AdEasy XL Adenoviral Vector System

Instruction Manual

Catalog #240010

Revision C1

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240010-12

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AdEasy XL Adenoviral Vector System

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MATERIALS PROVIDED

AdEasy XL Adenoviral Vector System (Catalog #240010) Materials Provided	Quantity
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-AD-1 electroporation competent cells[a] (Catalog #200157)	5 × 100 μl (Green Tubes)
Transformation Control plasmid (for BJ5183-AD-1 electroporation comp. cells 0.1 ng/μl in TE buffer)	10 μl
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)	10 μl
AD-293 cells (Catalog #240085; provided in 1-ml of DMEM + 40% FBS + 10% DMSO)	1×10^6 cells

[a] Each 100 μl aliquot is sufficient for two transformations.

[b] Each 100 μl aliquot is sufficient for one transformation.

STORAGE CONDITIONS

AdEasy Vectors: –20°C

Competent Cells (including Transformation Control plasmid, β-ME mix, and pUC18 control plasmid): Place at –80°C. immediately upon arrival. Do not place competent cells in liquid nitrogen.

AD-293 Cells: Place in liquid nitrogen immediately upon arrival.

ADDITIONAL MATERIALS REQUIRED

Pac I restriction enzyme
Pme I restriction enzyme
Chloroquine[§]
Transfection Reagent [ViraPack Transfection Kit (Agilent Catalog #200488) is recommended]
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)
Growth medium for AD-293 cells[§] [Invitrogen Life Technologies (Gibco) Catalog #11995]
SeaPlaque agarose [FMC Corporation]

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

Revision C1

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INTRODUCTION

Recombinant adenoviruses are versatile tools 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 (e.g. AD-293 cells). 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] This approach, which is incorporated into the AdEasy system, employs the efficient homologous recombination machinery in *E. coli*, to produce a recombinant adenovirus by a double-recombination event between the 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.

The AdEasy XL Adenoviral Vector System features two new components that facilitate the most time consuming steps of the original AdEasy system. The AdEasy XL system includes BJ5183 cells pre-transformed with the pAdEasy-1 plasmid (BJ5183-AD-1 cells). This feature dramatically decreases background caused by the non-recombinant shuttle plasmid, making it easier to obtain colonies that contain the recombinant adenoviral plasmid. Up to 80–90% of kanamycin-resistant colonies are recombinants using this single transformation system, compared to 20% recombinants using the traditional cotransformation procedure. In addition, the AdEasy XL system includes AD-293 cells, which are HEK293-derived cells with improved cell adherence properties, simplifying the process of producing recombinant adenovirus particles.

Overview of the AdEasy XL System

A schematic overview of the production of recombinant adenovirus is shown in Figure 1. In the AdEasy XL system, the DNA of interest is cloned into one of the four shuttle vectors: pShuttle-CMV (provided), pShuttle (provided), pShuttle-IRES-hrGFP-1 (available separately, Catalog #240081) or pShuttle-IRES-hrGFP-2 (available separately, Catalog #240082). Once constructed, the shuttle vector is linearized with *Pme* I and transformed into BJ5183-AD-1 competent cells. 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 (ITRs), and is then used to transfect AD-293 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 XL 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 XL 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).

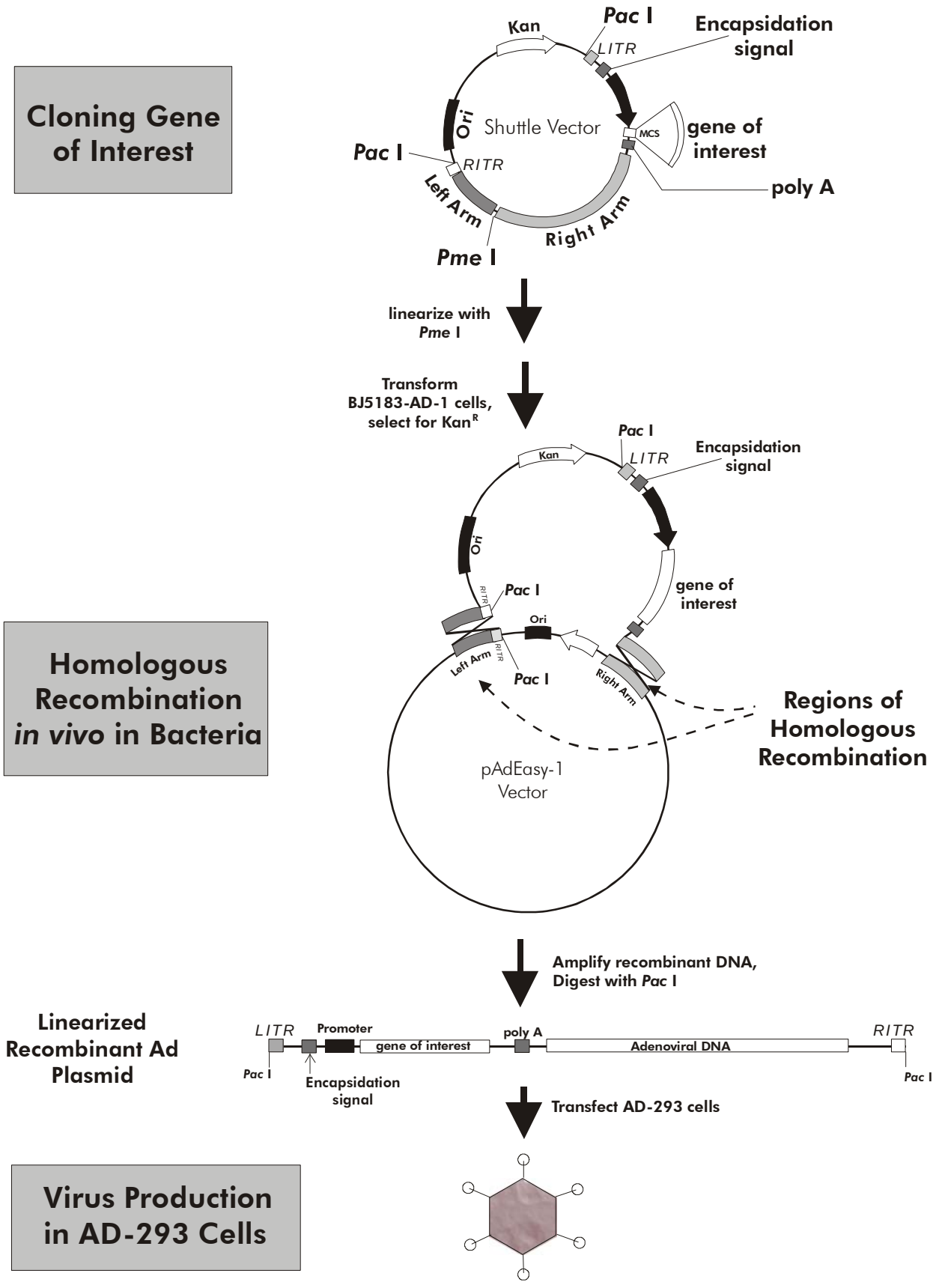


FIGURE 1 Production of recombinant adenovirus using the AdEasy XL adenoviral vector system

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 (provided with this kit) 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 (provided with this kit) 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]

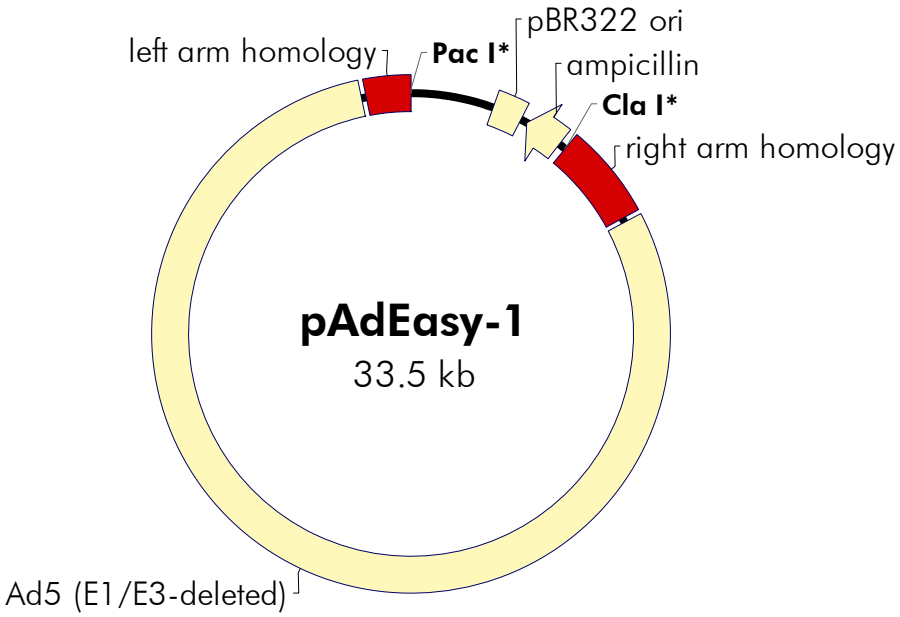
pShuttle-IRES-hrGFP-1 and pShuttle-IRES-hrGFP-2

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.

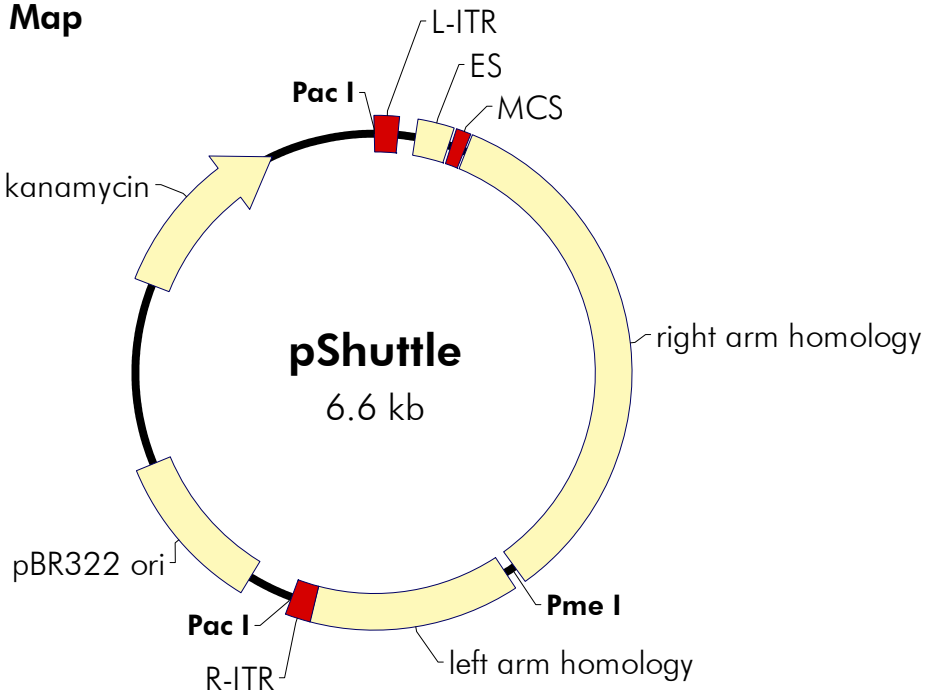
pAdEasy-1 Vector Map



Feature	Nucleotide Position
pBR322 origin of replication	1854–2521
ampicillin resistance (*bla*) 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

GAAGTGAAATCTGAATAATTTTGTGTTACTCATAGCGCGTAATACT...

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

...GGTACCGCGGCCGCCTCGAGTCTAGAGATATCGAATTCAAGCTTGTCGACTCGAAGATCT...

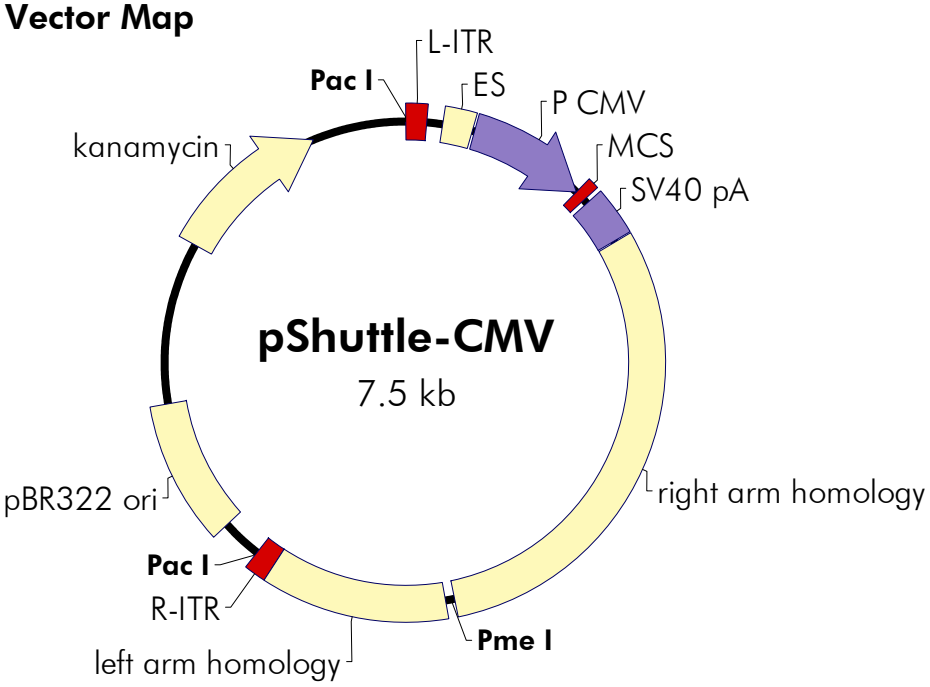
Reverse primer binding site

...GGGCGTGGTTAAGGGTGGGAAAGAATATATAAGGTGGGGGTCTTATGTAGTTTTG

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...

Kpn I Sal I Not I Xho I Hind III EcoR V

...AGATCTGGTACCGTCGACGCGGCCGCTCGAGCCTAAGCTTCTAGATAAGATATC...

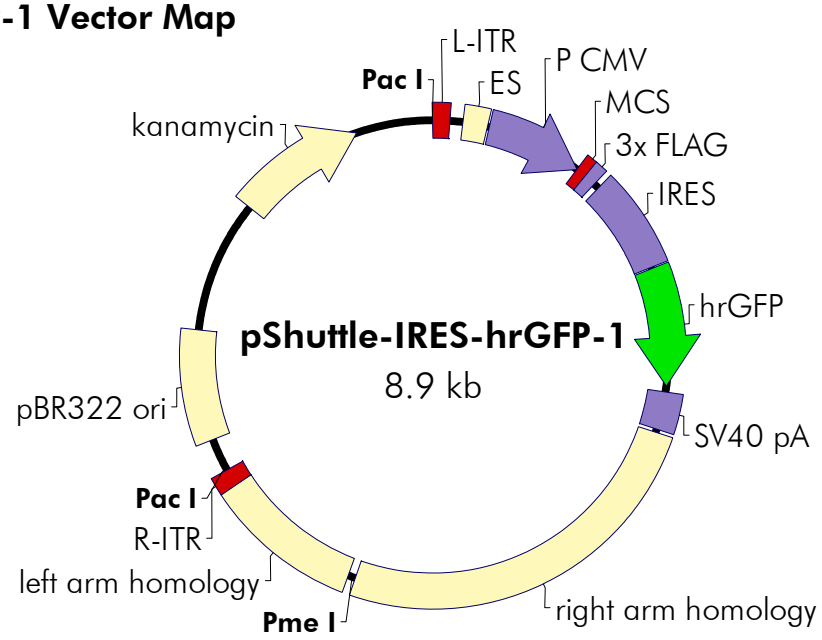
Reverse primer binding site

...CGATCCACCGGATCTAGATAACTGATCATAATCAGCCATACCAC

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-IRES-hrGFP-1 Vector Map



pShuttle-IRES-hrGFP-1 Multiple Cloning Site Region (sequence shown 933–1005)

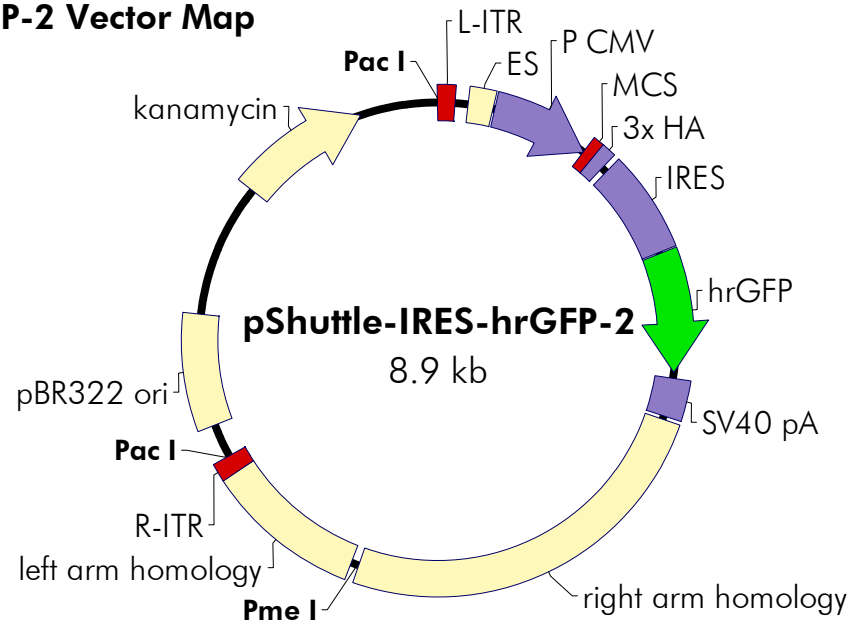
```
Bgl II*   Not I*        Sca I*   Nhe I*   Spe I*   EcoR V   Pvu I   Sal I   Srf I      Xho I                D  Y  K
A GAT CTG CGG CCG CAG TAC TGC TAG CAC TAG TGA TAT CCG ATC GGT CGA CGC CCG GGC CTC GAG GAC TAC AAG
                         STOP*    STOP*  STOP*                                        start of 3× FLAG
```

*The presence of stop codons in-frame with the 3× FLAG tag must be considered when inserting genes into the MCS. Do not use the *Bgl* II, *Not* I, *Sca* I, *Nhe* I or *Spe* I 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.

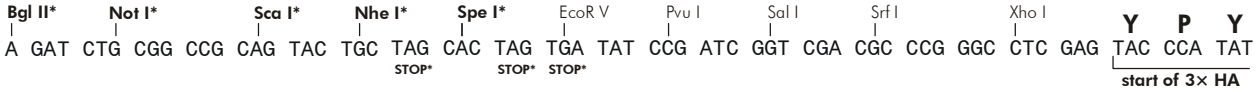
Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
CMV promoter	345–932
forward primer binding site	749–768
multiple cloning site	933–996
3× FLAG tag	997–1068
reverse primer binding site	1075–1094
internal ribosome entry site (IRES)	1104–1678
hrGFP ORF	1688–2404
SV40 polyA signal	2443–2670
Ad5 right arm homology	2693–4918
Ad5 left arm homology	4966–5845
right Ad5 inverted terminal repeat (ITR)	5846–5948
pBR322 origin of replication	6152–6819
kanamycin resistance ORF	7628–8419

FIGURE 5 The pShuttle-IRES-hrGFP-1 vector

pShuttle-IRES-hrGFP-2 Vector Map



pShuttle-IRES-hrGFP-2 Multiple Cloning Site Region (sequence shown 933–1005)

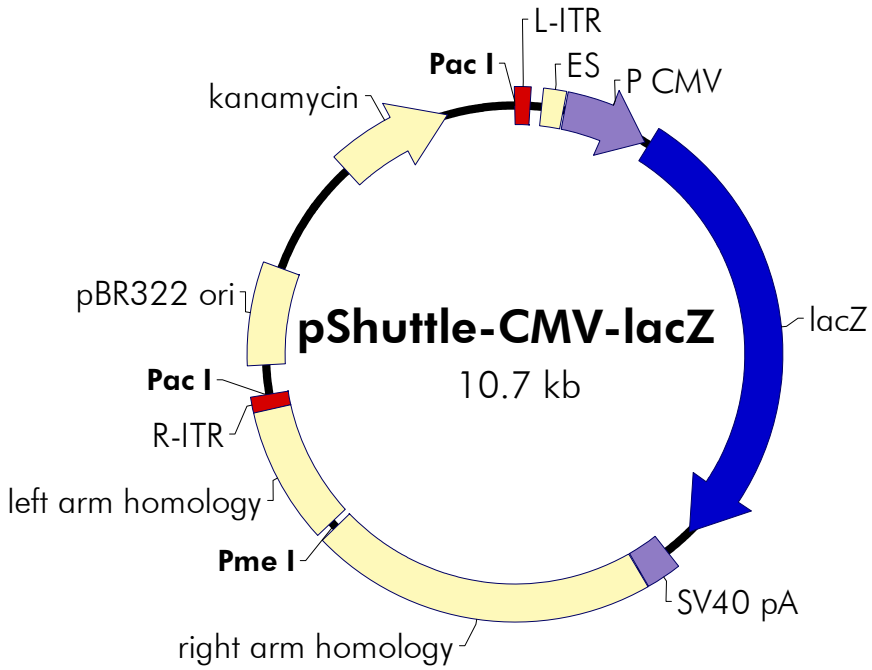


*The presence of stop codons in-frame with the 3× HA tag must be considered when inserting genes into the MCS. Do not use the *Bgl* II, *Not* I, *Sca* I, *Nhe* I or *Spe* I sites for cloning unless the cloning strategy removes the stop codons by double digestion using one of the upstream sites plus a site downstream of the stop codons.

Feature	Nucleotide Position
left Ad5 inverted terminal repeat (ITR)	1–103
encapsidation signal (ES)	183–331
CMV promoter	345–932
forward primer binding site	749–768
multiple cloning site	933–996
3× HA tag	997–1077
reverse primer binding site	1084–1103
internal ribosome entry site (IRES)	1113–1687
hrGFP ORF	1697–2413
SV40 polyA signal	2452–2679
Ad5 right arm homology	2702–4927
Ad5 left arm homology	4975–5854
right Ad5 inverted terminal repeat (ITR)	5855–5957
pBR322 origin of replication	6161–6828
kanamycin resistance ORF	7637–8428

FIGURE 6 The pShuttle-IRES-hrGFP-2 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 (lacZ) 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′ GAAGTGAAATCTGAATAATTTTGTG 3′	299–323
	Reverse	5′ CAAAACTACATAAGACCCCCAC 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 XL adenoviral system, two different prokaryotic host strains are required. The first, BJ5183-AD-1, is the BJ5183 strain pre-transformed with the pAdEasy-1 plasmid. The BJ5183-AD-1 strain is *recA* proficient and supplies the machinery necessary to execute the recombination event between the shuttle vector and the pAdEasy vector. The efficiency of recovery of recombinant vectors is greatly improved by using the BJ5183-AD-1 strain that is pre-transformed with the pADEasy-1 plasmid—up to 80–90% of kanamycin resistant colonies are recombinants using this single transformation system, compared to 20% recombinants using the traditional cotransformation procedure.

The second strain, provided as XL10-Gold ultracompetent cells, is used to amplify the recombined 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-AD-1 electrocompetent cells	4	*end*A1 *sbc*BC *rec*BC *gal*K *met thi*-1 *bio*T *hsd*R **(Str^r)** [pAdEasy-1 (Amp^R)]
XL10-Gold ultracompetent cells	5, 6	Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^R) Amy Cam^R]

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/vectorMapsAndSequence.jsp* 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 transformation of BJ5183-AD-1 bacteria and the recombinant Ad plasmid must be digested with *Pac* I before transfection of the AD-293 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 including the the Kozak initiation sequence. A complete Kozak sequence includes CCACC**ATG**G, although CC**ATG**G, 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	—	—	*Kpn* I, *Not* I, *Xho* I, *Xba* I, *Eco*R V, *Hind* III, *Sal* I, *Bgl* II	Ligate an entire expression cassette into MCS
pShuttle-CMV	6.6 kb	CMV	+	*Kpn* I *Sal* I, *Not* I, *Xho* I, *Hind* III, *Eco*R V	Ligate gene of interest into MCS between the CMV promoter and poly A
pShuttle-IRES-hrGFP-1 or –2 (available separately)	5.2 kb	CMV	+	*Bgl* II*, *Not* I*, *Sca* I*, *Nhe* I* *Spe* I*, *Eco*R V, *Pvu* I, *Sal* I, Srf I, *Xho* 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 *Pme* I-digestion and transformation steps (0.03–0.1 µg of linearized shuttle vector DNA is required for each transformation reaction).

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. Remove the enzyme and buffer using a method of choice. (We recommend the StrataPrep PCR Purification Kit.)

6. Treat the purified DNA with alkaline phosphatase for 30 minutes at 37°C.

   Note *We have achieved very similar recombination efficiencies using* Pme *I-digested vector that is not dephosphorylated.*

7. Gel-purify the linearized shuttle vector using agarose gel electrophoresis. Include 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. Isolate the linearized shuttle vector from the gel using a method of choice. Resuspend the purified DNA in sterile water at a final concentration of ~0.05 µg/µl.

   Notes *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.*

   Direct transformation of BJ5183-AD-1 cells with Pme *I-digested shuttle vector, without dephosphorylation or gel purification, often produces a sufficiently high recombination efficiency to allow recovery of recombinant adenoviral vectors. Longer digestion times (several hours to overnight) produce a greater proportion of linearized vector and increase the recombination efficiency. Transform with 1 µl (~0.05 µg) of digested DNA, after heat-inactivation of the enzyme.*

Transformation Guidelines for BJ5183-AD-1 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.

Transforming the BJ5183-AD-1 Cells to Produce Recombinant Ad Plasmid

Note *In this portion of the protocol, the BJ5183-AD-1 cells are transformed with the linearized shuttle vector (containing the gene of interest or containing the control gene,* lacZ*). A recombination event that takes place in the bacterial cells results in the production of recombinant AdEasy plasmid DNA.*

1. Prechill three DNase-free microcentrifuge tubes and three electroporation cuvettes (0.2 cm gap) on ice.
2. Remove two aliquots of BJ5183-AD-1 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 (~0.05 to 0.1 µg) of linearized shuttle vector. Mix by tapping the tube gently and keep on ice.
5. Into the second tube, pipet 1 µl (~0.05 to 0.1 µg) of linearized pShuttle-CMV-*lacZ* vector. Mix by tapping the tube gently and keep on ice.
6. Into the third tube, pipet, 1 µl of Transformation Control plasmid (supplied with the BJ5183-AD-1 electroporation comp. cells).
7. Set the electroporator to the following settings by referring to the instructions provided with the instrument: 200 Ω, 2.5 kV, 25 µF.
8. Transfer the contents of one microcentrifuge tube into one of the chilled electroporation cuvettes and tap the cuvette gently to settle the mixture to the bottom.
9. Slide the cuvette into the electroporation chamber until the cuvette connects with the electrical contacts.

10. 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.

11. Transfer the cell suspension to a sterile 14-ml BD Falcon polypropylene round-bottom tube.

12. Repeat the electroporation for the other two transformation reactions. Incubate all of the transformations at 37°C for 1 hour while shaking at 225–250 rpm.

13. For the recombination reactions (linearized DNA transformants), plate the entire volume of recovered cells on LB-kanamycin agar plates[§] (e.g. three plates containing 50 µl, 100 µl, and 850 µl of the transformed cell suspension, respectively).

    For the transformation using the Transformation Control plasmid, plate 10 µl and 100 µl of the recovered cells on LB-kanamycin agar plates.

    Note *When plating less than 100 µl, first place a 100-µl pool of LB broth on an LB-kanamycin agar plate. Pipet the transformed cells into the pool of LB broth, then use a sterile spreader to spread the mixture.*

14. Incubate the plates overnight at 37°C.

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

Testing Colonies for Recombinant Ad Plasmids

1. Examine the transformation plates. The linear shuttle vector transformants will appear as three populations: very large colonies, intermediate-, and small-sized colonies. The small and intermediate colonies are the potential recombinants and the very large colonies represent background from the shuttle vector. The ratio of small plus intermediate colonies to very large colonies should be approximately 10:1.

   Note *If the transformation was performed with non-gel purified shuttle vector, expect a ratio of small plus intermediate colonies to very large colonies of 5:1 or lower.*

2. Count colonies obtained on the Transformation Control plasmid transformation plates to determine transformation efficiency, if desired. Expect an efficiency of $\geq 1 \times 10^7$ cfu/µg Transformation Control DNA.

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 the cultures at 37°C overnight while shaking at 225–250 rpm.

5. Prepare miniprep DNA from 2 ml (or more) of overnight culture using a procedure suitable to purify large plasmids or cosmids. Resuspend the miniprep DNA in 50 µl of sterile dH_2O or TE buffer.

6. Cut 10 µl of the miniprep DNA with *Pac* I restriction enzyme and run the entire digest on a 0.8% agarose TAE[§] gel next to 10 µl of uncut miniprep DNA. As a control, also cut a small amount (~0.2 µg) of unrecombined shuttle vector (prepared in step 3 of *Cloning the Gene of Interest*) and run in an adjacent lane.

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

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.

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

More than 50% of the DNA minipreps should contain a recombinant plasmid close to 30 kb, when small colonies are selected for analysis.

There are often faint background bands in BJ5183-AD-1 minipreps. If the PREDOMINANT bands are the expected sizes, redigest potential recombinant DNA prepared from XL10-Gold cells (see *Amplifying Recombinant Ad Plasmids*). 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.

* 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 PLASMIDS

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 Ultracompetent 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 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. (Using an alternative source of β-ME may reduce transformation efficiency.)

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 Ultracompetent Cells Transformation Protocol

Note *Each 100 µl 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 tubes on ice.
3. Thaw the XL10-Gold ultracompetent cells on ice.
4. Gently mix the cells by hand. Aliquot 100 µl of the cells into prechilled 15-ml tubes.
5. Add 4 µl of the β-ME mix provided with the kit to the 100 µl of cells. (Using an alternative source of β-ME may reduce transformation efficiency.)
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 (generally 1 µl of miniprep DNA, with a maximum volume of 4 µl) to an aliquot of cells and swirl gently. To a separate tube of cells add 1 µl of the pUC18 control plasmid (diluted 1:10 in sterile dH_2O) 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 µl, 25 µl, and 100 µl of each putative recombinant transformation reaction on LB-kanamycin agar plates using a sterile spreader.

    Plate 5 µl of the pUC18 control plasmid transformation on LB-ampicillin agar plates. The pUC18 control transformation should yield an efficiency of > 10^9 cfu/µg pUC 18 DNA.

    Note *When plating less than 100 µl, first place a 100-µl pool of NZY+ broth on the agar plate. Pipet the transformed cells into the pool of NZY+ broth, then use a sterile spreader to spread the mixture.*

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. Grow the cultures overnight at 37°C while shaking at 225–250 rpm.

16. The next morning transfer 5 ml of each 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 µl 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 of ~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 µg of DNA is needed for each transfection.)

21. Run 0.2 µg 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 (e.g. the StrataPrep PCR Purification Kit).

23. Under sterile conditions, resuspend the DNA in 50 µl of sterile 0.1× TE buffer or dH_2O. Store the resuspended DNA at –20°C.

AD-293 CELL CULTURE GUIDELINES

Notes *All procedures must be performed using sterile technique in a laminar flow hood. For general information on mammalian cell culture and sterile technique, see reference 7.*

AD-293 cells may be passaged up to 30 times (with the supplied cells defined as passage number one). It is important to prepare a liquid nitrogen stock of early passage cell aliquots for long-range experiments.

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

Establishing AD-293 Cultures from Frozen Cells

1. Place 10 ml of growth medium (see *Preparation of Media and Reagents*) in a 15-ml conical tube.

2. Thaw the frozen cryovial of cells within 40–60 seconds by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath and decontaminate the cryovial by immersing it in 70% (v/v) ethanol (at room temperature).

3. Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium.

4. Collect the cells by centrifugation at 200 × *g* for 5 minutes at room temperature. Remove the growth medium by aspiration.

5. Resuspend the cells in the conical tube in 5 ml of fresh growth medium.

6. Add 10 ml of growth medium to a 75-cm^2 tissue culture flask. Transfer the 5 ml of cell suspension to the same tissue culture flask. Place the cells in a 37°C incubator at 5% CO_2.

7. Monitor cell density daily. Cells should be passaged when the cell culture is at ≤50% confluency. Proceed to either *Preparation of an AD-293 Cell Liquid Nitrogen Stock* or *Passaging of AD-293 Cells*.

Preparation of an AD-293 Cell Liquid Nitrogen Stock

1. When growing cells for the production of an AD-293 liquid nitrogen stock, cultures should be maintained at ≤50% confluence.

   Note *AD-293 cells grown at high confluence may lose the increased adherence phenotype. It is especially important to maintain cells propagated to establish a liquid nitrogen stock at≤50% confluence to ensure the integrity of the stock.*

2. Collect cells from a healthy, log-phase culture. Remove the culture medium by aspiration. Tyrpsinize cells for 1–3 minutes in 1.5 ml of Trypsin-EDTA Solution (see *Preparation of Media and Reagents*).

   Note *Incubate the cells in the Trypsin-EDTA solution for the minimum time required to release adherent cells from the flask. This process may be monitored using an inverted microscope. Excess trypsinization may damage or kill the cells.*

3. Dilute the cells with 8.5 ml of growth medium. The serum in the medium inactivates the trypsin. Transfer the suspension to a 15-ml conical tube, then collect the cells by centrifugation at 600 × *g* for 5 minutes at room temperature.

4. Remove the medium by aspiration. Resuspend the cell pellet in a minimal volume of growth medium (containing 10% fetal bovine serum). Count the cells present in an aliquot of the resuspension using a hemocytometer.

5. Dilute the cell suspension to 1×10^6 cells/ml in freezing medium (see *Preparation of Media and Reagents*), then dispense 1-ml aliquots of the suspension into 2-ml cryovials.

6. Freeze the cell aliquots gradually by placing the vials in a Styrofoam container and then placing the container in a –80° freezer overnight.

7. Transfer the vials of frozen cells to liquid nitrogen for long-term storage.

Passaging of AD-293 Cells

When the cell monolayer reaches 50% confluence, AD-293 cells should be split at a 1:10 ratio.

Note *If cell confluence exceeds 50%, AD-293 cells may lose the increased adherence phenotype.*

1. Remove the growth medium by aspiration. Wash cells once with 10 ml of phosphate-buffered saline.

2. Tyrpsinize cells for 1–3 minutes in 1.5-ml of Trypsin-EDTA Solution.

3. Dilute the cells with 8.5 ml of growth medium to inactivate the trypsin.

4. Transfer 1 ml of the cell suspension to a fresh 75-cm^2 tissue culture flask and add 9 ml fresh growth medium. Place the cells in a 37°C incubator at 5% CO_2. Monitor cell density daily.

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, we strongly recommend that the user 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

We recommend preparing and titering AdEasy recombinant virus stocks using the AD-293 cell line [provided with the AdEasy XL adenoviral vector system 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.[8] 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 × 10^5 cells per 60-mm tissue culture dish in growth medium[§] 24 hours prior to the transfection.

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

Transfecting AD-293 Cells

A variety of transfection protocols may be successfully used with these vectors and the AD-293 cell line. We have demonstrated success using the following 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.[9]

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 round bottom 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 µM 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.

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 × *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).

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.

* SeaPlaque GTG agarose (Catalog #50111) from BioWhittaker Molecular Applications (1-800-341-1574 or *www.bmaproducts.com*).

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 transformation of BJ5183-AD-1 cells with shuttle vector	Perform a control transformation of the electroporation competent BJ5183-AD-1cells using the Transformation Control DNA to ensure that the cells are highly competent ($\geq 1 \times 10^7$ cfu/μg). Be sure to plate the transformations on kanamycin plates.
	If the concentration of linearized shuttle vector recovered from gel purification is too low, the volume of DNA solution used to transform the BJ5183-AD-1 cells may be increased to a maximum of 6 μl of DNA per 40 μl of cells.
All of the colonies following transformation of BJ5183-AD-1 cells are large (comparable to supercoiled DNA transformants)	Gel-purify the *Pme* I cut shuttle vector (containing the gene of interest) and ensure that the DNA is resuspended in sterile dH_2O prior to transformation.
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 additional well- isolated **small** colonies from the original plates and test by restriction digestion There are often faint background bands in BJ5183-AD-1 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/μg). Be sure to plate the putative recombinant transformations on kanamycin plates.
No growth in liquid LB-kanamycin miniprep cultures	LB-kanamycin agar plates are 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 using a fresh kanamycin stock solution, and do not add the kanamycin until the molten agar solution is ≤ 48°C.

PREPARATION OF MEDIA AND REAGENTS

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

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

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 H_2O 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)

NZY+ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 using NaOH
Autoclave
Add the following filer-sterilized supplements prior to use:
12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

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 H_2O 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)

TE Buffer (1×)

10 mM Tris-HCl (pH 7.5)
1 mM EDTA
Autoclave

TE Buffer (0.1×)

100 µl TE Buffer (1×)
900 µl sterile dH_20

1× TAE Buffer

40 mM Tris-acetate
1 mM EDTA

PBS

137 mM NaCl
2.6 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
Adjust the pH to 7.4 with HCl

Growth Medium

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

Freezing Medium (100 ml)

50 ml DMEM (containing 4.5 g/L glucose, 110 mg/L sodium pyruvate and 4 mM L-glutamine)
40 ml heat-inactivated fetal bovine serum
10 ml dimethylsulfoxide (DMSO)
Filter sterilize

Stock Chloroquine Solution (25 mM final concentration)

Note *Chloroquine is toxic and should be opened in a fume hood only*

1.29 g of chloroquine diphosphate [$C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$]

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

Growth Medium (supplemented with 25 μM chloroquine)

Note *Chloroquine solution is toxic and should be opened in the laminar flow hood*

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.

Trypsin-EDTA Solution

0.53 mM tetrasodium ethylenediamine-tetraacetic acid (EDTA)
0.05% trypsin

MBS-Containing Medium

Note *Chloroquine solution is toxic and should be opened in the laminar flow hood*

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.

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ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.chem.agilent.com/en-US/search/library/Pages/MSDSSearch.aspx*. MSDS documents are not included with product shipments.

AdEasy XL Adenoviral Vector System

Catalog #240010

QUICK-REFERENCE PROTOCOL

Production of Recombinant Ad DNA

- Clone the gene of interest into the appropriate shuttle vector.
- Linearize the shuttle vector-plus-insert DNA and the control vector, pShuttle-CMV-*lacZ,* with *Pme* I. Confirm complete digestion, then remove the enzyme and buffer using method of choice (e.g. StrataPrep PCR Purification Kit).
- Treat the purified DNA with alkaline phosphatase for 30 minutes at 37°C, then gel-purify the linearized DNA by gel electrophoresis. Resuspend the DNA in sterile H_2O at ~0.05 μg/μl.

  Note Direct transformation of BJ5183-AD-1 cells with *Pme* I-digested DNA (~0.05 μg), without dephosphorylation or gel purification often produces a sufficient recombination efficiency to allow recovery of recombinant adenovirus DNA.

- Transform 1 μl of linearized shuttle vector-plus-insert DNA into 40 μl of BJ5183-AD-1 electrocompetent cells by electroporation. As a recombination control, transform 1 μl of linearized pShuttle-CMV-*lacZ* DNA into a second aliquot of the electrocompetent cells. After a 1-hr outgrowth in 0.9 ml of LB broth, plate the entire volume of each transformation reaction (e.g. in aliquots of 50 μl, 100 μl and 850 μl) on LB-kanamycin agar plates. Incubate the transformation plates at 37°C overnight.

  Note To measure transformation efficiency, transform a third aliquot of the electrocompetent cells with 1 μl of Transformation Control plasmid (supplied with the BJ5183-AD-1 electrocompetent cells), and plate 10 μl and 100 μl aliquots on LB-kanamycin agar plates. Expect a transformation efficiency of $\geq 1 \times 10^7$ cfu/μg Transformation Control DNA.

- Pick 10 or more of the **smallest**, well-isolated colonies from both the experimental and control transformation plates for restriction analysis to identify recombinant Ad DNA clones. Prepare miniprep DNA from 2-ml of overnight cultures for each clone, and resuspend the DNA in 50 μl of sterile H_2O or TE.
- Digest 10 μl of each DNA miniprep with *Pac* I, then run the entire digest on a 0.8% agarose TAE gel. Recombinant Ad plasmids cut with *Pac* I should yield fragments of ~30 kb plus either 3.0 kb or 4.5 kb. When small colonies are selected for analysis, more than 50% of the DNA minipreps should contain recombinant Ad plasmids.
- Once recombinant Ad DNA clones have been identified, amplify DNA from several clones by transforming 1–4 μl (0.1–50 ng) of the miniprep DNA from each clone into separate 100-μl aliquots of XL10-Gold ultracompetent cells (see *XL10-Gold Ultracompetent Cells Transformation Protocol* in the instruction manual).
- Prepare 10-ml overnight cultures of several independent transformants in LB-kanamycin broth. Prepare miniprep DNA from 5-ml of the overnight cultures and verify that digestion with *Pac* I produces the expected restriction pattern. Store the remaining 5-ml of each culture at 4°C.

- For positive recombinant clones, use the 5-ml of overnight culture stored at 4°C to inoculate a 500-ml culture of LB-kanamycin broth. Grow the culture at 37°C overnight, then prepare maxiprep DNA suitable for mammalian cell transfection.
- For each clone to be transfected, digest 5 μg of purified DNA with *Pac* I. Run 0.2 μg of digested DNA on a 0.8% agarose-TAE gel to confirm digestion and the expected restriction pattern.
- Purify the *Pac* I-digested DNA by phenol extraction/ethanol precipitation or using the StrataPrep PCR purification kit. Resuspend the purified DNA in 50 μl of sterile 0.1× TE or sterile dH_2O, then store the DNA solution at –20°C.

Transfection of AD-293 Cells and Primary Adenovirus Stock Preparation

- Plate AD-293 cells at 7–8 × 10^5 cells per 60-mm tissue culture dish in Growth Medium 24 hours prior to the transfection. Incubate the cells at 37°C with 5% CO_2 overnight. The cells should reach ~70% confluence after the overnight incubation.
- Prepare 4 ml of MBS-containing medium for each 60-mm plate just prior to transfection. Aspirate the growth medium, wash the cells twice with phosphate-buffered saline (PBS), then replace with 4 ml of MBS-containing medium in each plate. Incubate the plates at 37°C with 5% CO_2 for 20–30 minutes. Begin the preparation of transfection DNA mixtures approximately 10 minutes prior to the end of the 20–30 minute incubation period.
- For each transfection, pipet 5 μg of *Pac* I-digested DNA into a 5-ml BD Falcon polystyrene tube that contains sufficient dH_2O for a final volume of 225 μl. Add 25 μl of Solution I and 250 μl of Solution II from the ViraPack Transfection Kit. Immediately mix the contents gently by tapping the tube. Incubate the tube at room temperature for 10 minutes.
- Gently mix the DNA suspension by pipetting up and down, then add the DNA suspension to the AD-293 cell plates in a drop-wise fashion, swirling the plates gently. Incubate the cells at 37°C with 5% CO_2 for 3 hours.
- Remove the MBS-containing medium by aspiration, and replace it with 4 ml of Growth Medium supplemented with 25 μM chloroquine. Incubate the cells at 37°C with 5% CO_2 for 6–7 hours.
- Aspirate the medium from the plates and replace it with 4 ml Growth Medium (no chloroquine). Incubate the cells at 37°C with 5% CO_2 for 7–10 days, replenishing the growth medium as required.
- Aspirate the medium, wash the cells once with PBS, then harvest the cells in 0.5 ml of PBS, transferring the cell suspension to a 1.7-ml screw-cap tube.
- Subject the cell suspension to four rounds of freezing/thawing (using a dry-ice methanol bath and a 37°C water bath). Remove cellular debris by spinning the tube at 12,000 × *g* for 10 minutes at room temperature. Transfer the supernatant (primary virus stock) to a fresh tube and store at –80°C.