

Adeno-X™ Expression System 1 User Manual



Clontech

United States/Canada
800.662.2566

Asia Pacific
+1.650.919.7300

Europe
+33.(0)1.3904.6880

Japan
+81.(0)77543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

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Note: The viral supernatants produced by transfecting 293 cells with recombinant pAdeno-X Viral DNA could, depending on your DNA insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant adenovirus. **The user is strongly advised not to create adenoviruses capable of expressing known oncogenes.**

Appropriate NIH, regional, and institutional guidelines apply, as well as guidelines specific to other countries. NIH guidelines require that adenoviral production and transduction be performed in a Biosafety Level 2 facility. For more information, see appropriate HHS publications. Section IV in this User Manual contains a brief description of Biosafety Level 2 as well as other general information and precautions.

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I. Introduction & Protocol Overview

The **Adeno-X™ Expression System 1** provides an efficient method for constructing recombinant adenovirus. Our procedure uses conventional *in vitro* ligation (not homologous recombination) to incorporate a mammalian expression cassette into a replication-incompetent (Δ E1/ Δ E3) human adenoviral type 5 (Ad5) genome. This approach, originally developed by Mizuguchi & Kay (1998, 1999), enables you to produce recombinant adenovirus in less than three weeks.

Constructing recombinant adenovirus with the Adeno-X™ System 1

The assembly and production of recombinant adenovirus is completed in three stages (Figure 1). First, a mammalian expression cassette is made by cloning your gene of interest into pShuttle2. After amplification in *E. coli*, the expression cassette is excised from pShuttle2 and ligated to Adeno-X Viral DNA (the adenoviral genome). Because pShuttle2 and Adeno-X Viral DNA carry different antibiotic selection markers, you do not need to purify the expression cassette fragment for ligation with Adeno-X. In the final stage, the recombinant Adeno-X vector is packaged into infectious adenovirus by transfecting human embryonic kidney (HEK) 293 cells. Recombinant adenovirus is harvested by lysing transfected cells. Because you transfect with DNA from a single clone, you do not need to screen individual plaques after transfection. To create cell lines that transiently express your gene of interest, infect target cells with your recombinant adenovirus.

Replication-incompetent adenovirus provides added safety and control

To accommodate DNA inserts and to produce a replication-*incompetent* adenoviral vector, extensive portions of the Early Regions 1 (E1) and 3 (E3) of wild-type adenovirus have been deleted from the Ad5 genome in our Adeno-X Viral DNA (Appendix A). Together, the E1 and E3 deletions enable you to ligate up to 8 kb of foreign DNA to our Adeno-X Viral DNA without adversely affecting the efficiency of viral particle formation. Because the E1 elements have been eliminated, an early passage HEK 293 cell line is required to propagate and titrate recombinant adenoviruses derived from Adeno-X Viral DNA (Graham *et al.*, 1977; Aiello *et al.*, 1979). HEK 293 cells stably express the Ad5 E1 genes that are essential for replication and transcription of Adeno-X Viral DNA; the Adeno-X genome carries the remaining coding information necessary to produce fully functional viral particles.

In addition to creating room for DNA inserts, deleting E1 restricts the cytopathic activity of the recombinant adenoviral particles produced with this system (Graham *et al.*, 1977). This valuable safety feature means that when you clone your expression cassette into Adeno-X Viral DNA, you produce a replication-incompetent adenovirus, which propagates only in those cell types (e.g., HEK 293 cells) that express the E1-encoded *trans*-complementing factors. For all other somatic cell types susceptible to adenoviral infection, exposure to your recombinant adenovirus leads to a transient non-cytopathic (i.e., non-lytic) infection. The adenoviral genome is established as

I. Introduction & Protocol Overview *continued*

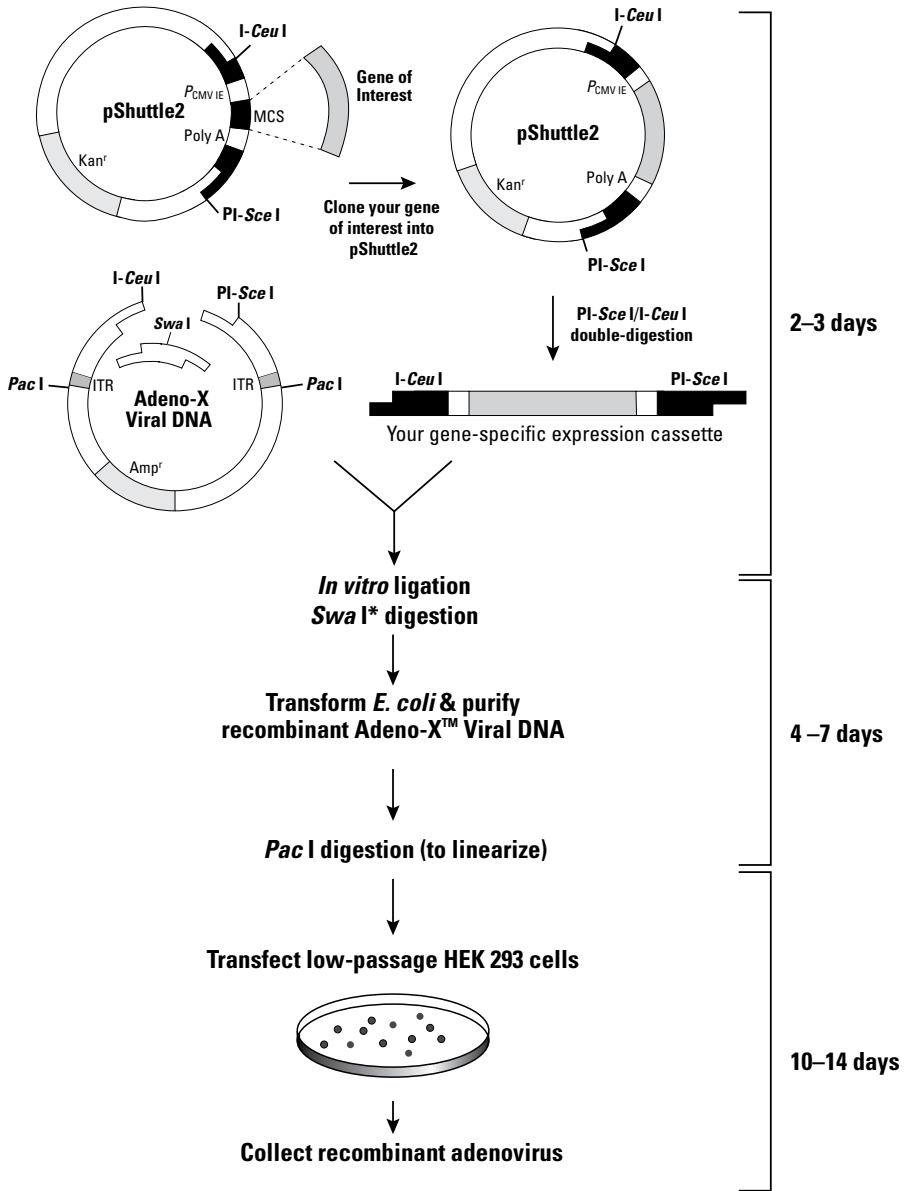


Figure 1. Constructing recombinant adenovirus with the Adeno-X™ Expression System 1.
 *The *Swa* I site is located between the *I-Ceu* I and *PI-Sce* I sites in the circular, nonlinearized Adeno-X Vector. We provide you with prelinearized (*PI-Sce* I- & *I-Ceu* I-digested) Adeno-X Viral DNA (also sold separately, Cat. No. 631026).

I. Introduction & Protocol Overview *continued*

an episome in the host cell's nucleus, but is neither replicated nor *actively* transcribed since the cell lacks the necessary transcription factors—the E1 gene products. Although the Ad genes remain inactive, your gene insert is still expressed at high levels because it is independently controlled by the constitutive cytomegalovirus promoter, P_{CMVIE} . Expression of your gene from the Adeno-X genome depends neither on the proliferation of the target cell line nor on the presence of any other viral genes or promoters.

Benefits of adenovirus-mediated gene delivery

Adenoviral gene transfer is one of the most reliable methods for introducing genes into mammalian cells. Because infection by adenovirus is not cell-cycle dependent, you can deliver your gene to primary as well as transformed cell lines. Following infection, your target gene is transiently expressed at high levels since many cells receive multiple copies of the recombinant genome. Expression is transient because adenoviral DNA normally does not integrate into the cellular genome.

Adenoviruses are capable of infecting a wide variety of proliferating and quiescent cell types from many different animal species including humans, non-human primates, pigs, rodents, mice, and rabbits (Table I). Published reports suggest that nearly all human cell types—including skin, muscle, bone, nerve, and liver cells—are susceptible to infection by adenovirus. In a more recent study, adenovirus was also shown to be effective for delivering genes to white blood cells—human lymphoma cells (Buttgereit, P. *et al.*, 2000).

TABLE I. ADENOVIRUS-MEDIATED GENE TRANSFER IN NON-HUMAN SPECIES*

Species	References
Chicken	Fisher & Watanabe, 1996; Thakur <i>et al.</i> , 2001
Monkey	Bout <i>et al.</i> , 1994; Zhong <i>et al.</i> , 2000
Mouse	Stratford-Perricaudet <i>et al.</i> , 1990; Lombardi <i>et al.</i> , 2001
Pig	French <i>et al.</i> , 1994; Patricia <i>et al.</i> , 2001
Rabbit	Donahue, <i>et al.</i> , 1998; Riew <i>et al.</i> , 1998; Yao <i>et al.</i> , 2001
Rat	Mastrangeli <i>et al.</i> , 1993; Skelly <i>et al.</i> , 2001
Sheep	Holzinger <i>et al.</i> , 1995; Klebe <i>et al.</i> , 2001

* To find out if a particular cell line can be infected by adenovirus, we suggest you search the literature. If you would like to test a particular cell line for infectivity, try one of our Adeno-X Marker Viruses (Related Products). These Marker Viruses encode well known reporter proteins—DsRed, and β -galactosidase—that can be easily detected by fluorescence microscopy or colorimetric staining, depending on the virus used.

II. List of Components

Storage Conditions

- Store all components at –20°C.
- Spin briefly to recover contents.
- Avoid repeated freeze-thaw cycles.

Adeno-X™ Expression System 1 (Cat. No. 631513)

- 20 µl Adeno-X™ Viral DNA (PI-*Sce* I/I-*Ceu* I digested; 250 ng/µl)
- 20 µg pShuttle2 Vector (500 ng/µl)
- 20 µg pShuttle2-lacZ Control Vector (500 ng/µl)
- 25 µl I-*Ceu* I (5 units/µl)
- 100 µl PI-*Sce* I (1 unit/µl)
- 250 µl 10X Double Digestion Buffer
- 100 µl 100X BSA
- 100 µl Adeno-X Forward PCR Primer (100 ng/µl)
- 100 µl Adeno-X Reverse PCR Primer (100 ng/µl)
- Adeno-X PCR Screening Primer Set Protocol-at-a-Glance (PT3507-2)
- pShuttle2 Vector Information Packet (PT3713-5)

The following kit components are also available separately:

- Adeno-X™ System 1 Viral DNA (linear; Cat. No. 631026)
- Adeno-X™ Accessory Kit (Cat. No. 631027)
Contains PI-*Sce* I and I-*Ceu* I enzymes, Double Digestion Buffer, and BSA
- Adeno-X™ PCR Screening Primer Set (Cat. No. 631030)
Includes the Adeno-X Forward and Reverse PCR Primers

III. Additional Materials Required

The following materials are required but not supplied.

Plasmid manipulations:

- Kanamycin (Kan)
Prepare a 50 mg/ml stock solution. Store at -20°C .
- Ampicillin (Amp)
Prepare a 50 mg/ml stock solution. Store at -20°C .
- LB Liquid and Agar Media
- Glycogen (20 mg/ml)
- RNase A (10 mg/ml) Store at -20°C .
- Agarose
- Sterile, deionized H_2O
- 10 M (saturated solution) Ammonium Acetate (NH_4OAc) or 3 M sodium acetate (NaOAc ; pH 5.2)
- Sodium Dodecyl Sulfate (SDS)
- Electrocompetent or Chemically Competent *E. coli* Cells
- Restriction Endonuclease *Swa* I (New England Biolabs)
- Restriction Endonuclease *Pac* I (New England Biolabs)
- Restriction Endonuclease *Xho* I (New England Biolabs)
- T4 DNA Ligase
- NucleoBond® Plasmid Midi Kit (Cat. No. 635929)
- NucleoSpin® Plus Plasmid Kit (Cat. No. 635987)
- 1XTE Buffer (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA)
- Phenol:chloroform:isoamyl alcohol (25:24:1)
Equilibrate with 100 mM Tris-HCl (pH 8.0)
- Ethanol (100% and 70%)

III. Additional Materials Required *continued*

Buffers for Mini-Scale Purification of Recombinant pAdeno-X DNA:

- Buffer 1: 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose
Autoclave and store at 4°C.
- Buffer 2: 0.2 M NaOH, 1% SDS
Prepare fresh, just prior to use. Keep tightly capped and at room temperature.
- Buffer 3: 5 M KOAc
Autoclave and store at 4°C.
- Buffer 4: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 µg/ml RNase (boiled to inactivate DNase)
Add RNase just before use. Store at -20°C after adding RNase (Shelf-life ≤ 6 months). In general, it is not advisable to store RNase solutions near RNase-free solutions.

Virus production and β-gal assays:

- Human Adenovirus 5-transformed Human Embryonic Kidney 293 cell line (HEK 293; ATCC, Rockville, MD, CRL 1573)
Used to package and propagate the recombinant adenoviral-based vectors produced with the Adeno-X Expression System. The HEK 293 Cell Line may be grown in DMEM or Minimum Essential Medium, α Modification (α-MEM). Supplement the medium with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mM L-glutamine and 10% fetal bovine serum.
- Dulbecco's Modified Eagle's Medium (DMEM) or Minimum Essential Medium, α Modification (α-MEM)
- Solution of 10,000 units/ml Penicillin G Sodium and 10,000 µg/ml Streptomycin Sulfate
- Fetal Bovine Serum
- Trypsin-EDTA
- Phosphate-Buffered Saline (PBS, without Ca²⁺ and Mg²⁺)
- Dulbecco's Phosphate-Buffered Saline (DPBS, with Ca²⁺ and Mg²⁺)
- Cell Freezing Medium
- Tissue culture plates and flasks (e.g., 60-mm plates, 6-well plates, T75 & T175 flasks)
- Neutral Red Stain (0.33%)
- Trypan Blue Dye (0.4%)
- Transfection Reagent (e.g., calcium phosphate or lipid)
- X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [25 mg/ml]) in dimethylformamide (DMF). Store in the dark at -20°C.
- Luminescent β-gal Reporter System 3 (Cat. No. 631713)

IV. Safety & Handling of Adenoviruses

The protocols in this User Manual require the production, handling, and storage of infectious adenovirus. It is imperative to fully understand the potential hazards of and necessary precautions for the laboratory use of adenoviruses.

The National Institute of Health and Center for Disease Control have designated adenoviruses as Level 2 biological agents. This distinction requires the maintenance of a Biosafety Level 2 facility for work involving this virus and others like it. The virus packaged by transfecting HEK 293 cells with the adenoviral-based vectors described here are capable of infecting human cells. These viral supernatants could, depending on your gene insert, contain potentially hazardous recombinant virus. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*.

For these reasons, due caution must be exercised in the production and handling of any recombinant adenovirus. **The user is strongly advised not to create adenoviruses capable of expressing known oncogenes.**

For more information on Biosafety Level 2, see the following reference:

- *Biosafety in Microbiological and Biomedical Laboratories*, 4th Edition (May 1999) U.S. Department of Health and Human Services, CDC, NIH. (Available at <http://bmb.lod.nih.gov>.)

Biosafety Level 2:

The following information is a brief description of Biosafety Level 2. *It is neither detailed nor complete.* Details of the practices, safety equipment, and facilities that combine to produce a Biosafety Level 2 are available in the above publication. If possible, observe and learn the practices described below from someone who has experience working with adenoviruses.

- Practices:
 - perform work in a limited access area
 - post biohazard warning signs
 - minimize aerosols
 - decontaminate potentially infectious wastes before disposal
 - take precautions with sharps (e.g., syringes, blades)
- Safety equipment:
 - biological safety cabinet, preferably Class II (i.e., a laminar flow hood with microfilter [HEPA filter] that prevents release of aerosols; *not* a standard tissue culture hood)
 - protective laboratory coats, face protection, double gloves
- Facilities:
 - autoclave for decontamination of waste
 - unrecirculated exhaust air
 - chemical disinfectants available for spills

V. Adeno-X™ Expression System 1 Overview

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Clone your gene of interest into pShuttle2 (Section VIII)

- Construct a gene-specific mammalian expression cassette by cloning your gene of interest into pShuttle2 using any of the unique restriction sites located in the MCS region.
- Transform competent *E. coli* cells with recombinant pShuttle2 plasmid DNA and select for kanamycin resistant transformants.
- Isolate putative recombinant pShuttle2 plasmid DNA and confirm that it contains your gene of interest.
- Verify expression of your protein by transfecting your cell line of choice with recombinant pShuttle2. If a suitable method is available, also check the activity of the expressed protein by biochemical assay.
- Excise your expression cassette from recombinant pShuttle2 plasmid DNA by digesting with I-Ceu I and PI-Sce I.

Produce recombinant adenoviral DNA containing your gene of interest (Section IX)

- Ligate your expression cassette to Adeno-X Viral DNA.
- Digest the ligation product with *Swa* I; transform *E. coli* cells with the product and select for ampicillin resistant transformants.
- Isolate putative recombinant adenoviral DNA and confirm that it contains your gene of interest.

Propagate and purify recombinant adenovirus (Section X)

- Digest recombinant adenoviral DNA with *Pac* I.
- Transfect low passage HEK 293 cells with *Pac* I-digested recombinant adenoviral DNA using standard transfection techniques.
- Harvest recombinant viral particles; [optional] purify virus using CsCl density gradient centrifugation or by using the Adeno-X Virus Purification Kit (Cat. No. 631532, 631533, or 631534).
- Determine adenoviral titer using the protocols in Appendix D or by using the Adeno-X Rapid Titer Kit (Cat. No. 631028).

Infect target cells (Section XI)

- Infect target cells with recombinant adenovirus to express your protein of interest.

V. Adeno-X™ Expression System 1 Overview *cont.*

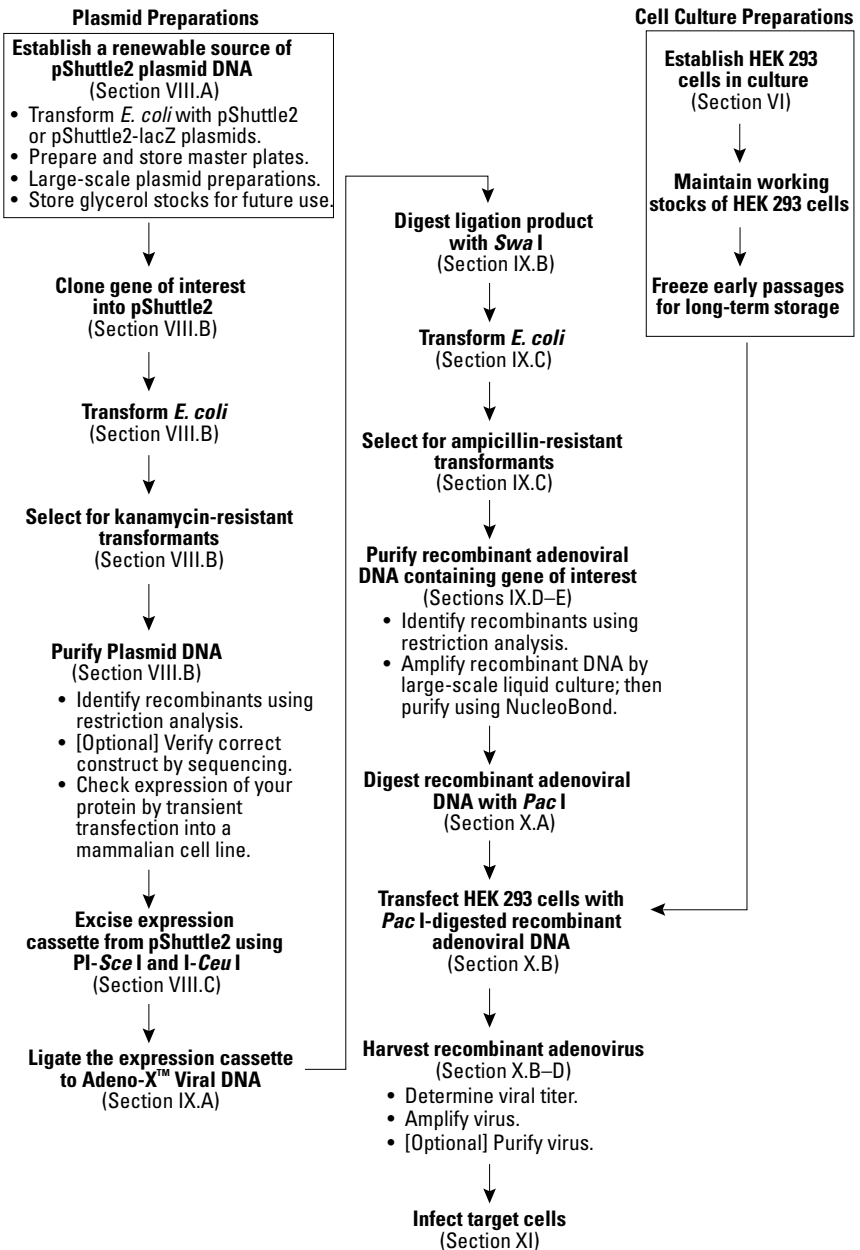


Figure 2. Overview of the Adeno-X™ Expression System 1 Protocol.

VI. Cell Culture Guidelines

A. General Considerations

The Human Adenovirus 5-transformed Human Embryonic Kidney 293 cell line (HEK 293; ATCC, Rockville, MD, CRL 1573) is used to package and propagate the recombinant adenoviral-based vectors produced with the Adeno-X Expression System. For more information on mammalian cell culture, we recommend the following references:

- *Culture of Animal Cells*, Fourth Edition, ed. by R.I. Freshney (2000, Wiley-Liss)
- *Current Protocols in Molecular Biology*, ed. by F.M. Ausubel *et al.* (1995 *et seq.*) John Wiley & Sons, Inc.

B. Maintaining HEK 293 Cells in Culture

HEK 293 cells should be grown in a monolayer, preferably in plastic petri dishes or flasks. Under optimum growth conditions (37°C, 5% CO₂), 293 cells double about every 36 hr. To maintain consistency, do not passage cells indefinitely. For best results, we recommend you use low passage 293 cells for transfection and titration procedures.

To prevent contamination, work with media and uninfected cells in a vertical laminar flow hood, using sterile technique. Keep this hood free of virus to prevent accidental infection of the stock cultures; ideally use another hood for all virus work. All virus-contaminated materials, including fluids, must be autoclaved or disinfected with 10% bleach or a chemical disinfectant before disposal.

1. To thaw 293 cells, place the vial of frozen cells in a 37°C water bath until just thawed. Sterilize the outside of the vial with 70% EtOH. For maximum viability upon plating, remove DMSO as follows:
 - a. Add 1 ml complete medium (prewarmed to 37°C). Transfer mixture to a 15-ml tube.
 - b. Add 5 ml complete medium and mix gently. Repeat. The final volume should be 12 ml.
 - c. Centrifuge at 125 x g for 10 min. Remove supernatant.
 - e. Gently resuspend cells in 10 ml complete medium: DMEM [or Minimum Essential Medium, α Modification (α -MEM)] supplemented with 100 units/ml penicillin G sodium, 100 μ g/ml streptomycin, 4 mM L-glutamine, and 10% fetal bovine serum.
2. Transfer cells (in 10 ml of growth medium) to a 100-mm culture plate.
3. Cells should be split every 2–4 days when they reach 80–90% confluency. Cells should not be allowed to become overly confluent nor should they be seeded too sparsely.
4. Split the cells as follows. Remove the medium and wash the cells once with sterile PBS (containing no Ca²⁺ or Mg²⁺). Add 1–2 ml of trypsin-EDTA solution and treat for 1–3 min, just long enough to

VI. Cell Culture Guidelines *continued*

detach cells (do not expose cells to trypsin for extended periods). Then add 5–10 ml of complete growth medium (to stop trypsinization) and resuspend the cells gently but thoroughly. Transfer the desired number of cells to a 100-mm plate containing 10 ml of medium. Gently rock the plate to distribute cells.

C. Preparing Frozen Cultures of HEK 293 Cells

We recommend you prepare frozen aliquots of early passages of the HEK 293 cell line to ensure a renewable source of cells.

1. Expand the cell line in the desired number of flasks or plates.
2. When the desired number of flasks/plates have reached ~80% confluence, wash the cells once with sterile PBS (containing no Ca^{2+} or Mg^{2+}), trypsinize, add 2–4 volumes complete medium to dilute trypsin, and harvest cells.
3. Count your cells and collect by centrifugation (~500 x g for 10 min).
4. Resuspend in 4°C Cell Freezing Medium at $1\text{--}2 \times 10^6$ cells/ml.
5. Dispense 1 ml aliquots into labeled freezing vials and place in a cell freezing container (reduces temperature ~1°C/min) at –80°C overnight.

Alternatively, place the vials on ice or at –20°C for 1–2 hours, transfer to an insulated container (foam ice chest), and place container in a –80°C freezer for several hours to overnight.

6. Transfer vials to liquid nitrogen.
7. Two or more weeks later, confirm the viability of frozen stocks by starting a fresh culture as described in Part B.

VII. Pilot Experiment

Construct lacZ-containing recombinant adenovirus (Adeno-X-lacZ)

Before you begin work on your own recombinant adenoviral vector, we recommend you perform the following pilot experiment to confirm that the Adeno-X Expression System 1 functions properly in your hands. Using the protocols in Sections VIII–XI and the DNA sources provided, construct recombinant adenovirus containing *lacZ*. Assess the functionality of your construct by infecting target cells and assaying for the expression of β -galactosidase as described in Section XI.

DNA sources provided:

- pShuttle2-lacZ Vector*
- Adeno-X Viral DNA (PI-*Sce* I- & I-*Ceu* I-digested)

* Constructed at Clontech by cloning the *E. coli* β -galactosidase (*lacZ*) gene into pShuttle2 using the *Xba* I and *Not* I sites.

VIII. Constructing Recombinant pShuttle2

The pShuttle2Vector (Appendix A) is used to construct a mammalian expression cassette containing your gene of interest. The *I-Ceu I* and *PI-Sce I* restriction sites, which flank the expression cassette in pShuttle2, are then used to excise the expression cassette for ligation to the Adeno-X genome.

A. Producing and Storing pShuttle2 Plasmid DNA

Before constructing recombinant pShuttle2 Vectors, you should transform a suitable *E. coli* host strain (e.g., DH5 α) with the pShuttle2 vectors provided with this kit, pShuttle2 and pShuttle2-lacZ, to ensure that you have renewable sources of these vectors for future experiments. Select for transformants by plating on LB agar/kanamycin (50 μ g/ml) plates. Streak out single colonies on fresh LB agar/kanamycin plates. After overnight incubation at 37°C, you can store the plates at 4°C for up to one month. Refer to Sambrook & Russell (2001) and Ausubel *et al.* (1995) for detailed information on making glycerol stocks and the conditions necessary for long-term storage of bacterial stock cultures.

B. Cloning Your Gene or DNA Fragment into pShuttle2

Construct your recombinant pShuttle2Vector using standard molecular biology techniques, as described below. For more detailed information, see Sambrook & Russell (2001) and Ausubel *et al.* (1995).

1. Digest pShuttle2 with the restriction enzyme(s) appropriate for your expression application. Consult the pShuttle2 Vector Information Packet (PT3713-5) supplied with this system to determine which multiple cloning site(s) are compatible with your DNA insert. Treat the digested plasmid with alkaline phosphatase, if desired, then purify.
2. Prepare and purify your target DNA fragment using any standard method. The ends of the DNA fragment must be compatible with one or more of the restriction sites present in the MCS region of pShuttle2.

One way to accomplish this is to generate your gene fragment using a suitable PCR protocol that utilizes primers bearing the necessary restriction sites.

Note: Check your gene insert for conflicting restriction sites.

- You should check your gene insert for the occurrence of *I-Ceu I*, *PI-Sce I*, *Swa I*, and *Pac I* recognition sequences (see following page). Although *I-Ceu I* and *PI-Sce I* sites are relatively rare, their presence will conflict with the excision of the expression cassette performed in Part C, below. *Swa I* and *Pac I* are used to process recombinant Adeno-X clones in Sections IX and X, respectively.

VIII. Constructing Recombinant pShuttle2 *continued*

I-*Ceu* I Recognition Sequence

5'TAAC TATAACGGTCCTAAGGTAGCGA3'
3'ATTGATATGCCAGGATTCATCGCT5'

Pac I Recognition Sequence

5'TTAATTA3'
3'AATTAAT5'

PI-*Sce* I Recognition Sequence

5'ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCA3'
3'TAGATACAGCCACACGCCTCTTCTCCATTACTTTACCGT5'

Swa I Recognition Sequence

5'ATTTAAAT3'
3'TAAATTA5'

3. Ligate the digested vector and the gene fragment.
4. Transform chemically or electrocompetent DH5 α cells with the ligation mixture.

Prepare a positive control strain by transforming a separate aliquot of competent cells with the control vector provided, pShuttle2-lacZ. Performing this transformation in parallel with your experimental sample(s) will help you evaluate the overall transformation efficiency of your host cell system and serve as a renewable source of positive control plasmid DNA that can be used to check the performance of the PI-*Sce* I/I-*Ceu* I double-digestion protocol used in Part C, below. In addition, pShuttle2-lacZ serves as a source of DNA for construction of the positive control adenoviral vector, pAdeno-X-lacZ.

5. Select for kanamycin-resistant (Kan^r) transformants by plating the transformation mixture on LB agar/Kan plates (50 μ g/ml kanamycin).
6. Inoculate a small-scale liquid culture with a single, well-isolated colony. We recommend you set up 5–10 such cultures to ensure you obtain at least one positive clone. After overnight incubation, isolate plasmid DNA using any standard method. For small-scale purification (≤ 20 μ g plasmid DNA), we recommend our NucleoSpin® Plus Plasmid Kit (Cat. No. 635987).
7. Identify the desired recombinant plasmid by restriction analysis. Verify the orientation and junctions of your insert by sequencing. Once a positive clone has been identified, inoculate a large-scale liquid culture to prepare greater quantities of your recombinant pShuttle2Vector. To ensure the purity of the DNA, isolate all plasmids for transfection using a NucleoBond® Plasmid Midi Kit (Cat. No. 635929) or by banding on a CsCl gradient (Sambrook & Russell, 2001).
8. Test for expression of your protein by transfecting your cell line of choice with recombinant pShuttle2. Verify expression by Western blotting, and, if possible, check the activity of your protein using a biochemical assay.

VIII. Constructing Recombinant pShuttle2 *continued*

C. *PI-Sce I* / *I-Ceu I* Digestion of Recombinant pShuttle2 Plasmid DNA

The unique restriction endonucleases *PI-Sce I* and *I-Ceu I* provided with this kit are used to excise your newly fashioned expression cassette from the recombinant pShuttle2 plasmid DNA. The excised expression cassette is then “shuttled” into Adeno-XViral DNA (Figure 1) by means of an *in vitro* ligation, described in Section IX.A.

1. Prepare a 30- μ l *PI-Sce I*/*I-Ceu I* double-digest of your recombinant pShuttle2 plasmid DNA. Combine the reagents shown in Table II in sterile 1.5-ml microcentrifuge tubes.

TABLE II. *PI-SCE I*/*I-CEU I* DOUBLE-DIGESTION OF RECOMBINANT pSHUTTLE2 PLASMID DNA

Reagent	Tube 1 Experiment	(Optional) Tube 2 lacZ Control
Sterile H ₂ O	19.5 μ l	19.5 μ l
10X Double Digestion Buffer	3.0 μ l	3.0 μ l
Recombinant pShuttle2 Plasmid DNA (500 ng/ μ l)	2.0 μ l	—
Positive Control pShuttle2-lacZ Plasmid DNA (500 ng/ μ l)	—	2.0 μ l
<i>PI-Sce I</i> Restriction Enzyme (1 unit/ μ l)	2.0 μ l	2.0 μ l
<i>I-Ceu I</i> Restriction Enzyme (5 units/ μ l)	0.5 μ l	0.5 μ l
10X BSA*	3.0 μ l	3.0 μ l

* **Note:** We provide you with 100X BSA. Before beginning this reaction, prepare 10X BSA by diluting a small aliquot of 100X BSA with sterile deionized water (1:10).

2. Mix well and spin briefly to collect liquid.
3. Incubate at 37°C for exactly 3 hours. **For best results, this incubation time must be strictly observed.**
4. Verify digestion by analyzing 3–5 μ l of your sample on a 1% agarose/EtBr gel. Be sure to include DNA size markers (e.g., a 1-kb ladder).

Note: Since *I-Ceu I* and *PI-Sce I* tend to remain bound to DNA, use a gel loading buffer that contains SDS (final concentration after combining with sample: 0.1%).

VIII. Constructing Recombinant pShuttle2 *continued*

5. Extract the digested DNA from the remaining volume using either the phenol:chloroform:isoamyl method (Part D) **or** a Nucleospin Extract II kit (Cat. No. 636972).

Note: The Extraction Kit has two advantages: It requires no organic solvents, and it extracts DNA quickly without the need for ethanol precipitation. If you wish to use the Extraction Kit, follow the NucleoSpin Extraction procedure "Isolation from PCR" given in the NucleoSpin Extraction User Manual (PT3631-1); **at the final step, elute your DNA with 30 µl of Buffer NE.**

D. Extraction with Phenol:Chloroform:Isoamyl Alcohol

1. To the remaining volume (~25 µl after Step C.4.) of the digested sample, add 70 µl 1XTE Buffer (pH 8.0) and 100 µl phenol:chloroform:isoamyl alcohol (25:24:1).
2. Vortex thoroughly.
3. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C to separate phases.
4. Carefully transfer the top aqueous layer to a clean 1.5-ml microcentrifuge tube. Discard the interface and lower phase into an organic waste container.
5. Add 400 µl 95% ethanol, 25 µl 10 M NH₄OAc (or 1/10 volume of 3 M NaOAc), and 1 µl glycogen (20 mg/ml).
6. Vortex thoroughly.
7. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C.
8. Remove and discard the supernatant.
9. Carefully overlay the pellet with 300 µl 70% ethanol.
10. Spin in a microcentrifuge at 14,000 rpm for 2 min at room temperature.
11. Carefully aspirate off the supernatant.
12. Air dry the pellet for approximately 15 min at room temperature to evaporate residual ethanol.
13. When the pellet is dry, dissolve the DNA precipitate in 10 µl sterile 1XTE Buffer (pH 8.0) and store at -20°C until use in Section IX.

IX. Constructing Recombinant Adenoviral DNA

A. Subcloning Your Expression Cassette into the Adeno-X™ Genome

To insert your expression cassette into the Adeno-X genome, use the following *in vitro* ligation reaction. Adeno-X Viral DNA has already been digested with *Pi-Sce I* and *I-Ceu I* and carefully tested to ensure its performance in this reaction. The ligation product you obtain is a circular recombinant E1/E3-deleted adenoviral genome that carries a *ColE1* origin of replication and an ampicillin resistance marker for propagation and selection in *E. coli*.

1. Combine the reagents shown in Table III in sterile 1.5-ml microcentrifuge tubes in the order shown.

TABLE III. LIGATING EXPRESSION CASSETTES TO ADENO-X™ DNA

Reagent	Tube 1 Experiment	(Optional) Tube 2 lacZ Control
<i>Pi-Sce I</i> / <i>I-Ceu I</i> -digested Recombinant pShuttle2 Plasmid DNA (from Section VIII.D.13)*	2 µl*	—
<i>Pi-Sce I</i> / <i>I-Ceu I</i> digested pShuttle2-lacZ Plasmid DNA	—	2 µl*
Sterile H ₂ O	3 µl	3 µl
10X DNA Ligation Buffer	1 µl	1 µl
Adeno-X Viral DNA (250 ng/µl)	3 µl	3 µl
DNA Ligase (1 unit/ µl)	1 µl	1 µl
Total Volume	10 µl	10 µl

* **Note:** If you used a NucleoSpin Extraction Kit to deproteinize your digested DNA (Section VIII.C.5), add 5 µl of DNA (dissolved in 30 µl NE Buffer) and omit the H₂O.

2. Gently mix, then spin briefly in a microcentrifuge.
3. Incubate at 16°C overnight.
4. To each sample, add 90 µl 1XTE Buffer (pH 8.0) and 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
5. Vortex gently but thoroughly.
6. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C to separate phases.
7. Carefully transfer the top aqueous layer to a clean 1.5-ml microcentrifuge tube. Discard the interface and lower phase.

IX. Constructing Recombinant Adenoviral DNA *continued*

8. Add 400 μ l 95% ethanol, 25 μ l 10 M NH_4OAc (or 1/10 volume 3 M NaOAc), and 1 μ l glycogen (20 mg/ml).
9. Vortex gently but thoroughly.
10. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C.
11. Remove and discard the supernatant.
12. Carefully overlay the pellet with 300 μ l 70% ethanol.
13. Spin in a microcentrifuge at 14,000 rpm for 2 min.
14. Carefully aspirate off the supernatant.
15. Air dry the pellet for approximately 15 min at room temperature.
16. Dissolve the DNA precipitate in 15 μ l sterile deionized H_2O . Proceed with Part B.

B. *Swa* I Digestion of Non-Recombinant Adeno-X™ DNA

Once the ligation is completed, the product should be treated with *Swa* I to linearize non-recombinant (i.e., self-ligated) pAdeno-X DNA. *Swa* I digestion reduces the frequency of non-recombinant clones formed during Step C, below.

1. Prepare a 20- μ l digest for each of your experimental and control samples as shown in Table IV.

TABLE IV. *Swa* I DIGESTION OF LIGATION REACTION PRODUCTS

Reagent	Volume
Ligation Product (from Step IX.A.16)	15 μ l
10X <i>Swa</i> I Digestion Buffer	2 μ l
10X BSA*	2 μ l
<i>Swa</i> I Restriction Enzyme (10 units/ μ l)	1 μ l
Total Volume	20 μ l

* **Note:** We provide you with 100X BSA. Before beginning this reaction, prepare 10X BSA by diluting a small aliquot of 100X BSA with sterile deionized water (1:10).

2. Incubate at 25°C for 2 hours.
3. To each sample, add 80 μ l 1XTE Buffer (pH 8.0) and 100 μ l phenol:chloroform:isoamyl alcohol (25:24:1).
4. Vortex gently.
5. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C to separate phases.
6. Carefully transfer the top aqueous layer to a clean 1.5-ml microcentrifuge tube. Discard the interface and lower phase.

IX. Constructing Recombinant Adenoviral DNA *continued*

7. Add 400 µl 95% ethanol, 25 µl 10 M NH₄OAc (or 1/10 volume 3 M NaOAc), and 1 µl glycogen (20 mg/ml).
8. Vortex gently.
9. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C.
10. Remove and discard the supernatant.
11. Carefully overlay the pellet with 300 µl 70% ethanol.
12. Spin in a microcentrifuge at 14,000 rpm for 2 min.
13. Carefully aspirate off the supernatant.
14. Air dry the pellet for approximately 15 min at room temperature.
15. Dissolve the DNA precipitate in 10 µl sterile 1XTE Buffer (pH 8.0). Store at -20°C until use in Part C.

C. Transforming *E. coli* with Recombinant Adeno-X™ DNA

1. Use standard molecular biology techniques to transform chemically or electrocompetent *E. coli* cells with the *Swa* I digestion product from Step IX.B.15. We recommend using a general purpose recombination deficient host strain.
2. Select for ampicillin-resistant (Amp^r) transformants by plating the transformation mixture on an LB agar/Amp plate (100 µg/ml ampicillin). Incubate at 37°C overnight.
3. Check colonies for recombinant pAdeno-X DNA by using PCR with the Adeno-X Forward and Reverse PCR Primers provided. Please refer to the Adeno-X™ PCR Screening Primer Set Protocol-at-a-Glance (PT3507-2) for conditions and set-up.

Notes:

- We have found that the smallest colonies, often mistaken for satellite colonies, frequently carry the desired recombinant adenoviral plasmid DNA.
 - Because colonies can be analyzed directly, without the need for DNA purification, PCR is probably the quickest and most convenient way to identify transformants containing pShuttle2-derived inserts.
4. Transfer a single colony to 5 ml of fresh LB/Amp (100 µg/ml). Incubate overnight at 37°C with continuous shaking.
 5. The next day, purify Adeno-X plasmid DNA using the mini-scale procedure described in Part D.

Note: pAdeno-X is a large plasmid (>32 kb) that is susceptible to damage and rearrangement in *E. coli*. For best results, always use fresh, log-phase cultures for purification of recombinant pAdeno-X DNA. Do not store your culture at room temperature, 4°C, or on ice for long periods (i.e., >24 hr) before starting the purification.

IX. Constructing Recombinant Adenoviral DNA *continued*

D. Mini-Scale Purification of Recombinant Adeno-X™ Plasmid DNA

This protocol utilizes a series of buffers (**Buffers 1–4**) that you will need to prepare beforehand. The compositions of **Buffers 1–4** are described in Section III.

1. Centrifuge 3–5 ml of fresh, log-phase culture (from Step IX.C.4) at 14,000 rpm for 15–30 sec. Carefully decant the supernatant.
Note: pAdeno-X is a large plasmid (>32 kb) that is susceptible to damage and rearrangement in *E. coli*. For best results, always use fresh, log-phase cultures for purification of recombinant pAdeno-X DNA. Do not store your culture at room temperature, 4°C, or on ice for long periods (i.e., >24 hr) before starting the purification.
2. Spin the pellet once again at 10,000 rpm for 1 min. Use a micropipette to aspirate the remaining supernatant.
3. Resuspend the pellet in 150 µl of **Buffer 1** by gently pipetting up and down.
4. Add 150 µl of **Buffer 2** to the suspension. Mix gently by inverting the tube several times. Incubate the cell suspension at room temperature for 5 min.
5. Add 150 µl of **Buffer 3** to the chilled suspension. Mix gently by inverting the tube several times. Place the cell suspension on ice for 5 min.
6. Centrifuge the suspension at 14,000 rpm for 5 min at 4°C.
7. Transfer the clear supernatant to a clean 1.5-ml microcentrifuge tube.
8. Add 450 µl of phenol:chloroform:isoamyl alcohol (25:24:1) to the supernatant. Mix by inversion.
9. Centrifuge for 5 min at 4°C to separate phases.
10. Carefully transfer the top aqueous layer to a clean 1.5-ml microcentrifuge tube. Discard the interface and lower phase into an organic waste container.
11. Add 1ml 95% ethanol. Mix thoroughly by inversion.
12. Centrifuge at 14,000 rpm for 10 min at 4°C.
13. Remove and discard the supernatant.
14. Add 1 ml 70% ethanol then centrifuge for 2 min at room temperature.
15. Remove and discard the supernatant.
16. Allow the pellet to dry at room temperature.
17. When the pellet is dry, dissolve the DNA precipitate in 15–30 µl of **Buffer 4**. Incubate at room temperature for 10 min.
18. Vortex gently.

IX. Constructing Recombinant Adenoviral DNA *continued*

19. Spin briefly to recover contents. Store at -20°C .
20. Identify the recombinant by restriction analysis or PCR or both (Part E).

E. Analyzing Putative Recombinant Adeno-X™ DNA

To identify recombinant pAdeno-X Plasmid DNA, use restriction analysis, PCR, or both. When you have identified a bacterial clone carrying the desired recombinant, inoculate 100 ml of LB/Amp medium with 2 ml of fresh, log-phase culture. Incubate the 100-ml culture at 37°C until it reaches log-phase. Then purify the plasmid using a NucleoBond® Plasmid Midi Kit (Cat. No. 635929). Follow the Low-Copy Plasmid Purification Protocol in the NucleoBond User Manual (PT3167-1). In following this protocol, **use filtration not centrifugation to clarify the bacterial lysate.**

Expected Yield: 30–50 μg plasmid DNA/100 ml of culture

Note: pAdeno-X is a large plasmid (>32 kb) that is susceptible to damage and rearrangement in *E. coli*. For best results, always use fresh, log-phase cultures for purification of recombinant pAdeno-X DNA. Do not store your culture at room temperature, 4°C , or on ice for long periods (i.e., >24 hr) before starting the purification or before inoculating a second culture.

Following NucleoBond purification, be sure to **reconfirm** the identity and integrity of the recombinant Adeno-X plasmid using one or both of the analyses listed below.

- **Restriction Analysis:** The presence of your expression cassette can be verified by digestion with *Xho* I or by double-digestion with *Pf-Sce* I and *I-Ceu* I (Table V). Analyze the digestion by electrophoresis on a 0.8–1% agarose/EtBr gel. Typical results of such restriction analyses are shown in Figure 6 in Appendix B.
- **PCR Analysis:** You can also screen pAdeno-X DNA for the presence of pShuttle2-derived expression cassettes by using PCR with the Adeno-X Forward PCR Primer and Reverse PCR Primer. These primers specifically amplify a 287-bp sequence that spans the *I-Ceu* I ligation site in pAdeno-X. Only recombinant pAdeno-X templates are amplified since nonrecombinants lack the Shuttle sequence needed for annealing with the reverse primer. Please refer to the

IX. Constructing Recombinant Adenoviral DNA *continued*

Adeno-X™ PCR Screening Primer Set Protocol-at-a-Glance (PT3507-2) for conditions and set-up.

1. PI-*Sce* I and I-*Ceu* I Restriction Analysis:

Set up 30- μ l PI-*Sce* I/I-*Ceu* I double-digests by combining the reagents shown in Table V in a sterile 1.5-ml microcentrifuge tube.

TABLE V. RESTRICTION ANALYSIS OF RECOMBINANT pADENO-X DNA

Reagent	Volume
Sterile H ₂ O	19.5 μ l
10X Digestion Buffer	3.0 μ l
Recombinant pAdeno-X DNA (500 ng/ μ l; from Step IX.D.19)	2.0 μ l
PI- <i>Sce</i> I Restriction Enzyme (1 unit/ μ l)	2.0 μ l
I- <i>Ceu</i> I Restriction Enzyme (5 units/ μ l)	0.5 μ l
10X BSA*	3.0 μ l
Total Volume	30.0 μ l

***Note:** Prepare 10X BSA by diluting a small aliquot of 100X BSA with sterile deionized water (1:10).

- Mix well and spin briefly to collect contents.
- Incubate at 37°C for exactly 3 hours. **Please note:** It is important that this incubation time be strictly observed.
- Verify the digestion by electrophoresis on a 0.8–1% agarose/EtBr gel.

Important: Since I-*Ceu* I and PI-*Sce* I tend to remain bound to DNA, use a gel loading buffer that contains SDS (final concentration after combining with sample: 0.1%).

X. Producing Recombinant Adenovirus

A. Preparing Recombinant Adeno-X™ DNA for Transfection

Before Adeno-X DNA can be packaged, the recombinant plasmid must be digested with *Pac* I to expose the inverted terminal repeats (ITRs) located at either end of the genome (Figure 3). The ITRs contain the origins of adenovirus DNA replication and must be positioned at the termini of the linear Ad DNA molecule to support the formation of the replication complex (Tamanoi & Stillman, 1982).

1. In a sterile 1.5-ml microcentrifuge tube, combine the following reagents (Table VI).

TABLE VI. *Pac* I DIGESTION OF RECOMBINANT pADENO-X DNA

Reagent	Volume
Sterile deionized H ₂ O	20 µl
Recombinant pAdeno-X Plasmid DNA (500 ng/µl)	10 µl
10X <i>Pac</i> I Digestion Buffer	4 µl
10X BSA ^a	4 µl
<i>Pac</i> I Restriction Enzyme (10 units/ µl)	2 µl
Total Volume ^b	40 µl

^aPrepare 10X BSA by diluting a small aliquot of 100X BSA with sterile deionized water (1:10).

^bEach 40 µl digest yields enough DNA to transfect one 60-mm culture plate. (The transfection protocol is given in Part B.) To transfect larger cultures, e.g., a series of 150-mm plates, scale the digest proportionally.

2. Mix contents and spin the tube briefly in a microcentrifuge.
3. Incubate at 37°C for 2 hr.
4. Add 60 µl 1XTE Buffer (pH 8.0) and 100 µl phenol:chloroform: isoamyl alcohol (25:24:1). Vortex gently.
5. Spin the tube in a microcentrifuge at 14,000 rpm for 5 min at 4°C to separate phases.
6. Carefully transfer the top aqueous layer to a clean sterile 1.5-ml microcentrifuge tube. Discard the interface and lower phase.
7. Add 400 µl 95% ethanol, 25 µl 10 M NH₄OAc (or 1/10 volume 3 M NaOAc), and 1 µl glycogen (20 mg/ml). Vortex gently.
8. Spin the tube in a microcentrifuge at 14,000 rpm and 4°C for 5 min.
9. Remove and discard the supernatant.

X. Producing Recombinant Adenovirus *continued*

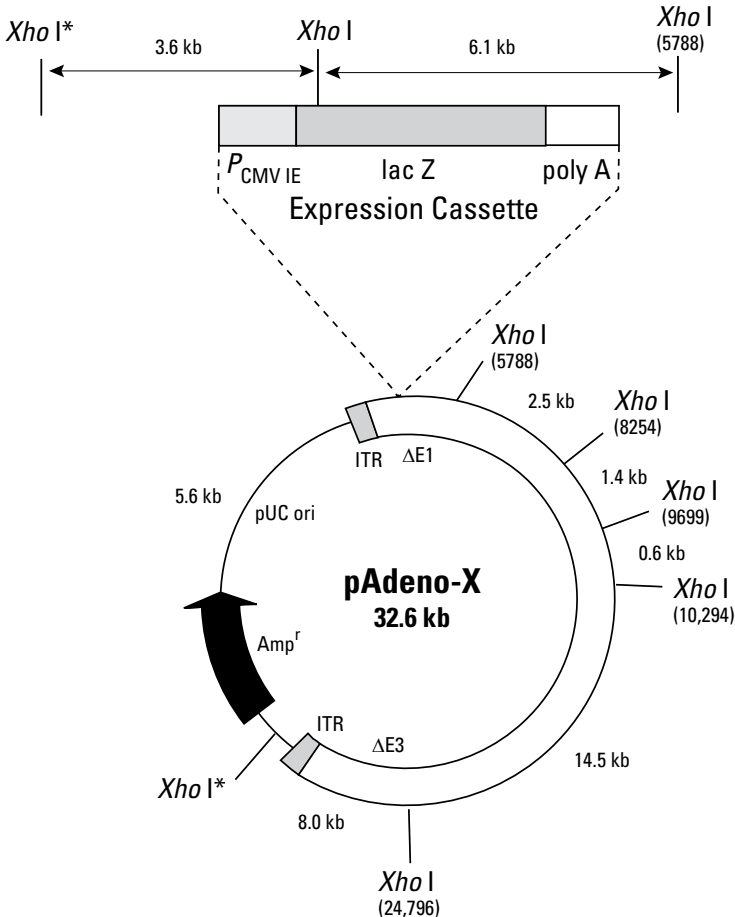


Figure 3. Producing recombinant adenovirus.

10. Wash the pellet with 300 μ l 70% ethanol.
11. Spin in a microcentrifuge at 14,000 rpm for 2 min.
12. Carefully aspirate off the supernatant.
13. Air dry the pellet for ~15 min at room temperature.
14. Dissolve the DNA precipitate in 10 μ l sterile 1XTE Buffer (pH 8.0). Proceed with Part B or store at -20°C .

X. Producing Recombinant Adenovirus *continued*

B. Transfecting HEK 293 Cells with *Pac* I-Digested Adeno-X™ DNA

1. Plate HEK 293 cells at a density of $1\text{--}2 \times 10^6$ cells per 60-mm culture plate (approximately 100 cells/mm²) 12–24 hr before transfection. For best results, cells should be 50–70% confluent, display a flat morphology, and adhere well to the plate just prior to transfection.

If you constructed a positive control vector (e.g., pAdeno-X-lacZ), be sure to seed sufficient plates to produce this virus as well.

Transfection control: To monitor the efficiency of your transfection procedure, transfect an additional plate with pShuttle2-lacZ. Approximately 48 hr after transfection, check for the expression of β -galactosidase by staining cells with X-Gal.

2. Incubate the plate(s) at 37°C in a humidified atmosphere maintained at 5% CO₂.
3. Transfect each 60-mm culture plate with 10 μ l of *Pac* I-digested Adeno-X DNA. Use any standard transfection method (e.g., calcium phosphate or lipid) to transfer DNA into HEK 293 cells.
4. One day later, and periodically thereafter, check for cytopathic effect (CPE).

Notes:

- Infected cells typically remain intact but round up and may detach from the plate. These changes are collectively referred to as the cytopathic effect (CPE). For a description of the CPE, please see the Adeno-X™ Frequently Asked Questions at www.clontech.com/clontech/techinfo/faqs/.
 - The time it takes for CPE to appear depends on the transfection efficiency—it may take up to two weeks for CPE to become evident. Because adenovirus remains associated with cells until late in the infection cycle, high titer virus is obtained by manually lysing cells with a series of freeze-thaw cycles as explained below in Steps 5–9.
5. One week later, transfer cells to a sterile 15-ml conical centrifuge tube. **Do not use trypsin:** Infected cells that still adhere to the bottom or sides of the culture plate can be dislodged into the medium by gentle agitation.
 6. Centrifuge the suspension at 1,500 x g for 5 min at room temperature.
 7. Resuspend the pellet in 500 μ l sterile PBS.
 8. Lyse cells with three consecutive freeze-thaw cycles: Freeze cells in a dry ice/ethanol bath; thaw cells by placing the tube in a 37°C water bath. **Do not allow the suspension to reach 37°C.** Vortex cells after each thaw.
 9. After the third cycle, briefly centrifuge to pellet debris. Transfer the lysate to a clean, sterile centrifuge tube and store the lysate at –20°C or use immediately for Step 10.

X. Producing Recombinant Adenovirus *continued*

10. Infect a fresh 60-mm culture by adding 250 μ l (50%) of the cell lysate from Step 9. Add the lysate directly to the medium, then incubate as normal. CPE should be evident within one week.

Note: If no CPE appears after one week, the viral titer of the cell lysate from Step 9 may be too low. Amplify the titer by repeating Steps 5–10.

11. When >50% of the cells have detached from the plate, prepare viral stock by following Steps 5–9. Name this stock "Primary Amplification". Store at -20°C .
 - Primary Amplification Stock is suitable for infecting target cells as described in Section XI. We suggest you evaluate the function of this viral stock before preparing High-Titer Stock (Part C).
 - The presence of your recombinant construct can be verified by PCR or Western blotting (Part D).

12. Determine adenoviral titer (Appendix D).

The **Adeno-X™ Rapid Titer Kit** (Cat. No. 631028) enables you to determine adenoviral titer using an anti-hexon antibody cell staining assay. See the April 2002 issue of *Clontechniques*, available from our web site, or download a free copy of the User Manual (PT3651-1) to learn more about this product.

C. Amplifying Recombinant Adenovirus: Preparing High-Titer Stocks

Note: The probability of producing replication competent adenovirus (RCA), although low, increases with each successive amplification. RCA is produced when Adeno-X DNA recombines with E1-containing genomic DNA in 293 cells. For this reason, we suggest you save aliquots of early amplifications. Use early amplification stocks whenever you need to produce additional quantities of adenovirus.

1. About 24 hours before infection, plate HEK 293 cells in a T75 flask. The cell monolayer should be 50–70% confluent when you infect.
2. Incubate cells overnight at 37°C in a humidified atmosphere maintained at 5% CO_2 .
3. On the following day, replace the medium with 5 ml of fresh growth medium that contains adenovirus: For best results, infect cells at a multiplicity of ≥ 5 (i.e., at ≥ 5 pfu/cell). For example, if the T75 flask contains $\sim 5 \times 10^6$ cells, add 2.5×10^7 pfu adenovirus.
4. Incubate for 90 min at 37°C in a humidified atmosphere maintained at 5% CO_2 .
5. Remove the flask and add 10 ml of fresh growth medium.
6. Incubate for 3–4 days at 37°C in a humidified atmosphere at 5% CO_2 .
7. Check for a cytopathic effect. When 50% of the cells have detached,

X. Producing Recombinant Adenovirus *continued*

transfer the suspension to a sterile 15-ml conical centrifuge tube. **Do not use trypsin:** Infected cells that still adhere to the bottom or sides of the flask can be dislodged into the medium by gentle agitation.

- Isolate virus using the freeze-thaw method given in Part B, Steps 5–9.
(At Step B.7, resuspend the pellet in 0.5–1 ml of PBS.)
- Determine adenoviral titer (Appendix D). We recommend the **Adeno-X™ RapidTiter Kit** (Cat. No. 631028). Expected titer: 10^8 – 10^9 pfu/ml.
- To produce a greater quantity of high-titer adenovirus, use the cell lysate from the first amplification to infect larger cultures (e.g., a series of T175 flasks).
- [Optional] Depending on how you intend to use your recombinant adenovirus, you may wish to refine your High-Titer Stock by banding on a CsCl density gradient. Please consult the following references for protocols on how to purify adenovirus using CsCl gradient centrifugation (Hitt *et al.*, 1998; Hitt *et al.*, 1995; Graham & Prevec, 1991; Spector & Samaniego, 1995; and Becker *et al.*, 1994).

Alternatively, use the **Adeno-X Virus Purification Kit** (Cat. Nos. 631532, 631533 & 631534). With this kit, adenovirus can be purified in less than 2 hours, and no ultracentrifugation steps are necessary. The purity is comparable to that achieved with CsCl centrifugation. See the July 2002 issue of *Clontechniques*, available from our web site, or download a free copy of the User Manual (PT3680-1) to learn more about this product.

D. Evaluating Recombinant Virus: Confirmation of Construct

A number of different methods can be used to verify that the encapsidated adenoviral genome contains a functional copy of your gene. The preferred methods detect synthesis of the target protein: e.g., Western blotting, ELISA, or a biochemical assay that specifically measures the enzymatic activity of the expressed protein. If an antibody is not available, Southern blotting can be used to confirm the presence of your gene.

Alternatively, PCR—e.g., using the Adeno-X Forward and Reverse PCR Primers or your own gene-specific primers—is a quick and efficient way to evaluate your construct. A small aliquot (e.g., 1 μ l) of viral stock can be sampled and used directly for PCR. The high temperatures associated with PCR denature the viral coat proteins and expose the DNA for hybridization with the primers.

XI. Infecting Target Cells with Adenovirus

A. Infecting Target Cells

We recommend infecting target cells at a multiplicity of between 10–100 pfu/cell. The multiplicity of infection (M.O.I.) needed to efficiently transmit your gene of interest to a particular host cell population depends on the biological properties of the target cell line and, therefore, must be determined empirically. An excessively high M.O.I. can be toxic to cells; however, an extremely low M.O.I. may not enable you to accurately evaluate the phenotype of an infected cell line. To infect some lymphoid cell lines, you may need to use higher M.O.I.s—e.g., 1000 pfu/cell. To infect the maximum number of cells, use the smallest volume needed to cover the cells.

1. Plate target cells in 6-well plates 12–24 hr before infection. The seeding density will depend on the growth characteristics of your cell line.

Note: Always use filtered pipette tips when handling viruses and cells.

Positive control infection: If you constructed a positive control recombinant adenovirus (e.g., one that contains Adeno-X-lacZ), be sure to seed sufficient plates to allow for this infection.

2. The next day, remove the growth medium and add 1.0 ml of virus (diluted to achieve the desired M.O.I.) to the center of each plate. Tip the plates to spread the virus evenly.
3. Cover the plates and incubate the cells in a humidified CO₂ (5%) incubator at 37°C for 4 hours to allow the virus to infect the cells.
4. Add fresh complete growth medium. Incubate in a humidified CO₂ incubator at the temperature appropriate for your cell line.
5. Analyze gene expression at different time points following viral infection. In general, detectable levels of your gene product should be evident 24–48 hr after infection.

B. Analyzing β -galactosidase Expression in Infected Cells

The expression of β -galactosidase in adherent cells infected with Adeno-X-lacZ can be observed by staining with X-Gal using any standard protocol (e.g., see Ausubel *et al.*, 1995 *et seq.*). To quantify β -galactosidase expression, we recommend using our Luminescent β -gal Reporter System 3 (Cat. No. 631713).

XII. Troubleshooting Guide

Constructing Recombinant pShuttle2

Problem	Possible Cause	Solution
Few or no colonies produced following transformation of <i>E. coli</i> with recombinant pShuttle2 plasmid DNA	<p>Wrong antibiotic; antibiotic concentration is too high</p> <p>Poor transformation efficiency</p>	<p>Use kanamycin at 50 µg/ml of LB agar medium.</p> <ul style="list-style-type: none"> • Check transformation efficiency using a control plasmid, e.g., pShuttle2-lacZ. • Use a different strain of <i>E. coli</i> or obtain DH5α from another commercial source.

Constructing Recombinant Adenoviral DNA

Problem	Possible Cause	Solution
Few or no colonies produced following transformation of <i>E. coli</i> with recombinant adenoviral DNA	<p>Wrong antibiotic</p> <p>Poor transformation efficiency</p> <p>Failure of ligation procedure due to inadequate or excessive (non-specific) digestion of pShuttle2 plasmid</p>	<p>Use ampicillin at 100 µg/ml of LB agar medium.</p> <ul style="list-style-type: none"> • Check transformation efficiency using a control plasmid, e.g., pShuttle2-lacZ or pAdeno-X-lacZ (if constructed). • Use a different strain of <i>E. coli</i>. <p>Check the fidelity and completeness of the <i>PI-Sce I/I-Ceu I</i> double-digestion of pShuttle2 by analyzing the product on an EtBr/agarose gel. If a "smear" of bands is observed, reduce the amount of <i>I-Ceu I</i> enzyme used and/or shorten the incubation time of the digest. If a large amount of uncut plasmid is observed, extend the incubation time until the digestion is complete.</p>

XII. Troubleshooting Guide *continued*

Constructing Recombinant Adenoviral DNA *cont.*

Problem	Possible Cause	Solution
Many ampicillin-resistant colonies produced but few that harbor recombinant adenoviral DNA	Incomplete digestion with <i>Swa</i> I	Check the <i>Swa</i> I reaction by digesting non-recombinant Adeno-X Viral DNA. The product of this digest should not confer ampicillin resistance to <i>E. coli</i> hosts. If background colonies do appear, digest DNA using a higher concentration of <i>Swa</i> I and/or longer incubation time.
Restriction analysis of DNA prepared from large-scale culture reveals more bands than expected.	DNA contamination	Use the plasmid DNA prepared by mini-scale purification (Section IX.D) to transform a fresh aliquot of competent <i>E. coli</i> as described in Section IX.C. Inoculate a 5-ml culture; incubate for just 6–8 hours; then, without delay, transfer 2–3 ml of the culture to 100 ml of fresh LB/Amp. Incubate overnight. Finally, purify plasmid DNA as suggested in Section IX.E.
Restriction enzymes do not cut DNA prepared from large-scale liquid culture	Inhibition of enzyme activity by contaminants derived from culture medium	Be sure to remove all traces of culture medium from the bacterial pellet before beginning the NucleoBond purification protocol.

XII. Troubleshooting Guide *continued*

Producing Recombinant Adenovirus *continued*

Problem	Possible Cause	Solution
No virus particles produced	Poor transfection efficiency	<ul style="list-style-type: none"> • Check the transfection efficiency. Use a suitable control plasmid, e.g., pShuttle2-lacZ. We normally observe transfection efficiencies in the range of 10–15% when we transfect a 60-mm plate with 2 µg of pShuttle2-lacZ plasmid DNA. • Adjust seeding density of cells to optimize confluency at time of infection. Check for abnormal growth characteristics, morphology.
	293 cell culture used for transfection may be too dense	Start a fresh culture of low passage cells (e.g., p ≤ 50). For best results, the 293 cells used in transfections should be at low passage, and about 50–70% confluent at the time of transfection.
	Low quality pAdeno DNA	Check the purity (A_{260}/A_{280}) and identity of the plasmid DNA used for transfection.
High rate of cell death	Too little or too much pAdeno DNA used	Titrate the amount of <i>Pac</i> I-digested Adeno-X recombinant DNA to achieve maximal transfection efficiency; as a starting point we recommend using 2–5 µg of Adeno-X DNA for a 60-mm plate of 293 cells (50–70% confluent).
	The protein encoded by your gene insert may be toxic to 293 cells.	Try using the Adeno-X™ Tet-Off or Tet-On Expression System 1 (Cat. No. 631022 or 631050). With these systems, you are able to modulate the expression of your gene.

XII. Troubleshooting Guide *continued*

Infecting Target Cells with Adenovirus

Problem	Possible Cause	Solution
High rate of cell death	<p>The multiplicity of infection (M.O.I.) may be too high.</p> <p>Your gene insert may be toxic to host cells</p>	<p>Infect at lower M.O.I.</p> <p>Try using the Adeno-X™ Tet-Off or Tet-On Expression System 1 (Cat. No. 631022 or 631050).</p>
Low expression of gene insert	<p>Low infection frequency of target cell population</p> <p>Target cells are not susceptible to infection by adenovirus</p>	<ul style="list-style-type: none"> • Infect at higher M.O.I. • Check activity of adenovirus stock. • Adjust seeding density of cells to optimize confluency at time of infection. Check for abnormal growth characteristics, morphology. <p>Try using retroviral-mediated gene delivery and expression. (See Related Products.)</p>

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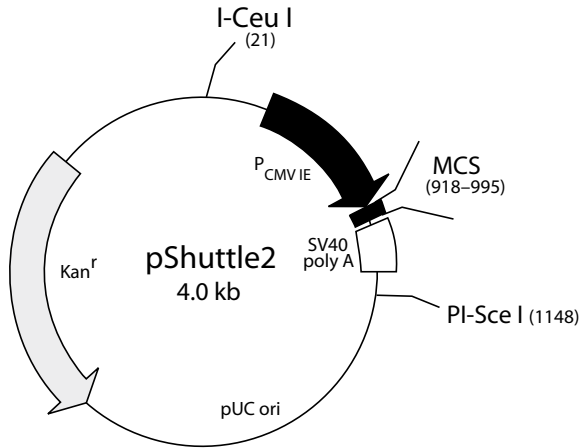
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Appendix A: Vector Information



pShuttle2 MCS

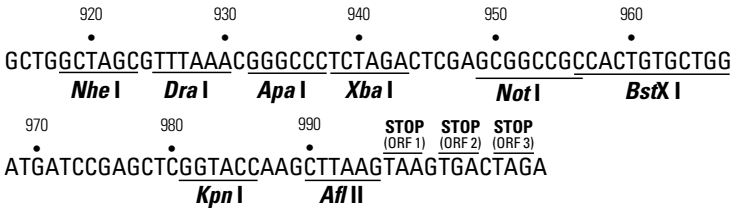


Figure 4. Plasmid map and multiple cloning site of pShuttle2. pShuttle2 allows you to clone your gene of interest into a mammalian expression cassette, which consists of the human cytomegalovirus immediate early promoter/enhancer (P_{CMVIE}), a multiple cloning site (MCS), and the SV40 polyadenylation signal (SV40 poly A). The entire cassette is flanked by unique I-Ceu I and PI-Sce I restriction sites so that it can be excised and ligated to Adeno-X Viral DNA. The vector backbone also possesses the pUC origin (pUC ori) and a kanamycin resistance gene (Kan^r) for propagation and selection in *E. coli*. To create your gene-specific expression cassette, insert your full-length cDNA into the MCS region of pShuttle2 using any of the unique restriction sites shown. Once recombinant pShuttle2 plasmid is formed *in vitro*, it can be cloned and amplified in *E. coli* using any standard transformation protocol. We recommend using a general purpose strain such as DH5 α . *E. coli* harboring recombinant plasmids can then be selected on LB agar/Kan (50 μ g/ml) plates and recultured to amplify the plasmid bearing the desired expression cassette.

pShuttle2-lacZ was constructed by cloning the *E. coli* β -galactosidase (*lacZ*) gene into the Xba I and Not I sites of pShuttle2.

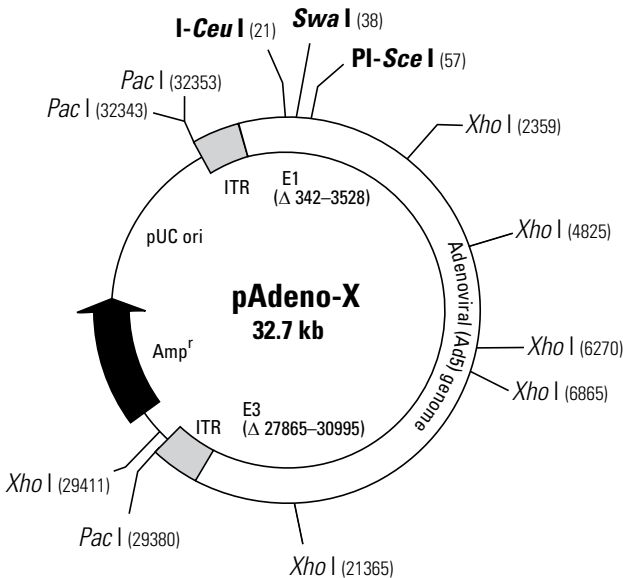
Appendix A: Vector Information *continued*

Figure 5. Plasmid map of pAdeno-X. The adenoviral genome used for the construction of recombinant adenovirus is shown in its circular form. Approximately 33 kb in length, pAdeno-X Viral DNA is derived from an adenovirus type 5 (Ad5) genome that has been altered by deleting extensive portions of the E1 and E3 regions of the Ad5 genome (Mizuguchi & Kay, 1998, 1999). The nucleotide sequences deleted (Δ) from each region in wild-type Ad5 DNA are indicated in the figure. Whereas E1 encodes proteins that are essential for viral replication and transcription, E3 is dispensable for the growth and propagation of the virus in culture (Kelley *et al.*, 1973). Inverted terminal repeats (ITR), which are necessary for the replication of adenoviral DNA, flank the E1/E3 deleted Ad5 genome. pAdeno-X also contains a pUC replication origin and an ampicillin resistance gene (Amp^r) for propagation and selection in *E. coli*. The linearized form of pAdeno-X supplied with this kit is generated by digesting pAdeno-X with PI-Sce I and I-Ceu I.

Appendix B: Typical Results of a Restriction Analysis

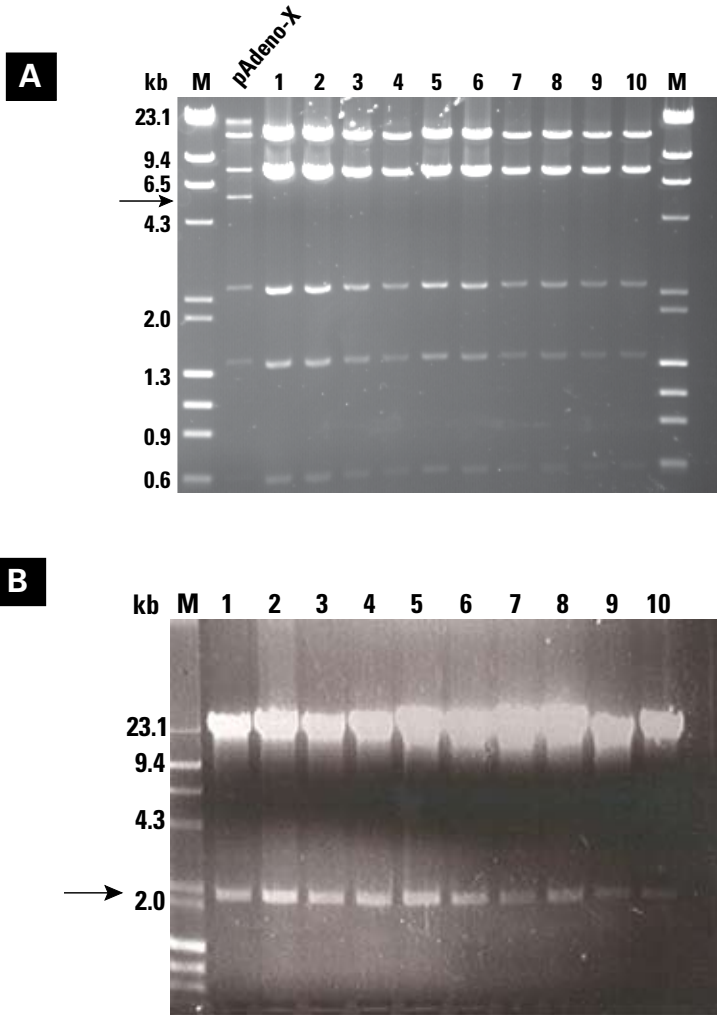


Figure 6. Restriction analysis of recombinant pAdeno-X Viral DNA. An expression cassette containing the Tet-On reverse transactivator (rtTA) was cloned into linearized Adeno-X Viral DNA (Pst I-Sce I & I-Ceu I-digested) and amplified in *E. coli* (DH5 α) according to the protocols in the User Manual. Following selection on LB/Amp, ten colonies were picked at random and analyzed by restriction with *Xho* I (**Panel A**) and Pst I-Sce I/I-Ceu I (**Panel B**). The digests were resolved on a 0.8% agarose/EtBr gel. All ten colonies tested positive for the presence of a DNA insert (arrow). In Panel A, the arrow indicates a band observed in *Xho* I digests of non-recombinant (circular) pAdeno-X. This band was eliminated by recombination with the pShuttle-derived insert.

Appendix C: Plaque Purification Protocol

For some studies, researchers prefer to use virus deriving from a single plaque on a monolayer of HEK 293 cells. The following protocol describes how to isolate virus from a single plaque.

1. Follow the agarose-overlay procedure in Appendix D.A to obtain a plate with well-isolated plaques.
2. Using a P200 pipette and sterile, filtered tips, select plaques as agarose plugs. Transfer to a 24-well plate containing 0.5 ml 293 cell growth medium.
3. To elute virus, incubate at 37°C in a humidified atmosphere at 5% CO₂ for 24 hr. Meanwhile, seed a second 24-well plate with HEK 293 cells (~1 x 10⁵ cells/well).
4. On the following day, aspirate medium from the 293 cell culture(s), then add 100 µl of eluted virus.
5. Incubate at 37°C for 90 min in a humidified atmosphere that is 5% CO₂.
6. Add 900 µl growth medium to each well.
7. Incubate at 37°C in a humidified atmosphere that is 5% CO₂ until cytopathic effect is complete (~5–10 days).
8. Transfer cells to a sterile 15-ml centrifuge tube. **Do not use trypsin.**
9. Centrifuge for 5 min.
10. Resuspend in 500 µl sterile PBS.
11. Lyse cells with three consecutive freeze-thaw cycles: Freeze cells in a dry ice/ethanol bath; thaw cells by placing the tube in a 37°C water bath, **Do not allow the suspension to be reach 37°C.** Vortex cells after each thaw.
12. After the third cycle, briefly centrifuge to pellet debris. Store at –20°C.
13. To amplify the virus further, repeat Steps 4–12. Use the amplification procedure in Section X.C to produce even greater quantities.

Appendix D: Determining Adenoviral Titer

This appendix provides three protocols for determining adenoviral titer:

1. Plaque Assay (requires ~1–3 weeks)
2. End-Point Dilution Assay (requires ~10 days)
3. OD₂₆₀ Assay (requires ~ 1 hour)

Methods 1 and 2 are biological assays; they measure the number of *infectious* viral particles. Method 3, on the other hand, is a physical assay; it measures the concentration of viral DNA and viral protein, and, therefore, does not distinguish between infectious and non-infectious viral particles.

As you evaluate your titer and compare it to that of another research group, keep in mind the following. Titers determined with biological assays may vary, depending on the individual performing the assay and the conditions under which the assay is carried out. Because the Plaque and End-Point Dilution Assays must be scored by eye, the titers measured with these methods are usually less precise than those obtained with a typical OD₂₆₀ Assay.

The titers determined with the OD₂₆₀ Assay, a physical method, are more precise because the assay is less sensitive to human bias. Samples are read with a spectrophotometer not by eye. However, the OD₂₆₀ Assay can only be used to measure virus that is free from growth medium since growth medium contains factors that interfere with the absorbance at 260 nm. If you wish to use the OD₂₆₀ method, you should first purify the virus by CsCl density gradient purification or using the Adeno-X Virus Purification Kit (Cat. Nos. 631532, 631533 or 631534).

A. Plaque Assay

Before starting this procedure, prepare 5% agarose as follows:

- (a) Dissolve 2.5 g SeaPlaque Agarose (FMC) in 50 ml DPBS (pH 7.4).
- (b) Sterilize by autoclaving.
- (c) Store the agarose in sterile, 50-ml conical tubes, 5 ml per tube.

The viral titer is determined by plaque assay as follows.

1. Approximately 24 hours before beginning the titration protocol, plate HEK 293 cells in 6-well plates. Seed the wells at a density of $0.5\text{--}1 \times 10^5$ cells per well in 4 ml of growth medium. To control for chance errors, seed 3–4 wells for each dilution of viral stock to be tested.
2. Prepare serial dilutions of your virus as follows:
 - a. Make a 1:100 dilution by adding 10 μ l viral stock to 990 μ l sterile DPBS.
 - b. Starting with a 1:100 dilution, prepare serial 1:10 dilutions by transferring 100 μ l of diluted virus to 900 μ l sterile DPBS.

In general, an appropriate range of dilutions for testing is $10^{-5}\text{--}10^{-10}$.

Appendix D: Determining Adenoviral Titer *continued*

3. Remove the cell culture plates from the incubator and inspect the wells to ensure that the cells have attached to form an even monolayer that is about 80–90% confluent. Remove the growth medium from the cell cultures and add 0.2 ml of adenovirus to each well taking care not to dislodge any cells. Tip the plates to spread the virus evenly over the monolayer.
4. Cover the plates and incubate the cells in a humidified CO₂ (5%) incubator for 60 min at 37°C to allow the virus to infect the cells.
5. During this incubation, prepare agarose overlay medium as follows.
 - a. Melt 5 ml of 5% agarose. Then cool to 44°C.
 - b. Warm 50–100 ml of HEK 293 growth medium to 44°C.
 - c. Add 45 ml growth medium to 5 ml 5% agarose. Mix well. This makes a 0.5% agarose solution, which is used to overlay the infected cell monolayer to prevent virus progeny from spreading to neighboring plaques.
6. Remove the virus inoculum from the cells by tilting the plate and aspirating from the edge.
7. Gently add 2–4 ml of 0.5% agarose solution to each well, taking care not to dislodge any cells.
8. When the agarose has set, incubate at 37°C in a humidified CO₂ (5%) incubator. Plaques should be visible within 7–10 days.
9. Prepare a 0.03% solution of neutral red in DPBS (1 ml 0.33% [w/v] neutral red stock + 10 ml DPBS). Add 1 ml of the 0.03% neutral red solution to each of the wells and incubate at 37°C for 2–3 hours.
10. Remove the stain by aspiration, then invert the dishes to allow the plaques to clear.

Note: Neutral red is taken up by healthy cells but not by dead cells. Therefore, plaques appear as clear circles against a red or pink background.

11. Calculate Viral Titer

The viral titer is a quantitative measurement of the biological activity of your recombinant virus and is expressed as plaque forming units (pfu) per ml. To calculate the viral titer, count the number of well isolated plaques. Then use the following formula to determine the titer (pfu/ml) of your viral stock.

$$\frac{\# \text{ plaques}}{d \times V} = \text{pfu/ml} \quad d = \text{dilution factor} \\ V = \text{volume of diluted virus added to the well}$$

Sample calculation:

- An average of 50 plaques formed in the 1:10,000 dilution wells
- Volume of diluted virus added: 0.2 ml

$$\frac{50}{0.0001 \times 0.2} = 2.5 \times 10^6 \text{ pfu/ml}$$

Appendix D: Determining Adenoviral Titer *continued*

B. End-Point Dilution Assay

1. Approximately 24 hours before beginning the titration protocol, plate HEK 293 cells in two 96-well plates. Carefully seed all wells in the same density ($\sim 10^4$ cells per well) in 100 μ l of growth medium. For best results, use a multi-channel pipette to transfer cells to the plate.

Note: Use sterile, cotton-plugged pipette tips.

2. Prepare serial dilutions of your virus as follows:
 - a. Make a 1:100 dilution by adding 10 μ l virus stock to 990 μ l sterile growth medium.
 - b. Starting with the 1:100 dilution, prepare serial 1:10 dilutions by transferring 100 μ l diluted virus to 900 μ l sterile growth medium

In general, an appropriate range of dilutions for testing is 10^{-3} – 10^{-10} .

3. Remove the 96-well culture plate from the incubator and inspect the wells to ensure that the cells have attached to form an even monolayer.
4. Add 100 μ l diluted virus to each well in columns 1–10 (Figure 7). Add 100 μ l of virus-free growth medium to wells in columns 11–12. These wells serve as controls for the viability of non-infected cells.

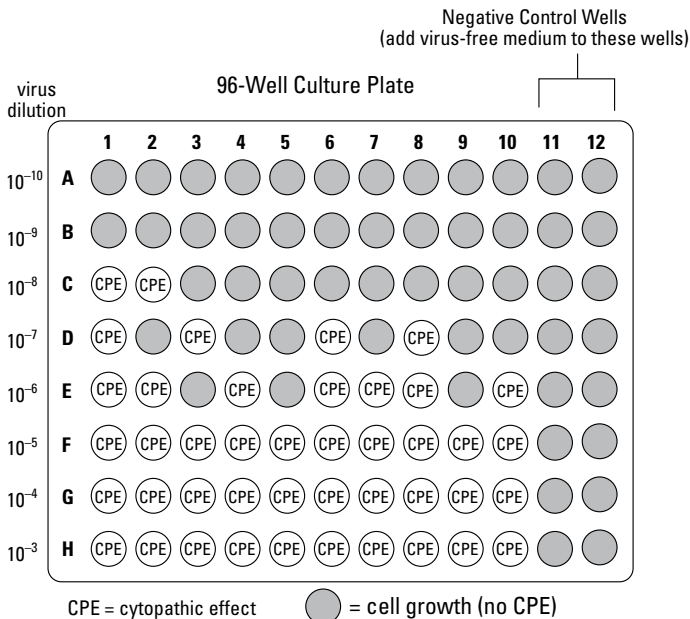


Figure 7. Determining adenoviral titer with the End-Point Dilution Assay.

Appendix D: Determining Viral Titer *continued*

5. Cover the plate and incubate in a humidified CO₂ (5%) incubator for 10 days at 37°C.
6. Using a microscope, check each well for cytopathic effect (CPE). For each row, count the number of wells having CPE. A well is scored as CPE positive even if only a few cells show cytopathic effects. If you are uncertain, compare the infected well with the non-infected control wells.
7. Calculate the fraction of CPE-positive wells in each row.

Figure 7 shows how you might score a typical End-Point Dilution Assay. In this example, the fraction of CPE-positive wells in each row can be written as follows:

<u>Dilution</u>	<u>Fraction of CPE-positive wells</u>
10 ⁻¹⁰	0 ÷ 10 = 0
10 ⁻⁹	0 ÷ 10 = 0
10 ⁻⁸	2 ÷ 10 = 0.2
10 ⁻⁷	4 ÷ 10 = 0.4
10 ⁻⁶	7 ÷ 10 = 0.7
10 ⁻⁵	10 ÷ 10 = 1
10 ⁻⁴	10 ÷ 10 = 1
10 ⁻³	10 ÷ 10 = 1

8. Calculate Viral Titer

$$\text{Titer (pfu/ml)} = 10^{(x + 0.8)}$$

x = the sum of the fractions of CPE-positive wells

Note: Even if you omit some of the lower dilutions—e.g., the 1:10 and 1:100 dilutions, as in the example above—you must count them as part of the sum. The formula given is based on the Spearman-Kärber method.

Sample calculation:

$$x = (1 + 1 + 1 + 1 + 1 + 0.7 + 0.4 + 0.2 + 0 + 0) = 6.3$$

$$\text{Titer} = 10^{7.1} = 1.3 \times 10^7 \text{ pfu/ml}$$

The assay is a reliable indicator of viral titer only if the following three conditions are met:

- The negative control wells show no visible signs of CPE or growth inhibition.
- Wells infected with the least dilute virus (10⁻³ in the example) are all CPE-positive.
- Wells infected with the most dilute virus (10⁻¹⁰ in the example) are all CPE-negative.

Appendix D: Determining Viral Titer *continued*

C. OD₂₆₀ Assay

This assay is for determining the titer of concentrated stocks of purified adenovirus. It should not be used for measuring virus in crude cell lysates or in culture supernatant since serum and other factors in growth media interfere with the absorbance at 260 nm.

1. Purify and concentrate your recombinant adenovirus by banding in a CsCl density gradient or by using the Adeno-X Virus Purification Kit (Cat. Nos. 631532, 631533 or 631534).

Purification protocols are provided in the following references: Hitt *et al.*, 1998; Hitt *et al.*, 1995; Graham & Prevec, 1991; Spector & Samaniego, 1995; and Becker *et al.*, 1994.

2. Prepare dilutions of your virus as follows:

<u>Dilution</u>	<u>Virus stock</u>	<u>0.1% SDS Buffer*</u>
1:10	50 µl	450 µl
1:25	20 µl	480 µl
1:50	10 µl	490 µl

* Prepare 0.1% SDS as a solution in any suitable buffer (e.g., 1XTE or PBS).

In general, appropriate dilutions for assay are those having an OD₂₆₀ in the range 0.1–1.0.

Remember to make 0.5-ml blanks for each dilution by substituting TE buffer (or equivalent “dialysis” buffer) for your virus stock.

3. Record the absorbance at 260 nm (OD₂₆₀). Always start by reading the absorbance of the most dilute sample.
 - a. Fill the cuvette with the blank for the 1:50 dilution.
 - b. Record the OD₂₆₀.
 - c. Discard the blank.
 - d. Add the 1:50 dilution and read its absorbance.
 - e. Rinse the cuvette once before adding the blank for the next dilution—the 1:25 dilution.
 - f. Repeat Steps a–e for the 1:25 and 1:10 dilutions.
4. Record the absorbance at 280 nm (OD₂₈₀).
5. Calculate viral titer and purity

$$\text{viral titer (opu/ml)} = \text{OD}_{260} \times \text{viral dilution} \times 1.1 \times 10^{12}$$

opu = opticle particle unit

Note: Because opticle particle units (opu) and plaque forming units (pfu) define different properties, these measurements cannot be directly compared.

$$\text{purity} = \text{OD}_{260} / \text{OD}_{280} \text{ (typically } \sim 1.2\text{--}1.3 \text{ after CsCl purification)}$$

Notes

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