transfection efficiency and virus production by GFP expression, visible with fluorescence microscopy. Maintain the transfected cells in the 37°C, 5% CO_2 incubator for 10 to 12 days. During this period, it is not necessary to change the medium.

    In general, no obvious plaques or cytopathic effect (CPE) are observed by standard microscopy up to 2 weeks post-transfection. However, GFP plaques are usually observed under fluorescence microscopy starting 5 to 7 days after transfection. Whether the comet-like plaques are observed largely depends on transfection efficiency. Lower transfection efficiency (10% to 30%) may produce "comet-like" plaques, whereas high transfection efficiency (>50%) may generate an intense "scattered stars" phenomenon.

21. Prepare viral lysates as follows.

    a. Scrape cells off flasks with a rubber policeman (do not use trypsin) at 10 to 12 days post-transfection and transfer to 50-ml conical tubes.

        If the transfection efficiency is low (<30%), it is more desirable to harvest the cells at >15 days after transfection to ensure a reasonable initial viral titer. In this case, feed the cells with 2 ml fresh medium at ~10 days after transfection.

    b. Centrifuge cells in a benchtop centrifuge 10 min at 500 × *g*, 4°C, and resuspend the pellet in 3.0 ml HBSS or sterile PBS.

    c. Freeze cells in dry ice/methanol bath, and thaw in a 37°C water bath to release virus from cells. Vortex vigorously. Repeat freeze/thaw/vortex for three more cycles. Remove tubes from water bath as soon as they thaw to avoid warming virus supernatants, which can reduce titer.

    d. Centrifuge samples briefly at 500 × *g*, 4°C, to pellet the cell debris.

        The viral lysates are ready to be used for amplification and preparation of high titer viruses.

    e. Store viral lysates at −20°C or −80°C if they are not immediately used for infection.

GENERATE RECOMBINANT ADENOVIRUS PLASMIDS USING AdEasier CELLS

ALTERNATE PROTOCOL

Although the method described above (see Basic Protocol) works effectively for generating recombinant adenoviruses, the homologous recombination step in BJ5183 cells has become rate limiting for many users. For the past few years, the author's laboratory, as well as those of others, has efficiently generated numerous recombinant adenoviruses using so-called AdEasier cells which are derived from BJ5183 cells already containing the pAdEasy backbone plasmids. The BJ5183 cells harboring pAdEasy-1 are designated AdEasier-1, whereas those containing pAdEasy-2 are designated AdEasier-2. As outlined in Figure 12.4.2, efficient homologous recombination occurs when linearized shuttle vectors are transformed into the competent AdEasier cells. This protocol replaces steps 6 to 10 of the Basic Protocol; the rest of the protocol is the same as the Basic Protocol.

There are at least three advantages to using AdEasier cells. First, the cells are extremely efficient for generating recombinants, because their use circumvents the relatively low efficiency of cotransforming BJ5183 cells with two large plasmids. Second, this approach does not require preparation of high quality pAdEasy plasmids. Third, it is possible to generate recombinants using conventional chemical transformation methods instead of electroporation.

STRATAGENE is now selling competent AdEasier-1 cells (which they call BJ5183-AD-1). For users planning to make only a few adenoviral constructs, this is particularly convenient.

Additional Materials *(also see Basic Protocol)*

pAdEasy-2 plasmid (optional; Quantum Biotechnologies or Stratagene)
LB agar plates containing 50 µg/ml ampicillin and 30 µg/ml streptomycin (*APPENDIX 2D*)
Restriction endonucleases (*Hin*dII or *Pst*I)
LB medium without antibiotics (*APPENDIX 2D*)
LB medium containing 25 µg/ml kanamycin (*APPENDIX 2D*)

Generate competent AdEasier cells

1. Transform 50 ng pAdEasy-1 or pAdEasy-2 plasmid into electro-competent BJ5183 cells following the conditions described (see Basic Protocol, step 8).
2. Plate the transformation mix (usually 5% to 20%) on LB agar plates containing 50 µg/µl ampicillin and 30 µg/ml streptomycin. Incubate 15 to 20 hr at 37°C.
3. Pick 10 to 20 colonies and grow each in 2 ml LB medium containing ampicillin and streptomycin with continuous shaking at 37°C overnight.
4. Purify the plasmid DNA from each culture following the alkaline lysis procedure described (see Support Protocol 4).
5. Use 20% to 30% miniprep DNA for restriction digestion (e.g., *Hin*dIII, *Pst*I) to confirm the integrity of the clones. Pick one confirmed clone (designated as AdEasier-1 or 2) for subsequent use.

   Because BJ5183 cells have a relatively high frequency of homologous recombination, unwanted or detrimental rearrangements and/or recombinations of the pAdEasy sequences in AdEasier cells can occur. It is thus important to pick individual clones and characterize the clones with extensive restriction digestions (e.g., HindIII and/or PstI). The digestion pattern can be compared with the pAdEasy stock DNA made in a nonrecombinant strain (e.g., DH10B). A restriction digest characterization should optimally be carried out on DNA from the large-scale culture that is used to prepare competent cells.

6. Prepare electrocompetent AdEasier cells as described (see Support Protocol 3), except grown in LB medium containing ampicillin and streptomycin.
7. Store electrocompetent AdEasier cells in 20 µl/tube aliquots kept up to 6 months at –80°C. When ready for transformation, thaw aliquots and keep competent cells on ice.

Clone gene into shuttle vector

8. Clone the gene into the shuttle vector and digest with *Pme*I as described (see Basic Protocol, steps 1 to 5).

Generate recombinants using AdEasier cells

9. To 20 µl electrocompetent AdEasier cells, add 8.0 µl *Pme*I-digested shuttle vector. Limit the final volume to ~30 µl.
10. Carefully transfer the bacteria/DNA mix to an ice-cold 2-mm electroporation cuvette, avoiding formation of bubbles and keeping the cuvette on wet ice. Deliver the pulse at 2500 V, 200 Ω, and 25 µFD in a Bio-Rad Gene Pulser electroporator.
11. Resuspend the transformation mix in 500 µl LB medium. Plate 10% to 20% of the transformation mix onto 1 to 2 LB/kanamycin plates, and grow overnight (16 to 20 hr) at 37°C.

    Optionally, incubate 10 to 20 min at 37°C, depending on vector background and AdEasier competency.

    The conventional chemical transformation method can also be used for AdEasier transformation experiments if the chemically competent AdEasier cells are used.
12. Pick 10 to 20 of the smallest colonies, and grow each in 2 ml LB medium containing 25 µg/ml kanamycin for 10 to 15 hr in a 37°C orbital shaker. Purify, characterize, and regrow plasmid DNA as described (see Basic Protocol, steps 11 through 13).

    Generate recombinant adenoviruses in the 293 packaging line by following the procedure described above (see Basic Protocol, steps 14 to 21).

SUPPORT PROTOCOL 1

PREPARATION AND PURIFICATION OF HIGH-TITER ADENOVIRUSES

This protocol describes the amplification steps for adenoviruses starting from the initial transfection lysates. In most cases, it takes two to four rounds of amplification to arrive at a large-scale preparation of high titer viruses. However, the number of amplification rounds is largely dependent on the initial titers of the primary transfection lysates.

Additional Materials *(also see Basic Protocol)*

Primary transfection viral supernatant (see Basic Protocol)
Cesium chloride (CsCl)
Mineral oil
Chlorine bleach
2× storage buffer (see recipe)
Blank solution: 1.35 g/ml CsCl mixed with equal volume 2× storage buffer
TE buffer (*APPENDIX 2D*) containing 0.1% SDS

75-cm^2 tissue culture flasks
Benchtop centrifuge
50-ml conical centrifuge tubes
Sorvall refrigerated centrifuge with HS-4 rotor
12-ml polyallomer tubes for SW 41 Ti rotor
Beckman ultracentrifuge (or equivalent) with SW 41 Ti rotor
Ring stand and clamp
3-ml syringe and 18-G needle

Round one: Amplify from primary transfection lysates

1. Plate 293 cells in 25-cm^2 tissue culture flasks at 80% to 90% confluency (~3×10^6 cells/flask in 6 ml complete DMEM) at 12 to 15 hr prior to infection.

2. Infect 25-cm^2 tissue culture flasks of 293 cells by adding 30% to 50% of the primary transfection viral supernatants to each flask.

   The amount of the primary transfection lysates used in each infection is largely determined by their initial titers (usually in a range of 10^6 to 10^8 infectious particles/ml). The rest of the viral lysates should be kept at −20°C or −80°C. A cytopathic effect (CPE) or genuine cell lysis should become evident at 2 to 4 days post-infection. Productive infections should be easily observed using the GFP expression incorporated in pAdTrack-based vectors.

3. Scrape and collect cells when 30% to 50% of the infected cells are detached, usually at 3 to 5 days post-infection.

   If the infected cells become sick within two days post-infection, it indicates that the titer of the primary transfection lysate is high, and less virus lysate should be used for infection or more 293 cells should be infected (e.g., use one 75-cm^2 tissue culture flask). On the other hand, if the infected cells do not show an obvious CPE by 5 days post-infection, it usually suggests that the primary transfection lysates have relatively low titers. In this case, more viral lysate should be used for infection and the infected cells should be collected at a much later time (e.g., 1 week after infection). Alternatively, higher-titer lysates can be prepared by repeating the transfection (with high transfection efficiency).

4. Transfer the scraped cells to a 15-ml conical centrifuge tube, and centrifuge in a benchtop clinical centrifuge cells 10 min at ~$500 \times g$, 4°C. Remove all but 5 ml medium and resuspend cells by vortexing.

5. Perform four cycles of freezing in a dry ice/methanol bath and thawing at 37°C to release the viruses from the cells. Perform the next round of amplification with cleared lysates or keep up to 1 year to −80°C.

Rounds 2 and 3: Perform intermediate-scale amplification

6. Plate 293 cells in 75-cm^2 tissue culture flasks at ~90% confluency 12 to 15 hr prior to infection (~$5–7 \times 10^6$ cells/flask in 16 ml complete DMEM).

7. Add 2 to 4 ml viral lysate prepared in step 5 to one 75-cm^2 tissue culture flask of 293 cells. Return cells to 37°C, 5% CO_2 incubator.

   After ~30 to 48 hr of incubation, the CPE caused by the amplification of adenovirus should be readily observed. The infected cells will appear round and refractile, and will begin to lift off the surface of the flasks.

8. Scrape and collect cells when 30% to 50% of the infected cells are detached, usually at 2 to 4 days post-infection.

9. Transfer the scraped cells to a 50-ml conical centrifuge tube, and centrifuge 10 min at ~$500 \times g$, 4°C in a benchtop clinical centrifuge. Remove all but 10 ml medium and resuspend cells by vortexing. Perform four cycles of freezing in dry ice/methanol bath and thawing at 37°C to release the viruses from cells.

   Cleared lysates are ready for the next round (round 3) of amplification or can be kept at −80°C.

   The virus-containing waste should be disinfected with chlorine bleach.

10. Repeat steps 6 to 9 for another round of amplification. When collecting the infected cells, resuspend in 25 ml sterile PBS or HBSS. Perform three to four cycles of freezing/thawing to release the viruses from cells. Use the cleared viral lysates for a final round of large-scale amplification or keep at −80°C.

For this additional round of amplification, the viral lysates will be used to infect three to five 75-cm^2 tissue culture flasks.

For optimal amplification, ~30% to 50% of the infected cells should demonstrate obvious CPE at 2 to 3 days after infection. Under these circumstances, each round of amplification should yield at least ten-fold more virus than is present in the previous round.

Titers can be measured at any time, which is particularly easy with AdTrack-based vectors. Simply infect 293 cells with various dilutions of viral supernatant and see how many are GFP-expressing cells 18 hr later. Without AdTrack, viruses can be plaque titered (see Support Protocol 2) or titered by limiting dilution using standard methods; the author finds these methods much less simple and quantitative than employing the GFP marker, but these have to be used if GFP is not present. After three rounds of amplification, viral titer should reach 10^9 to 10^{10} infectious particles (or plaque-forming units, pfu) per milliliter of lysate.

Final round: Perform large-scale amplification and CsCl gradient purification

11. Plate 293 cells in 75-cm^2 tissue culture flasks to be 90% to 100% confluent at the time of infection (~1×10^7 cells/flask).

    Usually, 15 to 20 75-cm^2 tissue culture flasks are sufficient to make a high-titer stock.

12. Infect cells with viral supernatant at a multiplicity of infection (MOI) of 10 pfu per cell.

13. When all infected cells have rounded up and about half of the cells are detached (usually at 3 to 4 days post-infection), harvest and combine infected cells from all flasks. Centrifuge 5 min at ~500 × *g* in a benchtop centrifuge and remove supernatant.

    The virus-containing waste should be disinfected with chlorine bleach.

14. Resuspend the cell pellet in 8.0 ml sterile PBS. Perform four cycles of freezing in a dry ice/methanol bath and thawing at 37°C to release viruses from cells. Centrifuge viral lysate 5 min in a Sorvall refrigerated centrifuge at 7000 × *g* (HS-4 rotor at 6000 rpm), 4°C.

    It is important to resuspend the viral lysate in PBS because it provides a better visualization of the viral band on the CsCl gradient without interference from the phenol red.

15. Weigh 4.4 g CsCl into a 50-ml conical tube, transfer 8.0 ml cleared virus supernatant to the tube (avoiding the pellet), and mix well by vortexing.

16. Transfer the CsCl solution (~10 ml, density of 1.35 g/ml) to a 12-ml polyallomer tube for SW 41 Ti rotor. Overlay with 2 ml mineral oil to fill tube. Prepare a balance tube.

    It is important to fill the tubes with mineral oil to prevent crashes during high-speed centrifugation.

17. Centrifuge in a Beckman ultracentrifuge with an SW 41 Ti rotor for 18 to 24 hr at 176,000 × *g* (SW 41 Ti rotor at 32,000 rpm), 10°C.

18. Remove tubes from ultracentrifuge and clamp onto a ring stand above a beaker of chlorine bleach. Note the position of the virus band, which appears as a narrow opaque white band ~1 to 2 cm below mineral oil interface. Collect virus fraction (~0.5 to 1.0 ml) with a 3-ml syringe and 18-G needle by puncturing the side of the tube under the band to extract it into syringe. Do not collect any bands above it.

19. Mix virus fraction with equal volume 2× storage buffer. Store virus stocks at −80°C.

20. Check viral titer by GFP, plaque assays (see Support Protocol 2), or immunohistochemical staining, or simply read OD_{260}. To read OD, add 15 µl virus to 15 µl blank solution and 100 µl TE/0.1% SDS, vortex 30 sec, centrifuge 5 min, and measure OD_{260}.

    One OD unit contains ~10^{12} viral particles/ml (particles:infectious particles = ~20:1). However, the OD_{260} calculation is based on an estimate of viral DNA content and does not imply either competent viral packaging or transgene expression. Therefore, it is advisable to combine this estimate with the other approaches described.

    The thickness of viral band on CsCl gradient is largely determined by the efficiency of amplification. In an optimal infection for amplification, ~30% to 50% of the infected cells show obvious CPE at 2 to 3 days after infection. If the infected cells exhibit significant CPE within 24 hr or after 5 days post-infection, it is most likely that the virus amplification is not optimal and the resultant viral titers are likely to be lower.

    It is best to keep the concentrated virus stock at −80°C because the viral particles are generally more stable in high-salt conditions. For in vitro applications where the virus stock is highly diluted, the purified virus preparation can be directly used. However, because CsCl may interfere with or cause toxicity in some other applications, it is best to desalt the virus stocks, immediately before use, by using desalting columns or quick dialysis with agarose-tubes (see Support Protocol 6).

ADENOVIRUS PLAQUE ASSAY

SUPPORT PROTOCOL 2

This protocol can be used for several purposes. The procedure can be used at any stage to plaque-purify the recombinant virus from any background vectors that may be present. It can also be used to determine the infectious titer of virus stock at any step. The infectivity titer of adenoviruses is expressed as plaque-forming units (pfu) per milliliter. Additionally, this procedure can also be performed at the beginning of the primary transfection of recombinant adenoviral plasmids to prepare plaques, if desired, to ensure amplification begins with a clonal virus population.

Additional Materials *(also see Basic Protocol)*

Adenovirus
2.8% Bacto agar (Becton Dickinson)
2× Basal Medium Eagle (BME; Life Technologies)
1 M HEPES
1.0 M $MgCl_2$ (*APPENDIX 2D*)
Fetal bovine serum (FBS)
100× penicillin/streptomycin solution (e.g., Life Technologies)
100× neutral red stock (Life Technologies)

6-well plates
45°C water bath

1. Plate 293 cells in 6-well plates at 50% to 70% confluency (~2-5 × 10^5 cells/well in 5 ml complete DMEM).

2. Determine an appropriate range of 6 ten-fold dilutions based on the approximate adenovirus titer (typically, a range of 10^{-3} to 10^{-8} µl/well is chosen).

    The diluted adenovirus should be in a reasonable volume (e.g., 10 to 50 µl) to infect cells. Prepare enough of each dilution to run duplicate assays.

3. In each well of the 6-well plates, remove all but 2 ml complete DMEM. Add the serially diluted adenovirus to each well to infect for 6 to 16 hr. Set up duplicate wells for each dilution.

4. Prepare the overlay agar by autoclaving 100 ml of 2.8% Bacto agar and keep warm in a 45°C water bath.

5. Prepare a 100-ml overlay mix (~25 ml overlay mix is needed for one 6-well plate) as follows:

   50.0 ml 2× Basal Medium Eagle (final 1×)
   2.0 ml 1.0 M HEPES (final 20.0 mM)
   1.25 ml 1.0 M $MgCl_2$ (final 12.5 mM)
   10.0 ml FBS (final 10% v/v)
   1.0 ml 100× penicillin/streptomycin solution (final 1×)
   Mix well and warm in a 37°C water bath.
   36.0 ml 2.8% Bacto-agar (final 1.0%)
   Mix well and swirl in a 37°C water bath.

   To melt 2.8% agar stock, microwave or heat it in a boiling water bath. Prepare 50 ml overlay at a time to prevent premature solidification.

6. Aspirate complete DMEM from wells, and overlay each well with 4 ml warmed overlay mixture by slowly adding the solution down the side of each well, taking care not to dislodge cells.

7. Allow agar to solidify 10 to 20 min at room temperature. Return plates to the 37°C incubator.

   To prevent the pre-drying of agar before plaque formation, it is helpful to add sterile PBS or HBSS in the space between wells, and wrap the plates with plastic wrap.

8. On day 5, overlay 1.5 ml agar overlay mix on top of existing agar in each well to feed cells and maintain monolayer integrity. After solidification, return plates to the incubator.

9. On day 9, prepare neutral red-containing agar overlay mix by adding 500 µl of 100× neutral red stock to 50 ml overlay mix.

10. Overlay each well with 2 ml neutral red-containing agar mix.

11. Allow agar to solidify for 10 min at room temperature, and then return plates to the 37°C incubator.

12. After 12 to 20 hr, remove plates from incubator and hold up to light or place onto a light box, observing the monolayer from the bottom of the plate. For each well, count plaques, which will appear as clear pale orange areas amid a darker reddish-orange monolayer.

13. Determine plaque counts for each dilution by averaging the duplicate wells. This average will determine the titer of adenoviral stock as expressed in plaque forming units per milliliter (pfu/ml).

    If plaque-purification is desired, pick up 5 to 10 well-isolated plaques for each virus by punching out agar plugs with a sterile Pasteur pipet. Store agar plugs in 200 µl HBSS medium. Perform four cycles of freezing/thawing to release viruses. Recovered viruses can be used to gradually scale up amplification by starting with infection of one well of a 24-well plate.

    For viruses encoding GFP, titer infectious units (i.e., those resulting in expression of GFP) can be simply determined by counting GFP expressing foci using fluorescence microscopy.

PREPARATION OF ELECTROCOMPETENT BJ5183 CELLS

This protocol provides detailed instructions for the preparation of electrocompetent BJ5183 cells and the determination of quality of the competent cells. It is critical to carefully follow this protocol because preparation of high-efficiency, electrocompetent BJ5183 cells is one of the most important steps in successfully producing recombinant adenoviruses with this system, and BJ5183 cells exhibit a relatively low transformation efficiency.

Additional Materials *(also see Basic Protocol)*

LB medium containing 30 µg/ml streptomycin (*APPENDIX 2D*)
10% (v/v) sterile glycerol, ice cold
10 ng/µl pAdEasy-1 plasmid DNA
LB agar plates with 50 µg/ml ampicillin (*APPENDIX 2D*)

50-ml conical centrifuge tubes
250-ml sterile centrifuge tubes (for IEC centrifuge)
IEC centrifuge (or equivalent)
1.5-ml microcentrifuge tubes, prechilled at −80°C

1. Use a fresh colony or frozen stock of BJ5183 cells to inoculate 10 ml LB medium containing 30 µg/ml streptomycin in a 50-ml conical tube. Grow cells overnight in a 37°C environmental shaker.
2. Dilute 1 ml of cells grown overnight into 1000 ml LB medium containing 30 µg/ml streptomycin. Shake vigorously for 4 to 6 hr with good aeration in 37°C environmental shaker, until A_{550} is ~0.8.
3. Collect cells in four 250-ml sterile centrifuge tubes and incubate on ice 1 to 3 hr.

   The longer the cells are incubated, the higher the competency.
4. Centrifuge 10 min at 2600 × *g* (3000 rpm in an IEC centrifuge), 4°C.
5. Remove supernatant. Wash the cell pellet by resuspending in 1000 ml sterile ice-cold 10% glycerol.
6. Centrifuge cell suspension 20 min at 2500 × *g* (3000 rpm in an IEC), 4°C.
7. Repeat steps 5 and 6 for one more wash.
8. Pour off most of the supernatant, then gently pipet off most of residual supernatant, leaving ~10 ml per 250-ml centrifuge tube.
9. Combine cells and transfer cell suspension to a 50-ml centrifuge tube. Centrifuge for 10 min at 2500 × *g* (3000 rpm in IEC), 4°C.
10. Remove most of the supernatant and add 40 ml ice-cold 10% glycerol. Resuspend cells and centrifuge 10 min at 2500 × *g* (3000 rpm in IEC), 4°C.
11. Pipet out all but 2 ml of the supernatant and resuspend cell pellet. Pipet 20-µl aliquots per prechilled 1.5-ml microcentrifuge tube. Store the aliquots at −80°C.
12. To verify the competency of prepared BJ5183 cells, add 1.0 µl of 10 ng/µl pAdEasy-1 plasmid DNA to 20 µl BJ5183 competent cells.
13. Transfer cell/DNA mix to an ice-cold 2-mm cuvette. Perform electroporation with Bio-Rad gene pulser at 200 Ω/25 µF/2.5 kV.
14. In a 50-ml conical tube, add 1 ml LB medium to cells and shake for 1 hr at 37°C.
15. Make 100- and 1000-fold serial dilutions of the cells in LB medium.
16. Plate 100 µl diluted cells on LB-agar plates with 50 µg/ml ampicillin.

17. Incubate overnight in a 37°C incubator.

Titer should be >10^8 colonies/μg DNA.

SUPPORT PROTOCOL 4

ALKALINE LYSIS PROCEDURE FOR PLASMID MINIPREPARATION

This protocol describes a commonly used procedure for plasmid minipreparation. The author has found the miniprep DNAs purified with this procedure always yield reliable and consistent results for the homologous recombination step of the AdEasy system.

Materials

Plasmid-containing bacterial cells
Resuspension buffer (see recipe)
Lysis solution (see recipe)
Precipitation solution (see recipe)
2-propanol
70% ethanol

1. Grow 2 ml plasmid-containing bacterial cells in a 2.0-ml microcentrifuge tube overnight in an orbital shaker at 37°C. Microcentrifuge 1 min at room temperature.
2. Discard the supernatants. Add 200 μl resuspension buffer and vortex briefly.
3. Add 200 μl lysis solution and gently mix by inverting the tubes several times.
4. Add 200 μl precipitation solution and mix well by inverting the tubes several times.
5. Microcentrifuge 3 min at maximum speed, room temperature.
6. Pour supernatants into a new set of 1.5-ml microcentrifuge tubes. Add 500 μl 2-propanol and mix well.
7. Microcentrifuge 5 min at maximum speed, room temperature.
8. Discard the supernatants. Add 500 μl of 70% ethanol, vortex, and microcentrifuge 1 min, room temperature.
9. Discard the supernatants. Briefly spin down and aspirate the residual liquid in the tubes.
10. Add 70 μl distilled, deionized water to resuspend plasmid DNA.

SUPPORT PROTOCOL 5

PREPARATION OF ADENOVIRAL DNA

Adenoviral DNA can be isolated from virions by digestion of the capsid proteins with proteinase K in the presence of SDS, followed by deproteinization with phenol and ethanol precipitation. The purified DNA can then be analyzed by restriction enzyme analysis (including Southern blotting) or PCR analysis.

Materials

Viral lysate or CsCl gradient purified virus stock
10% SDS (*APPENDIX 2D*)
0.5 M EDTA (*APPENDIX 2D*)
20 mg/ml PCR grade proteinase K (Life Technologies)
7.5 M ammonium acetate (*APPENDIX 2D*)
seeDNA (Amersham Pharmacia Biotech)
PC-8 (Fisher) or 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol
70% and 100% ethanol
55°C water bath

1. To 100 µl viral lysate or 10 µl CsCl gradient purified virus stock, add 7 µl of 10% SDS, 3 µl of 0.5 M EDTA, and 20 µl of 20 mg/ml proteinase K.
2. Mix well and incubate 3 hr in a 55°C water bath. Heat the mix 5 min at 95°C.
3. Bring the viral DNA solution to a total volume of 200 µl with deionized, distilled water, and then add 100 µl of 7.5 M ammonium acetate and 2 µl seeDNA.
4. Extract the mix twice with 300 µl PC-8.
5. Transfer top phase to a new 1.5-ml microcentrifuge tube, avoiding the interface. Add 600 µl of 100% ethanol.
6. To precipitate viral DNA, microcentrifuge 10 min at maximum speed. Wash pellet two times with 70% ethanol.
7. Dissolve viral DNA pellet in 50 µl water.

The prepared DNA is ready for restriction digestion analysis or PCR.

QUICK AGAROSE-TUBE DIALYSIS

SUPPORT PROTOCOL 6

This protocol describes a simple and efficient homemade dialysis system to remove CsCl salt from purified virus stock. This modified procedure was previously described by Atrazhev and Elliott (1996). Because CsCl may exert significant toxicity in many applications, it is always desirable to desalt the virus stock immediately before use. Other commercially available microdialysis/desalting systems can also be used for this purpose.

Materials

Agarose, molecular-biology grade
2-ml microcentrifuge tubes
200-µl filter tips

1. Prepare 1% agarose by melting the agarose in deionized, distilled water in a microwave oven at full power.
2. Make a dialysis-tube apparatus by pipetting 1 ml melted 1% agarose into a 2-ml microcentrifuge tube. Stick a beveled 200-µl filter tip to the very bottom of the tube.
3. After 1 hr at room temperature, remove the pipet tip. Add 50 µl ddH_2O to the hole to keep the gel wet. Store the tubes at 4°C.
4. To dialyze, remove the 50 µl water and add the virus stock solution (generally ≤25 µl) with a needle-nosed pipet tip.
5. After an appropriate time period (usually 10 to 20 min), remove the solution with a needle-nosed pipet tip and either use directly or add to a new agarose dialysis apparatus if further dialysis is desired.

REAGENTS AND SOLUTIONS

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

Lysis solution

0.2 N NaOH
1% (w/v) SDS
Store up to two weeks at room temperature

Precipitation solution

60.0 ml 5 M potassium acetate
11.5 ml glacial acetic acid
28.5 ml ddH_2O
Total 100 ml
Store up to 3 months at 4°C

Resuspension buffer

50 mM glucose
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2D*)
10 mM EDTA, pH 8.0 (*APPENDIX 2D*)
Store up to 3 months at 4°C

Storage buffer, 2×

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2D*)
100 mM NaCl
0.1% bovine serum albumin (BSA)
50% (v/v) glycerol
Filter to sterilize
Store up to 1 year at 4°C

COMMENTARY

Background Information

Recombinant adenovirus vectors have become one of the most useful gene-delivery vehicles in studies of gene therapy, vaccine therapy, and basic biology (Berkner, 1988; Graham and Prevec, 1991; Miller, 1992; Morgan and Anderson, 1993). Several features intrinsic to adenoviruses make them particularly attractive as vectors for somatic gene transfer. Adenoviruses can be purified as high-titer preparations that achieve a high level of transgene expression in a broad spectrum of host cells and tissues, including nondividing cells. However, the traditional methods for generating such vectors have been time-consuming and inefficient, which has hampered more widespread use of adenoviral technology (Graham and Prevec, 1991; Precious and Russell, 1995). Based on several technological and conceptual advances made in the past few years, particularly the successful generation of infectious adenoviruses through homologous recombination in a yeast system (Ketner et al., 1994), the author has developed a system for rapid and efficient generation of recombinant adenoviruses using recombination in bacterial cells (He et al., 1998).

Adenoviruses are nonenveloped DNA viruses whose capsid is primarily composed of pentons (penton base and fiber monomers) and hexons. The viral genome consists of 36 kb double-stranded linear DNA with inverted terminal repeat sequences at each end. DNA substantially larger than this cannot ordinarily be packaged into competent viral particles, thus limiting the size of exogenous inserts that can be used in adenoviral vectors. Decades of study of adenovirus biology have resulted in a detailed picture of the viral life cycle and the functions of the majority of viral proteins. The viral life cycle begins with the attachment of the fiber to cell surface receptors and the interaction with specific integrins. After receptor-mediated endocytosis, the virus escapes from the endosomes to the cytoplasm and translocates into the nucleus where viral transcription and replication begin. Completion of the viral life cycle triggers cell death and the release of

virion progeny. Based on their temporal expression relative to the onset of viral DNA replication, viral transcription units are conventionally referred to as early (E1a, E1b, E2, E3, and E4), delayed early (proteins IX and Iva2), or late genes (L1 to L5). The early gene products are mostly involved in viral gene transcription, DNA replication, host immune suppression, and inhibition of host cell apoptosis, while the late gene products are required for virion assembly. For further details on the biology of adenovirus and its life cycle, readers are referred to other more specialized publications (Becker, 1994; Precious and Russell, 1995; Shenk, 1996).

Among the early genes, E1 (especially E1a) is the first to be expressed after infection and is the most essential transcriptional activator for subsequent adenoviral gene expression and viral DNA replication. Thus, many adenoviral vector systems have substituted the exogenous transgene cassette for E1, thereby rendering the virus defective for replication and incapable of producing infectious viral particles in target cells. Such E1-deleted vectors can be propagated in cell lines that express essential E1 gene products, such as 293 or 911 cells (Graham et al., 1977; Fallaux et al., 1996). In contrast, the E3 region is not essential for viral replication and therefore many vectors also contain E3 deletions to increase the possible size of inserted exogenous sequences. It should be noted, however, that the E3 region exerts a variety of immunomodulatory effects (Tollefson et al., 1991) and, at least in some settings, retaining E3 region in the adenoviral backbone appears to reduce the inflammatory response elicited by the vectors in vivo (Wen et al., 2001).

Additional room for insertion of exogenous sequences can be produced by deletion of E4, another essential early region. However, as noted above, generation of E4-expressing packaging lines has been problematic and it is often difficult to produce high titer preparations of E4-deleted adenoviruses.

Given the relatively large size of the adenoviral genome and relative paucity of unique restriction sites, most vector systems have relied on subcloning the gene of interest into a shuttle vector, which is subsequently combined with the rest of the adenoviral sequences or backbone to generate a single DNA molecule encoding all the sequences necessary for virus production in an appropriate cell line. This has traditionally been achieved through homologous recombination in mammalian cells by using purified, restriction-digested adenoviral DNA as the source of viral "backbone." Graham and colleagues used plasmid vectors to supply this viral DNA but still relied on recombination after cotransfection in 293 cells. Given the inefficient and unpredictable nature of homologous recombination in mammalian cells, this was often the rate-limiting step to production of recombinant adenoviral vectors. The AdEasy system described in this unit uses a similar plasmid-based strategy. However, this system exploits the higher efficiency of homologous recombination in specific bacterial strains and selectable antibiotic resistance to simplify recombinant vector production. In the author's opinion, this results in a robust and efficient approach that is significantly faster than traditional strategies.

Other methods for generating recombinant adenoviruses have also been described elsewhere and the readers are referred to these publications for additional information (Ketner et al., 1994; Imler et al., 1995; Chartier et al., 1996; Fisher et al., 1996; Kochanek et al., 1996; Lieber et al., 1996; Miyake et al., 1996; Parks et al., 1996).

Critical Parameters

One of the most critical steps for the successful use of the AdEasy technology is to generate recombinant adenovirus plasmids in BJ5183 cells. Preparation of high-quality electrocompetent BJ5183 cells is essential for the efficient generation of adenovirus recombinants. Because BJ5183 cells exhibit lower transformation efficiency than most of the conventional strains used for molecular cloning, and both shuttle vectors and AdEasy backbone vectors are large molecules (>10 kb and 30 kb, respectively), it is important to optimize the cotransformation conditions. One common mistake is to use a density of competent BJ5183 lower than that required for efficient transformation. In this regard, users are strongly recommended to follow the detailed procedure for preparing competent BJ5183 cells as described in Support Protocol 3. Recently, high-quality competent BJ5183 cells have become commercially available from Stratagene and Quantum Biotechnologies.

Alternatively, investigators are encouraged to use the AdEasier cells for generating recombinant adenovirus plasmids. The author's laboratory has recently tested this approach, and it has worked extremely efficiently in generating recombinants. However, it is recommended that a thorough analysis of BJ5183/AdEasy clones be carried out before and after their

Table 12.4.2 Troubleshooting Guide for Using the AdEasy Technology

Problems	Possible cause(s)	Solution(s)
Low number or no colonies after cotransformation in BJ5183	Transformation conditions are not optimal	Follow the protocol provided in this unit
		Consult the manufacturer for specifications of the electroporator
	Incorrect antibiotic is used or antibiotic concentration is too high	Plate the transformation mix on LB plates containing 25 μg/ml kanamycin
	Wrong strain of bacteria is used, or BJ5183 cells are contaminated	Use BJ5183 for homologous recombination
		Grow BJ5183 cells in the presence of streptomycin to eliminate contaminating strains
	DNA preparations are not optimal	Purify the AdEasy backbone vectors by CsCl gradient
		Avoid gel-purifying the *Pme*I-digested shuttle vectors
		Purify shuttle plasmids using alkaline lysis miniprep procedure; avoid using commercial miniprep kits
	Competence of BJ5183 is not sufficient	Keep BJ5183 cells concentrated
		Check the competence of BJ5183 cells
		Prepare high-quality competent BJ5183 by carefully following Support Protocol 3
		Avoid repeated freezing/thawing of the competent cell stock
		Obtain the competent BJ5183 commercially
		Introduce AdEasy plasmid into BJ5183 to make competent BJ5183/AdEasy cells (see text for precautions)
Too many colonies after cotransformation in BJ5183	Too much shuttle vector DNA is used for transformation	Reduce the quantity of shuttle plasmids used (usually 0.2-0.5 μg is sufficient)
	*Pme*I digestion is incomplete	Check digested products on agarose gel. Use less DNA.
		Make sure the enzyme is active.
	Incubation after transformation is too long	Minimize the length of incubation to no longer than 30 min at 37°C (in most cases, no incubation after electroporation is needed)
No virus plaques observed after transfection in 293 cells	Plasmid DNA preparation is not appropriate	Prepare DNA using CsCl gradient procedure
		Double check concentrations of the plasmids
	293 cell passages are too high	Use earlier passages or fresh stocks of 293 cells
	Recombinant plasmid is not linearized with *Pac*I	Digest the viral plasmid with *Pac*I
	Transfection efficiency is too low	Improve transfection efficiency by optimizing conditions or using different types of transfection reagents

continued

Table 12.4.2 Troubleshooting Guide for Using the AdEasy Technology, *continued*

Problems	Possible cause(s)	Solution(s)
	Did not wait long enough	Wait for longer time, e.g., ≤2 weeks (this is particularly important if transfection efficiency is low)
	There is a defect in the adenoviral backbone	Perform comprehensive restriction analysis of the recombinant plasmid along with control vectors
	Insert exceeds the packaging limit of adenovirus	Consult Table 12.4.1 for proper selection of the AdEasy vectors
No transgene expression detected	The integrity of transgene is not maintained	Make sure the transgene cassette is intact by restriction analysis or PCR
	Efficiency of transient transfection is not high enough	Improve transfection efficiency by optimizing conditions or using different types of transfection reagents
		Make sure detection system works properly by including positive controls
	Transgene is not efficiently expressed	Make sure to include a Kozak sequence in front of the coding sequence

large-scale growth, because there is a relatively high tendency for rearrangement and recombination of the AdEasy backbone vector in BJ5183 cells. Nevertheless, if proper cautions are exercised, this approach can be an important alternative to circumvent the relative inefficiency of a two-plasmid cotransformation system such as the one described here (see Basic Protocol).

Standard CsCl gradient purification for pAdEasy-1 and -2 vectors is highly recommended. For efficient homologous recombination in BJ5183 cells, it is critical to maintain the integrity of the shuttle vector DNAs. The author has found that plasmids purified with commercial DNA minipreparation kits contain significant numbers of nicked DNAs, which are detrimental to efficient and faithful recombination. The conventional alkaline lysis procedure has given the author's laboratory the most consistent and reliable results. However, for transfection of the 293 cells, miniprep DNAs made by commercial kits (e.g., Nucleobond) may be acceptable.

Anticipated Results

The AdEasy system has been validated as an efficient and robust technology for generating recombinant adenoviruses by >1000 research laboratories worldwide. The key step is to generate adenoviral recombinants in BJ5183 cells. Once the recombinants are obtained, generation of adenoviruses in 293 cells is virtually guaranteed. Among several dozens of viruses produced, the author has never failed in generating recombinant adenoviruses in 293 cells once the recombinant plasmids are obtained from BJ5183 bacterial cells. Thus, provided that homologous recombination in bacterial cells works, the overall success rate for generating adenoviruses is high. Depending on transfection efficiency of 293 cells, one could expect virus titer of the initial viral lysate from one 25-cm^2 flask ranges from 10^8 to 10^{10} pfu/ml. If the transfection efficiency is not high (e.g., <30% cells are transfected), it is desirable to collect the cells 12 to 14 days post-transfection with a medium change on day seven. It usually requires two to three rounds of further amplification in 293 cells to reach a virus titer of 10^{12} or 10^{13} pfu/ml.

Table 12.4.2 lists potential problems that can arise in using the AdEasy technology along with their possible causes and solutions.

Time Considerations

If every step works well, one can expect to get the initial virus production in 2 weeks and the subsequent large-scale purification in an additional 1 to 2 weeks.

Cloning genes of interest into shuttle vectors requires mostly conventional molecular biology techniques. Thus, the user's experience in that area would likely determine the length of the experiments. In general, the most time-limiting step is to obtain recombinants in

BJ5183 cells. Depending on the quality of competent BJ5183 and user's experience, one can expect to take 2 days to weeks to obtain the recombinants; however, once recombinants are made, it should only take 2 to 4 weeks to generate adenoviruses and to prepare high-titer virus stocks.

Literature Cited

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Internet Resources

http://www.coloncancer.org/adeasy.htm

This site provides important and updated information regarding DNA sequences and vector maps, as well as modifications for the AdEasy system. The FAQs section has also provided helpful information for trouble-shooting this technology.

http://www.qbiogene.com/products/gene-expression/adeasy.html

http://www.stratagene.com/vectors/expression/adeasy.htm

Commercial Web pages that provide detailed instructions for using the AdEasy system and on the availability of the high quality of purified AdEasy backbone vectors and electrocompetent BJ5183 cells.

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