



AdEasy Adenoviral Vector System

Instruction Manual

Catalog #240009 (AdEasy Adenoviral Vector System)

#240005 (pAdEasy-1 vector)

#240006 (pShuttle vector)

#240007 (pShuttle-CMV vector)

#240008 (pShuttle-CMV-*lacZ* vector)

#240081 (pShuttle-IRES-hrGFP-1 vector)

#240082 (pShuttle-IRES-hrGFP-2 vector)

Revision D1

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ADeASY ADENOVIRAL VECTOR SYSTEM

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AdEasy Adenoviral Vector System

MATERIALS PROVIDED

AdEasy Adenoviral Vector System (Catalog #240009) Materials Provided	Quantity
pAdEasy-1 vector (Catalog #240005; 100 ng/μl in TE buffer)	2.5 μg
pShuttle vector (Catalog #240006; 1 μg/μl in TE buffer)	20 μg
pShuttle-CMV vector (Catalog #240007; 1 μg/μl in TE buffer)	20 μg
pShuttle-CMV-lacZ control vector (Catalog #240008; 1 μg/μl in TE buffer)	10 μg
BJ5183 electroporation competent cells ^a (Catalog #200154)	5 × 100 μl (Clear tubes)
XL10-Gold ultracompetent cells ^b (Catalog #200314)	5 × 100 μl (Gold tubes)
XL10-Gold β-mercaptoethanol mix (β-ME)	50 μl
pUC18 DNA control plasmid (0.1 ng/μl in TE buffer ^b)	10 μl

^a Each 100 μl aliquot is sufficient for two transformations.

^b Each 100 μl aliquot is sufficient for one transformation.

pShuttle-IRES-hrGFP-1 (Catalog #240081) Materials Provided	Quantity
pShuttle-IRES-hrGFP-1 vector (1 μg/μl in TE buffer)	20 μg

pShuttle-IRES-hrGFP-2 (Catalog #240082) Materials Provided	Quantity
pShuttle-IRES-hrGFP-2 vector (1 μg/μl in TE buffer)	20 μg

STORAGE CONDITIONS

AdEasy Vectors: –20°C

Competent Cells (including β-ME mix and pUC18 control plasmid): –80°C

Place cells at –80°C immediately upon arrival. Do not place cells in liquid nitrogen.

ADDITIONAL MATERIALS REQUIRED

Pac I restriction enzyme
Pme I restriction enzyme
Alkaline phosphatase, molecular biology grade
Chloroquine[§]
ViraPack Transfection Kit [Agilent Catalog #200488]
StrataPrep PCR Purification Kit [Agilent Catalog #400771]
Electroporation cuvettes, 0.2 cm gap
Electroporator
14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
5-ml BD Falcon polystyrene round bottom tubes (BD Biosciences Catalog #352054)
AD-293 cells (**recommended**) [Agilent Catalog #240085] or HEK293 cells
Growth medium for AD-293 cells[§] [Invitrogen Life Technologies (Gibco) Catalog #11995]
SeaPlaque agarose [FMC Corporation]

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[§] See *Preparation of Media and Reagents*.

INTRODUCTION

Recombinant adenoviruses are a versatile tool for gene delivery and expression. Several features of adenovirus biology have made such viruses the vectors of choice for certain applications. For example, adenoviruses are capable of infecting a broad range of cell types and infection is not dependent on active host cell division. Additionally, high virus titers and high-level gene expression can be obtained, which are important considerations for protein production techniques in mammalian cells. The most commonly used adenoviral vector is human adenovirus serotype 5, which is rendered replication defective by the deletion of the E1 and E3 genes. The E1 gene is essential for the assembly of infectious virus particles and is complemented in vivo by an adenovirus packaging cell line (e.g. AD-293). The E3 gene encodes proteins involved in evading host immunity and is dispensable. Not only do these deletions render the virus incapable of replicating itself, but they also create space for up to 7.5 kb of foreign DNA.¹

Two methods have traditionally been used to generate recombinant adenoviruses. The first involves direct ligation of the gene of interest into the adenoviral genome. Because the adenovirus genome is large (36 kb) and contains few useful restriction sites, this method is technically very challenging. The second, and more commonly used method, involves cloning the gene of interest into a shuttle vector and transferring the gene into the adenovirus genome by means of homologous recombination in an adenovirus packaging cell line.² The isolation of recombinant adenovirus by this method involves performing multiple plaque isolations and is extremely laborious and time consuming. More recently, a different approach has been developed by T.C. He and colleagues.³ Employing the efficient homologous recombination machinery in *E. coli*, a recombinant adenovirus is produced by a double-recombination event between cotransformed adenoviral backbone plasmid vector, pAdEasy-1, and a shuttle vector carrying the gene of interest. This eliminates the need to manipulate the large adenovirus DNA molecule in vitro (in restriction and ligation reactions). There is no need to carry out laborious plaque purification rounds. The time needed to generate a recombinant adenovirus is reduced by several weeks.

Note *The AdEasy XL Adenoviral Vector System is also available (Agilent Catalog #240010). The XL kit includes BJ5183 cells pre-transformed with the pAdEasy-1 plasmid (BJ5183-AD-1 cells), providing a streamlined recombinant Ad DNA production method. This kit also includes the AD-293 cells recommended for use with the AdEasy vector system.*

Overview of the AdEasy System

A schematic overview of the production of recombinant adenovirus is shown in Figure 1. In the AdEasy system, the DNA of interest is cloned into any of the four shuttle vectors (pShuttle-CMV, pShuttle, pShuttle-IRES-hrGFP-1 (Catalog #240081) or pShuttle-IRES-hrGFP-2 (Catalog #240082)). Once constructed, the shuttle vector is linearized with *Pme* I and cotransformed into BJ5183 together with pAdEasy-1, the supercoiled viral DNA plasmid. Transformants are selected for kanamycin resistance, and recombinants are subsequently identified by restriction digestion. Once a recombinant is identified, it is produced in bulk using the recombination-deficient XL10-Gold strain. Purified recombinant Ad plasmid DNA is digested with *Pac* I to expose its inverted terminal repeats (ITR), and then used to transfect AD-293 (or HEK293) cells where deleted viral assembly genes are complemented *in vivo*.

Advantages of Recombinant Adenovirus for Gene Expression

Broad range of infectivity and high titer

Adenoviruses can infect a broad range of mammalian cells and have been used successfully to express human and non-human proteins. Recombinant adenoviruses can produce high titers (10^7 to 10^8 pfu/ml following transient transfection).

Infection does not require an actively dividing host cell

Recombinant adenovirus can infect both dividing and non-dividing cells.

Expressed human proteins are properly folded and modified

Because the AdEasy vector system employs human adenovirus and human host cell lines, human proteins expressed using this system are abundant and have the correct posttranslational modification and folding.

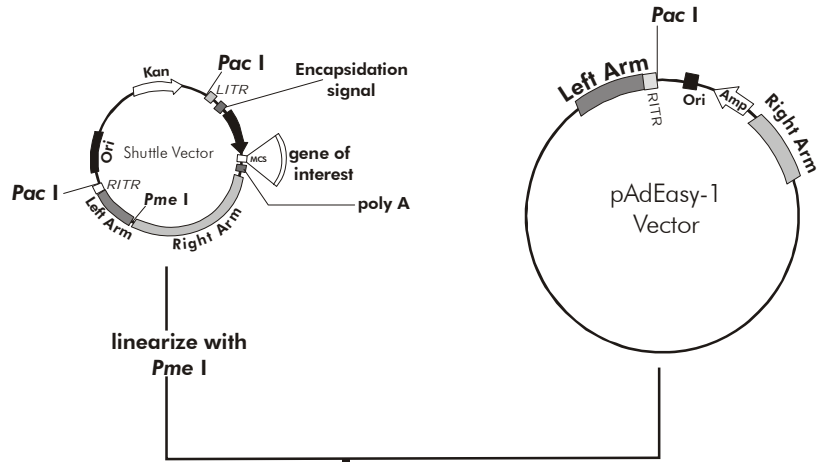
Large insert size

The AdEasy vector system allows for the insertion of up to 7.5 kb of foreign DNA.

AdEasy vector is non-insertional

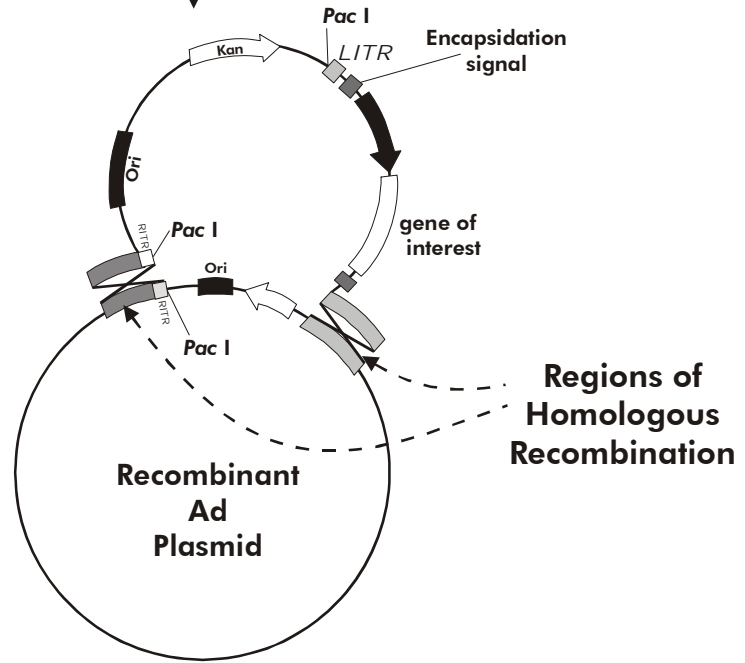
Because the recombinant adenovirus remains epichromosomal in the human host cell, there is only a remote possibility of activation or inactivation of host cell genes resulting from interruption by the transfected gene(s).

Cloning Gene of Interest

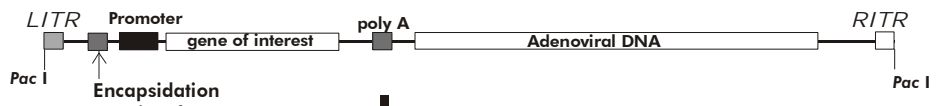


Homologous Recombination in vivo in Bacteria

cotransform BJ5183 cells, select for Kan^R



Digest with Pac I



Transfect AD-293 (or HEK293) cells

Virus Production in AD-293 Cells

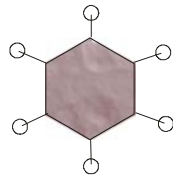


FIGURE 1 Production of recombinant adenovirus

Vector Features

pAdEasy-1 and the set of shuttle vectors contain different resistance cassettes: ampicillin and kanamycin, respectively. All plasmids have the pBR322 origin of replication. The circular map of the pAdEasy-1 vector is shown in Figure 2, and circular maps of the shuttle vectors that may be used in the AdEasy adenoviral vector system are shown in Figures 3–7.

pAdEasy-1

The plasmid pAdEasy-1, containing most of the human adenovirus serotype 5 (Ad5) genome, is deleted for the genes E1 and E3. The removal of these two viral genes creates space for foreign DNA and eliminates self-replication capabilities. The E1 deletion renders the viruses defective for replication and incapable of producing infectious viral particles in target cells (provided there is no complementation by the host cell); the E3 region encodes proteins involved in evading host immunity and is dispensable. The deletion of both genes creates room for up to 7.5 kb of foreign DNA that can be inserted into the Ad5 genome. The E1 gene, which is necessary for production of viral particles, is provided in *trans* by AD-293 cells. pAdEasy-1 carries the ampicillin resistance gene, which is lost after recombination with a shuttle vector.

pShuttle and pShuttle-CMV

The vector pShuttle-CMV contains a multiple cloning site sandwiched between the CMV promoter and the SV40 polyadenylation signal and is suitable for insertion of a large cDNA (up to 6.6 kb). pShuttle contains only a multiple cloning site. This allows for the insertion of an entire expression cassette, including specialized promoters and termination signals (up to 7.5 kb). The regions indicated as arms are the stretches of sequence homology with pAdEasy-1 where the homologous recombination occurs. The R-ITR and L-ITR regions are short inverted terminal repeats (Left and Right) which have a role in replication of the viral DNA.²

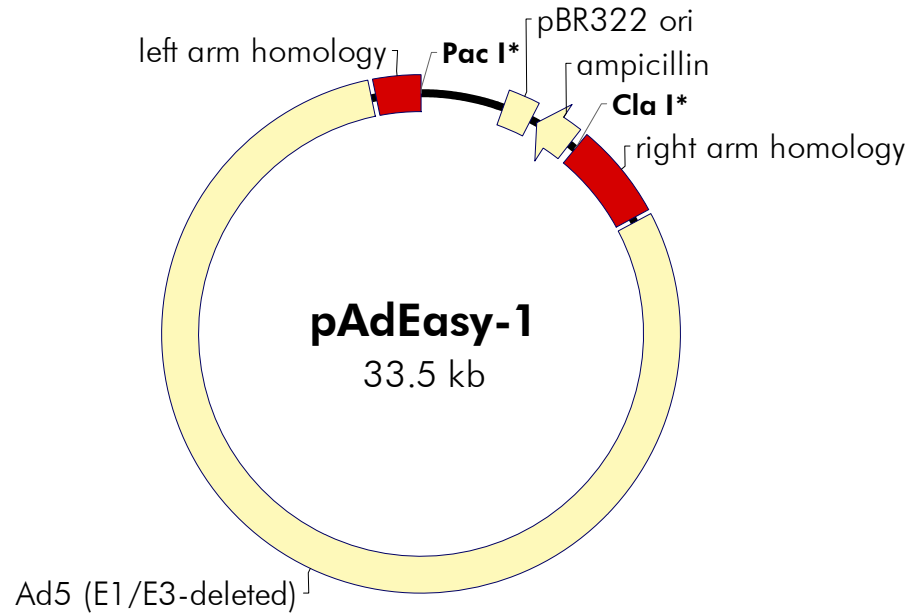
pShuttle-IRES-hrGFP-1 and pShuttle-IRES-hrGFP-2

Two additional shuttle vectors, pShuttle-IRES-hrGFP-1 (Catalog #240081) and pShuttle-IRES-hrGFP-2 (Catalog #240082) are available separately. Both of these vectors contain the CMV promoter and a dicistronic expression cassette in which the multiple cloning site (MCS) is followed by the EMCV-IRES, which directs translation of a humanized recombinant green fluorescent protein (hrGFP) from a novel marine organism as a second open reading frame. This design allows the expression of the gene of interest (up to 5.2 kb) to be monitored at the single-cell level due to expression of the hrGFP on the same transcript. The gene of interest may be fused to three contiguous copies of either the FLAG[®] epitope (pShuttle-IRES-hrGFP-1) or the HA epitope (pShuttle-IRES-hrGFP-2).

pShuttle-CMV-lacZ

The *lacZ* gene was inserted in the MCS site of the pShuttle-CMV to produce pShuttle-CMV-*lacZ*. This construct is provided as a control for the production of recombinant adenovirus.

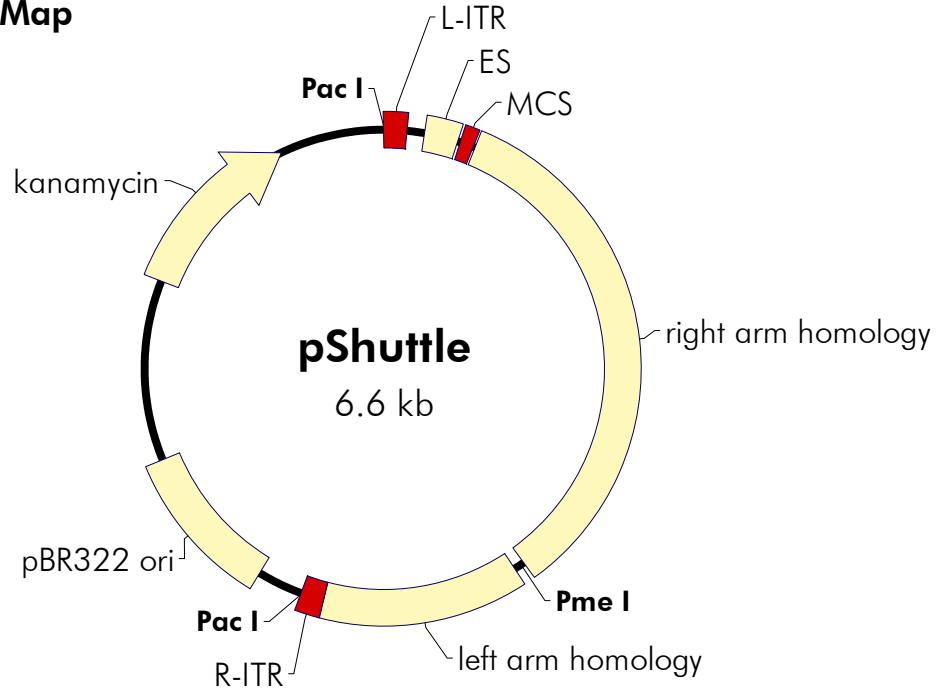
pAdEasy-1 Vector Map



Feature	Nucleotide Position
pBR322 origin of replication	1854–2521
ampicillin resistance (<i>bla</i>) ORF	2669–3529
Ad5 right arm homology	3716–5721
Ad5 left arm homology	32468–33455

FIGURE 2 The pAdEasy-1 vector

pShuttle Vector Map



pShuttle Multiple Cloning Site Region (sequence shown 299–459)

Forward primer binding site

GAAGTGAAATCTGAATAATTTTGTGTTACTCATAGCGGTAATACT...

Kpn I
Not I
Xho I
Xba I
EcoR V
Hind III
Sal I
Bgl II
 ...GGTACCGCGGCCCGCCTCGAGTCTAGAGATATCGAATTC AAGCTTGTGACTCGAAGATCT...

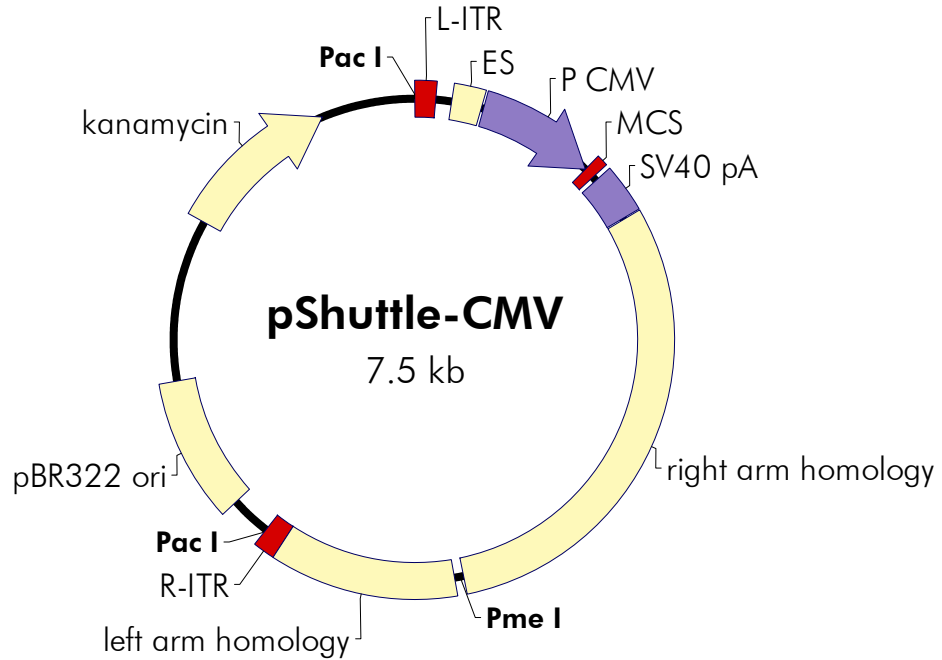
Reverse primer binding site

...GGGCGTGGTTAAGGGTGGGAAAGAATATATAAGGTGGGGTCTTATGTAGTTTTG

Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
forward primer binding site	299–323
multiple cloning site	345–404
Ad5 right arm homology	414–2652
reverse primer binding site	438–459
Ad5 left arm homology	2701–3580
right Ad5 inverted terminal repeat (ITR)	3581–3683
pBR322 origin of replication	3887–4554
kanamycin resistance ORF	5363–6154

FIGURE 3 The pShuttle vector

pShuttle-CMV Vector Map



pShuttle-CMV Multiple Cloning Site Region (sequence shown 888–1031)

Forward primer binding site

GGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAG...

...AGATCTGGTACCGTCGACGCGCCGCTCGAGCCTAAGCTTCTAGATAAGATATC...

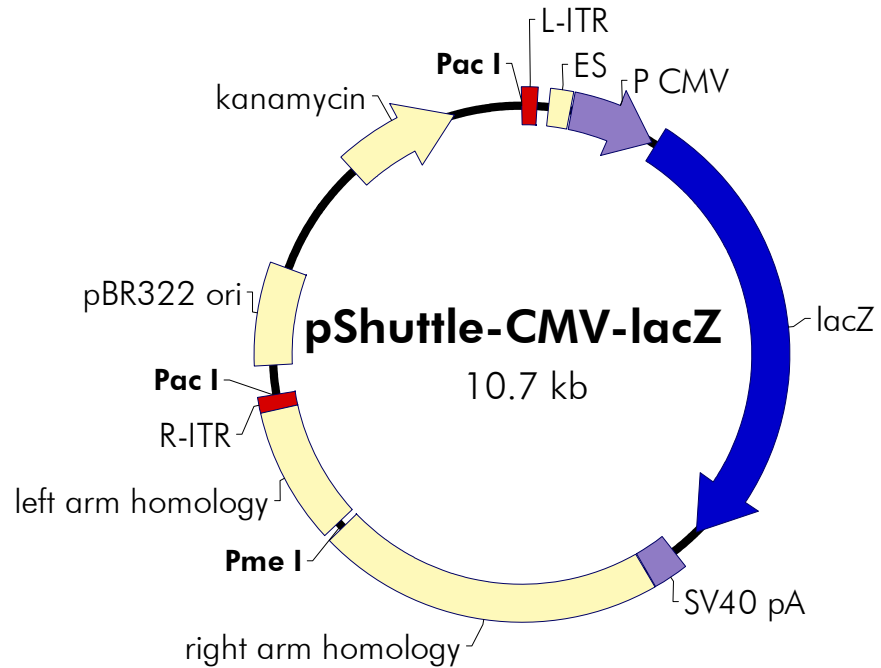
Reverse primer binding site

...CGATCCACGGATCTAGATAACTGATCATAATCAGCCATACCAC

Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
CMV promoter	341–933
forward primer binding site	888–907
multiple cloning site	940–987
reverse primer binding site	1009–1031
SV40 polyA signal	1011–1238
Ad5 right arm homology	1243–3497
Ad5 left arm homology	3545–4428
right Ad5 inverted terminal repeat (ITR)	4429–4531
pBR322 origin of replication	4735–5402
kanamycin resistance ORF	6211–7002

FIGURE 4 The pShuttle-CMV vector

pShuttle-CMV-lacZ Vector Map



Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1-103
encapsidation signal (ES)	183-331
CMV promoter	345-932
β -galactosidase (<i>lacZ</i>) ORF	967-4014
SV40 polyA signal	4217-4444
Ad5 right arm homology	4453-6692
Ad5 left arm homology	6740-7619
right Ad5 inverted terminal repeat (ITR)	7620-7722
pBR322 origin of replication	7926-8593
kanamycin resistance ORF	9402-10193

FIGURE 7 The pShuttle-CMV-*lacZ* vector

Recommended Primer Sequences

Recommended sequences of primers flanking the MCS suitable for PCR amplification and/or sequencing applications are given in Table I.

TABLE I

Vector	Direction	Sequence	Position in Vector
pShuttle	Forward	5' GAAGTCAAATCTGAATAATTTTGTG 3'	299-323
	Reverse	5' CAAACTACATAAGACCCCCAC 3'	438-459
pShuttle-CMV	Forward	5' GGTCTATATAAGCAGAGCTG 3'	888-907
	Reverse	5' GTGGTATGGCTGATTATGATCAG 3'	1009-1031
pShuttle-IRES-hrGFP-1	Forward	5' CTCACGGGGATTTCCAAGTC 3'	749-768
	Reverse	5' ATGCAGTCGTCGAGGAATTG 3'	1075-1094
pShuttle-IRES-hrGFP-2	Forward	5' CTCACGGGGATTTCCAAGTC 3'	749-768
	Reverse	5' ATGCAGTCGTCGAGGAATTG 3'	1084-1103

FEATURES OF BACTERIAL STRAINS

In order to produce and amplify recombinant adenovirus with the AdEasy adenoviral system, two different prokaryotic host strains are required. The first, BJ5183, is *recA* proficient and supplies the machinery necessary to execute the recombination event between the shuttle vector and the pAdEasy vector.

The second strain, provided as XL10-Gold ultracompetent cells, is used to amplify the recombinant adenovirus plasmid. This strain is both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The *endA1* mutation greatly improves the quality of plasmid miniprep DNA, and the *recA* mutation helps ensure insert stability.

In the following table, the genes indicated in italics signify that the bacterium carries a mutant allele. The genes present on the F' episome represent the wild-type bacterial alleles.

Host strain	References	Genotype
BJ5183	4	<i>endA1 sbcBC recBC galK met thi-1 bioT hsdR (Str^r)</i>
XL10-Gold ultracompetent cells	5, 6	Tet ^r Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lac^qZΔM15 Tn10 (Tet^r) Amy Cam^R]</i>

GENERATING ADEASY RECOMBINANTS

Cloning Considerations

Refer to Figures 3–6 for circular maps and corresponding MCS sequences for the AdEasy shuttle vectors and to www.genomics.agilent.com for the complete nucleotide sequence and restriction maps for the pShuttle and pShuttle-CMV vectors. Each vector sequence for vectors in the AdEasy system has been verified for accuracy at the cloning junctions. The remainder of the sequence of each of the AdEasy vectors has been compiled from existing data.

Absence of *Pme* I and *Pac* I Sites in the Insert DNA

The shuttle vector must be linearized using *Pme* I before cotransformation of BJ5183 bacteria and the recombinant Ad plasmid must be digested with *Pac* I before transfection of the packaging cell line. Ensure that the gene of interest does not contain either of these restriction sites. If these sites exist in the gene of interest, it will be necessary to perform site-directed mutagenesis before proceeding to the cloning steps.

Inclusion of Transcriptional and Translational Control Sequences in the Insert DNA

The choice of shuttle vector (pShuttle, pShuttle-CMV, pShuttle-IRES-hrGFP-1 or pShuttle-IRES-hrGFP-2) depends on the user's desired application. The pShuttle vector allows insertion of an entire expression cassette, such that gene expression is controlled by insert-provided transcriptional promoter and terminator sequences as well as translation initiation and stop codons. If transcription via the CMV promoter is desired, choose from among the pShuttle-CMV, pShuttle-IRES-hrGFP-1 or pShuttle-IRES-hrGFP-2 vectors. If the gene of interest is cloned into one of these three CMV-containing vectors, the insert must include an initiation codon. We recommend the use of the Kozak initiation sequence. A complete Kozak sequence includes CCACCATGG, although CCATGG, or the core ATG, is sufficient. If the pShuttle-CMV vector is used, the insert must also contain a stop codon. Conversely, the pShuttle-IRES-hrGFP-1 and pShuttle-IRES-hrGFP-2 vectors already contain in-frame stop codons at the C-terminus of the fusion tags. Proteins may also be expressed from either of the pShuttle-IRES-hrGFP-1 or pShuttle-IRES-hrGFP-2 vectors in the absence of a fusion tag if an in-frame stop codon is included in the insert DNA that is cloned into the MCS upstream of the tag.

Cloning Capacity of the Shuttle Vectors

It is important to adhere to the upper size limit of insert DNA (cloning capacity) for any of the shuttle vectors. Inserting larger fragments results in considerable decreases in efficiency of the AdEasy system. See the table below for shuttle vector cloning capacities and for an outline of the general features for the AdEasy shuttle vectors.

Shuttle Vector Features

Vector	Cloning capacity	Promoter	Poly A	MCS restriction sites	Description
pShuttle	7.5 kb	—	—	<i>Kpn I, Not I, Xho I, Xba I, EcoR V, Hind III, Sal I, Bgl II</i>	Ligate an entire expression cassette into MCS
pShuttle-CMV	6.6 kb	CMV	+	<i>Kpn I Sal I, Not I, Xho I, Hind III, EcoR V</i>	Ligate gene of interest into MCS between the CMV promoter and poly A
pShuttle-IRES-hrGFP-1 or -2	5.2 kb	CMV	+	<i>Bgl II*, Not I*, Sca I*, Nhe I* Spe I*, EcoR V, Pvu I, Sal I, Srf I, Xho I</i>	Ligate gene of interest into MCS, in-frame with the FLAG or HA tag. Dicistronic transcript encoding hrGFP allows monitoring of the expression of the gene of interest by GFP fluorescence

*These restriction sites are upstream of in-frame stop codons. Do not use these sites for cloning unless the cloning strategy removes the stop codons by double digestion using one of the upstream sites plus a site downstream of the stop codons.

Cloning the Gene of Interest

1. Clone the gene of interest into the shuttle vector of choice using appropriate site(s) in the MCS.
2. Confirm the presence of the insert by restriction digestion or sequence analysis.
3. Purify DNA (shuttle vector plus gene of interest) in sufficient quantity for the subsequent cotransformation steps (2 µg of linearized shuttle vector DNA is required for each cotransformation reaction; 1 µg for each test and control transformation reactions).
4. Linearize shuttle plasmid DNA using *Pme* I and confirm complete digestion by agarose gel electrophoresis. It is necessary to prepare linearized samples of both the test plasmid DNA (shuttle vector plus gene of interest) and the control shuttle vector, pShuttle-CMV-*lacZ*.
5. Once complete digestion with *Pme* I is confirmed, remove the enzyme and buffer by method of choice. The Agilent StrataPrep PCR Purification Kit is suitable for this purpose.
6. Treat the purified DNA with alkaline phosphatase for 30 minutes at 37°C.
7. Separate the linearized, dephosphorylated shuttle vector using agarose gel electrophoresis. Put samples of uncut shuttle vector in adjacent lanes for comparison. Ensure that the gel is run long enough to visualize good separation between uncut and cut DNA.
8. Gel purify the linearized shuttle vector by method of choice.

Note *Ensure that the method chosen to purify the shuttle vector results in a DNA sample that does not contain salts, which will severely damage cells during electroporation.*
9. Resuspend the purified DNAs in sterile dH₂O to a final concentration of 1 µg/µl.

Transformation Guidelines for BJ5183 Cells

Storage Conditions

Electroporation competent cells are sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Electroporation competent cells should be placed at –80°C directly from the dry ice shipping container. When aliquoting, keep electroporation competent cells on ice at all times.

Cotransforming the BJ5183 Cells to Produce Recombinant Ad Plasmid

Note *In this portion of the protocol, the BJ5183 cells are cotransformed with the linearized shuttle vector (containing the gene of interest or containing the control gene, lacZ) and the pAdEasy-1 vector. A recombination event that takes place in the bacterial cells results in the production of recombinant AdEasy plasmid DNA. It is very important that this cotransformation takes place in BJ5183 cells (supplied in this kit) and not in the XL10-Gold cells (also supplied in this kit). Only the BJ5183 cells have the cellular components necessary to carry out recombination.*

1. Prechill four DNase-free microcentrifuge tubes and four electroporation cuvettes (0.2 cm gap) on ice.
2. Remove two aliquots of BJ5183 electroporation competent cells from -80°C storage and thaw on ice.
3. Gently pipet 40 μl of the competent cells into each of the chilled microcentrifuge tubes.
4. Into one of the tubes, pipet 1 μl (1 μg) of linearized, dephosphorylated shuttle vector and 1 μl of pAdEasy-1 supercoiled vector (100 ng/ μl). Mix by tapping the tube gently and keep on ice.
5. Into the second tube, pipet 1 μl (1 μg) of linearized, dephosphorylated pShuttle-CMV-*lacZ* vector and 1 μl of pAdEasy-1 supercoiled vector (100 ng/ μl). Mix by tapping the tube gently and keep on ice.
6. Into the third tube, pipet (1 μl) 1 μg of linearized, dephosphorylated shuttle vector. Mix by tapping the tube gently and keep on ice. This controls for background contributed by the shuttle vector.
7. Into the fourth tube, pipet, 1 μl of pUC18 DNA control plasmid (diluted 1:10 in sterile dH_2O).
8. Set the electroporator to the following settings by referring to the instructions provided with the instrument: 200 Ω , 2.5 kV, 25 μF .
9. Transfer the contents of one microcentrifuge tube from step 4 into one of the chilled electroporation cuvettes and tap the cuvette gently to settle the mixture to the bottom.
10. Slide the cuvette into the electroporation chamber until the cuvette connects with the electrical contacts.
11. Pulse the sample once, then quickly remove the cuvette. **Immediately** add 1 ml of sterile LB broth (see *Preparation of Media and Reagents*) and pipet up and down to resuspend the cells.

12. Transfer the cell suspension to a sterile 14-ml BD Falcon polypropylene round-bottom tube.
13. Repeat the electroporation for the other three transformation reactions. Incubate all of the transformations at 37°C for 1 hour while shaking at 225–250 rpm.
14. For the three recombination reactions and associated controls, plate the entire volume of recovered cells onto 3–5 LB-kanamycin plates[§] (e.g. four plates containing 50 µl, 100 µl, 250 µl, and 600 µl respectively).
15. For the pUC18 transformation, first place a 100-µl pool of LB broth on an LB–ampicillin agar plate.[§] Add 5 µl of the transformed cells to the pool of LB broth. Use a sterile spreader to spread the mixture. The pUC18 control transformation should yield an efficiency of > 10⁸ cfu/µg.
16. Incubate the plates overnight at 37°C.

Testing Colonies for Recombinant Ad Plasmid

1. Examine the transformation plates. The plates containing the transformation of linearized shuttle vector alone have uniform-sized colonies arising from uncut/recircularized vector. When compared to the control shuttle vector plates, the transformants on the plates containing the pAdEasy-1 recombinants will appear as two populations: normal size and tiny at an approximate ratio of 3:1. The tiny colonies are the potential recombinants and the normal-sized colonies are background from the shuttle vector.
2. Examine the pUC18 transformation to determine transformation efficiency, if desired.
3. Pick 10 or more of the smallest, well isolated colonies each from the test recombination plate (shuttle vector plus gene of interest) and the control recombination plate (pShuttle-CMV-lacZ) into 3–5 ml cultures of LB-kanamycin broth.[§]

Note *The recombinants will be low copy number plasmids approximately 40 kb in size. As implied by the small colony size on the test plates, cultures will grow slowly and plasmid yields will be low. Miniprep procedures should be adapted accordingly. Procedures suitable for purification of cosmids or large plasmids are recommended.*

4. Incubate at 37°C overnight while shaking at 225–250 rpm.

[§] See *Preparation of Media and Reagents*.

5. Prepare miniprep DNA from 2 ml (or more) of overnight culture using a procedure suitable to purify large plasmids or cosmids. The final volume of miniprep DNA should be 50 μ l and the DNA should be resuspended in sterile dH₂O or TE buffer.
6. Cut 10 μ l of the miniprep DNA with Pac I and run the entire digest on a 0.8% agarose TAE^s gel next to 10 μ l of uncut miniprep DNA. As a control, also cut a small amount (~0.2 μ g) unrecombined shuttle vector (prepared in step 3 of *Cloning the Gene of Interest*) and run in an adjacent lane.

Interpretation of Results

Restriction of recombinant Ad plasmid DNA with *Pac* I should yield a large fragment of ~30 kb*, and a smaller fragment of either 3.0 kb (if recombination took place between the left arms) or 4.5 kb (if recombination took place at the origins of replication). Uncut recombinants will give a large smear at the top of the gel very close to the wells (and often have a smaller band that runs just below 23 kb). Potential recombinants may be difficult to identify (in some instances you can only visualize the 30 kb band) if the quality or yield of the miniprep is low. If this is the case, prepare DNA from a greater volume of culture with a procedure adapted for purification of large plasmids or cosmids. More than 20% of the colonies should contain a recombinant plasmid close to 40 kb. There are often faint background bands in BJ5183 minipreps. If the PREDOMINANT bands are the expected sizes, redigest potential recombinants prepared from XL10-Gold cells. If after that second preparation there are still bands that are unaccounted for, discard the clone. For this reason it is recommended that more than one potential recombinant be amplified in XL10-Gold cells.

Note *Remember to reserve a small amount of each recombinant Ad plasmid DNA sample for transforming in the subsequent protocol.*

* Due to limitations in the resolution of large DNA fragments on 0.8% agarose gels, the 30 kb band can be observed to migrate next to the 23 kb marker of the λ Hind III DNA size ladder.

AMPLIFYING RECOMBINANT AD PLASMID

In this part of the protocol, individual positive recombinant Ad plasmids, identified by restriction digest, are used to transform XL10-Gold ultracompetent cells so that the recombinant adenovirus plasmid DNA can be amplified. It is possible that some clones identified as positive by restriction digest will produce low titers of packaged virus or will express the gene of interest poorly. **It is recommended that more than one positive clone be used to transform XL10-Gold cells so that a few individuals can be tested in human cell transfection.**

Transformation Guidelines for XL10-Gold Cells

Storage Conditions

Ultracompetent cells are sensitive to even small variations in temperature and must be stored at the bottom of a -80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Ultracompetent cells should be placed at -80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep ultracompetent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 100 μl of ultracompetent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon Polypropylene Round-Bottom Tubes

It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol, since other tubes may be degraded by the β -mercaptoethanol used in step 3 of the *Transformation Protocol*. In addition, the incubation period during the heat-pulse step is critical and has been optimized specifically for the thickness and shape of 14-ml BD Falcon polypropylene tubes.

Use of β -Mercaptoethanol

β -Mercaptoethanol (β -ME) has been shown to increase transformation efficiency. The XL10-Gold β -mercaptoethanol mix provided in this kit is diluted and ready to use. For optimum efficiency, use 4 μl of the β -ME mix. (We cannot guarantee highest efficiencies with β -ME from other sources.)

Length and Temperature of the Heat Pulse

There is a defined window of highest efficiency resulting from the heat pulse during transformation. Optimal efficiencies are observed when cells are heat-pulsed for 30 seconds. Do not exceed 42°C .

XL10-Gold Transformation Protocol

Note Each 100 μ l aliquot of XL10-Gold ultracompetent cells is sufficient for one transformation. Thaw as many aliquots as required to transform the test recombinant adenovirus plasmid(s) (shuttle vector plus gene of interest recombined with pAdEasy-1) and the control recombinant adenovirus plasmid (pShuttle-CMV-lacZ recombined with pAdEasy-1). It is recommended that more than one test recombinant adenovirus plasmid be transformed in this step so that a few individuals can be tested in human cell transfection experiments.

1. Prepare a 42°C water bath
2. Prechill 14-ml BD Falcon polypropylene round-bottom tubes.
3. Thaw the XL10-Gold ultracompetent cells on ice.
4. Gently mix the cells by hand. Aliquot 100 μ l of the cells into prechilled 15-ml tubes.
5. Add 4 μ l of the β -ME mix provided with the kit to the 100 μ l of cells. (We cannot guarantee highest efficiencies with β -ME from other sources.)
6. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
7. For each plasmid to be transformed, add 0.1–50 ng of DNA to one of the aliquots of cells and swirl gently. To a separate tube of cells add 1 μ l of the pUC18 control plasmid (diluted 1:10 in sterile dH₂O) and swirl gently.
8. Incubate the tubes on ice for 30 minutes.
9. Prewarm NZY⁺ broth (see *Preparation of Media and Reagents*) in a 42°C water bath for use in step 12.

Note Transformation of XL10-Gold ultracompetent cells has been optimized with NZY⁺ broth.

10. Heat-pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is *critical* for obtaining the highest efficiencies. Do not exceed 42°C.
11. Incubate the tubes on ice for 2 minutes.
12. Add 0.9 ml of prewarmed (42°C) NZY⁺ broth to each tube and incubate the tubes at 37°C for 1 hour with shaking at 225-250 rpm.

13. Plate 5 μ l, 25 μ l, and 100 μ l of each transformation reaction on LB-kanamycin agar plates using a sterile spreader.

To plate the cells transformed with the pUC18 control plasmid, first place a 195- μ l pool of NZY⁺ broth on an LB-ampicillin agar plate. Add 5 μ l of the control transformation reaction to the pool of NZY⁺ broth. Use a sterile spreader to spread the mixture. This pUC18 control transformation should yield an efficiency of $> 10^9$ cfu/ μ g.

14. Incubate the plates overnight at 37°C.
15. The next afternoon, pick one colony from each transformation and transfer into 10 ml LB-kanamycin broth and grow overnight at 37°C while shaking at 225-250 rpm.
16. The next morning transfer 5 ml of the overnight culture into a clean tube and store at 4°C.
17. **(OPTIONAL)** Prepare miniprep DNA from some or all of the remaining 5 ml using any standard miniprep procedure. Digest 5 μ l of the miniprep DNA with *Pac* I and analyze by agarose gel electrophoresis (using a 0.8% agarose TAE gel). Confirm the desired restriction pattern (one band ~30 kb and a second band of either 3.0 kb or 4.5 kb) prior to inoculating a 500-ml flask in step 18.
18. Use the 5 ml of overnight culture stored at 4°C to inoculate a flask containing 500 ml LB-kanamycin broth. Grow the culture overnight at 37° while shaking at 225–250 rpm.
19. The following morning, prepare maxiprep DNA from the liquid culture. This DNA will be used to transfect human cells and must be of suitable quality and purity (e.g. prepared using standard cesium chloride density gradient centrifugation or affinity column purification that produces DNA of equivalent quality).
20. Digest a sufficient amount of each purified recombinant adenovirus plasmid with *Pac* I. (5 μ g of DNA is needed for each transfection.)
21. Run 0.2 μ g of each cut DNA on a 0.8% agarose TAE gel and confirm the desired restriction pattern (one band ~30 kb and a second band of either 3.0 kb or 4.5 kb).
22. Remove buffer and enzyme from the remainder of the restriction reactions by phenol extraction/ethanol precipitation or using a similar DNA purification kit, such as the Agilent StrataPrep PCR Purification Kit.
23. Under sterile conditions, resuspend the DNA in 50 μ l of sterile 0.1 \times TE buffer or dH₂O. Store the resuspended DNA at –20°C.

PREPARATION OF PRIMARY ADENOVIRUS STOCK WITH RECOMBINANT AD PLASMID

Safety Considerations

Note *The safety guidelines presented in this section are not intended to replace the BSL 2+ safety procedures already in place at your facility. The information set forth below is intended as an additional resource and to supplement existing protocols in your laboratory.*

Prior to use of the AdEasy vectors, the user should become thoroughly familiar with the safety considerations concerning the production and handling of adenovirus. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site <http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm>. Production of adenovirus and use of adenoviral vectors fall within NIH Biosafety Level 2 criteria. For more information regarding BSL-2+ practices, consult the UCSD Environmental Health and Safety Web site <http://www-ehs.ucsd.edu/ADENO.HTM>.

Note *The steps performed in this section, Preparation of Primary Adenovirus Stock with Recombinant Ad Plasmid, need to be carried out under sterile conditions in a laminar flow hood which is designated for use with virus. For handling adenovirus-containing solutions, use disposable pipets or pipettors with filter tips to prevent the transfer of contaminated aerosols.*

Description of AD-293 Cells

Prepare and titer AdEasy recombinant virus stocks using the AD-293 cell line [provided with the AdEasy XL adenoviral vector system (Catalog #240010) and available separately (Catalog #240085)]. AD-293 cells are derived from the commonly used HEK293 cell line, but have improved cell adherence and plaque formation properties. HEK293 cells are human embryonic kidney cells transformed by sheared adenovirus type 5 DNA.⁷ AD-293 cells, like HEK293 cells, produce the adenovirus E1 gene in *trans*, allowing the production of infectious virus particles when cells are transfected with E1-deleted adenovirus vectors such as the pAdEasy-1 vector. Standard HEK293 cells do not adhere well to tissue culture dishes, hindering adherent cell culture and plaque assay procedures. AD-293 cells demonstrate improved adherence to tissue culture dishes, making AD-293 cell monolayers less susceptible to disruption.

Note *Despite the improved adherence of AD-293 cells, it is important to minimize monolayer disruption during passaging and plaque assays by gently pipeting liquids down the side of the culture dish instead of pipetting directly onto the cells.*

Preparing AD-293 Cells for Transfection

Plate AD-293 cells at $7-8 \times 10^5$ cells per 60-mm tissue culture dish in growth medium[§] 24 hours prior to the transfection.

Notes *To achieve optimal titers, it is important that the AD-293 cells are healthy and plated at optimal density. Cells should be passaged at $\leq 50\%$ confluence, and ideally passaged no more than 30 times. It is thus prudent to initially prepare a large number of frozen vials of the cells while they are at a low passage and healthy. Care should be taken to avoid clumping of the cells during passaging and plating for transfection.*

HEK293 cells may also be used for these procedures, but these cells display decreased adherence compared to AD-293 cells and additional care must be taken during the following manipulations.

Transfecting AD-293 Cells

A variety of transfection protocols may be successfully used with these vectors and the AD-293 cell line, including the protocol below. This protocol is a modification of the Agilent ViraPack Transfection Kit protocol. As a general guideline, the following protocol is expected to produce a viral titer of approximately 10^7 plaque forming units (pfu)/ml when transducing AD-293 cells. Titers of $10^{11}-10^{13}$ can be achieved by concentrating the virus by CsCl gradient banding.[§]

Note *The procedure in this section, Transfecting AD-293 Cells, will take a minimum of 10 hours to complete.*

Adding the MBS-Containing Medium to the Cells

1. Inspect the host cells that were split the day before; they should be approximately 70% confluent.
2. Prepare the MBS-containing medium.[§] This must be done immediately prior to the transfection. For each 60-mm tissue culture plate, 4 ml of MBS-containing medium must be prepared.
3. Aspirate growth medium, wash the cells twice with phosphate-buffered saline (PBS), then replace with 4 ml of MBS-containing medium in each 60-mm plate. Return the plates to the 37°C incubator. This must be done 20–30 minutes before the addition of the DNA suspension.

[§] See *Preparation of Media and Reagents*.

Adding the DNA Suspension to the Cells

Note *Begin the preparation of the transfection DNA mixtures, as described in this section, approximately 10 minutes prior to the end of the 20–30 minute incubation from the previous section.*

1. Remove the resuspended, *Pac* I digested recombinant Ad plasmid DNA samples from storage at -20°C and transfer them to the laminar flow hood.
2. For each transfection, pipet 5 μg of *Pac* I digested, recombinant Ad plasmid DNA in a 5-ml BD Falcon polystyrene tube containing sterile dH_2O such that the final volume of dH_2O plus DNA is 225 μl .

Note *The volumes above are for a single transfection. If duplicates are desired, the volumes of DNA and dH_2O may be scaled up proportionally.*

3. Add 25 μl Solution I and 250 μl Solution II from the ViraPack Transfection Kit to the tubes containing the DNA. **Immediately** following the addition of Solutions I and II, gently mix the contents of the tube by tapping the tube.
4. Incubate the DNA mixture at room temperature for 10 minutes.
5. Remove the plates containing AD-293 cells in MBS-containing medium from the incubator. Gently mix the DNA suspension by pipetting up and down to resuspended any DNA precipitate, then add the DNA suspensions to the plates in a dropwise fashion. Swirl the plate gently while adding the DNA suspension to prevent lifting of cells from the plate and to distribute the DNA suspension evenly.

Note *From this point on, it should be assumed that adenovirus is present in plates containing the transfected cells. Gloves and disposable lab coats should be worn while working with the virus. When pipetting solutions and transferring plates to and from the laminar flow hood, contamination with aerosols should be avoided. In case of spills, follow the procedures recommended at your facility. Additionally, consult the Web sites described in Safety Considerations.*

6. Return the tissue culture plates to the 37°C incubator.
7. After incubating for 3 hours, remove the medium from the plates and replace it with 4 ml of growth medium supplemented with 25 μM chloroquine (see *Preparation of Media and Reagents*). Return the plates to the 37°C incubator.

8. After incubating for an additional 6–7 hours, remove the growth medium containing 25 μ M chloroquine and replace with 4 ml growth medium—no chloroquine.
9. Incubate the culture plates at 37°C for 7–10 days, replenishing the growth medium when needed (based on media color). If the cells appear to be well attached to the plate, replace the medium with 4 ml of fresh medium, taking care not to dislodge the cells. Alternatively, if detached cells are observed in the growth medium, add an equal volume of fresh medium to the existing medium. These cells will be used to prepare the primary viral stocks. If using one of the hrGFP-containing vectors, the progress of the transfection may be monitored by fluorescence microscopy (see *Detection of hrGFP*).

Preparing the Primary Viral Stocks

1. Prepare a small dry ice-methanol bath and a small 37°C water bath and place them in the laminar flow hood.
2. Carefully remove growth medium from adenovirus-producing AD-293 plates and wash the cells once with PBS. Take care not to lose any clusters of floating and partially attached cells during this process.

Note *If the cells are already mostly detached, pipet up and down gently in the growth medium until cells become completely resuspended. Transfer cell suspension to a screw cap centrifuge tube and pellet the cells by low speed centrifugation. Aspirate medium, and wash the cells once with 0.5 ml of sterile PBS. Resuspend the cell pellet in a fresh 0.5 ml sterile PBS (per 60-mM dish) and proceed to Step 5.*

3. Add 0.5 ml of PBS to each plate of cells to be harvested. Collect the cells by holding the plate at an angle and scraping the cells into the pool of PBS with a cell lifter.
4. Transfer the cell suspension to a 1.7-ml screw-cap microcentrifuge tube. If duplicate DNA samples were transfected, the cells from duplicate samples may be combined in the microcentrifuge tube at this stage.
5. Subject the cell suspension to four rounds of freeze/thaw by alternating the tubes between the dry ice-methanol bath and the 37°C water bath, vortexing briefly after each thaw.

Note *Each freeze and each thaw will require approximately 5 minutes' incubation time.*

6. Collect cellular debris by microcentrifugation at 12,000 \times g for 10 minutes at room temperature.
7. Transfer the supernatant (primary virus stock) to a fresh screw-cap microcentrifuge tube. Viral stocks can be stored for more than one year at -80°C .

GUIDELINES FOR INFECTION CONDITIONS AND AMPLIFICATION OF THE PRIMARY VIRAL STOCK

Titer Considerations

Primary viral stocks produced with the above protocol are generally expected to be in the 10^7 – 10^8 pfu/ml range. However, there is significant variation in titer achieved based on differences in constructs and recombinant clones, as well as user methodology and fluctuations in transfection efficiency. The titer of the virus stock can be determined by plaque assay (see *Appendix*).

In practice, it generally saves time to proceed with the virus stock amplification, monitor the infection visually (see *Monitoring the Infection*), and then titer the amplified virus stock for use in subsequent applications.

Amplification Guidelines

Amplification of a virus stock is achieved by infection of AD-293 cultures with a low passage virus stock. One round of amplification generally produces a 10-fold increase in titer.

Minimizing the Production of Replication-Competent Adenovirus (RCA)

Since AD-293 cells possess integrated human Ad5 DNA, there is a low frequency of homologous recombination between the E1-deleted vector and the host DNA resulting in the production of some replication competent adenovirus (RCA). The frequency of occurrence is very low, but the percentage of RCA in a given virus stock goes up with each amplification of that stock. **The primary viral stock contains the lowest numbers of RCA, and it is recommended that all amplifications be initiated with virus stock at the lowest possible passage number.**

Infection Procedure Guidelines

Infection of AD-293 cells may be achieved simply by adding a solution of viral particles to adherent cells in tissue culture dishes. To amplify a virus stock, prepare cultures of AD-293 cells that are 50–70% confluent (see *Optimizing Infection Conditions*). Dilute the primary virus stock into a minimal volume of growth medium (just enough to cover cells) and add the virus suspension to the cell culture dishes. Incubate the infection reactions for 2 hours, preferably on a rocking platform to disperse the solution evenly. After two hours, supply additional growth medium to the culture. After the desired number of days of incubation,* harvest the cells in a minimal volume of PBS. Prepare the amplified virus stock by 4 rounds of freeze/thaw as described in *Preparing the Primary Viral Stock*.

* The number of days infection/amplification is allowed to proceed will depend on the confluency of the cells at the time of infection and on the initial ratio of virus particles to cells (multiplicity of infection). See *Monitoring the Infection* for additional information.

Optimizing Infection Conditions

The multiplicity of infection (MOI) is the number of virus per cell used to infect a culture. At high MOIs (10–20), cells should be plated at high densities (near confluence) as once the virus takes over the cell machinery, the cell will cease to divide. The opposite is optimal for low MOI infections; cells should be infected near 50% confluence as only a fraction of the cells will become infected initially and the uninfected cells can continue to grow until they become infected.

Confluence (%)	Cells needed for a 60-mm plate	Cells needed for a 100-mm plate
50%	1.5×10^6	3.5×10^6
75%	2.5×10^6	5.5×10^6
100%	3.5×10^6	7.5×10^6

Monitoring the Infection

To monitor the progress of an adenoviral infection, it is necessary to observe phenotypic changes to infected cells. The cells will show evidence of a cytopathic effect (CPE): cells will round up and detach from the plate, and the nucleus will occupy a major part of the cell due to the high level of virus production. High MOI infections will show complete CPE and can be harvested as soon as three days post-infection, whereas low MOI infections will need to incubate for longer periods until CPE is observed (up to 10 days).

If pShuttle-IRES-hrGFP-1 or pShuttle-IRES-hrGFP-2 was used as the shuttle vector, infection may also be monitored by hrGFP fluorescence in infected cells. The time of appearance of hrGFP fluorescence depends on the MOI of the infection, but hrGFP fluorescence should precede the appearance of CPE in the cellular phenotype. hrGFP fluorescence of cells in culture plates may be observed one day post-infection for high MOI infections, and may require incubation up to five days post-infection for detection for low MOI infections.

DETECTION OF HRGFP

Monitoring Transfection and Infection of Cells

AD-293 (or HEK293) cells transfected with recombinant Ad DNA derived from pShuttle-IRES-hrGFP-1 or pShuttle-IRES-hrGFP-2 should show expression of hrGFP 24–72 hours after transfection. The time of observation of hrGFP fluorescence in cultured cells after infection depends on the multiplicity of infection (MOI). hrGFP fluorescence may be observed as early as one day post-infection for high MOI infections and may become detectable as late as five days post-infection for low MOI infections.

Fluorescing cells growing in tissue culture dishes can be observed using an inverted fluorescence microscope. Fluorescence of populations of harvested cells can also be measured using FACS analysis or fluorometer assays. The table below lists excitation and emission spectra peaks for the Agilent hrGFP as compared to the widely used EGFP.

Specifications for hrGFP and EGFP Excitation and Emission Spectra

GFP Form ^a	Excitation/Emission Spectra Maxima (nm)
hrGFP	500/506
EGFP	488/509 ^b

^a Both forms of GFP compared in this table have been codon-optimized for maximum expression in human cells.

^b The emission spectrum for EGFP also shows a shoulder at 540 nm.

Note *Filter sets compatible with the detection of hrGFP and EGFP are sold by Omega Optical, Inc. (Phone: 802 254 2690, or see www.omegafilters.com):*

Exciter filter: XF1073

Emitter filter: XF3084

Beam splitter: XF2010

Microscope cube set with the exciter filter, emitter filter and beam splitter: XF100-2

LACZ CONTROL: DETECTION AND APPLICATIONS

The control vector pShuttle-CMV-*lacZ* can be used to monitor your success at various points during the AdEasy procedure. To detect the presence of LacZ the cells are stained with X-gal, using the X-gal staining protocol of choice. The β -galactosidase activity is easily detected, making this a useful control for the recombinant Ad plasmid transfection.

Transfection Control

A minimum of two days post-transfection with *Pac I* digested LacZ recombinant Ad plasmid, cells can be stained with X-gal to evaluate the success of the transfection. Keep in mind that if transfected into an E1-complementing cell line such as AD-293, adenovirus will be present and the staining procedure should be performed under BSL-2+ guidelines.

Virus Control

An X-gal stain can be performed on adenovirus infected cells to (1) confirm that the transfection was successful and that infectious virus particles were produced (2) estimate titer of stocks produced from transfection or amplification and (3) test the ability of adenovirus vectors to infect a potential target cell. Three days of incubation post-infection is adequate to detect the presence of LacZ.

APPENDIX: PLAQUE ASSAY USING AGAROSE OVERLAY

The following protocol may be used to determine the titer (pfu/ml) of a viral stock. In addition, although the AdEasy system essentially obviates the need to plaque purify clones for a viral stock, if desired, the following protocol can also be used to isolate a single virus clone.

Preparing Viral Stock Dilutions

1. Plate AD-293 cells at a density of 5×10^5 per well of 6-well tissue culture plates.
2. Incubate overnight at 37°C.
3. Dilute viral stocks in 1-ml volumes over a 10-fold series from 10^{-5} to 10^{-9} in growth medium. Carry dilutions in duplicate.
4. Add 1 ml of each dilution to a separate well of the 6-well plate. Leave one well “medium only” (no virus added) as a control.
5. Incubate at 37°C for 2 hours. Gentle rocking during the incubation is beneficial but not required.
6. Proceed to *Overlaying the Infected Cells with Agarose*.

Overlaying the Infected Cells with Agarose

Note *The agarose overlay should be applied so that it spreads fast enough to cover the plate before solidifying but not so fast that the cells are disrupted. It may be necessary to practice the overlaying technique on uninfected cells prior to performing the plaque assay. Prior to the addition of the agarose overlay, inspect the plates containing the cells to ensure adequate adherence.*

1. Prepare a solution of 5% SeaPlaque agarose in sterile PBS, autoclave, and store in 10-ml aliquots at 4°C in 50-ml sterile conical tubes.
2. Prior to use, melt the agarose by placing the tube in a beaker of boiling water. Do not microwave the tube of agarose.
3. Once melted, cool the agarose to 45°C.
4. Add 30 ml of growth medium previously equilibrated to 37°C and mix. This makes the final agarose concentration 1.25%. Proceed immediately to the next step.
5. Completely remove the growth medium from the wells that will receive the overlay.
6. Gently pipet 3 ml of agarose/growth medium mix very gently along the side of the well and allow it to completely cover the bottom of the well.

7. Incubate the plate at 37°C. Plaques, having the appearance of small white spots, should be visible to the naked eye within 12–21 days. During that time, if the agarose/growth medium overlay becomes yellow, pour additional overlays at a volume of 1.5 ml per addition.
8. To determine titer, count plaques from wells where isolated plaques are clearly visible and countable. Average the counts from duplicate wells and multiply that number by the dilution factor to estimate pfu/ml.

Plaque Isolation

1. Under sterile conditions, core-out well isolated plaques (as agarose plugs) with a sterile Pasteur pipette and transfer to 250 µl of growth medium in a sterile microcentrifuge tube.
2. Elute virus for 24 hours at 37°C.
3. Use this virus stock to perform an additional round of plaque purification if desired, or amplify for later use.

TROUBLESHOOTING

Observation	Suggestion
No colonies following cotransformation of BJ5183 cells with shuttle vector and pAdEasy-1 vectors	Perform a control transformation of the electroporation competent BJ5183 cells using the pUC18 control DNA to ensure that the cells are highly competent ($\geq 10^8$ cfu/ μ g). Be sure to plate pUC transformations on ampicillin plates.
All of the colonies following cotransformation of BJ5183 cells are normal size	Ensure that the <i>Pme</i> I cut shuttle vector (containing the gene of interest) is gel purified and dephosphorylated, and that the DNA is resuspended in sterile dH ₂ O prior to cotransformation.
	This results from poor <i>Pme</i> I digestion or too much shuttle vector used in the cotransformation. Ensure that the shuttle vector is completely digested with <i>Pme</i> I and dephosphorylated, and that no more than 1 μ g is used in the cotransformation.
Restriction digestion to confirm correct recombination produces bands of sizes other than 3.0, 4.5, and 30 kb following agarose gel electrophoresis	Provided the quality and yield of the miniprep DNA is adequate to make an evaluation, plasmids not producing bands of expected sizes following restriction digestion should be discarded. Choose more isolated small colonies from the original plates and test by restriction digestion. There are often faint background bands in BJ5183 minipreps. If the PREDOMINANT bands are the expected sizes, redigest potential recombinants prepared from XL10-Gold cells. If after that second preparation there are still bands that are unaccounted for, discard the clone. For this reason it is recommended that more than one potential recombinant be amplified in XL10-Gold cells.
No colonies following transformation of XL10-Gold ultracompetent cells using recombinant Ad plasmid DNA	Perform a control transformation of the XL10-Gold cells using the pUC18 control DNA to ensure that the cells are competent ($\geq 5 \times 10^9$ cfu/ μ g).
No growth in liquid LB-kanamycin miniprep cultures	LB-kanamycin agar plates weak or bad. Small colonies will grow alone or as satellites if the kanamycin plates are not at full strength. Prepare fresh LB-kanamycin agar plates, and do not add the kanamycin until the molten agar solution is $\leq 42^\circ\text{C}$.
Western analysis does not detect the FLAG- or HA-fusion protein	Insert is cloned out of frame. Sequence to ensure correct reading frame. Reclone if insert is out of reading frame.
	Assay is not sufficiently sensitive or is being performed incorrectly. Use a positive control. Perform western analysis after viral amplification to ensure sufficient fusion protein is present for detection.

PREPARATION OF MEDIA AND REAGENTS

<p>LB Broth (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract Add deionized H₂O to a final volume of 1 liter Adjust to pH 7.0 with 5 N NaOH Autoclave</p>	<p>LB-Kanamycin Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Adjust pH to 7.0 with 5 N NaOH Add deionized H₂O to a final volume of 1 liter Autoclave Cool to 48°C Add 5 ml of 10-mg/ml-filter-sterilized kanamycin Pour into petri dishes (~25 ml/100-mm dish)</p>
<p>LB-Kanamycin Broth (per Liter) Make 1 liter of LB Broth. Autoclave Cool to 48°C Add 5 ml of 10-mg/ml-filter-sterilized kanamycin</p>	<p>LB-Ampicillin Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Adjust pH to 7.0 with 5 N NaOH Add deionized H₂O to a final volume of 1 liter Autoclave Cool to 48°C Add 10 ml of 10-mg/ml-filter-sterilized ampicillin Pour into petri dishes (~25 ml/100-mm dish)</p>
<p>NZY+ Broth (per Liter) 10 g of NZ amine (casein hydrolysate) 5 g of yeast extract 5 g of NaCl Add deionized H₂O to a final volume of 1 liter Adjust to pH 7.5 using NaOH Autoclave Add the following filter-sterilized supplements prior to use: 12.5 ml of 1 M MgCl₂ 12.5 ml of 1 M MgSO₄ 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)</p>	<p>TE Buffer (1 ×) 10 mM Tris-HCl (pH 7.5) 1 mM EDTA Autoclave</p>
<p>1 × TAE Buffer 40 mM Tris-acetate 1 mM EDTA</p>	<p>TE Buffer (0.1 ×) 100 μl TE Buffer (1 ×) 900 μl sterile dH₂O</p> <p>PBS 137 mM NaCl 2.6 mM KCl 10 mM Na₂HPO₄ 1.8 mM KH₂PO₄ Adjust the pH to 7.4 with HCl</p>

<p>Growth Medium</p> <p>DMEM (containing 4.5 g/L glucose and 110 mg/L sodium pyruvate and 4 mM L-glutamine), supplemented with 10% (v/v) heat-inactivated fetal bovine serum</p>	<p>Stock Chloroquine Solution (25 mM final concentration)</p> <p>Note <i>Chloroquine is toxic and should be opened in a fume hood only</i></p> <p>1.29 g of chloroquine diphosphate [C₁₈H₂₆CIN₃ • 2H₃PO₄]</p> <p>Add 100 ml of 1 × PBS; dissolve the solid chloroquine. Filter-sterilize and store in aliquots at -20°C. Discard aliquots that are older than one month. Dilute 1:1000 into media for use during the transfection</p>
<p>Growth Medium (supplemented with 25 μM chloroquine)</p> <p>Note <i>Chloroquine solution is toxic and should be opened in the laminar flow hood</i></p> <p>Prepare growth medium as above. Add chloroquine from stock chloroquine solution to a final concentration of 25 μM. Filter sterilize. Prepare just before use and keep at 37°C until required.</p>	<p>MBS-Containing Medium</p> <p>Note <i>Chloroquine solution is toxic and should be opened in the laminar flow hood</i></p> <p>Add stock chloroquine solution to DMEM containing 7% (v/v) modified bovine serum (Solution III from the ViraPack Transfection Kit) to a final concentration of 25 μM. Filter sterilize. Prepare just before use and keep at 37°C until required.</p>

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ENDNOTES

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