

Efficient gene transfer and high-level protein expression within a variety of cells

# Generate Adenovirus Vectors in *E. coli* by Homologous Recombination with the AdEasy™ Adenoviral Vector System

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Stratagene

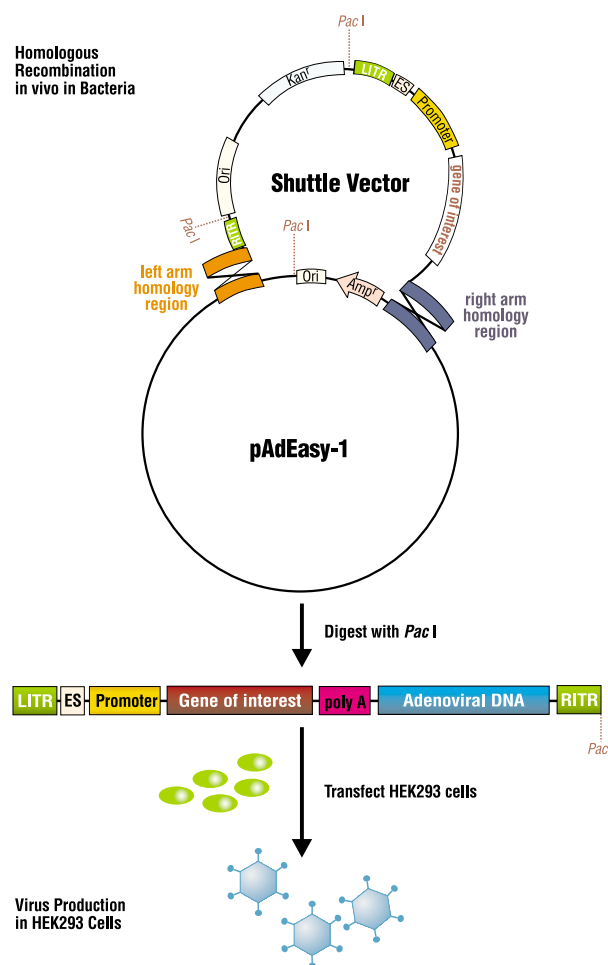
Recombinant adenoviruses provide a versatile means of gene delivery and expression. We describe the AdEasy™ adenoviral vector system,<sup>\*\*\*,2</sup> which simplifies the production of such viruses. Constructing a recombinant adenoviral vector using the AdEasy system is a two-step process in which the desired expression cassette is first subcloned into a shuttle vector, then transferred into the adenoviral genome by homologous recombination in *E. coli*. This method allows recombinant adenovirus vectors to be selected more quickly and easily than traditional methods.

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 many applications. For example, adenovirus can infect a broad spectrum of cell types, and infection is not dependent on active cell division. The ability to overexpress human proteins in human cell lines allows purification of recombinant proteins with the correct posttranslational processing. Additionally, high titers (amplified titers up to  $10^{11}$  pfu/ml) and high-level gene expression can be obtained. Consequently, with all these attributes, successful protein production in mammalian cells can be predicted.

The most commonly used adenoviral vector, human adenovirus serotype 5, 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, such as HEK293. The E3 gene encodes proteins involved in evading host immunity and is dispensable. Not only do these deletions prevent the virus from replicating itself, they also create space for up to 7.5 kb of foreign DNA.<sup>1</sup>

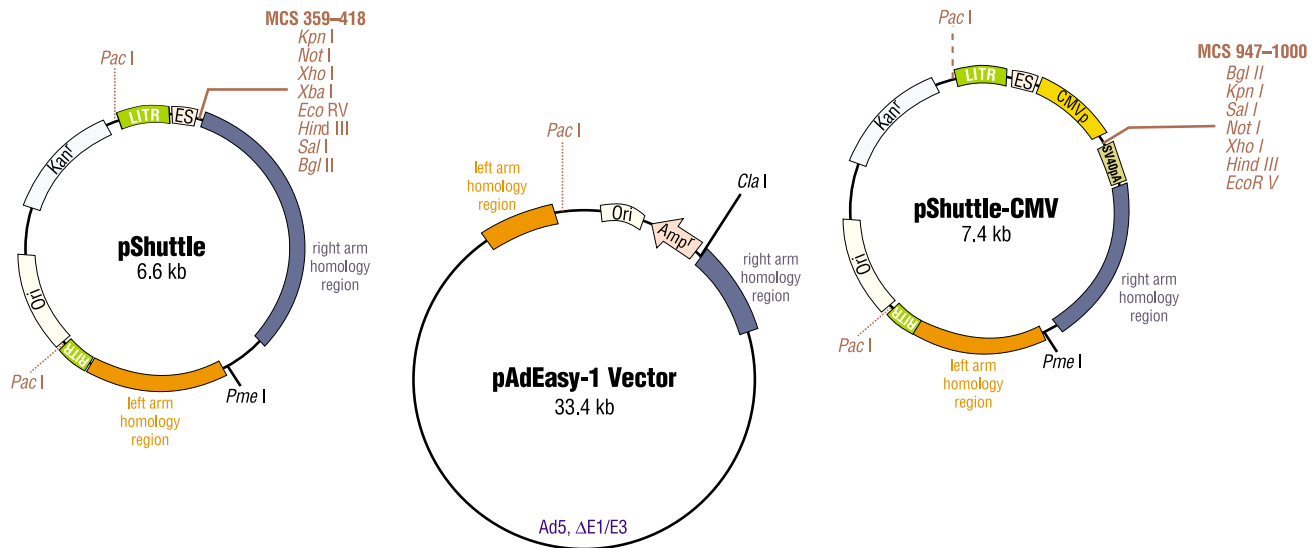
Two methods have traditionally been used to generate recombinant adenoviruses. The first involves direct ligation of the gene of interest into the adenoviral genome. The scarcity of unique restriction sites and the prohibitive size of the genome (36 kb) make this method technically challenging. The second and more widely used method involves cloning the gene of interest into a shuttle vector and transferring the gene into the

**Figure 1**  
**Homologous Recombination In Vivo in Bacteria**



The gene of interest or expression cassette is first cloned into a shuttle vector (either pShuttle or pShuttleCMV). The resultant plasmid is linearized by *Pme* I and cotransformed into *E. coli* strain BJ5183 with the adenoviral backbone plasmid pAdEasy-1. Recombinant adenoviral plasmids are selected on kanamycin and confirmed by restriction digest. The recombinant adenoviral plasmid is then digested with *Pac* I and transfected into HEK293 cells where they are packaged into virus particles.

**Figure 2**  
**Maps of AdEasy Plasmid Vectors**



The two shuttle vectors pShuttle and pShuttle-CMV, and the adenoviral backbone vector pAdEasy-1, are shown. A transfer vector, pShuttle, contains a multiple cloning site in which an entire expression cassette can be inserted. The transfer vector pShuttle-CMV contains a multiple cloning site sandwiched between the CMV promoter and the SV40 polyadenylation

signal. The pAdEasy-1 plasmid contains most of the human adenovirus serotype 5 genome with deletions in the E1 and E3 regions. Upon homologous recombination between a shuttle vector and pAdEasy-1, a recombinant adenoviral plasmid is generated in which the expression cassette is inserted into the original E1 region of the adenovirus genome.

adenovirus genome by means of homologous recombination *in vivo*.<sup>2</sup> To isolate and identify recombinant adenovirus by this method, multiple plaque isolations must be performed, which is an extremely laborious and time-consuming process.

The AdEasy system uses a method, developed by T.C. He and colleagues, where homologous recombination between an adenoviral backbone plasmid vector and a shuttle vector carrying the gene of interest is performed in *E. coli*.<sup>3</sup> This method generates recombinant adenoviral plasmid vectors that obviate the need for plaque purification and significantly decrease the time required to generate virus.

### Overview of the AdEasy™ System

In the AdEasy system, the cDNA or expression cassette of interest is cloned into either of two shuttle vectors, pShuttle-CMV<sup>\*\*\*</sup> or pShuttle. pShuttle is used if an alternate promoter, other than CMV, is preferred. Once constructed, the shuttle vector is linearized with *Pme*I and cotransformed into *E. coli* strain BJ5183 with pAdEasy-1 viral DNA plasmid. Transformants are selected for kanamycin resistance, and recombinants are subsequently identified by restriction digest. Once a recombinant adenoviral plasmid is identified, it is digested with *Pac*I to expose the inverted terminal repeats (ITR), then transfected into HEK293 cells where the E1 gene (necessary for virus assembly) is complemented *in vivo* (Figure 1).

### Vector Description

The pAdEasy-1 vector contains most of the human adenovirus serotype 5 (Ad5) genome and is deleted for the genes E1 and E3 (Figure 2). The expression cassette from the shuttle vector is inserted into the original E1 region of the adenovirus genome by homologous

recombination. The pAdEasy-1 vector carries the ampicillin resistance gene, which is lost after recombination with a shuttle vector.

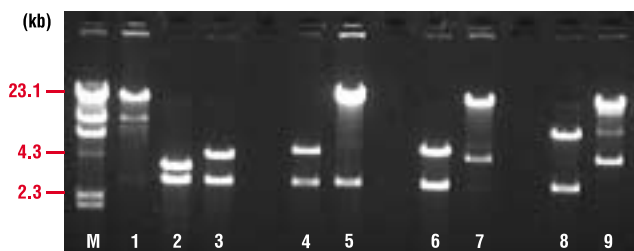
The pShuttle-CMV vector contains a multiple cloning site between the CMV promoter and the SV40 polyadenylation signal. The pShuttle vector contains only a multiple cloning site in which an entire expression cassette can be inserted. The regions indicated as arms are the stretches of sequence homology with pAdEasy-1 vector where homologous recombination occurs. The RITR and LITR regions are short inverted terminal repeats (Right and Left) and have a role in replication of the viral DNA.<sup>2</sup> Both of these shuttle vectors contain the kanamycin resistance gene.

Three test shuttle vectors, pShuttle-CMV-GFP, pShuttle-CMV-LacZ, and pShuttle-CG were prepared to validate the AdEasy system described below. The pShuttle-CMV-GFP vector was prepared by cloning the sequence for green fluorescent protein (GFP) between the *Bgl* II and *Xho* I sites into pShuttle-CMV vector. The pShuttle-CMV-LacZ vector was prepared by cloning the sequence for *E. coli* LacZ between the *Xho* I and *Hind* III sites in pShuttle-CMV vector. The pShuttle-CG vector was prepared by inserting an entire expression cassette, which included the CMV promoter, and the green fluorescent protein between the *Xho* I and *Sal* I sites in the pShuttle vector.

### Efficient Recombination in *E. Coli* BJ5183

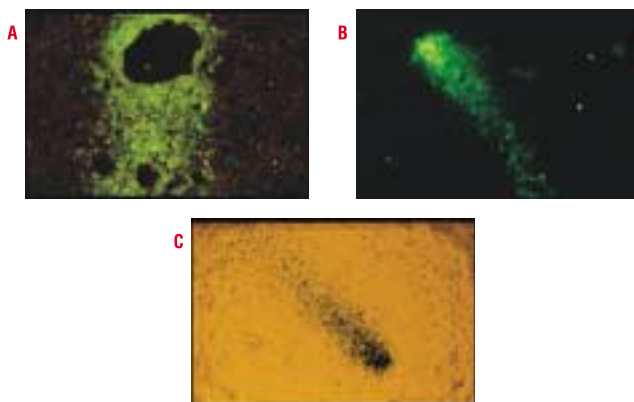
The AdEasy system is unique to other adenoviral vector systems because homologous recombination between shuttle vector and adenovirus genome is performed in *E. coli*. The *E. coli* strain BJ5183 was selected for its recombination capabilities and higher transformation

**Figure 3**  
**Pac I Digests of Positively Identified Adenoviral Plasmid Recombinants**



The shuttle vectors pShuttle-CG, pShuttleCMV-GFP, and pShuttleCMV-LacZ were subjected to homologous recombination with pAdEasy-1 in the *E. coli* strain BJ5183. Positive recombinants were selected on kanamycin and confirmed by digestion with *Pac*I. The digest of a recombinant should yield a large fragment (~30 kb) and a smaller fragment (3.0 kb or 4.5 kb depending on whether recombination occurred at the left arm or the origin of replication). Shown is a 0.8% agarose TAE gel of *Pac*I digested samples as indicated: M: Lambda *Hind* III markers; 1: pAdEasy-1; 2: pShuttle; 3: pShuttleCMV; 4: pShuttle-CG; 5: pAd-CG; 6: pShuttleCMV-GFP; 7: pAdCMV-GFP; 8: pShuttleCMV-LacZ; 9: pAdCMV-LacZ.

**Figure 4**  
**Viral Delivery and Gene Expression of GFP and LacZ**



Photographs represent transduction of HEK293 cells with an adenovirus stock. A: High magnification of an AdCMV-GFP plaque; B: Low magnification of an Ad-CG plaque; C: Low magnification of an AdCMV-LacZ-plaque cells stained with X-gal.

efficiency. To validate production of recombinant adenoviral plasmids in BJ5183, the shuttle vectors described in the previous section were linearized with *Pme*I, dephosphorylated, and gel purified. Linear shuttle vectors were then electrotransformed into BJ5183 cells, both alone to serve as controls, and cotransformed with the pAdEasy-1 vector to generate recombinants. Miniprep DNA was prepared from picked kanamycin-resistant colonies and analyzed by digestion with *Pac*I. Positive recombinants were identified by the presence of a large fragment of approximately 30 kb and a smaller fragment of either 3.0 kb or 4.5 kb (depending on whether recombination occurred at the left arm or the origin of replication). Representative *Pac*I digests of one positively identified recombinant for each of the three shuttle vectors is shown in Figure 3.

## High-Titer Virus Production and High-Level Gene Expression

Once recombinant adenoviral plasmid clones were identified, they were digested with *Pac*I and transfected into HEK293 cells using Stratagene's MBS Mammalian Transfection Kit, with modifications described by Pear and colleagues.<sup>4</sup> HEK293 cells are human embryonic kidney cells that have been transformed by sheared Ad5 DNA.<sup>5</sup> The cells contain and express the transforming genes of Ad5, including E1, which complements the deleted E1 gene from the pAdEasy-1 vector for assembly of replication-defective adenovirus. To confirm the presence of infectious virus, primary viral stocks were subsequently used to transduce HEK293 cells. Three days after transduction, cells were analyzed for expression of their respective reporter genes. Cells transduced with AdCMV-GFP and Ad-CG (both harboring a GFP reporter gene) were visualized by fluorescence microscopy. Cells transduced with AdCMV-LacZ were stained with X-gal using Stratagene's In Situ  $\beta$ -Galactosidase Staining Kit. From representative photographs of HEK293 cells transduced with the test vectors (Figure 4), the positive identification of the reporter gene expression in transduced cells indicates that infectious adenovirus were produced using the AdEasy vector system.

## Conclusions

Stratagene's AdEasy vector system simplifies and speeds up the process of generating a recombinant adenovirus, compared to traditional methods of preparation. With the AdEasy system's unique two-step process, a gene of interest, or expression cassette, is subcloned into a shuttle vector, then transferred into the adenoviral genome by homologous recombination in *E. coli*. This method eliminates the need for laborious plaque isolations, since the resulting adenoviral plasmid can be directly transfected into HEK293 cells to prepare adenovirus. With the AdEasy system, adenoviral-mediated gene transfer and expression are realized efficiently and conveniently.

## REFERENCES

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AdEasy™ Adenoviral Vector System	
pAdEasy-1 Vector pShuttle Vector pShuttle-CMV Vector pShuttle-CMV-LacZ Vector BJ5183 electroporation competent cells XL10-Gold® ultracompetent cells	#240009
AdEasy™ Adenoviral Vector System Components	
<b>pAdEasy-1 vector</b> (2.5 µg, 100 ng/µl in TE buffer)	#240005
<b>pShuttle vector</b> (20 µg, 1 µg/µl in TE buffer)	#240006
<b>pShuttle-CMV vector</b> (20 µg, 1 µg/µl in TE buffer)	#240007
<b>pShuttle-CMV-LacZ control vector</b> (10 µg, 1 µg/µl in TE buffer)	#240008
<b>BJ5183 electroporation competent cells</b> (5 x 100 µl)	#200154