p*Silencer*™ adeno 1.0-CMV System

(Part Number AM5790)

Protocol

P/N 5790M Revision B **Revision Date: December 5, 2008**

I. Product Description and Background

A. Brief Product Description

One of the biggest challenges in the use of RNA interference (RNAi) to study gene function in mammalian systems is delivering the small interfering RNAs (siRNA) or siRNA expression vector into cells. The p*Silencer*™ adeno 1.0-CMV System employs adenoviral mediated gene transfer—a well characterized and useful tool for the delivery of DNA sequences both in vivo and in vitro. Once inside a mammalian cell, a modified CMV promoter drives the expression of a hairpin siRNA for RNAi studies. Using this kit, researchers can produce a replication-deficient adenovirus containing a hairpin siRNA template that targets their gene of interest. It has been shown that adenoviral vectors are capable of expressing functional siRNAs that enter the RNAi pathway and reduce target gene expression in both tissue culture cells and animals (Xia 2002).

IMPORTANT

The recombinant adenovirus produced with this kit are classified as a Biosafety Level 2 hazard. We strongly recommend that you implement the recommendations for safe handling of Biosafety Level 2 hazards described in the following CDC/NIH publication: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition May 1999. (See section I.G for information on obtaining this publication.)

Because a significant amount of time and effort are required to produce recombinant adenovirus, we recommend that you identify siRNA sequences with the desired gene silencing activity using one of our other RNAi products. Then create your virus to encode a known optimized siRNA sequence. See the RNA Interference Resource on the web for more information on our RNAi product line:

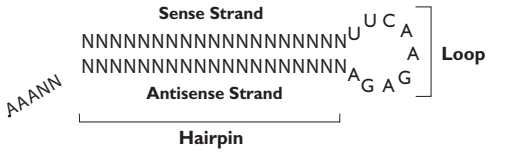
www.ambion.com/techlib/resources/RNAi

B. siRNA and RNA Interference

siRNAs are short, double-stranded RNA molecules that can target mRNAs with complementary sequence for degradation via a cellular process termed RNA interference (RNAi) (Elbashir 2001). Researchers in many disciplines employ RNAi to analyze gene function in mammalian cells. The siRNAs used in early studies were typically prepared in vitro and transfected into cells. More recent publications feature plasmids, and expression cassettes made by PCR, that include RNA polymerase promoters upstream of a hairpin siRNA template. This strategy of introducing an siRNA template into cells, and taking advantage of endogenous RNA polymerases to transcribe dsRNA can also be used with adenoviral vectors (Xia 2002). Upon introduction into mammalian cells, the siRNA template is transcribed, producing an ~19-mer

hairpin siRNA (Figure 1). The hairpin siRNA is recognized by Dicer, the nuclease responsible for activating dsRNAs for the RNAi pathway, and is cleaved to form functional siRNA (Brummelkamp 2002). Adenoviral vectors are episomal, thus, using the p*Silencer* adeno 1.0-CMV System, siRNAs are expressed transiently.

Figure 1. Hairpin siRNA



C. Expression Via the CMV Promoter and RNA Polymerase II

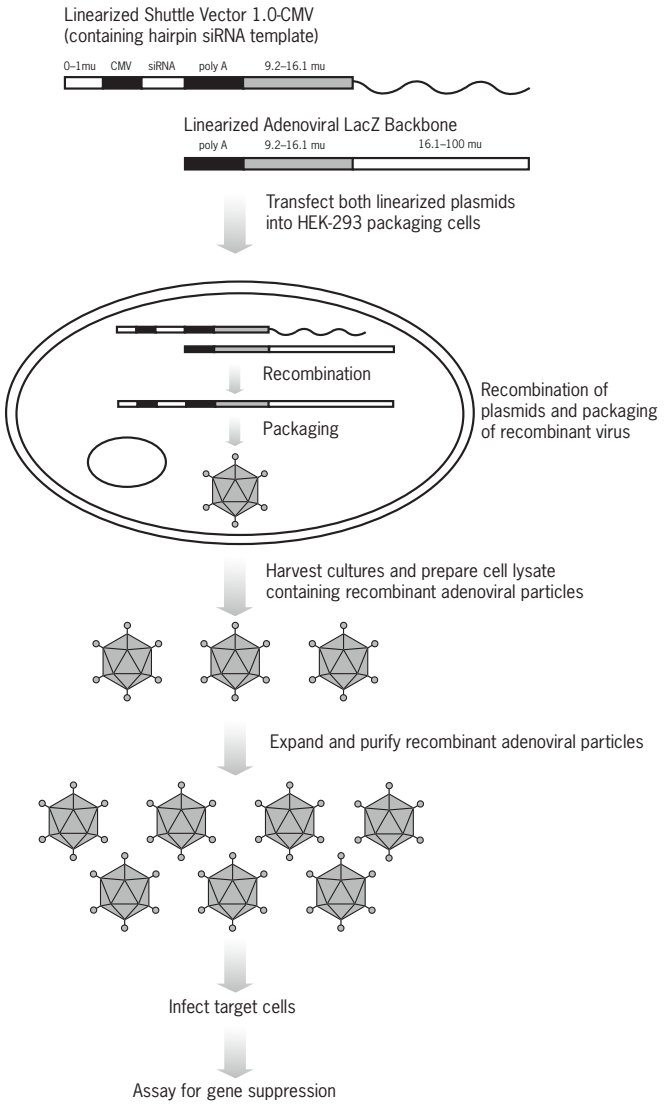
Many commonly used systems for expressing siRNA in cells use an RNA polymerase III (RNA pol III) promoter such as U6 or H1. However it has recently been shown that RNA polymerase II (RNA pol II) promoters are capable of expressing high levels of functional siRNA in cells (Xia 2002). The p*Silencer* adeno 1.0-CMV System employs a modified Cytomegalomavirus (CMV) promoter to drive expression with RNA pol II, and includes a modified simian virus-40 (SV40) polyadenylation signal downstream of the siRNA template to terminate transcription.

The CMV promoter is considered to be a stronger promoter than the other common RNA pol II promoters used in mammalian expression vectors such as Simian virus-40 (SV40) and Rous sarcoma virus (RSV) (Foecking, 1986). In vivo, RNA pol II is primarily responsible for transcription of mRNA within the cell. The CMV promoter has the advantage of being highly active in a broad range of cell types, and it does not interfere with other transcription events as may be the case with the RNA pol III U6 and H1 promoters in some situations.

D. Overview of the p*Silencer* adeno 1.0-CMV System

The recombinant adenovirus produced by this kit is based on a two vector system derived from the wild type Adenovirus serotype 5. Figure 2 shows an overview of how the system works.

Figure 2. Overview of Recombinant Adenovirus Production with the p*Silencer* adeno 1.0-CMV System

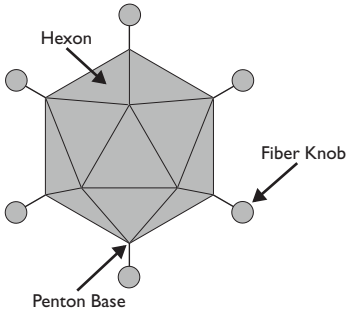


Adenovirus structure

Capsid structure

Adenoviruses share a common architecture consisting of a nonenveloped icosahedral capsid surrounding a linear dsDNA genome. The viral capsid (see Figure 3) is composed of three structural proteins: hexon, fiber, and penton base. Hexon is the major structural component; it forms the 20 facets of the icosahedron. Pentons are complexes of penton base with fiber; they form the 12 vertices (O'Riordan et al. 2003). An in-depth description of adenovirus is beyond the scope of this protocol and therefore we recommend that the reader refer to one of the many excellent sources of adenoviral information such as O'Riordan 2002.

Figure 3. Adenovirus Capsid



Adenoviral genome structure

The adenoviral genome is 36 kb, and is traditionally divided into 100 map units, each representing approximately 360 bp (see Figure 4). Functionally, the genome is divided into two major overlapping regions, early (E) and late (L). The proteins encoded by the early regions E1a and E1b regulate viral replication by transactivating viral gene expression and deregulating the host cell cycle (Schenk 1996). Like most adenoviral vectors, the p*Silencer* adeno 1.0-CMV System is designed to produce recombinant virus that lacks the E1a and E1b regions, so that the virus cannot replicate in normal cells.

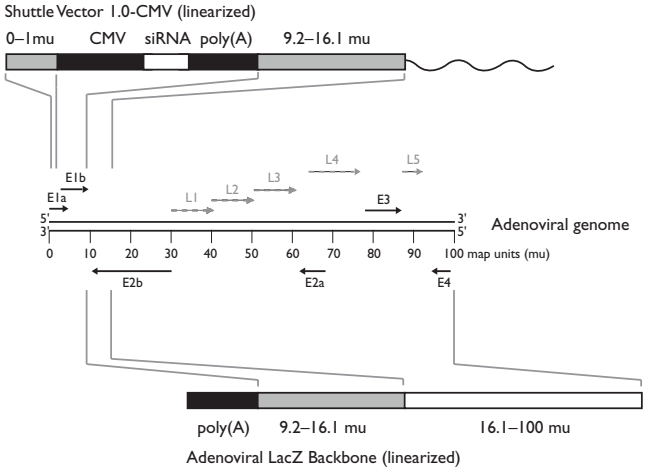
HEK-293 cells are used to produce recombinant virus

In order to make recombinant virus, linearized Shuttle Vector and Adenoviral LacZ Backbone plasmid are cotransfected into the HEK-293 packaging cell line. There are several types of 293 cells, but not all of them are suitable for adenoviral production; we recommend using ATCC Cat #CRL-1573 293 cells. These cells supply the missing E1a and E1b regions of the Adenovirus 5 (Ad 5) genome to enable production of recombinant adenovirus.

Upon transfection into HEK-293 cells, the Shuttle Vector and the Adenoviral LacZ Backbone recombine in the region where they share sequence identity. After recombination, infective (but replication-deficient) recombinant virus are packaged by the HEK-293 cells. Figure 4

below outlines the composition of the plasmids in the p*Silencer* adeno 1.0-CMV System, and their relationship to the adenoviral genome and to each other.

Figure 4. Adenoviral Genome and Structure of the p*Silencer*™ adeno 1.0-CMV System Plasmids



Adenovirus infection and expression of the cloned siRNA template

Adenoviral infection is mediated in part through the coxsackievirus and adenovirus receptor (CAR), and the Integrin αvβ1 and αvβ5 receptors. The ability to infect via either receptor type contributes to the wide range of cell types, including terminally differentiated cells, that can be infected with adenoviral vectors for DNA delivery (Bergelson 1997, Swati 2001). Infection is independent of cell cycle, so recombinant adenovirus made with this kit can be used to express siRNA in both dividing and nondividing cells. Integration of the adenoviral DNA into the host genome is rare, which means that there is little chance of insertional mutagenesis. Because of this feature, and the fact that most recombinant adenoviruses elicit an immune response in animal systems, these viral vectors are appropriate for transient RNA expression.

E. Procedure Overview

1. Clone your siRNA template sequence into Shuttle Vector 1.0-CMV

a. Design sense and antisense DNA oligonucleotides that encode a hairpin siRNA template for your gene of interest, and anneal them to form the siRNA template insert.

b. Ligate the siRNA template insert into the linear Shuttle Vector 1.0-CMV supplied with this kit.

c. Transform *E. coli* with the ligation products, and plate the cells on media containing ampicillin or carbenicillin to select for transformants.

d. Conduct small-scale plasmid preparations of clones, and screen for clones that contain the insert by digestion with *Xho* I and *Spe* I.

e. Perform a 100–500 µg scale, transfection-quality plasmid preparation of positive clones. Sequence the insert with the provided sequencing primers to verify that the hairpin siRNA template has the correct sequence. If desired, test the clone for it ability to induce RNAi by transfecting it into a cell line known to express the target.

2. Generate and purify recombinant Adenovirus

The process for generating adenoviral particles carrying the hairpin siRNA template is shown schematically in Figure 7 on page 17. Briefly, the Adenoviral LacZ Backbone provided with the kit and the Shuttle Vector 1.0-CMV containing the siRNA template are linearized by restriction enzyme digestion, and transfected into HEK-293 cells. In the cells, the Shuttle Vector and the Adenoviral LacZ Backbone recombine and recombinant adenoviral particles are produced. The viral particles are then harvested with a simple freeze-thaw procedure, and the recombinant virus is expanded by infecting five larger cultures of HEK-293 cells. At this point, the recombinant adenovirus is tested for the ability to induce RNAi, and if desired, it can be further expanded and purified by ultracentrifugation through a cesium chloride gradient (or using a commercially available purification product).

F. Reagents Provided with the p*Silencer* adeno 1.0-CMV System and Storage

The p*Silencer* adeno 1.0-CMV System includes reagents to produce five preparations of adenovirus containing a hairpin siRNA template for the target of your choice.

Store at –20°C

Store the p*Silencer* adeno 1.0-CMV System at –20°C in a non-frost-free freezer.

Component list

Amount	Component
20 µL	Shuttle Vector 1.0-CMV (linearized, 0.2 mg/mL)
40 µL	Adenoviral LacZ Backbone (circular, 0.5 mg/mL)
1 mL	DNA Annealing Buffer
3 mL	HEPES-Buffered-Saline (HEBS)
250 µL	2.5 M Calcium Chloride
10 µL	GAPDH Control Insert (80 ng/µL)
20 µL	Negative Control Shuttle Vector (circular, 100 ng/µL)
10 µL	Forward Sequence Primer (10 pmol/µL)
10 µL	Reverse Sequence Primer (10 pmol/µL)

G. Other Required Material

To prepare Shuttle Vector 1.0-CMV containing your hairpin siRNA template

Ligation and transformation

- Two complementary oligonucleotides targeting the gene of interest for RNAi (design and ordering is discussed in section II starting on page 10).
- DNA ligase, ligase reaction buffer, and competent *E. coli* cells are needed to subclone the siRNA inserts.
- Ampicillin or carbenicillin containing plates and liquid media will also be needed to propagate the plasmids.

Plasmid purification reagents

- Small scale plasmid purification reagents to prepare plasmid for restriction and sequence analysis are needed.
- 100–500 µg scale plasmid purification reagents that yield transfection-grade plasmid are needed to purify Shuttle Vector after cloning.

Restriction analysis reagents and equipment

For initial clone verification, the restriction enzymes *Xho* I, *Spe* I, and *Eco*R I or *Hin*d III are used. The reaction products can be visualized by routine agarose gel electrophoresis.

Sequence analysis services

Putative clones containing Shuttle Vector 1.0-CMV with your hairpin siRNA template must be sequenced before using the plasmid for transfection and recombinant adenovirus production.

Restriction enzymes to linearize plasmids for recombinant adenovirus production

Both the Shuttle Vector containing your siRNA template insert, and the Adenoviral LacZ Backbone must be linearized with *Pac* I to prepare them for transfection into HEK-293 cells. If your siRNA template insert contains a *Pac* I restriction enzyme site, *Nhe* I can be used instead.

Biosafety level 2 cell culture facility

The recombinant adenovirus produced with this kit are classified as a Biosafety Level 2 hazard. We strongly recommend that you read the information on safe handling of Biosafety Level 2 hazards in the following CDC/NIH publication: *Biosafety in Microbiological and Biomedical Laboratories, 4th Edition* May 1999.

- This publication is available on the internet at this address: http://bmbl.od.nih.gov
- It is also available for sale by the Superintendent of Documents, US Government Printing Office (GPO), stock #017-040-00547-4. Contact the GPO by telephone (7:30 AM to 4:30 PM EST) at (202) 512-1800, by fax at (202) 512-2250, or write to: Superintendent of Documents, US GPO, Washington D.C. 20402þ

HEK-293 cells, transfection reagents, and supplies

- HEK-293 cells: ATCC #CRL-1573 is recommended.
- 2% FBS culture medium: Dulbecco's Modified Eagle Medium (DMEM) containing 2% heat-inactivated fetal bovine serum (FBS) and 50 Units penicillin/50 µg streptomycin
- 10% FBS culture medium: DMEM containing 10% heat-inactivated FBS and 50 Units penicillin/50 µg streptomycin
- Typical mammalian cell culture facilities and supplies are needed to transfect HEK-293 cells, and to expand recombinant adenovirus.

Viral purification reagents and equipment

Cell lysates containing recombinant adenovirus are used for expansion of virus. Preparation of cell lysates requires typical lab equipment, supplies, and reagents such as sterile tubes, a low speed centrifuge, and Tris solutions (see the procedure in section III.H on page 25 for specific requirements).

Ultracentrifugation equipment and supplies: recombinant adenovirus can be concentrated and purified with two successive ultracentrifugations through cesium chloride (see the procedure in section V.B on page 34 for specific requirements).

H. Related Products Available from Applied Biosystems

T4 DNA Ligase P/N AM2130, AM2132, AM2134	T4 DNA Ligase (E.C. 6.5.1.1) catalyzes the formation of phosphodiester bonds between adjacent 3' hydroxyl and 5' phosphate groups in double-stranded DNA. T4 DNA ligase will join both blunt-ended and cohesive-ended DNA and can also be used to repair nicks in duplex DNA. Includes 10X Ligase Reaction Buffer.
Silencer® siRNAs see our web or print catalog www.ambion.com/siRNA	Ambion *Silencer* Pre-designed siRNAs, Validated siRNAs, and siRNA Libraries are designed with the most rigorously tested siRNA design algorithm in the industry. *Silencer* siRNAs are available for >100,000 human, mouse, and rat targets from our searchable online database. Because of their carefully optimized design, *Silencer* siRNAs are very effective, and they are guaranteed to reduce target mRNA levels by 70% or more. Furthermore, their exceptional potency means that *Silencer* siRNAs effectively induce RNAi at very low concentrations, minimizing off-target effects.
Silencer® siRNA Construction Kit P/N AM1620	The *Silencer* siRNA Construction Kit (patent pending) synthesizes siRNA by in vitro transcription, producing transfection-ready siRNA at a fraction of the cost of chemical synthesis. The *Silencer* siRNA Construction Kit includes all reagents for transcription, hybridization, nuclease digestion, and clean up of siRNA (except gene specific oligonucleotides for template construction).

Antibodies for siRNA Research see our web or print catalog	For select *Silencer* Control and Validated siRNAs, Ambion offers corresponding antibodies for protein detection. These antibodies are ideal for confirming mRNA knockdown results by analyzing concomitant protein levels.
Silencer® siRNA Controls see our web or print catalog www.ambion.com/siRNA	The *Silencer* siRNA Controls are ready-to-use, chemically synthesized, purified siRNAs targeting mRNAs frequently used as internal controls in RT-PCR, Northern blot, RPA, and other experiments designed to monitor gene expression. Corresponding scrambled siRNA negative controls are included with the gene-specific siRNAs. Also offered are Negative Control siRNAs #1, #2, and #3. They are ideal for use in any siRNA experiment as controls for nonspecific effects on gene expression. *Silencer* siRNA Controls are ideal for developing and optimizing siRNA experiments and have been validated for use in human cell lines. The GAPDH and cyclophilin siRNAs are also validated for use in mouse cell lines.

II. Cloning siRNA Template into the Shuttle Vector

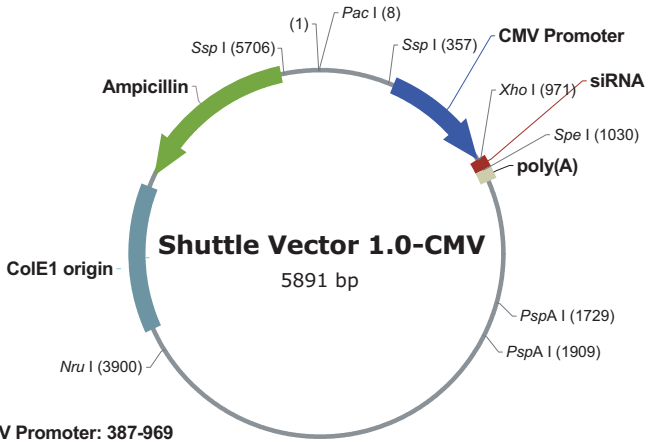
A. Description of the Shuttle Vector

The p*Silencer* adeno 1.0-CMV Shuttle Vector was designed for directional cloning into *Xho* I and *Spe* I restriction enzyme sites. It is supplied in a linear form, ready for ligation of your siRNA template. Figure 5 shows the Shuttle Vector. More specific sequence information, including the sequence of the cloning site, is available on our website by following the link at:

www.ambion.com/catalog/CatNum.php?5790

Once your siRNA template is successfully cloned into the p*Silencer* adeno 1.0-CMV Shuttle Vector, you can test its ability to induce RNAi by transfecting it into a cell line that expresses your target RNA, and measuring target RNA and/or protein levels.

Figure 5. Shuttle Vector 1.0 CMV Map



CMV Promoter: 387-969
Ampicillin: 4830-5689
ColE1 origin: 4021-4768
polyA terminator: 1030-1093

B. Web-Based siRNA Design Resources

Web-based target sequence converter

The easiest way to design hairpin siRNA template oligonucleotides is to enter your siRNA target sequence into the web-based insert design tool at the following address:

www.ambion.com/techlib/misc/psilencer_converter.html

Current, detailed target site selection and hairpin siRNA template design information

Targeted gene silencing using siRNA is a rapidly evolving tool in molecular biology. The instructions in this booklet are based on both the current literature, and on empirical data from scientists at Ambion. For an in depth discussion of information gleaned from the current literature and from experiments performed at Ambion regarding hairpin siRNA stem length and loop design, as well as our most current general recommendations on hairpin siRNA template design, see the "siRNA Design Guidelines" page on our website:

www.ambion.com/techlib/misc/siRNA_design.html

C. siRNA Target Site Selection

Scanning downstream from the known start of transcription of your target mRNA, select a few 21 nucleotide sequences as potential siRNA target sites. Research at Ambion has found that typically more than half of randomly designed siRNAs provide at least a 50% reduction in target mRNA levels and approximately 1 of 4 siRNAs provide a 75–95% reduction. Use the following criteria to help choose target sequences:

- The restriction enzyme *Pac* I will be used to linearize the Shuttle Vector clone before transfection into the packaging cells. For recombination with the Adenoviral LacZ Backbone, it is important to choose a target sequence that ***will not*** be cut with *Pac* I. Alternatively, *Nhe* I can be used for the linearization as long as its recognition site is not present in the target sequence. Note that an *Nhe* I inactivation step will have to be added just before transfection if the Shuttle Vector is linearized with *Nhe* I instead of *Pac* I.
- Target sequences should also be designed ***without*** *Eco*R I or *Hin*d III restriction enzyme recognition sites. After cloning into the Shuttle Vector, we recommend digesting with one of these enzymes to corroborate that your clone is valid. These sites are part of the multiple cloning site that is removed during the manufacturing process, and before sequencing, it is a good idea to confirm that the clone lacks either site.

- Since some regions of mRNA may be either highly structured or bound by regulatory proteins, we generally select siRNA target sites at different positions along the length of the gene sequence. We have not seen any correlation between the position of target sites on the mRNA and siRNA potency.
- Ambion researchers find that siRNAs with 30–50% GC content are more active than those with a higher GC content.
- Compare the potential target sites to the appropriate species' genome database and eliminate from consideration any target sequences with more than 16–17 contiguous base pairs of homology to other coding sequences. We suggest using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST.

D. Hairpin siRNA Template Oligonucleotide Design & Ordering

The 21 nt target sequence(s) will serve as a basis for the design of the two complementary 55-mer oligonucleotides that must be synthesized, annealed, and ligated into the Shuttle Vector for each target gene. These instructions describe how to design the siRNA template oligonucleotides.

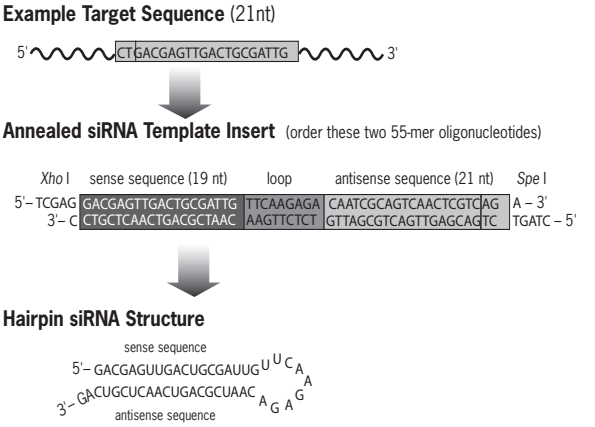
Negative Controls

It is important to plan for negative controls when designing siRNA experiments. One commonly used control is an siRNA with the same base composition as the experimental siRNA, but which lacks sequence identity to genes expressed in the organism under study. Typically, a "scrambled" siRNA is used for these controls. The Negative Control Shuttle Vector supplied with the kit contains an siRNA template sequence that lacks significant homology to the mouse, human, and rat genome databases. For many experimental systems, recombinant adenovirus made with the Negative Control Shuttle Vector can serve as a negative control.

Oligonucleotide design instructions: see Figure 6

Figure 6 below shows an example target sequence, and how to design corresponding hairpin siRNA template oligonucleotides that will be annealed to form the DNA insert. The sense and antisense template oligonucleotides should encode a hairpin structure with a 19-mer stem and a 2 nt overhang derived from the 21 nt mRNA target site. Several different loop sequences have been successfully used in hairpin siRNA templates (Sui 2002, Lee 2002, Paddsion 2002, Brummelkamp 2002, Paul 2002), and we have observed no particular benefit in using one or another. The loop sequence shown here, 5'-TTCAAGAGA-3', is one that we have used successfully. The 5' ends of the two oligonucleotides form the *Xho* I and *Spe* I restriction site overhangs that facilitate efficient directional cloning into the Shuttle Vector.

Figure 6. Example siRNA Template Oligonucleotide Design



Ordering the siRNA template oligonucleotides

Order a 25–100 nM scale synthesis of each oligonucleotide; no extra purification of the oligonucleotides is necessary for use with the Shuttle Vector. It is important, however, that the oligonucleotides are mostly full-length. Choose a supplier that reliably delivers oligonucleotides that are of high quality in terms of sequence and purity.

E. Cloning siRNA Template Insert into the Shuttle Vector

1. Prepare a 1 µg/µL solution of each oligonucleotide

a. Dissolve the siRNA template oligonucleotides in approximately 100 µL of nuclease-free water.

b. Dilute 1 µL of each oligonucleotide 1:100 to 1:1000 in TE (10 mM Tris, 1 mM EDTA) and measure the absorbance in a spectrophotometer set to 260 nm. Calculate the concentration (µg/mL) of the hairpin siRNA oligonucleotides by multiplying the A_{260} by the dilution factor and then by the extinction coefficient (~33 µg/mL).

c. Dilute each oligonucleotide solution to ~1 µg/µL.

2. Anneal the siRNA template oligonucleotides

a. Assemble the 50 µL annealing mixture as follows:

Amount	Component
2 µL	sense siRNA template oligonucleotide
2 µL	antisense siRNA template oligonucleotide
46 µL	1X DNA Annealing Solution

b. Heat the mixture to 90°C for 3 min, then cool to 37°C and incubate at 37°C for 1 hr.

c. The annealed siRNA template insert can either be ligated into the Shuttle Vector immediately, or stored at –20°C.

3. Ligate annealed siRNA template insert into the Shuttle Vector

a. Set up two 15 µL ligation reactions; a plus-insert ligation, and the minus-insert negative control. To each tube, add the following reagents:

plus-insert	minus-insert	Component
1.25 µL	– –	annealed siRNA insert (80 ng/µL)
– –	1.25 µL	DNA Annealing Buffer
11.25 µL	11.25 µL	nuclease-free water
1.5 µL	1.5 µL	10X T4 DNA Ligase Buffer
0.5 µL	0.5 µL	Shuttle Vector 1.0-CMV
0.5 µL	0.5 µL	T4 DNA ligase (5 U/µL)

b. The recommended incubation time and temperature for ligation reactions varies widely among different sources of T4 DNA ligase. Follow the recommendation provided by the manufacturer of your DNA ligase. Using Ambion T4 DNA Ligase (P/N AM2134), incubate overnight (≥12 hr) at 16°C.

4. Transform *E. coli* with the ligation products

a. Transform an aliquot of *E. coli* cells with the plus-insert ligation products, and transform a second aliquot with the minus-insert ligation products. Use an appropriate amount of ligation product according to how the competent cells were prepared and the transformation method. (For chemically competent cells, we routinely transform with 5 µL of the ligation reaction.)

b. Plate the transformed cells on LB plates containing 50–200 µg/mL ampicillin or carbenicillin and grow overnight at 37°C. Generally it is a good idea to plate 2–3 different amounts of transformed cells so that at least one of the plates will have distinct colonies.
Always include a nontransformed competent cell control: this negative control is a culture of your competent cells plated at the same density as your transformed cells.

c. Examine each plate and evaluate the number of colonies promptly after overnight growth at 37°C (or store the plates at 4°C until they are evaluated).

5. Expected results

Nontransformed control culture:
The nontransformed control culture should yield no colonies (indicating that the ampicillin or carbenicillin in the culture medium is effective at inhibiting the growth of *E. coli* that do not contain the Shuttle Vector 1.0-CMV plasmid).

Plus- and minus-insert ligation transformations:
Identify the dilution of plus- and minus-insert ligation transformations that yield well-spaced (countable) colonies, and count the colonies on those plates. The minus-insert ligation will probably result in some ampicillin resistant colonies (background), but ***the plus-insert ligation should yield 2–10 fold more colonies than the minus-insert ligation***. (Remember to take the dilution into account when calculating the proportion of background colonies.)

6. Identify clones with the siRNA template insert

Pick clones, isolate plasmid DNA, and digest the plasmid with *Xho* I and *Spe* I, and run the digestion products on a 12% acrylamide gel to confirm the presence of the ~57 bp siRNA template insert. To confirm that the insert is not an artifact of the plasmid manufacturing process, digest the plasmid DNA with *Eco*R I or *Hin*d III to make sure that its restriction enzyme site has been eliminated. Analyze the products of this digestion on a typical agarose gel alongside a control lane containing undigested plasmid for comparison. The table below summarizes the expected results when an siRNA template insert has been successfully cloned into the Shuttle Vector.

Enzyme(s)	Expected Result
Xho I, *Spe* I	~57 bp insert + ~5800 kb vector band
*Eco*R I or *Hin*d III	No effect: the plasmid should *not* be digested. These sites are removed during linearization of the Shuttle Vector. If your clone is digested with either enzyme, then it is probably an artifact of the manufacturing process.

Links to the Shuttle Vector 1.0-CMV restriction map and the entire plasmid sequence are provided at:
www.ambion.com/catalog/CatNum.php?5790

7. Prepare ~100–500 µg of the verified plasmid, and sequence the insert using the primers provided with the kit

Culture a larger volume of the *E. coli* clone containing the verified plasmid, and purify 100–500 µg of transfection-quality plasmid. Commercially available plasmid purification products designed for preparation of DNA for transfection can be used to obtain Shuttle Vector plasmid DNA for use in this procedure.

Use the Forward and Reverse Sequence Primers (supplied) to sequence the insert. It is important to verify that the siRNA template insert has the expected sequence before proceeding with recombinant adenovirus production.

8. (optional) Transfect the Shuttle Vector containing your siRNA template into cells to verify that it can induce RNAi

At this point, you may want to test your Shuttle Vector clone for the ability to induce the desired siRNA effect. This can be done by transfecting the purified plasmid into a cell line that expresses the target gene, and looking for gene knockdown.

We recommend using Ambion siPORT™ *XP-1* transfection agent (P/N AM4506, AM4507) to deliver Shuttle Vector 1.0-CMV plasmids into mammalian cells for the purpose of testing whether the Shuttle Vector alone can induce RNAi. Follow the instructions for using siPORT *XP-1* provided with the product. Check our web site for more information on siPORT *XP-1*.

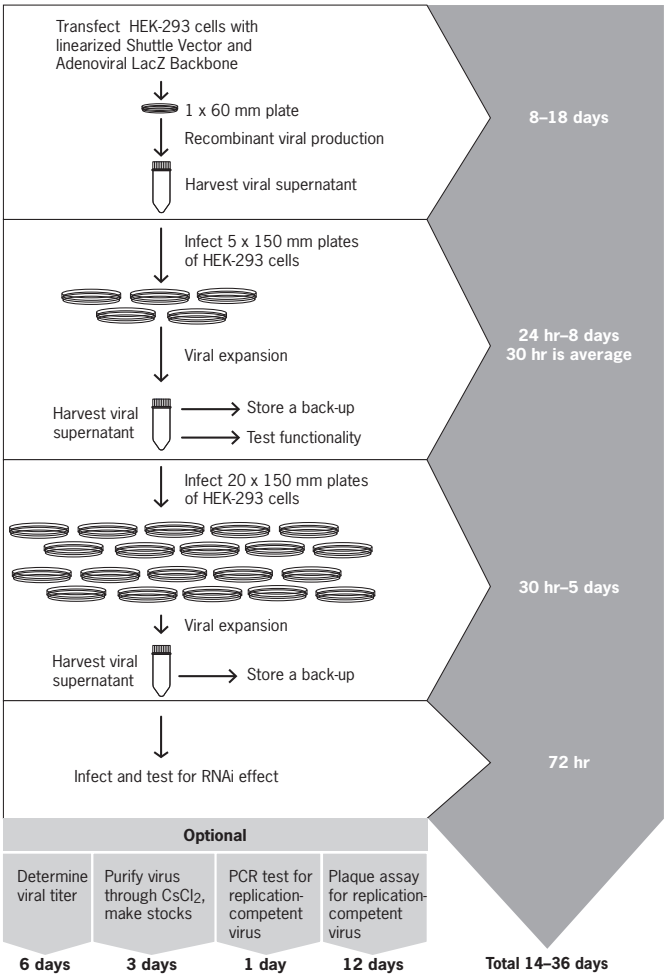
www.ambion.com/catalog/CatNum.php?4506

Other transfection agents suitable for use with plasmids can alternatively be used to transfect cells with the Shuttle Vector. Follow the transfection instructions provided with the reagent.

III. Recombinant Adenovirus Production

A. Overview of Recombinant Adenovirus Production

Figure 7. Recombinant Adenovirus Production



B. Safe Handling of Adenovirus

The recombinant adenovirus produced using this kit are capable of infecting any cell type with the required receptors. The NIH has classified them as a Biosafety Level 2 hazard. We strongly recommend that you read the information on safe handling of Biosafety Level 2 hazards in the following CDC/NIH publication: *Biosafety in Microbiological and Biomedical Laboratories, 4th Edition* May 1999.

- This publication is available on the internet at this address: http://bmbl.od.nih.gov
- It is also available for sale by the Superintendent of Documents, US Government Printing Office (GPO), stock #017-040-00547-4. Contact the GPO by telephone (7:30 AM to 4:30 PM EST) at (202) 512-1800, by fax at (202) 512-2250, or write to: Superintendent of Documents, US GPO, Washington D.C. 20402.

C. Linearize Plasmids

The Adenoviral LacZ Backbone and the Shuttle Vector containing your siRNA template must be linearized before transfection in order to get efficient recombination of the plasmids in the HEK-293 packaging cell line.

- We recommend digesting the plasmids in separate reactions.
- Each restriction enzyme digestion reaction volume must be 50 µL or less. Reaction volumes >50 µL could compromise transfection efficiency.

Digest 4 µg Adenoviral LacZ Backbone with *Pac* I at 37°C for 1.5 hr

The Adenoviral LacZ Backbone plasmid is supplied at 0.5 µg/µL, so digest 8 µL of the plasmid with the restriction enzyme *Pac* I to obtain 4 µg of linearized plasmid. We typically conduct the digestion in a 20 µL reaction.

Digest 15 µg of your Shuttle Vector clone with *Pac* I (or with *Nhe* I) at 37°C for 1.5 hr

Check the sequence of your siRNA insert for *Pac* I and *Nhe* I restriction enzyme recognition sites. Use *Pac* I to linearize the Shuttle Vector containing your siRNA template insert unless the insert contains a *Pac* I site. In this case linearize with *Nhe* I instead (assuming that the insert will not be cut by *Nhe* I—inserts must be designed to lack either *Pac* I or *Nhe* I recognition sites).

We typically conduct this digestion in a 50 µL reaction.

Pac I digestion reaction products can be transfected without any further treatment. If the Shuttle Vector was linearized with *Nhe* I however, heat inactivate the restriction enzyme at 65°C for 20 min to prevent the enzyme from digesting the Adenoviral LacZ Backbone plasmid.

D. Transfection into HEK-293 Cells to Produce Recombinant Adenovirus

IMPORTANT

We strongly recommend using the transfection agents included with the kit for this cotransfection. Other transfection agents may efficiently deliver the linearized plasmid DNA to the cells, but production of recombinant virus will be compromised.

The linearized Shuttle Vector containing your siRNA template, and the linearized Adenoviral LacZ Backbone plasmid are mixed and transfected in HEK-293 cells using a calcium phosphate method. Inside the cells, recombination of the two plasmids will occur, and the HEK-293 cells will produce recombinant adenovirus. As virus is produced, more cells will become infected with the recombinant adenovirus, and plaques will form in the layer of HEK-293 cells as infected cells detach from the plate.

Culture media for transfection and infection

In this procedure, HEK-293 cells are grown in either "10% FBS" culture medium, or "2% FBS" culture medium. Both media consist of Dulbecco's Modified Eagle Medium (DMEM) with the optional addition of 50 Units penicillin/50 µg streptomycin.

- 10% FBS culture medium contains 10% heat-inactivated fetal bovine serum (FBS).
- 2% FBS culture medium contains 2% heat-inactivated FBS.

1. Plate HEK-293 cells in a 60 mm dish 24 hr before transfection

24 hr before transfection, plate ~1.5 x 10^6 HEK-293 cells in 10% FBS culture medium in a 60 mm tissue culture dish so that they will be about 60% confluent on the day of transfection.

2. Prepare transfection complex

a. To make the ***Backbone plasmid complexing mixture***, add the following to a 12 x 75 mm polystyrene tube, and vortex briefly:

Amount	Component
500 µL	HEPES-Buffered-Saline (HEBS)
4 µg	Adenoviral LacZ Backbone linearized with *Pac* I

It is a good idea to prepare a master mix of the backbone plasmid complexing mixture if more than one Shuttle Vector clone will be transfected in the experiment.

b. Add 15 µg of linearized Shuttle Vector clone DNA to the Backbone plasmid complexing mixture, and vortex briefly.

c. Add 25 µL of 2.5 M Calcium Chloride, and vortex briefly.

d. Incubate the complete transfection complex for 25 min at room temp.

3. Replace the culture medium with 2 mL 2% FBS medium, add transfection complex, and incubate 4 hr

a. Aspirate the medium from the HEK-293 cells, and ***immediately*** replace it with 2 mL 2% FBS culture medium. Work quickly so that the cells do not dry out during this process. HEK-293 cells are dislodged easily so add the medium to the side of the dish slowly, and allow it to roll over the cells.

b. Pipet the transfection complex onto the plate in a slow spiral motion, and incubate the cells in normal cell culture conditions (37°C, 5% CO_2) for 4 hr.

4. Wash cells, and culture overnight in 10% FBS culture medium

a. After 4 hours, remove the culture medium, and carefully wash the transfected cells 2–3 times with either culture medium or with PBS to remove the visible precipitate. Be careful to avoid lifting the cells, and be sure to keep the cells moist at all times while rinsing them.

b. Add 4 mL 10% FBS culture medium and incubate overnight in normal cell culture conditions.

5. Change medium and culture cells for 1 week

a. The day after transfection, remove the culture medium, and ***immediately*** replace it with 4 mL 10% FBS culture medium.

b. Incubate in normal cell culture conditions for 7 days.

6. Harvest cultures when >60% of the cells are detached or on day 18 after transfection

Feed the cells with 1 mL 2% FBS culture medium on days 8, 11, and 15 (if they haven't been harvested)

Beginning on day 8, you may begin to see adenoviral foci. As cells become infected they will ball-up and float off of the plate, making clear spaces in the monolayer of HEK-293 cells. In many cases, the foci trail of to one side, giving them a characteristic comet shape (see Figure 9 on page 38).

- Harvest cultures when >60% of the cells have become infected and detached from the culture plate. The virus harvest procedure is in section III.H on page 25.
- Check the percentage of detached cells 8 and 11 days after transfection; if they are not ready to be harvested (<60% detached from the culture plate) check ***every*** day after day 11.
- Feed the cells by adding 1 mL 2% FBS culture medium on days 8, 11, and 15 after transfection if they haven't already been harvested.
- On day 18 conclude the experiment. Follow the recommendations below according to the appearance of the plates:
  - If >60% of the cells were detached from the plate, harvest the culture, and use the resulting lysate to infect ***five*** 150 mm culture plates (see below).
  - If <60% of the cells were detached from the plate, harvest the culture, and use the resulting lysate to infect ***one*** 150 mm culture plate (see below).
  - If there are no visible plaques either discard the culture and repeat the transfection, or harvest the culture and use the resulting lysate to infect one 150 mm culture plate (see below).

7. Use the harvested virus for the initial expansion of adenovirus

After harvesting the culture as described in section III.H on page 25, use the cell lysate to infect five 150 mm culture plates following the instructions in section III.E below. We do not recommend testing the virus harvested at this stage for siRNA function because low viral titer can make it difficult to see an RNAi effect, and remaining plasmid DNA can express siRNA and lead to false positives.

E. Initial Expansion of Recombinant Adenovirus

1. Plate five 150 mm dishes of HEK-293 cells 24 hr before infection

24 hr before infection, plate ~1.2 x 10^7 HEK-293 cells per 150 mm tissue culture dish in 10% FBS culture medium, so that they will be approximately 60% confluent on the day of infection. Prepare 5 plates for this initial virus expansion.

2. Dilute 1 mL adenovirus into 75 mL 2% FBS culture medium

Dilute 1 mL of the cell lysate containing recombinant adenovirus (harvested from the initial transfection) into 75 mL 2% FBS culture medium (DMEM with 2% FBS and 1% pen/strep). Swirl gently or invert the container several times to mix thoroughly.

3. Infect cells by replacing their culture medium with medium containing virus

a. Aspirate and discard the culture media from the plates, and ***immediately*** replace it with 15 mL of the medium containing recombinant adenovirus.

b. Incubate in normal cell culture conditions for 24 hr.

4. Harvest cultures when >60% of the cells are detached

At 24 hr, look for viral foci caused by infected cells balling up and detaching from the surface of the plate. As in the initial transfection, harvest cultures when >60% of the cells are detached from the culture plate using the virus harvest procedure described in section III.H on page 25.

Typically cultures will be ready to harvest about 30 hr after infection. Check plates daily, and if they haven't already been harvested, feed the cells by adding 1 mL 2% FBS culture medium on days 8, 11, and 15 after infection.

Conclude the experiment on day 18 at the latest by harvesting the cultures.

- If, at day 18, <60% of the cells were detached from the plate, your harvested virus prep will contain fewer infective recombinant adenovirus particles than cultures which had 60% or more of the cells detached. Therefore, you will need to use more of your lysate for testing siRNA functionality or for the large scale virus expansion (use approximately 2.5-fold more lysate than what is recommended in the procedures).
- If, at day 18, there are no visible plaques, and no detectable detachment of cells from the plate, you should repeat the procedure for recombinant virus production starting at section III.D (page 19).

F. Testing Recombinant Adenovirus for siRNA Functionality

After harvesting your recombinant adenovirus from the initial expansion on five 150 mm plates, it is a good idea to test whether the virus can induce the desired RNAi response by infecting cells that express the target gene, and looking for a reduction in target mRNA and/or protein levels. Ideally you should purify virus from the cell lysate, using the ultracentrifugation method described in section V.B on page 34, or an adenovirus purification method of your choice. With purified virus, the effect of infection with known amounts of your experimental adenovirus and your scrambled negative control adenovirus can be compared to determine if gene silencing is induced. General recommendations for infection with recombinant adenovirus are provided in section III.I on page 26. We include this alternative method for infecting cells with a crude cell lysate for users who do not want to purify virus to screen for siRNA functionality.

Recommended cell lines

Typically experiments to test recombinant adenovirus are done using a cell line that contains the CAR receptor, is capable of being infected by adenovirus, and which expresses the gene that is targeted for RNAi. The cell lines with "good" infectability according to the chart below are all good choices for this testing; we do not recommend using the HEK-293 packaging cell line.

Table 1. Infectability of Selected Cell Lines with Adenovirus

Cell Line	Infectability	Cell Line	Infectability
HeLa	Good	Myoblasts	Good
A549	Good	Keratinocytes	Poor
293	Good	Suspension cells	Poor
MCF7	Good	HUVEC	Poor
HepG2	Good	B-cells	No

1. Plate cells into 20 wells of a 24 well plate 24 hr before infection

Plate cells in a 24 well plate so that they will be at ~50% confluency on the following day. For HeLa cells, plate 50,000 cells.

This experimental setup includes 10 samples that will be infected with the recombinant adenovirus, and 10 samples that will be infected with a scrambled negative control recombinant adenovirus. If you don't have a recombinant adenovirus with a scrambled version of your siRNA template, you could include a nontreated negative control instead.

2. Make ten, 10 fold serial dilutions of the virus

a. Assemble 2 sets of ten 1.5–2 mL microfuge tubes with 1350 µL of reduced serum medium appropriate for your cell line (we use DMEM with 2% FBS and 1% pen/strep for HeLa cells). One set is for the experimental recombinant adenovirus, and the other set is for the negative control recombinant adenovirus.

b. Label the tubes 10^{-1}, 10^{-2}, etc., through 10^{-10}.

c. Add 150 µL of cell lysate containing recombinant adenovirus (harvested from the initial viral expansion) to the tube labeled 10^{-1} and briefly vortex.

d. Take 150 µL from the tube labeled 10^{-1}, add it to the tube labeled 10^{-2}, and briefly vortex.

e. Repeat this process until all the tubes contain a dilution of recombinant adenovirus.

3. Infect cells by replacing their culture medium with medium containing virus

a. Aspirate and discard the culture media from the plates, and ***immediately*** replace it with 1 mL of the medium containing recombinant adenovirus. If your experiment does not include a set of infections with a negative control recombinant adenovirus, include a noninfected negative control culture instead (add culture medium without virus).

b. Incubate in normal cell culture conditions for 4 hr.

c. Remove the media containing virus, and replace it with normal growth media. For HeLa cells normal growth medium is typically DMEM with 10% FBS and 1% pen/strep.

d. Incubate in normal cell culture conditions for 48 hr.

4. Assay for gene knockdown

Test each dilution infected with experimental recombinant adenovirus for a reduction in target gene expression compared to the culture infected with the same dilution of negative control adenovirus. Assay for gene knockdown using your preferred assay (e.g. Northern blot, Western blot, immunofluorescence, real-time RT-PCR).

HeLa cells are sensitive to adenoviral infection. If the titer of your recombinant adenovirus is high, then cells infected with the least dilute virus may die. If the titer of your virus is very low, then only the cells infected with the least dilute virus may show any gene knockdown.

G. Large Scale Expansion of Virus

Once your recombinant adenovirus has been functionally verified you may want to conduct a large scale virus expansion.

1. Plate twenty 150 mm dishes of HEK-293 cells 24 hr before infection

24 hr before infection, plate ~1.2 x 10^7 HEK-293 cells per 150 mm tissue culture dish in 10% FBS culture medium, so that they will be approximately 60% confluent on the day of infection. Prepare 20 plates for this large scale virus expansion.

2. Dilute adenovirus into 302 mL 2% FBS culture medium

Starting with a cell lysate harvested from the initial expansion on 5 plates:

In a sterile 500 mL flask, dilute 1 mL of the cell lysate containing recombinant adenovirus into 302 mL of 2% FBS culture medium (DMEM with 2% FBS and 1% pen/strep). Pipette vigorously, or gently invert the flask several times to mix thoroughly.

Starting with a purified viral prep:

You will want to infect at a multiplicity of infection (MOI) equal to 2.5. Since you will be infecting 20 plates, each containing approximately 2.5 x 10^7 HEK-293 cells, there will be a total of 5 x 10^8 cells. Multiplied by 2.5, you will need about 1.25 x 10^9 plaque forming units (pfu) of your recombinant virus diluted in 2% FBS culture medium (DMEM with 2% FBS and 1% pen/strep) for a total volume of 303 mL. For this virus expansion, you can assume that 100 viral particles (pt) equals 1 plaque forming unit (pfu).

3. Infect cells by replacing their culture medium with medium containing virus

a. Aspirate and discard the culture media from the plates, and ***immediately*** replace it with 15 mL of the medium containing recombinant adenovirus.

b. Incubate in normal cell culture conditions for 24 hr.

4. Harvest cultures when >60% of the cells are detached

Check for viral foci, harvest, and feed the cells as described in section *E. Initial Expansion of Recombinant Adenovirus*, step 4 on page 21. Typically cultures will be ready to harvest about 30 hr after infection.

H. Adenovirus Harvest Procedure

1. Collect cells and recover the culture media

a. Transfer cells and media to a conical tube(s). Do not trypsinize the cells; recover cells that remain adhered to the plate by pipetting up and down. Pool material from cultures containing the same recombinant adenovirus.

b. Collect cells by low speed centrifugation (~150 x g) for 10 min. It is important to spin the cells at a low speed so that they do not rupture. Remove the culture medium.
If desired, freeze the culture medium at –80°C. It will have some recombinant adenovirus, but the titer will be much lower than that of the cell lysate. Many researchers save the harvested culture medium as a backup viral stock; it can be thawed once and used to infect cells if needed.

2. Wash cells in 1X PBS

a. Resuspend cells in 1 mL 1X PBS per plate harvested, and pool cells containing the same recombinant adenovirus.

b. Centrifuge 5 min at ~150 x g, and aspirate and discard the PBS without disturbing the pellet.

c. Resuspend the cell pellet(s) in 10 mM Tris pH 8.1; the table below shows the amount of Tris to use with different sources of transfected or infected cells. Pool material from cultures containing the same recombinant adenovirus.

	10 mM Tris
initial transfection	1 mL
five 150 mm plates	5 mL
twenty 150 mm plates	12 mL

3. Freeze/thaw the cells 3 times, centrifuge, and recover the adenovirus

a. Freeze the cells in a dry ice/ethanol bath, and thaw at 37°C with occasional mixing to lyse the cells. Repeat this freeze/thaw treatment twice more for a total of 3 freeze/thaw treatments.

b. Centrifuge the infected cell lysate at 1500 x g for 10 min.

c. Transfer the supernatant to a fresh tube. This cell lysate contains the recombinant adenovirus; store it at –20°C. Cell lysates can be stored for up to a year when they will be used for viral expansion. However, if they will be used for gene silencing experiments without further purification, they may lose activity over time in storage.

Source of lysate	Next step
Initial transfection	• Save the culture medium as a backup source of virus. • Use the lysate for the initial 5 plate viral expansion (section III.E on page 21).
Five 150 mm plates	• Archive 500 µL of the lysate as a backup source of virus. • *If you need purified virus for your experiments:* test the virus for its ability to induce RNAi (section III.F) before proceeding to the large scale virus expansion. • *If an infected cell lysate is sufficiently pure for your experiments:* proceed directly to the large scale virus expansion (section III.G).
Twenty 150 mm plates	• *If you need purified virus for your experiments:* purify the virus using the ultracentrifugation procedure in section V.B or with a purification method of your choice. • *If an infected cell lysate is sufficiently pure for your experiments:* proceed directly to your experiments (see below for general recommendations).

I. Using Recombinant Adenovirus in RNAi Experiments

Target cells/organism

Adenovirus can be used to infect many different higher organisms. The method used to infect animals is entirely dependent on the animal, and on the purpose of the experiment. Refer to the literature to find guidelines for adenovirus infectability and methods for your experimental system.

Both primary and transformed cell lines from many species can also be infected with adenovirus (see Table 1 on page 22 for a partial list). We suggest that you check the literature to find guidelines for adenovirus infectability and methods for your target cells or cell line. Cells that cannot be infected with adenovirus can in most cases be transfected with the virus instead; this is called "cadfection". See Fasbender et al. 1998 for more information on cadfection.

Determine how much adenovirus to use

It is important to optimize the amount of recombinant adenovirus to use for the best expression of your hairpin siRNA. Typically the optimal amount of virus is that which provides the highest level of gene silencing with the least cellular toxicity.

Infected cell lysate

If an infected cell lysate is sufficiently pure for your experiments, conduct a titration experiment as described in section III.F on page 22 to determine the optimal amount of lysate to use. Note that for injection into animals, highly purified virus is typically used to minimize toxicity.

Purified virus

If your application requires titered virus to begin optimization experiments, the instructions for measuring viral titer empirically are provided in section V.E on page 37. Alternatively, use a commercially available product to determine the viral titer, or roughly estimate that the titer will be 100-fold lower than the viral particle (pt) value calculated from the A_{260} reading (section V.C on page 36).

Using the viral titer, expressed as plaque forming units (pfu), plan optimization experiments to test multiplicity of infection (MOI) values from 10–600. The optimal MOI will depend on the cell line or organism, and the abundance of the target message, however, 10–600 is typically a good range to start with.

$$\text{MOI} = (\text{pfu})(\text{cell number})$$

Example MOI calculation:

- You want to infect 5×10^5 cells at an MOI of 80.
- The titer of your viral stock is: 1×10^{12} pfu/mL.

$$80 \text{ MOI} \times 5 \times 10^5 \text{ cells} = 4 \times 10^7 \text{ pfu}$$

$$\frac{4 \times 10^7 \text{ pfu}}{1 \times 10^{12} \text{ pfu/mL}} = 4 \times 10^{-5} \text{ mL or } 4 \text{ µL of a 1:100 dilution of the viral stock}$$

Collecting data

Evidence of gene silencing can be obtained by measuring a reduction in mRNA levels, protein levels, or both. The method most appropriate for your system will depend on the experimental system and on the tools available. Check this web-based RNAi resource for ideas:

www.ambion.com/techlib/resources/RNAi

IV. Troubleshooting

The first step in troubleshooting most problems with the kit is to conduct the control reactions using the GAPDH Control Insert; this is described in section A below. Before doing this, however, you may want to first scan this chapter of the Protocol to see if your problem is described.

A. Making Control Recombinant Adenovirus

1. Description of the GAPDH Control Insert

The GAPDH Control Insert (80 ng/µL) is a double-stranded DNA fragment with *Xho* I and *Spe* I sticky ends surrounding an siRNA template that targets the GAPDH mRNA in human cell lines tested.

2. Ligation instructions

a. Dilute 2 µL of the GAPDH Control Insert with 18 µL nuclease-free water for a final concentration of 8 ng/µL.

b. Ligate 1 µL of the diluted GAPDH Control Insert into the Shuttle Vector 1.0-CMV Shuttle Vector using the instructions starting at step II.E.3.a on page 14.

3. Expected result of the positive control ligation and *E. coli* transformation

If the ligation reaction and subsequent *E. coli* transformation procedure are functioning properly, then the ligation reaction with the GAPDH Control Insert (the plus-insert reaction) should provide 2–10 times as many colonies as the minus-insert ligation reaction. You should also isolate plasmid DNA and verify putative clones by the restriction digests described in step II.E.6 on page 15.

4. Using the Negative Control Shuttle Vector to produce Negative Control adenovirus

The Negative Control Shuttle Vector supplied with the kit is a circular plasmid encoding a hairpin siRNA whose sequence is not found in the mouse, human, or rat genome databases; nor is it found in the adenoviral genome. It is provided at 100 ng/µL, and can be propagated in *E. coli* to prepare plasmid DNA for cotransfection and recombinant Negative Control adenovirus production. To do this, transform the Negative Control Shuttle Vector into competent *E. coli* cells, grow the cells in medium containing 50–200 µg ampicillin, and isolate plasmid DNA using a method that yields transfection-quality DNA. Then produce Negative Control adenovirus by using the Negative Control Shuttle Vector DNA in the procedure for adenovirus production in section III starting on page 17.

5. GAPDH Control adenovirus production

If desired, the Shuttle Vector with the GAPDH Control Insert can be used to create a recombinant GAPDH Control adenovirus. To do this, use the Shuttle Vector with the GAPDH Control Insert in the procedure for adenovirus production in section III starting on page 17.

6. Expansion and purification of control adenovirus

Proceed with adenovirus expansion through the initial viral expansion as described in section III.E on page 21.

Purify control recombinant adenovirus using the same procedure as used for your experimental adenovirus.

B. Using the Kit Controls in Your Experiments

1. Negative Control adenovirus

Negative Control adenovirus made from the Negative Control Shuttle Vector supplied with the kit can be used to identify effects of introducing recombinant adenovirus into your experimental system. Samples infected with the recombinant adenovirus expressing your target-specific siRNA can be compared to cells infected with the Negative Control recombinant adenovirus.

2. GAPDH Control adenovirus

GAPDH Control adenovirus expresses a hairpin siRNA which targets GAPDH in the human cell lines tested. Knockdown of GAPDH slows the growth rate of the cells and reduces the rate of cell proliferation of most cell types. To assess whether siRNA-mediated gene silencing is occurring, levels of GAPDH RNA, levels of GAPDH protein, and/or cell proliferation can be monitored.

Either of the following assays for assessing siRNA-mediated reduction in GAPDH gene expression can be used:

a. **Quantitate mRNA levels by Northern analysis or RT-PCR.** GAPDH mRNA levels are typically reduced 50–90% 48 hr after infection.

b. **Analyze protein levels by Western blot, immunohistochemistry, or immunofluorescence.** GAPDH protein levels are typically reduced 50–90% 48 hr after infection.

C. Problems Cloning into the Shuttle Vector

If you get few or no Shuttle Vector clones containing your insert, or if the minus- and plus-insert transformations yield equal numbers of colonies, these troubleshooting suggestions may help.

1. Low quality competent cells

Cells could either be nonviable or exhibit low transformation competency. This can be tested by transforming a circular plasmid that has been used successfully in the past.

2. Competent *E. coli* cells were handled poorly

Competent cells tend to be fragile, so handle them gently throughout the transformation and plating process.

3. Poor ligation efficiency

If the ligation reaction (section II.E.3 on page 14) is inefficient, then there will be relatively few Shuttle Vector plasmids to transform. Possible causes of poor ligation efficiency include:

a. The concentration of the annealed siRNA template insert is lower than expected.
Evaluate ~5 µL of the insert DNA (from step II.E.2.c on page 14) using a 12% native polyacrylamide gel and compare its ethidium bromide staining to bands from a molecular weight marker or another standard of known concentration.

b. The ligase or ligase reaction buffer have become inactive.
Test your ligation components using another vector and insert or replace your ligation components and retry the siRNA insert cloning.

c. One or both of the hairpin siRNA template oligonucleotides have high levels of less than full-length products.
The size of oligonucleotides can be evaluated on a 12% native polyacrylamide gel.

d. The oligonucleotide annealing reaction was ineffective.
A low concentration of one of the oligonucleotides or incomplete denaturation of individual oligonucleotides could have reduced the relative amount of dsDNAs.

Compare the annealed siRNA template insert to each of the single-stranded oligonucleotides using native 8–12% polyacrylamide gel electrophoresis. If the annealed insert has bands corresponding to the single-stranded oligonucleotides, then equalizing the concentrations of the single-stranded DNA molecules and heat-denaturing at a higher temperature during siRNA insert preparation (step II.E.2 on page 13) might improve the percentage of dsDNA products. Alternatively, in some cases, gel purifying the band corresponding to annealed insert may result in better ligation.

e. Ligation inhibitors in the oligonucleotide preparations
EDTA and high concentrations of salts or other small molecules can inhibit ligation efficiency. Ethanol precipitate the oligonucleotides prior to using them in the cloning procedure (either before or after annealing).

f. Incompatible ends on the insert
Verify that the sequences of the hairpin siRNA template oligonucleotides include 5' *Xho* I and 3' *Spe* I overhanging sequences for cloning (see Figure 6 on page 13).

4. The concentration or activity of the ampicillin is too low or high

If there are large numbers of clones derived from both minus- and plus-insert ligations, then confirm that the ampicillin is active and at 50–200 µg/mL in the medium. If there are low numbers of clones for each, try transforming a plasmid that has an ampicillin resistance gene and confirm that the ampicillin concentration in the plates is not too high to allow the growth of transformed cells.

D. Troubleshooting Recombinant Virus Production

1. After transfection, the HEK-293 cells never detach from the culture plate

a. Transfection failed.
Try simply repeating the transfection. There are many variables involved in efficient transfection of mammalian cells and occasionally transfection may be poor or may fail entirely.

b. Transfection efficiency was poor.
Very low efficiency transfection generally results in production of little or no recombinant virus. If some recombinant adenovirus was generated, albeit at a level much lower than expected, then an additional expansion step in a single 60 mm plate of HEK-293 cells may be beneficial.

If transfection efficiency is very low, recombination may not occur, and thus no virus will be produced. You may want to consider transfecting a reporter gene such as GFP, Luciferase, β-gal (see section D.4 on page 33), CAT, or SEAP using the transfection reagents included with this kit to analyze your transfection efficiency.

Transfection efficiency is influenced by many factors including the condition and density of the cells, the quality of the DNA, the quality of the transfection reagents and the ratio of DNA to transfection reagents. The parameters recommended in this protocol were optimized empirically, and tested many times; they should work if followed exactly as written in section III.D starting on page 19.

c. The Shuttle Vector clone DNA quality is poor.
The quality of the Shuttle Vector DNA is critical for efficient recombination in the packaging cells. DNA prepared using commercially available plasmid purification kits has been used successfully many times, however, if you are experiencing difficulty it may be necessary to purify your DNA using a cesium chloride banding protocol. Detailed protocols for cesium chloride plasmid purification can be found in comprehensive laboratory protocol reference books such as *Molecular Cloning*, Sambrook et al. or *Protocols in Molecular Biology,* Ausubel et al.

d. The plasmid DNA used for transfection was not linear.
Virus production requires recombination of the Adenoviral LacZ Backbone and the Shuttle Vector containing your siRNA template insert to occur in the packaging cell line. For efficient recombination to occur, both plasmids must be in a linear form when transfected into the packaging cells. Instructions for plasmid linearization are in section III.C on page 18.

- You can check your restriction digests by simply running a small sample (~250–500 ng) of the digested plasmid DNA on an ethidium bromide stained 0.8% agarose gel.

- If you used *Nhe* I to linearize your Shuttle Vector clone, be sure to inactivate the *Nhe* I by heating to 65°C for 15 min before using it for transfection complex formation.

e. Serum (FBS) levels are >2% in the culture medium during transfection.
FBS can inhibit viral expansion by binding to viral particles and preventing them from infecting cells. To minimize this effect, we recommend using media containing only 2% FBS for viral expansion procedures.

f. Knockdown of the target gene kills the cells.
If the recombinant adenovirus expressing your siRNA is being made efficiently, but down regulation of the target gene is lethal to the cells, it may be impossible to produce or expand the recombinant adenovirus.

2. Recombinant virus was produced in the initial transfection, but attempts to expand the virus fail

a. Serum (FBS) levels are >2% in the culture medium during transfection.
(See section 1.e above)

b. The virus are no longer infectious.
Adenovirus is killed by multiple rounds of freeze/thawing. The three rounds used in the harvest procedure will not compromise virus viability; however, any additional freeze/thaw treatments will decrease the number of pfu 10-fold per freeze/thaw cycle.

c. Only a packaging cell line can be used for viral expansion.
The p*Silencer* adeno 1.0-CMV System is designed so that viral production can only occur in a packaging cell line and not in other cell types. We recommend using HEK-293 cells (ATCC Cat #CRL-1573). HEK-293 cells contain some of the necessary genes for virus production, which are missing from the recombinant adenovirus. The recombinant adenovirus produced with this kit are capable of ***infecting*** any cell line that has the required receptors. However they can only ***replicate*** effectively in the HEK-293 or other appropriate packaging cell lines.

3. Recombinant virus does not induce RNAi in infected samples

a. The siRNA sequence expressed by your recombinant adenovirus does not elicit RNAi.
We strongly recommend testing several different RNAi sequences in a system as similar to the "real" experimental system as possible to identify siRNA sequences with the desired gene knockdown activity. Use only optimized siRNA sequences as a basis for producing recombinant adenovirus for your experiments. If desired, the GAPDH positive control adenovirus can be used to demonstrate knockdown of GAPDH mRNA and protein levels in a variety of cell types.

b. The siRNA template sequence is not correct.
We strongly recommend that you sequence the inserts from a sample of the Shuttle Vector clone plasmid prep that will be used to produce recombinant adenovirus. This will assure that the adenovirus produced contains the expected hairpin siRNA template sequence.

4. The cells lift as expected during viral expansion but viral foci are not visible during the titering procedure

It can be very difficult to visualize viral foci in HEK-293 cells without previous experience. As an alternative, the Adenoviral LacZ Backbone in this kit contains the β-gal reporter gene LacZ and can be used to visualize the presence of virus in your cells. Any routine β-gal assay can be used; we supply instructions for a typical assay in section V.F on page 39.

V. Additional Information and Procedures

A. HEK-293 Cell Plating Density Chart for Infection

The table below provides guidelines for plating cells in several different size culture plates. Note that we recommend that HEK-293 always be plated 24 hr before infection with recombinant adenovirus.

Plate Size	Time to Use	# of Cells
150 mm	ready next day	1.2×10^7
	ready in 2 days	5×10^6
	ready in 3 days	2×10^6
	ready in 4 days	1.5×10^6
100 mm	ready next day	2 million
60 mm	ready next day	1.5 million
6 well	ready next day	500,000
24 well	ready next day	50,000
96 well	ready next day	10,000

B. Viral Particle Purification by Ultracentrifugation

Ultracentrifugation is a rigorous virus particle purification method that is commonly used because it yields very pure virus suitable for any application. For some applications, such as determining viral titer, using a simpler virus purification procedure, or a commercially available kit will typically yield virus that is sufficiently pure.

1. Prepare the following solutions:

- 10 mM Tris pH 8.1 (autoclave, store at room temp)
- Light cesium chloride: 1.2 g/mL in 10 mM Tris pH 8.1 (autoclave, store at room temp)
- Heavy cesium chloride: 1.45 g/mL in 10 mM Tris pH 8.1 (autoclave, store at room temp)
- 3% sucrose/PBS: Prepare 3 L per purification using clean containers and sterile water.

2. Assemble the cesium chloride gradient

Use ultraclear open-top ultracentrifuge tubes. Select the appropriate size ultracentrifuge tubes based on the lysate/sample volume.

Table 2. Sample Volume and $CsCl_2$ Gradient Size

Sample volume	Tube capacity	Light $CsCl_2$	Heavy $CsCl_2$
1 mL	~3 mL	1 mL	1 mL
3 mL	~13 mL	3.5 mL	3.5 mL
12 mL	~38 mL	12 mL	12 mL

a. Measure the light $CsCl_2$ into the ultracentrifuge tubes; prepare an even number of tubes to balance the rotor.

b. Using the smallest pipette possible (i.e. a 10 mL pipette for 12 mL of solution) aspirate the heavy $CsCl_2$ solution. Place the pipette tip at the bottom of the tube, and slowly dispense the heavy $CsCl_2$ under the light $CsCl_2$. Be careful to avoid introducing air bubbles because they could disrupt the gradient.

c. Aspirate the viral prep into a pipette. Place the pipette tip against the side of the tube above the $CsCl_2$ gradient, and very gently release the cell lysate on top of the gradient. Add 10 mM Tris (pH 8.1) to fill the tubes to about 1/4 inch below the top of the tube, and to balance the tubes.

3. Spin at 50,000 x g at 25°C for ≥2.5 hr

Centrifuge in the ultracentrifuge at 50,000 x g at 25°C for a minimum of 2.5 hr.

4. Recover the viral band and dilute ≥2-fold with 10 mM Tris

a. Assemble a sterile 18 gauge, 1.5 inch needle on a syringe for each tube; use a 5 mL syringe for ~38 mL tubes, a 3 mL syringe for ~13 mL tubes, or a 1 mL syringe for ~3 mL tubes.
Insert the needle through the tube about 0.5 cm below the viral band. Tilt the needle so that the opening is directly under the viral band, and aspirate the entire viral band in the smallest possible volume. Do not collect bands formed by incomplete viruses and cellular proteins (see Figure 8).

b. Transfer the viral band to a sterile tube with a secure closure, and dilute with an equal volume of 10 mM Tris pH 8.1. Mix gently by inverting the tube a few times.

5. Prepare a smaller second $CsCl_2$ gradient

For this second ultracentrifugation, prepare a $CsCl_2$ gradient in the next smaller ultracentrifuge tube size shown in Table 2 on page 34. For example, use an ~13 mL tube for this final centrifugation if you started with 12 mL of lysate from 20 plates, and spun in an ~38 mL tube for the initial centrifugation.

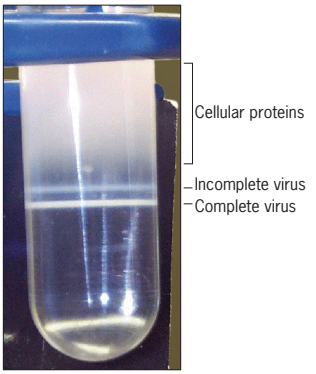
Follow the instructions in step 2 on page 34 to prepare the gradient. Using the same technique as for the first gradient, layer the viral prep from step 4.b onto the $CsCl_2$.

6. Spin at 50,000 x g at 4°C overnight, and recover the viral band

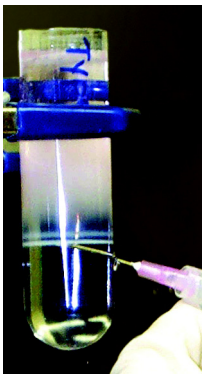
a. Centrifuge in the ultracentrifuge at 50,000 x g at 4°C for a minimum of 16 hr.

b. Recover the band containing intact viral particles as described in step 4 on page 35.

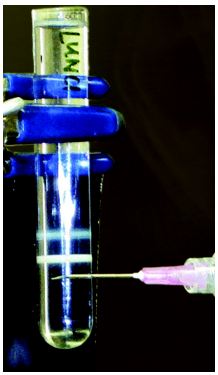
Figure 8. Removing Bands of Adenovirus from Cesium Chloride Gradients



Appearance of the gradient after the first centrifugation



Recovering the viral band from the gradient after the first centrifugation



Recovering the viral band from the gradient after the second centrifugation

7. Dilute the virus with 0.5 volume 3% sucrose/PBS

Transfer the viral band to a sterile tube with a secure closure, and dilute with 0.5–1 mL of 3% sucrose/PBS. If the virus is too concentrated, it will precipitate.

8. Dialyze in 3 changes of 3% sucrose/PBS at 4°C

Dialyze the diluted viral prep in a 10,000 molecular weight cut-off sterile dialysis membrane in 1 L of 3% sucrose/PBS for 1 hr at 4°C. Cover the dialysis setup to minimize the introduction of contaminants that could jeopardize cell cultures. Change the 3% sucrose/PBS once an hour, for a total of 3 changes.

Using sterile technique, remove the dialyzed virus prep from the dialysis membrane, and measure its volume.

C. Determining Approximate Viral Particle Concentration by Spectrophotometer

1. Dilute a sample of the viral prep 1:75 in 10 mM Tris pH 8.1 (i.e. 13 µL of virus in 987 µL of 10 mM Tris). Prepare a solution to blank the spectrophotometer using 3% sucrose/PBS in place of the viral prep.
2. Set the spectrophotometer to 260 nm, and blank the instrument. Then measure the A_{260} of your diluted viral prep.
3. Use the following equation to determine the approximate number of viral particles (pt) per mL:

$$A_{260} \text{ X } 75 \text{ (dilution) X } 10^{12} = \text{pt/mL}$$

D. Preparation of Viral Stock Solutions

Purified adenovirus can be stored in either glycerol or sucrose. Glycerol stocks have the advantage of remaining viable for years at –20°C, but they cannot be used for animal studies because glycerol causes tissue damage when injected into animals. Prepare glycerol stocks of virus to be used for further viral expansion and for studies involving infection of cell lines. Sucrose stocks are useful for whole animal injection, but they have the disadvantage of remaining stable for only 1–2 years at –80°C.

Sucrose stocks (for injection into animals):
1–1.5 x 10^{12} pt/mL in 0.45 µm filter-sterilized 3% sucrose/PBS

Glycerol stocks (for viral expansion, or for infection of tissue culture cells):
3–4 x 10^{12} pt/mL in sterile Glycerol/BSA (bovine serum albumin):

Concentration	Component
50 %	glycerol
10 mM	Tris pH 8.1
100 mM	NaCl
mix the first 3 ingredients, autoclave the mixture, then add:	
0.1 %	BSA (0.2 µm filter sterilized)

Measure the A_{260} of the prepared stock solutions, and calculate the viral particle count per mL as described in section V.C a second time to get a final concentration.

E. Determining Viral Titer

Determining the viral titer may be unnecessary for many researchers, but in some experimental systems, it is important to know the viral titer in order to use recombinant adenovirus successfully.

1. Plate 3 wells of HEK-293 cells per virus in 6 well plates

For each virus to be titered, plate 3 wells containing 5 x 10^5 HEK-293 cells per well in a 6 well tissue culture plate 24 hr before starting the titering procedure.

2. Prepare serial dilutions of the virus from 10^{-9} to 10^{-11}

Using purified virus, prepare 1.5 mL serial dilutions down to 10^{-9} through 10^{-11} pt/mL for each viral sample you wish to titer using 2% FBS culture medium (DMEM with 2% FBS and 1% pen/strep).

Each virus preparation will vary; however, typically 10^{-9}, 10^{-10}, 10^{-11} pt/mL dilutions should give interpretable results.

3. Infect cells with diluted virus and incubate for 6 days

a. Aspirate the media from the HEK-293 cells, and immediately replace it with 1 mL of diluted virus. Do not allow the cells to dry out during this process.

b. Add 2 mL of 2% FBS culture medium to each well, bringing the final culture media volume to 3 mL per well.

c. Incubate in normal cell culture conditions for 6 days.

4. Reading the results:

Examine the plates under a microscope after 6 days of incubation. Count the number of viral foci seen in each of the wells. Foci appear as holes in the cell monolayer with rounded up cells along the edge. They are often comet-shaped, with a large hole and a tail trailing off behind it. Figure 2 shows a viral focus made more visible by staining for β-galactosidase activity. If no viral foci are visible 6 days after infection, check again 8 days after infection.

The viral titer equals the number of viral foci in the well infected with the lowest dilution that yields viral foci. For example, if there were no viral foci in the 10^{-11} well, and the 10^{-10} well contained 3 viral foci. it would indicate a titer of 3×10^{10} pfu/mL. Typically the viral titer is approximately 100-fold lower than the pt/mL determined using the spectrophotometer.

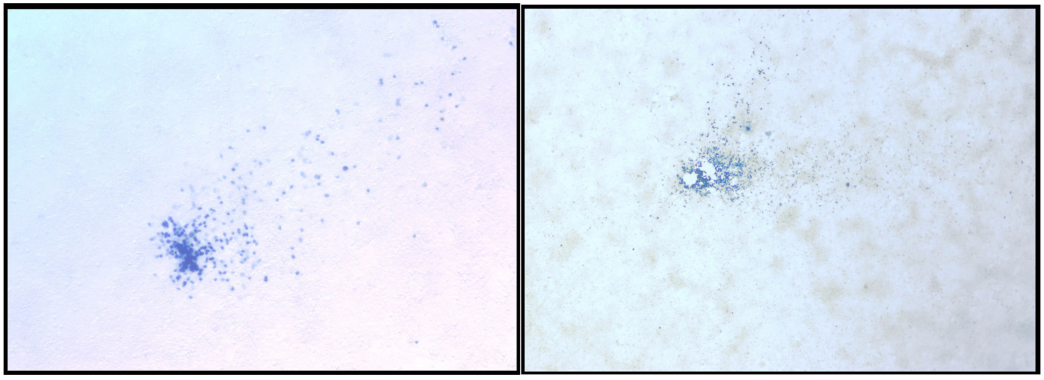


Figure 9. Adenoviral Foci After Staining for β-galactosidase Activity

Adenoviral foci can be made more visible by staining for β-gal activity because of the lacZ gene present in recombinant adenovirus. In the left panel, the cells have not yet detached from the plate, but a comet-shaped area of infected cells is evident. The photo on the right shows a culture with more extensive infection—a whole in the monolayer of cells can be seen, and a faint double tail is visible.

F. Whole Cell Staining for β-gal Activity in a 24 well Plate

Cells infected with recombinant adenovirus made with this kit should have β-gal activity because of the LacZ gene introduced by the Adenoviral LacZ Backbone. This assay can be used to determine if cells were infected when viral foci are not visible. Conduct the assay approximately 48–72 hr after infection with recombinant adenovirus.

1. Remove media from cells and wash with 1 mL 1X PBS.
2. Remove the PBS by aspiration, and fix the cells by adding 0.5 mL of 0.25% glutaraldehyde (v/v) directly to the plate, and incubating 15 min at room temp.
3. Gently wash the cells three times with 1 mL of 1X PBS for each wash. It is important to remove the glutaraldehyde entirely because it can inhibit the reaction.
4. Add 1 mL of β-gal stain to each sample.

Concentration	Component
1 X	PBS
5 mM	$K_3Fe(CN)_6$
5 mM	$K_4Fe(CN)_6$
2 mM	$MgCl_2$
0.2 %	X-Gal (v/v) made in dimethyl formamide

5. Incubate at 37°C until blue color develops. The incubation should not exceed 24 hr.
6. Examine using any standard light field microscope. Cells that are infected with recombinant adenovirus should turn blue.

G. Screening for Replication-Competent Adenovirus

There is some evidence to suggest that serial amplification of first generation adenoviruses in HEK-293 cells increases the probability that replication-competent adenovirus will be produced. This can happen through recombination of homologous adenoviral sequence in the HEK-293 genome and your recombinant adenovirus (Anderson 2000). The result is a recombinant adenovirus that has the E1 gene, and thus can replicate in any host cell, not just HEK-293 cells. We recommend archiving viral preps from both the initial and large scale recombinant adenovirus expansion steps to make viral stocks that can be amplified a single time to provide virus for your experiments. It is possible to screen for replication-competent virus by either PCR or with a soft agar plaque assay. Here we provide instructions for the much simpler PCR method of detection.

PCR assay

In this assay, viral DNA is amplified with two sets of primers. One set amplifies a portion of DNA from the adenoviral E1 gene, which is normally carried by the HEK-293 cells, and should not be present in your recombinant virus. The other PCR primer set amplifies a portion of the E3 gene which is expected to be in your recombinant virus, and serves as a positive control. The primer sequences are shown below:

Primer	Sequence
E1 forward	5'–GGAGCGAAGAAACCCATCTGAG–3'
E1 reverse	5'–CCTATCCTCCGTATCTATCTCCAC–3'
E3 forward	5'–TGCAGCCAGGAGGAAGCTGCAATACCAGAG–3'
E3 reverse	5'–GTCCAACTACAGCGACCCACCCTAACAGAG–3'

The limitation of the PCR assay is that it is very sensitive, but does not provide information about the amount of replication-competent virus in the prep, and thus the health risk involved. If your viral prep tests positive for replication-competent adenovirus using this PCR assay, we recommend following up with a soft agar plaque assay to help determine the level of health risk your prep represents.

Soft agar plaque assay

A procedure for the soft agar plaque assay is included in section V.H starting on page 42.

1. Prepare viral DNA for PCR

Starting with material from the adenovirus harvest procedure (infected cell lysate):

a. Make sure that cell lysates have been freeze/thawed 3 times. Aliquot 100 µL of each lysate from the adenovirus harvest procedure into 1.5 mL microfuge tubes.

b. Add 0.5–1 µL DNase I (10 mg/mL) to each sample and incubate at 37°C for 15–30 min.

Starting with purified viral particles (from ultracentrifugation or from a commercially available prep):

Aliquot 50 µL of each viral prep into 1.5 mL microfuge tubes, and add 100 µL sterile water for a final volume of 150 µL.

2. Digest with Pronase for 2–16 hr at 37°C

a. Thaw 2X Pronase reagent.

b. Add an equal volume of 2X Pronase to each sample and incubate for 2 hr to overnight at 37°C.

3. Phenol/chloroform extract and ethanol precipitate the viral DNA

a. Add an equal volume of phenol/chloroform, vortex briefly, and centrifuge at top speed in a microfuge for 10 min at room temp.

b. Transfer the aqueous phase to a fresh tube, making sure to leave the interface behind.

c. Add 5 M NaCl to bring the aqueous phase to 0.4 M NaCl, mix, add 2.5 volumes of 100% ethanol, and vortex to mix.

d. Incubate at –20°C for 20 min to overnight.

e. Spin at top speed in a microcentrifuge for 10 min. Then, remove the ethanol from the DNA pellet, and wash with 300 µL of 70% ethanol.

f. Spin again for 5 minutes, then remove the ethanol without disturbing the DNA pellet. Spin again to adhere the DNA pellet to the bottom of the tube if necessary.

g. Air dry or vacuum dry the DNA pellet, and resuspend in 50 µL nuclease-free water.

h. The DNA can be stored at –20°C if desired at this point.

4. PCR primers and conditions

a. Assemble both an E1 and an E3 amplification reaction for each virus prep. Also include a no-template control for each primer set.

Amount	Reagent
to 50 µL	Nuclease-free Water
5 µL	10X PCR Buffer (final [$MgCl_2$] is typically optimal at 1.5 mM)
5 µL	10 mM dNTP mix
0.25 µL	Forward primer (100 pmoles/mL)
0.25 µL	Reverse primer (100 pmoles/mL)
0.25 µL	Thermostable DNA polymerase (~1 Unit)
5 µL	*DNA template (~10–100 ng)

* Include a no-template control for each reaction using nuclease-free water in place of DNA template.

b. Cycle as follows:

initial denaturation:	94°C 4 min	
35 cycles:	94°C 30 sec	(denature)
	57°C 30 sec	(annealing)
	72°C 1 min	(extension)

After the cycling is finished, hold the reactions in the thermal cycler at 4°C until you are ready to load the gel, or store at –20°C for later analysis.

5. Analysis and interpretation

Run ~5 µL of the PCRs on a 1% agarose gel stained with ethidium bromide.

Primer set	Product size	Interpretation
E1	639 bp	If this adenoviral gene product can be amplified, it means that your viral prep may contain replication-competent adenovirus. *Either discard the prep, or conduct a soft agar plaque assay to determine whether the positive result is biologically significant* (see section *H. Soft Agar Plaque Assay for Replication-Competent Adenovirus* below).
E3	1499	This is a positive control to show that the DNA from your viral prep can be amplified, and that the PCR reagents and equipment are working. *If no product is amplified in this reaction, consider the prep untested for replication-competent adenovirus.*

H. Soft Agar Plaque Assay for Replication-Competent Adenovirus

The purpose of this assay is to determine the titer of replication-competent virus present in your purified recombinant adenoviral prep. We recommend running this assay if the PCR assay described in section V.G indicates that an adenovirus prep contains DNA from the E1 or E2 adenoviral genes. The titer of replication-competent virus will provide an indication of how safe it is to conduct experiments with your viral prep.

1. Plate 6 wells of 549 cells per viral prep in 24 well plates

For each viral prep to be titered, plate 6 wells containing 5 x 10^4 549 cells per well in a 24 well tissue culture plate 24 hr before starting the titering procedure.

2. Prepare ~1.5 mL of culture medium containing 10^{-4} pt/mL purified virus

Using purified virus, prepare 1.5 mL serial dilutions down to 10^{-4} pt/mL for each viral prep to be tested using 2% FBS culture medium (MEM with 2% FBS and 1% pen/strep).

3. Infect cells with diluted virus and incubate for 1 day

a. Aspirate the media from the cells, and immediately replace it with 200 µL of virus diluted to 10^{-4} pt/mL. Do not allow the cells to dry out during this process.

b. Incubate in normal cell culture conditions for 24 hr.

4. Overlay each well with 1 mL 4% FBS soft agar and incubate for 5 days

a. Prepare the 4% Fetal Bovine Serum (FBS) soft agar overlay:

Amount	Component
50%	1.6% agarose (autoclaved)
50%	Modified Eagle Medium (MEM), 1% pen/strep, 8% FBS

You will need 1 mL 4% FBS soft agar per well. Melt the 1.6% agarose in a microwave oven, then place it in a 45°C waterbath to cool. Measure the volume of MEM/8% FBS needed, and place it in a sterile tube at 45°C as well. When both solutions have reached 45°C,

mix an equal volume of agarose with culture medium to make enough soft agar to add 1 mL per well. The soft agar will remain liquid for about 10–15 min at room temp.

b. Overlay each well with 1 mL 4% FBS soft agar.
Remove the culture media from the infected cells no more than 6 wells at a time. Slowly add 1 mL of the 4% FBS soft agar to each well.

Cover the plates and leave them at room temp until the agar overlay has solidified.

c. Incubate in normal cell culture conditions for 5 more days.

5. On day 6 overlay each well with 500 µL 4% FBS soft agar, and incubate for 5 days

a. Prepare 4% FBS soft agar.
Prepare 4% FBS soft agar as described in step 4.a above.

b. Overlay each well with 500 µL 4% FBS soft agar.
Add 500 µL of 4% FBS soft agar to each well.

Cover the plates and leave them at room temp until the agar overlay has solidified.

c. Incubate in normal cell culture conditions for 5 more days.

6. On day 11, overlay each well with 300 µL 2.5% FBS soft agar containing neutral red, and incubate for 1 day

a. Prepare the 2.5% FBS soft agar with neutral red overlay:

Amount	Component
47%	MEM with 1% pen/strep and 5% FBS
3%	1.4% neutral red (this dye stains living cells)
50%	1.6% agarose (autoclaved)

You will need 300 µL 2.5% FBS soft agar per well. Melt the 1.6% agarose in a microwave oven, then place it in a 45°C waterbath to cool. Measure the volume of MEM/5% FBS needed, place it in a sterile tube at 45°C. When both solutions have reached 45°C, add 0.45 µm filter-sterilized neutral red to the MEM/5% FBS, then immediately add an equal amount of agarose to the culture medium to make enough soft agar to add 300 µL per well. Once the neutral red is added, proceed quickly or the dye could precipitate. The soft agar will remain liquid for about 10–15 min at room temp.

b. Overlay each well with 300 µL 2.5% FBS soft agar with neutral red.
Add 300 µL 2.5% FBS soft agar with neutral red to each well.

Cover the plates and leave them at room temp until the agar overlay has solidified.

c. Incubate in normal cell culture conditions for 24 hr.

7. Read the results on day 12

Examine the plates under an inverted microscope at 12 days. You should see an even cell monolayer, with living cells stained red. For accurate results in this assay it is important that the A549 cells are healthy (as evidenced by picking up the neutral red dye), and that they grow to form a uniform monolayer. If the cell monolayer does not stain red, or if the red-stained cells appear only in patches, then the experiment should be repeated with fresh, healthy A549 cells.

Adenoviral foci in the monolayer appears as a clear, almost perfectly round circle. Foci can often be seen by eye on the back side of the plate.

- If no foci are visible in any of the wells, it indicates that the titer of replication-competent virus is $<10^{-4}$. This indicates that it the viral prep does ***not*** represent a significant health hazard, and is considered safe to use. Any prep that test positive for replication-competent virus (by the PCR assay or this soft agar plaque assay), should not be expanded further as this could increase the titer of replication-competent virus.
- If any foci are seen, or if there are so many foci that they cannot be counted, it indicates that your prep contains $\geq 10^4$ pfu/mL replication-competent virus. Such preps ***do indeed represent a significant health hazard, and should be discarded*** following the recommendations for biohazard level 2 hazards.

VI. Appendix

A. References

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B. Quality Control

Functional Testing

The GAPDH Control Insert is used to make a GAPDH recombinant adenovirus following the instructions in section II.E and chapter III. The recombinant adenovirus produced is shown to form adenoviral foci in infected cells.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

C. Safety Information

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety goggles, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.