BLOCK-iT™ Adenoviral RNAi Expression System

A Gateway®-adapted, adenoviral destination vector for high-level, transient expression of short hairpin RNA (shRNA) in dividing and non-dividing mammalian cells

Catalog nos. K4941-00 and V492-20

Invitrogen™
life technologies

www.invitrogen.com
tech_service@invitrogen.com

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the kits listed below.

Product	Catalog no.
BLOCK-iT™ Adenoviral RNAi Expression System	K4941-00
pAd/BLOCK-iT™-DEST RNAi Gateway® Vector Kit	V492-20

Kit Components

The BLOCK-iT™ Adenoviral RNAi Kits include the following components. For a detailed description of the contents of each component, see page vi. For a detailed description of the contents of the BLOCK-iT™ U6 RNAi Entry Vector Kit and how to use the reagents supplied, see the BLOCK-iT™ U6 RNAi Entry Vector Kit manual. For detailed instructions to grow and maintain the 293A Cell Line, see the 293A Cell Line manual.

Component	Catalog no.	
	K4941-00	V492-20
pAd/BLOCK-iT™-DEST Gateway® Vector Kit	✓	✓
Gateway® LR Clonase™ II Enzyme Mix	✓	
293A Cell Line	✓	
BLOCK-iT™ U6 RNAi Entry Vector Kit	✓	

Shipping/Storage

The BLOCK-iT™ Adenoviral RNAi Kits are shipped on dry ice. Upon receipt, store each component as detailed below. For detailed information about the reagents supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual.

Note: The pAd/BLOCK-iT™-DEST RNAi Gateway® Vector Kit includes Box 1 **only**.

Box	Component	Storage
1	pAd/BLOCK-iT™-DEST Gateway® Vector Kit	-20°C
2	Gateway® LR Clonase™ II Enzyme Mix	-20°C
3	293A Cell Line	Liquid nitrogen
4	BLOCK-iT™ U6 RNAi Entry Vector Kit	U6 RNAi Entry Vector Reagents: -20°C One Shot® TOP10 Chemically Competent *E. coli*: -80°C

continued on next page

Kit Contents and Storage, continued

pAd/BLOCK-iT™-DEST Vector Kit

The following reagents are included with the pAd/BLOCK-iT™-DEST Gateway® Vector Kit (Box 1). **Store the vectors at -20°C.**

Vector	Composition	Amount
pAd/BLOCK-iT™-DEST	150 ng/µl in TE Buffer, pH 8.0	40 µl
pAd-GW/U6-lamin^{shRNA} control plasmid	1 µg/µl in TE Buffer, pH 8.0	10 µl

LR Clonase™ II Enzyme Mix

The following reagents are included with the Gateway® LR Clonase™ II Enzyme Mix (Box 2). **Store Box 2 at -20°C for up to 6 months.** For long-term storage, store at -80°C.

Reagent	Composition	Amount
Gateway® LR Clonase™ II Enzyme Mix	Proprietary	40 µl
Proteinase K Solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl
pENTR™-gus Positive Control	50 ng/µl in TE Buffer, pH 8.0	20 µl

Note: The pENTR™-gus control included with the LR Clonase™ II Enzyme Mix may be used as a positive control for the LR recombination reaction **only** (see page 16). **Do not** use the resulting expression clone to produce adenovirus for expression purposes as the pAd/BLOCK-iT™-DEST vector does not contain a eukaryotic promoter and the *gus* gene will not be expressed in mammalian cells.

293A Cell Line

Each ViraPower™ Adenoviral Expression Kit includes the 293A Cell Line to facilitate production of adenovirus. Refer to the 293A Cell Line manual for detailed information about the amount of cells provided and instructions on how to culture and maintain the cell line. The 293A Cell Line manual is supplied with each ViraPower™ Adenoviral Expression Kit, and may also be downloaded from our Web site (www.invitrogen.com) or requested from Technical Service (see page 47).

BLOCK-iT™ U6 RNAi Entry Vector Kit

The BLOCK-iT™ Adenoviral RNAi Expression System includes the BLOCK-iT™ U6 RNAi Entry Vector Kit to facilitate production of a Gateway® entry construct containing a U6 RNAi cassette for expression of your short hairpin RNA (shRNA) of interest. The BLOCK-iT™ U6 RNAi Entry Vector Kit contains:

- U6 RNAi Entry Vector Reagents
- One Shot® TOP10 Chemically Competent *E. coli*

Refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual for a detailed description of the reagents provided with the kit and instructions to produce the Gateway® entry construct.

Accessory Products

Introduction

The products listed in this section may be used with the ViraPower™ Adenoviral Expression Kits. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 47).

Accessory Products

The reagents supplied in the ViraPower™ Adenoviral Expression Kits as well as other products suitable for use with the kits are available separately from Invitrogen. Ordering information is provided below.

Item	Quantity	Catalog no.
BLOCK-iT™ U6 RNAi Entry Vector Kit	20 constructions	K4945-00
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µl	C4040-03
	40 x 50 µl	C4040-06
Library Efficiency® DH5α™ Chemically Competent *E. coli*	5 x 0.2 ml	18263-012
293A Cell Line	3 x 10^6 cells, frozen	R705-07
Lipofectamine™ 2000	0.75 ml	11668-027
	1.5 ml	11668-019
Opti-MEM® I Reduced Serum Medium	100 ml	31985-062
	500 ml	31985-070
Phosphate-Buffered Saline (PBS), pH 7.4	500 ml	10010-023
	1 L	10010-031
UltraPure™ Agarose	100 g	15510-019
	500 g	15510-027
PureLink™ HQ Mini Plasmid Purification Kit	100 purifications	K2100-01

continued on next page

Accessory Products

BLOCK-iT™ RNAi Products

Other BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis. If you wish to stably express your shRNA in any mammalian cell line, we recommend using the BLOCK-iT™ Lentiviral RNAi Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System. The BLOCK-iT™ RNAi TOPO® Transcription Kit allows generation of double-stranded RNA (dsRNA) for use in invertebrate RNAi analysis. The dsRNA may also be used as a substrate with the BLOCK-iT™ Dicer RNAi Kits to produce diced siRNA (d-siRNA) using the Dicer Enzyme. Ordering information for these products is provided below. For more information, see the RNAi Central application portal at www.invitrogen.com/rnai or call Technical Service (see page 47)

Item	Quantity	Catalog no.
BLOCK-iT™ Lentiviral RNAi Expression System	20 constructions	K4944-00
BLOCK-iT™ Lentiviral RNAi Gateway® Vector Kit	6 µg	K4943-00
BLOCK-iT™ Inducible H1 Lentiviral RNAi System	20 constructions	K4925-00
BLOCK-iT™ Lentiviral RNAi Zeo Gateway® Vector Kit	6 µg	V488-20
BLOCK-iT™ RNAi TOPO® Transcription Kit	5 genes	K3500-01
BLOCK-iT™ Dicer RNAi Transfection Kit	5 genes x 150 transfections	K3600-01
BLOCK-iT™ Complete Dicer RNAi Kit	5 genes x 150 transfections	K3650-01

Introduction

Overview

Introduction

The BLOCK-iT™ Adenoviral RNAi Expression System combines Invitrogen's BLOCK-iT™ RNAi and ViraPower™ Adenoviral technologies to facilitate creation of a replication-incompetent adenovirus that transiently delivers a short hairpin RNA (shRNA) of interest to dividing or non-dividing mammalian cells for RNA interference (RNAi) analysis. The System includes:

- The BLOCK-iT™ U6 RNAi Entry Vector Kit for production of an entry clone that contains elements required for expression of a double-stranded oligonucleotide (ds oligo) encoding an shRNA of interest in mammalian cells (*i.e.* human U6 promoter and RNA Polymerase III (Pol III) terminator). The entry vector containing this U6 RNAi cassette (U6 promoter + ds oligo + Pol III terminator) is used to transfer the U6 RNAi cassette into the adenoviral expression plasmid (see below) using Gateway® Technology.
- A promoterless pAd/BLOCK-iT™-DEST destination vector into which the U6 RNAi cassette of interest is transferred. This expression plasmid contains elements that allow packaging of the construct into virions.
- An optimized cell line, 293A, which allows production and subsequent titering of the recombinant adenovirus.

For more information about the BLOCK-iT™ RNAi, ViraPower™ Adenoviral, and Gateway® technologies, see page 2.

Advantages of the BLOCK-iT™ Adenoviral RNAi Expression System

Use of the BLOCK-iT™ Adenoviral RNAi Expression System to facilitate DNA virus-based delivery of shRNA to mammalian cells provides the following advantages:

- The pENTR™/U6 entry vector provides a rapid and efficient way to clone ds oligo duplexes encoding a desired shRNA target sequence into a vector containing an RNA Pol III-dependent expression cassette (*i.e.* U6 RNAi cassette) for use in RNAi analysis
- The vectors in the System are Gateway™-adapted for easy transfer of the U6 RNAi cassette from the pENTR™/U6 vector into a full-length pAd/BLOCK-iT™-DEST adenoviral vector, bypassing the need for a shuttle vector and inefficient homologous recombination in human or bacterial cells
- Allows generation of high titer adenoviral stocks (*i.e.* 1×10^9 pfu/ml in crude preparations and 1×10^{11} pfu/ml in concentrated preparations)
- Efficiently delivers the shRNA of interest to actively dividing and non-dividing mammalian cells in culture or *in vivo*
- Generates adenoviral constructs with such a high degree of efficiency and accuracy that the system is amenable for use in high-throughput applications
- Allows production of a replication-incompetent virus that enhances the biosafety of the system and its use as an shRNA delivery vehicle

Overview, continued

The BLOCK-iT™ RNAi Technology

A variety of BLOCK-iT™ RNAi products are available from Invitrogen to facilitate RNAi analysis in mammalian and invertebrate systems. The BLOCK-iT™ U6 RNAi Entry Vector Kit supplied with the BLOCK-iT™ Adenoviral RNAi Expression System uses a vector-based approach to allow efficient generation of U6 RNAi cassettes for expression of shRNA molecules in mammalian cells. Other BLOCK-iT™ RNAi products are available to facilitate production and delivery of synthetic short interfering RNA (siRNA), diced siRNA (d-siRNA) or double-stranded RNA (dsRNA) for RNAi analysis in mammalian cells or invertebrate organisms, as appropriate. For more information about any of the BLOCK-iT™ RNAi products, see the RNAi Central application portal at www.invitrogen.com/rnai or contact Technical Service (see page 47).

The ViraPower™ Adenoviral Technology

The ViraPower™ Adenoviral Technology facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene or RNA to dividing and non-dividing mammalian cells using a replication-incompetent adenovirus. Based on the second-generation vectors developed by Bett *et al.*, 1994, the ViraPower™ Adenoviral Technology takes advantage of the Gateway® Technology to simplify and greatly enhance the efficiency of generating high-titer, recombinant adenovirus. The main components of the ViraPower™ Adenoviral Expression System include:

- A pAd-based expression vector (*e.g.* pAd/BLOCK-iT™-DEST) into which the U6 RNAi cassette of interest will be cloned. This vector contains elements required to allow packaging of the expression construct into virions. For more information, see page 6.
- An optimized 293A producer cell line to facilitate optimal production, amplification, and titering of virus.

The Gateway® Technology

Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems. To express your shRNA of interest in mammalian cells using the BLOCK-iT™ Adenoviral RNAi Expression System and Gateway® Technology, simply:

1. Clone a double-stranded oligonucleotide encoding your shRNA of interest into the pENTR™/U6 entry vector to create an entry clone. Transfect this entry clone directly into mammalian cells for initial screening, if desired.
2. Generate an expression clone by performing an LR recombination reaction between the pENTR™/U6 entry clone and the pAd/BLOCK-iT™-DEST vector.
3. Use your expression clone and the reagents supplied in the kit to produce an adenoviral stock.
4. Transduce the adenovirus into mammalian cells to express the shRNA of interest and perform RNAi analysis.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).

continued on next page

Overview, continued

Purpose of this Manual

This manual provides an overview of the BLOCK-iT™ Adenoviral RNAi Expression System and provides instructions and guidelines to:

1. Use the pAd/BLOCK-iT™-DEST vector and a pENTR™/U6 entry clone in an LR recombination reaction to generate an expression clone containing the U6 RNAi cassette of interest.
2. Transfect the pAd/BLOCK-iT™-DEST expression construct into the 293A Cell Line to produce an adenoviral stock.
3. Amplify the adenoviral stock.
4. Titer the adenoviral stock.
5. Use the amplified adenoviral stock to transduce mammalian cells and perform transient RNAi analysis.

For details and instructions to generate a pENTR™/U6 entry clone containing the U6 RNAi cassette, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual. For instructions to culture and maintain the 293A producer cell line, refer to the 293A Cell Line manual. Both of these manuals are supplied with the BLOCK-iT™ Adenoviral RNAi Expression System, but are also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).

Important

The BLOCK-iT™ Adenoviral RNAi Expression System is designed to help you create an adenovirus to deliver and transiently express an shRNA of interest in mammalian cells for RNAi analysis. Although the system has been designed to help you express your shRNA of interest in the simplest, most direct fashion, use of the system is geared towards those users who are familiar with the biology of DNA viruses, adenoviral vectors, and gene silencing. We highly recommend that users possess a working knowledge of viral and tissue culture techniques and the RNAi pathway. For more information about the following topics, refer to these published reviews:

- Adenovirus biology: see Russell, 2000
- Adenoviral vectors: see Hitt *et al.*, 1999 and Wivel, 1999
- RNAi pathway and expression of shRNA in mammalian cells: see Brummelkamp *et al.*, 2002, McManus and Sharp, 2002, Paddison *et al.*, 2002, Paul *et al.*, 2002, Sui *et al.*, 2002, and Yu, 2002

The BLOCK-iT™ Adenoviral RNAi Expression System

Components of the System

The BLOCK-iT™ Adenoviral RNAi Expression System facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene to dividing and non-dividing mammalian cells using a replication-incompetent adenovirus, and includes the following major components:

- The BLOCK-iT™ U6 RNAi Entry Vector Kit containing the pENTR™/U6 vector for production of an entry clone that contains elements required for expression of a double-stranded oligonucleotide encoding an shRNA of interest in mammalian cells. The entry vector containing this U6 RNAi cassette (*i.e.* human U6 promoter + double-stranded oligonucleotide + Polymerase III terminator) may be transfected into dividing mammalian cells for initial RNAi screening or used to transfer the U6 RNAi cassette into the pAd/BLOCK-iT™-DEST expression plasmid (see below) using Gateway® Technology. For more information about the U6 RNAi cassette, see page 9. For detailed information about the pENTR™/U6 vector and instructions to generate an entry clone, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual.
- The E1 and E3-deleted pAd/BLOCK-iT™-DEST expression vector into which the U6 RNAi cassette will be cloned. The vector contains the elements required to allow packaging of the expression construct into virions (*e.g.* 5′ and 3′ ITRs, encapsidation signal, adenoviral late genes). For more information about the pAd/BLOCK-iT™-DEST vector, see page 6 and pages 43-44.
- An optimized 293A Cell Line that facilitates initial production, amplification, and titering of replication-incompetent adenovirus. The 293A cells contain a stably integrated copy of E1 that supplies the E1 proteins (E1a and E1b) *in trans* required to generate adenovirus. For more information about the 293A Cell Line, refer to the 293A Cell Line manual.

You will transfect the pAd/BLOCK-iT™-DEST vector containing the U6 RNAi cassette into 293A cells to produce a replication-incompetent adenovirus. You will next use the crude adenoviral stock to infect 293A cells to produce an amplified adenoviral stock. Once the adenoviral stock is amplified and titered, this high-titer stock can be used to transduce the recombinant adenovirus into the mammalian cell line of choice to express the shRNA of interest for transient RNAi analysis.

How Adenovirus Works

Adenovirus enters target cells by binding to the Coxsackie/Adenovirus Receptor (CAR) (Bergelson *et al.*, 1997). After binding to the CAR, the adenovirus is internalized via integrin-mediated endocytosis (Russell, 2000) followed by active transport to the nucleus. Once in the nucleus, the early events are initiated (*e.g.* transcription and translation of E1 proteins), followed by expression of the adenoviral late genes and viral replication. Note that expression of the late genes is dependent upon E1. In the BLOCK-iT™ Adenoviral RNAi Expression System, the 293A producer cells supply E1. The viral life cycle spans approximately 3 days.

For more information about the adenovirus life cycle and adenovirus biology, refer to published reviews (Russell, 2000).

continued on next page

The BLOCK-iT™ Adenoviral RNAi Expression System, continued

shRNA Expression

After adenovirus is transduced into the target cell and is transported to the nucleus, it does not integrate into the host genome. Therefore, expression of your shRNA of interest:

- Typically occurs within 24 hours after transduction.
- Is transient and will only persist for as long as the viral genome is present. For more information, see page 33.

pAd-GW/U6-lamin^{shRNA} Control

The BLOCK-iT™ Adenoviral RNAi Kits also include the pAd-GW/U6-lamin^{shRNA} plasmid for use as a positive control for adenovirus production. Once generated, the control adenoviral construct may be transduced into certain mammalian cell lines (see **Note** below), where it expresses an shRNA targeted to the human lamin A/C gene (Fisher *et al.*, 1986; Lin and Worman, 1993). Lamin A/C is a structural component of the nuclear envelope and has been shown to be non-essential for cell viability (Harborth *et al.*, 2001).

Note

Use of the pAd-GW/U6-lamin^{shRNA} adenoviral construct for RNAi analysis is limited by the following factors:

- Not all mammalian cell lines express the lamin A/C gene, and the control adenoviral construct may **only** be used to block lamin A/C expression in cell lines that express the lamin A/C gene. Cell lines that are known to express Lamin A/C **and** that have been used successfully in knockdown experiments include HeLa, HEK 293, A549, HT1080, and COS-7.

  Note: Cell lines that are known to express Lamin A/C, but that have not been tested for lamin A/C knockdown include CHO-S, K562, and MDCK.
- The shRNA produced from the control adenoviral construct targets the human lamin A/C gene. Although this particular target sequence is active in facilitating knockdown of the human lamin A/C gene (Elbashir *et al.*, 2001; Harborth *et al.*, 2001), it is not known whether this particular shRNA is able to facilitate knockdown of the lamin A/C gene in non human-derived cell lines. A non human-derived cell line that has been used successfully in a knockdown experiment is COS-7.

continued on next page

The BLOCK-iT™ Adenoviral RNAi Expression System, continued

Features of the pAd/BLOCK-iT™-DEST Vector

The pAd/BLOCK-iT™-DEST vector contains the following elements:

- Human adenovirus type 5 sequences (Ad 1-458 and 3513-35935) encoding genes and elements (*e.g.* Left and Right Inverted Terminal Repeats (ITRs), encapsidation signal sequence, late genes) required for proper packaging and production of adenovirus (Hitt *et al.*, 1999; Russell, 2000)
- Two recombination sites, *att*R1 and *att*R2, for recombinational cloning of the U6 RNAi cassette from the pENTR™/U6 entry clone using Gateway® Technology
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- The *ccd*B gene located between the *att*R sites for negative selection
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

Important

Note that the pAd/BLOCK-iT™-DEST vector does not contain a eukaryotic promoter. The promoter used to control expression of the shRNA of interest is contained within the U6 RNAi cassette that is transferred from the pENTR™/U6 entry clone into pAd/BLOCK-iT™-DEST after LR recombination. For more information about the features of the U6 RNAi cassette, see page 9.

Infection vs. Transduction

Note that we refer to viral **infection** in some procedures in this manual, and viral **transduction** in other procedures. These terms are defined below.

- **Infection:** Applies to situations where viral replication occurs and infectious viral progeny are generated. Only cell lines that stably express E1 can be infected.
- **Transduction:** Applies to situations where no viral replication occurs and no infectious viral progeny are generated. Mammalian cell lines that do not express E1 are transduced. In this case, you are using adenovirus as a vehicle to deliver shRNA.

Using shRNA for RNAi Analysis

The RNAi Pathway

RNAi describes the phenomenon by which dsRNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA), and is functionally similar to the processes of post-transcriptional gene silencing (PTGS) or cosuppression in plants (Cogoni *et al.*, 1994; Napoli *et al.*, 1990; Smith *et al.*, 1990; van der Krol *et al.*, 1990) and quelling in fungi (Cogoni and Macino, 1999; Cogoni and Macino, 1997; Romano and Macino, 1992). In plants, the PTGS response is thought to occur as a natural defense against viral infection or transposon insertion (Anandalakshmi *et al.*, 1998; Jones *et al.*, 1998; Li and Ding, 2001; Voinnet *et al.*, 1999).

In eukaryotic organisms, dsRNA produced *in vivo* or introduced by pathogens is processed into 21-23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases (Bernstein *et al.*, 2001; Ketting *et al.*, 2001). Each siRNA then incorporates into an RNA-induced silencing complex (RISC), an enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation (Hammond *et al.*, 2000; Nykanen *et al.*, 2001). In addition to dsRNA, other endogenous RNA molecules including short temporal RNA (stRNA) (see below) and micro RNA (miRNA) (Ambros, 2001; Carrington and Ambros, 2003) have been identified and shown to be capable of triggering gene silencing.

For more information about the RNAi pathway and the mechanism of gene silencing, refer to recent reviews (Bosher and Labouesse, 2000; Dykxhoorn *et al.*, 2003; Hannon, 2002; Plasterk and Ketting, 2000; Zamore, 2001).

stRNA and shRNA

Small temporal RNA (stRNA), a subclass of micro RNA (miRNA), were originally identified and shown to be endogenous triggers of gene silencing in *C. elegans* (Grishok *et al.*, 2001; Lee *et al.*, 1993). Short temporal RNA including *let-7* (Grishok *et al.*, 2001) and *lin-4* (Lee *et al.*, 1993) encode hairpin precursors that are processed by the Dicer enzyme into 21-23 nucleotide siRNA duplexes (Hutvagner *et al.*, 2001; Ketting *et al.*, 2001) that then enter the RNAi pathway and result in gene silencing by blocking translation.

Short hairpin RNA (shRNA) are an artificially designed class of RNA molecules that can trigger gene silencing through interaction with cellular components common to the RNAi and miRNA pathways. Although shRNA are a structurally simplified form of miRNA, these RNA molecules behave similarly to siRNA in that they trigger the RNAi response by inducing cleavage and degradation of target transcripts (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002).

continued on next page

Using shRNA for RNAi Analysis, continued

Structural Features of shRNA

Exogenous short hairpin RNA can be transcribed by RNA Polymerase III (Paule and White, 2000) and generally contain the following structural features:

- A short nucleotide sequence ranging from 19-29 nucleotides derived from the target gene, followed by
- A short spacer of 4-15 nucleotides (*i.e.* loop) and
- A 19-29 nucleotide sequence that is the reverse complement of the initial target sequence.

The resulting RNA molecule forms an intramolecular stem-loop structure that is then processed into an siRNA duplex by the Dicer enzyme.

Hallmarks of RNA Polymerase III-Based Expression

RNA Polymerase III transcribes a limited number of genes including 5S rRNA, tRNA, 7SL RNA, U6 snRNA, and a number of other small stable RNAs that are involved in RNA processing (Paule and White, 2000). Some of the hallmarks of RNA Polymerase III-based transcription are that:

- Transcription initiates and terminates at fairly precise points
- There is little addition of unwanted 5′ and 3′ sequences to the RNA molecule

For more information about RNA Polymerase III transcription, refer to published reviews or reference sources (Paule and White, 2000; White, 1998).

Using a Vector-Based System to Express shRNA

Use of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is limited by their transient nature. To address this limitation, a number of groups have developed vector-based systems to facilitate expression of siRNA and shRNA in mammalian cells (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002). At Invitrogen, we have developed the Gateway®-adapted pENTR™/U6 vector (supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit) to facilitate generation of an entry clone containing a ds oligo encoding an shRNA of interest within the context of an RNA Polymerase III-driven expression cassette (*i.e.* U6 RNAi cassette; see the next page). The resulting pENTR™/U6 entry construct may be introduced into dividing mammalian cells for transient expression of the shRNA of interest and initial RNAi screening, if desired. Once initial screening is complete, the U6 RNAi cassette may then be easily and efficiently transferred into the pAd/BLOCK-iT™-DEST vector (or other suitable destination vector) by LR recombination.

For more information about the BLOCK-iT™ U6 RNAi Entry Vector Kit, its components, and how to generate the pENTR™/U6 construct, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual.

continued on next page

Using shRNA for RNAi Analysis, continued

Features of the U6 RNAi Cassette

The U6 RNAi cassette contains all of the elements required to facilitate RNA Polymerase III-controlled expression of your shRNA of interest from pAd/BLOCK-iT™-DEST (or pENTR™/U6) including a:

- Human U6 promoter (see below for more information)
- Double-stranded oligo encoding an shRNA to your target gene of interest
- Polymerase III (Pol III) terminator consisting of a cluster of six thymidine (T) residues (Bogenhagen and Brown, 1981)

See the diagram below for an illustration of the U6 RNAi cassette.

Human U6 Promoter

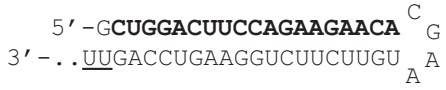
Expression of the shRNA of interest from pAd/BLOCK-iT™-DEST (or pENTR™/U6) is controlled by the human U6 promoter. The endogenous U6 promoter normally controls expression of the U6 RNA, a small nuclear RNA (snRNA) involved in splicing, and has been well-characterized (Kunkel *et al.*, 1986; Kunkel and Pederson, 1988; Paule and White, 2000). We and other groups have chosen this particular promoter to control vector-based expression of shRNA molecules in mammalian cells (Paddison *et al.*, 2002; Paul *et al.*, 2002) for the following reasons:

- The promoter is recognized by RNA Polymerase III and controls high-level, constitutive expression of shRNA
- The promoter is active in most mammalian cell types
- The promoter is a type III Pol III promoter in that all elements required to control expression of the shRNA are located upstream of the transcription start site (Paule and White, 2000)

Structure of the shRNA

Once you have used the BLOCK-iT™ Adenoviral RNAi Expression System to generate an adenoviral construct containing the U6 RNAi cassette, you will transduce the adenovirus into mammalian cells to express the shRNA of interest. The shRNA forms an intramolecular stem-loop structure similar to the structure of miRNA that is then processed by the endogenous Dicer enzyme into a 21-23 nt siRNA duplex.

Example: The figure below illustrates the structure of the shRNA generated from the pAd-GW/U6-lamin^{shRNA} construct. The 19 bp lamin A/C target sequence is indicated in bold. The underlined bases are derived from the Pol III terminator.



Note: The length of the stem and loop may differ depending on how you design the oligonucleotides encoding your target sequence. For guidelines to design the oligonucleotides, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual.

Biosafety Features of the System

Introduction

The BLOCK-iT™ Adenoviral RNAi Expression System is a second-generation system based on an adenoviral vector developed by Bett *et al.*, 1994. This second-generation adenoviral system includes a number of safety features designed to enhance its biosafety. These safety features are discussed below.

Information for European Customers

The 293A Cell Line is genetically modified and carries adenovirus type 5 sequences. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Biosafety Features of the BLOCK-iT™ Adenoviral RNAi Expression System

The BLOCK-iT™ Adenoviral RNAi Expression System includes the following safety features:

- The entire E1 region is deleted in the pAd/BLOCK-iT™-DEST expression vector. Expression of the E1 proteins is required for the expression of the other viral genes (*e.g.* late genes), and thus viral replication only occurs in cells that express E1 (Graham *et al.*, 1977; Kozarsky and Wilson, 1993; Krougliak and Graham, 1995).
- Adenovirus produced from the pAd/BLOCK-iT™-DEST expression vector is replication-incompetent in any mammalian cells that do not express the E1a and E1b proteins (Graham *et al.*, 1977; Kozarsky and Wilson, 1993; Krougliak and Graham, 1995).
- Adenovirus does not integrate into the host genome upon transduction. Because the virus is replication-incompetent, the presence of the viral genome is transient and will eventually be diluted out as cell division occurs.

Biosafety Level 2

Despite the presence of the safety features discussed above, the adenovirus produced with this System can still pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat adenoviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published guidelines for BL-2.** Furthermore, exercise extra caution when creating adenovirus that express shRNA targeting human genes involved in controlling cell division (*e.g.* tumor suppressor genes) or when producing large-scale preparations of virus (see the next page).

For more information about the BL-2 guidelines and adenovirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories", 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded from the Web at the following address:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

continued on next page

Biosafety Features of the System, continued

Additional Cautions When Producing Large-Scale Preparations of Virus

The genomic copy of E1 in all 293 cell lines contains homologous regions of overlap with the pAd/BLOCK-iT™-DEST vector. In rare instances, it is possible for homologous recombination to occur between the E1 genomic region in 293 cells and the viral DNA, causing the U6 RNAi cassette to be replaced with the E1 region, and resulting in generation of a "wild-type", replication-competent adenovirus (RCA) (Lochmuller *et al.*, 1994). This event is most likely to occur during large-scale preparation or amplification of virus, and the growth advantages of the RCA allow it to quickly overtake cultures of recombinant adenovirus. To reduce the likelihood of propagating RCA-contaminated adenoviral stocks:

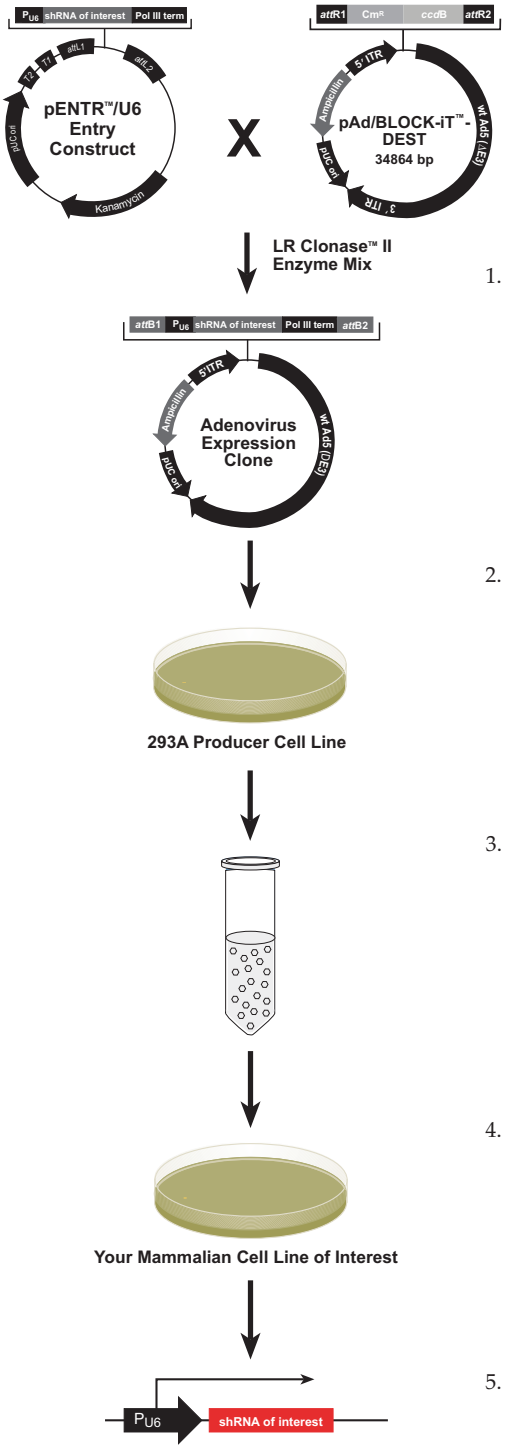
- Use caution when handling all viral preparations, and treat as BL-2 (see the previous page and page 26 for more details).
- Perform routine screening for the presence of wild-type RCA contamination after large-scale viral preparations. Suitable methods to screen for RCA contamination include PCR screening (Zhang *et al.*, 1995) or supernatant rescue assays (Dion *et al.*, 1996).
- If RCA contamination occurs, perform plaque purification to re-isolate the recombinant adenovirus of interest.

Note: As an alternative, E1-containing producer cell lines such as 911 (Fallaux *et al.*, 1996) or PER.C6 (Fallaux *et al.*, 1998) which contain no regions of homologous overlap with the adenoviral vectors can be used to help reduce the incidence of RCA generation.

Experimental Outline

Flow Chart

The diagram below describes the general steps required to express your shRNA of interest using the BLOCK-iT™ Adenoviral RNAi Expression System.



1. Perform an LR recombination reaction between the pENTR™/U6 entry construct and pAd/BLOCK-iT™-DEST to generate the pAd/BLOCK-iT™ expression construct. Digest the purified plasmid with *Pac* I to expose the ITRs.

2. Transfect the 293A producer cell line with your adenoviral expression clone. Harvest cells and prepare a crude viral lysate.

3. Amplify the adenovirus by infecting 293A producer cells with the crude viral lysate. Determine the titer of your adenoviral stock.

4. Add the viral supernatant to your mammalian cell line of interest.

5. Assay for knockdown of the target gene.

Methods

Generating an Entry Clone

Introduction

To express your shRNA of interest from pAd/BLOCK-iT™-DEST, you will first need to generate an entry clone in the pENTR™/U6 vector using the BLOCK-iT™ U6 RNAi Entry Vector Kit. General guidelines are provided below.

Important

Note that you must use the pENTR™/U6 entry vector to generate entry clones containing your shRNA sequence. Although a large selection of Gateway® entry vectors exists to facilitate generation of entry clones, **only** the pENTR™/U6 entry vector contains the elements required to facilitate proper expression of shRNA molecules in mammalian cells. These elements include:

- The human U6 promoter, an RNA Polymerase III-dependent promoter that facilitates high-level, constitutive expression of the shRNA of interest in mammalian cells (Kunkel *et al.*, 1986; Kunkel and Pederson, 1988).
- A Polymerase III (Pol III) terminator for efficient transcription termination of the shRNA molecule.

The BLOCK-iT™ U6 RNAi Entry Vector Kit is supplied with Catalog no. K4944-00, but is also available separately from Invitrogen (see page vii for ordering information).

Using pENTR™/U6

To generate an entry clone in pENTR™/U6, you will:

- Design and synthesize two complementary oligonucleotides encoding your shRNA target sequence according to specified guidelines
- Anneal the oligonucleotides to create a double-stranded oligonucleotide
- Clone the double-stranded oligonucleotide into pENTR™/U6 using an optimized 5-minute ligation procedure
- Transform competent *E. coli* and select for entry clones

For detailed instructions and guidelines to generate your entry clone, refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual. This manual is supplied with Catalog no K4944-00, but is also available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 47).

Creating Expression Clones

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction using your pENTR™/U6 entry construct and pAd/BLOCK-iT™-DEST to create an expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reaction** (pages 16-19) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing pENTR™/U6 entry clone and the *att*R-containing pAd/BLOCK-iT™-DEST vector. **Note:** Both the entry clone and the destination vector should be supercoiled (see **Important Note** below).
2. Transform the reaction mixture into a suitable *E. coli* host (see page 16).
3. Select for expression clones (see the next page for an illustration of the recombination region of expression clones in pAd/BLOCK-iT™-DEST.

Important

The pAd/BLOCK-iT™-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual has previously recommended using a linearized destination vector for more efficient recombination, further testing at Invitrogen has found that linearization of pAd/BLOCK-iT™-DEST is **not** required to obtain optimal results for any downstream application.

Destination Vectors

The pAd/BLOCK-iT™-DEST vector is supplied in solution at a concentration of 150 ng/µl in TE Buffer, pH 8.0, and is ready-to-use in the LR recombination reaction.

Propagating the Destination Vectors

If you wish to propagate and maintain the pAd/BLOCK-iT™-DEST vector, we recommend using One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli* from Invitrogen (Catalog no. C7510-03) for transformation. The *ccd*B Survival T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain integrity of the vector, select for transformants in media containing 50-100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance as these strains are sensitive to CcdB effects.

continued on next page

Creating Expression Clones, continued

Recombination Region of pAd/BLOCK-iT™-DEST

The recombination region of the expression clone resulting from pAd/BLOCK-iT™-DEST x pENTR™/U6 entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the pENTR™/U6 entry clone into the pAd/BLOCK-iT™-DEST vector by recombination. Non-shaded regions are derived from the pAd/BLOCK-iT™-DEST vector.

  Note: The DNA sequences transferred from the pENTR™/U6 entry clone consist of a U6 RNAi cassette containing the human U6 promoter + your ds oligo encoding the shRNA of interest + Pol III terminator.

- The transcriptional start site is indicated. Note that transcription starts at the first nucleotide following the end of the human U6 promoter sequence.
- Bases 519 and 2202 of the pAd/BLOCK-iT™-DEST sequence are marked.

Performing the LR Recombination Reaction

Introduction

Follow the guidelines and instructions in this section to perform the LR recombination reaction using the pENTR™/U6 entry clone and the pAd/BLOCK-iT™-DEST vector to generate an expression clone. We recommend including a negative control (no LR Clonase™ II) in your experiment to help you evaluate your results.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, or equivalent for transformation (see page vii for ordering information). **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is supplied with the kit (Catalog no. K4941-00 only) or available separately from Invitrogen to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 18 to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix provided in this manual.

Positive Control

The pENTR™-gus plasmid is included with the LR Clonase™ II enzyme mix for use as a positive control for the LR recombination reaction. You may use this entry clone in your LR recombination reaction to verify the efficiency of the reaction. However, the resulting expression clone **cannot** be used as an expression control because neither the pAd/BLOCK-iT™-DEST vector nor pENTR™-gus include a eukaryotic promoter to control expression of the *gus* gene in mammalian cells.

continued on next page

Performing the LR Recombination Reaction, continued

Materials Needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your pENTR™/U6 entry clone (50-150 ng/µl in TE Buffer, pH 8.0)
- pAd/BLOCK-iT™-DEST vector (150 ng/µl in TE Buffer, pH 8.0)
- LR Clonase™ II enzyme mix (supplied with Catalog no. K4941-00, Box 2; keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with Catalog no. K4941-00, Box 2; thaw and keep on ice until use)
- pENTR™-gus positive control (if desired, supplied with Catalog no. K4941-00, Box 2)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates containing 100 µg/ml ampicillin to select for expression clones

Important

Use care when handling the pAd/BLOCK-iT™-DEST plasmid DNA. The pAd/BLOCK-iT™-DEST plasmid is large (> 34 kb in size) and excessive manipulations can shear the DNA, resulting in reduced LR recombination efficiency. When working with pAd/BLOCK-iT™-DEST plasmid DNA, **do not vortex or pipet the solution vigorously**.

continued on next page

Performing the LR Recombination Reaction, continued

Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR reaction between the pAd/BLOCK-iT™-DEST vector and your pENTR™/U6 entry clone. If you want to include a negative control, set up a separate reaction but omit the LR Clonase™ II enzyme mix.

1. Add the following components to 0.5 ml microcentrifuge tubes at room temperature and mix.

Component	Sample	Positive Control
Entry clone (50-150 ng/reaction)	1-7 µl	--
pENTR™-gus (50 ng/µl)	--	2 µl
pAd/BLOCK-iT™-DEST vector (150 ng/µl)	1 µl	1 µl
TE Buffer, pH 8.0	to 8 µl	5 µl

2. Remove the LR Clonase™ II enzyme mix from -20°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 2 µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II enzyme mix to -20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 2-3 µl of the LR recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

   Note: You may store the LR reaction at -20°C for up to 1 week before transformation, if desired.

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1×10^8 cfu/µg, the LR reaction should give greater than 5000 colonies if the entire LR reaction is transformed and plated.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccdB* gene will be ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

continued on next page

Sequencing

Sequencing of the expression construct is not required as transfer of the U6 RNAi cassette from pENTR™/U6 into the pAd/BLOCK-iT™-DEST vector preserves the orientation of the cassette. However, if you wish to sequence your pAd/BLOCK-iT™-DEST expression construct, we recommend using the following primers. Refer to the diagram on page 15 for the location of the primer binding sites in the expression vector.

Note: For your convenience, Invitrogen has a custom primer synthesis service. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 47).

Primer	Sequence
pAd forward priming site	5′-GACTTTGACCGTTTACGTGGAGAC-3′
pAd reverse priming site	5′-CCTTAAGCCACGCCCACACATTTC-3′

Producing Adenovirus in 293A Cells

Introduction

Once you have created a pAd/BLOCK-iT™-DEST expression clone, you will transfect the expression clone into 293A cells to produce an adenoviral stock. The following section provides protocols and instructions to generate an adenoviral stock.

Preparing the Expression Clone for Use – *Pac* I Digestion

Before you can transfect your pAd/BLOCK-iT™-DEST expression clone into 293A cells, you must expose the left and right viral ITRs to allow proper viral replication and packaging. The pAd/BLOCK-iT™-DEST vector contains *Pac* I restriction sites (refer to the map of the vector on page 43 for the location of the *Pac* I sites). Digestion of the vector with *Pac* I allows exposure of the left and right viral ITRs and removal of the bacterial sequences (*i.e.* pUC origin and ampicillin resistance gene). **Important:** Make sure that your DNA sequence of interest does not contain any *Pac* I restriction sites.

1. Digest at least 5 µg of purified plasmid DNA of your pAd/BLOCK-iT™-DEST expression construct with *Pac* I (New England Biolabs, Catalog no. R0547S). Follow the manufacturer's instructions.
2. Purify the digested plasmid DNA using phenol/chloroform extraction followed by ethanol precipitation or a DNA purification kit (*e.g.* Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit; Catalog no. K2100-01). **Note:** Gel purification is not required.
3. Resuspend or elute the purified plasmid, as appropriate in sterile water or TE Buffer, pH 8.0 to a final concentration of 0.1-3.0 µg/µl.

Materials Needed

You should have the following materials on hand before beginning:

- *Pac* I-digested pAd/BLOCK-iT™-DEST expression clone containing your DNA sequence of interest (0.1-3.0 µg/µl in sterile water or TE, pH 8.0)
- *Pac* I-digested pAd-GW/U6-lamin^{shRNA} positive control vector (if desired, supplied with the kit; see the next page for more information)
- 293A cells cultured in the appropriate medium (see the next page for more information)
- Transfection reagent suitable for transfecting 293A cells (*e.g.* Lipofectamine™ 2000; see page 22 for more information)
- Opti-MEM® I Reduced Serum Medium (if using Lipofectamine™ 2000; pre-warmed; see page 22)
- Fetal bovine serum (FBS)
- Sterile, 6-well and 10 cm tissue culture plates
- Sterile, tissue culture supplies
- 15 ml sterile, capped, conical tubes
- Table-top centrifuge
- Water bath (set to 37°C)
- Cryovials

continued on next page

Producing Adenovirus in 293A Cells, continued

293A Cell Line

The human 293A Cell Line is included with the BLOCK-iT™ Adenoviral RNAi Expression System to facilitate adenovirus production from the E1-deleted pAd/BLOCK-iT™-DEST vector. The 293A Cell Line, a subclone of the 293 cell line, supplies the E1 proteins *in trans* that are required for expression of adenoviral late genes, and thus viral replication. The cell line exhibits a flattened morphology, enabling easier visualization of viral plaques. For more information about how to culture and maintain 293A cells, refer to the 293A Cell Line manual. The 293A Cell Line manual is supplied with the BLOCK-iT™ Adenoviral RNAi Expression System, but is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).

Positive Control

The pAd-GW/U6-lamin^{shRNA} plasmid is included with the BLOCK-iT™ Adenoviral RNAi Kits as a control for adenovirus production. We recommend including the positive control vector in your transfection experiment to generate a control adenoviral stock. Once generated, the control adenovirus may be transduced into certain mammalian cell lines (see **Note** on page 5) to express an shRNA targeted to the human lamin A/C gene, and may be used as a control for the RNAi response in these cell lines. To use pAd-GW/U6-lamin^{shRNA} as a positive control, you will need to digest the vector with *Pac* I using the protocol on page 20. Once digested with *Pac* I, use the linearized plasmid in your transfection experiment to generate an adenoviral stock.

For details about the vector, see page 45. To propagate and maintain the plasmid:

1. Use the 1 µg/µl stock solution provided to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α-T1^{R}, or equivalent. Use 10 ng of plasmid for transformation.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Important

Reminder: Use care when handling your pAd/BLOCK-iT™-DEST expression clone and pAd-GW/U6-lamin^{shRNA} plasmid DNA. The adenoviral plasmids are large (> 34 kb in size) and excessive manipulations can shear the DNA, resulting in reduced transfection efficiency and lower viral titers. When working with the plasmids, **do not vortex or pipet the solution vigorously**.

continued on next page

Transfection Reagent

You may use any suitable transfection reagent to introduce the pAd/BLOCK-iT™-DEST expression construct into 293A cells. We recommend using the cationic lipid-based Lipofectamine™ 2000 Reagent (Ciccarone *et al.*, 1999) available from Invitrogen (see page vii for ordering information). Using Lipofectamine™ 2000 to transfect 293A cells offers the following advantages:

- Provides the highest transfection efficiency in 293A cells
- DNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum
- Removal of complexes or medium change or addition following transfection are not required, although complexes can be removed after 4-6 hours without loss of activity

Note: To facilitate optimal formation of DNA-Lipofectamine™ 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium available from Invitrogen (see page vii for ordering information). For more information about Opti-MEM® I, see our Web site (www.invitrogen.com) or call Technical Service (see page 47).

Recommended Transfection Conditions

We generally produce adenoviral stocks in 293A cells using the following optimized transfection conditions below. The amount of adenovirus produced using these recommended conditions is approximately 10 ml of crude viral lysate with a titer ranging from 1 x 10^7 to 1 x 10^8 plaque-forming units (pfu)/ml.

Note: We use Lipofectamine™ 2000 for transfection. If you are using another transfection reagent, follow the manufacturer's instructions.

Condition	Amount
Tissue culture plate size	6-well (one well per adenoviral construct)
Number of 293A cells to transfect	5 x 10^5 cells (see **Note** below)
Amount of *Pac* I-digested pAd/BLOCK-iT™-DEST expression plasmid	1 µg
Amount of Lipofectamine™ 2000	3 µl

Note

293A cells should be plated 24 hours prior to transfection in complete medium, and should be 90-95% confluent on the day of transfection. Make sure that cells are healthy at the time of plating.

continued on next page

Producing Adenovirus in 293A Cells, continued

Transfection Procedure

Follow the procedure below to transfect 293A cells using Lipofectamine™ 2000. Remember that you may keep the cells in culture medium during transfection. We recommend including a positive control and a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help you evaluate your results.

1. The day before transfection, trypsinize and count the 293A cells, plating them at 5 x 10^5 cells per well in a 6-well plate. Plate cells in 2 ml of normal growth medium containing serum.
2. On the day of transfection, remove the culture medium from the 293A cells and replace with 1.5 ml of normal growth medium containing serum (or Opti-MEM® I Medium containing serum). **Do not include antibiotics.**
3. Prepare DNA-Lipofectamine™ 2000 complexes **for each transfection sample** by performing the following:
   a. Dilute 1 µg of *Pac* I-digested pAd/BLOCK-iT™-DEST expression plasmid DNA in 250 µl of Opti-MEM® I Medium without serum. Mix gently.
   b. Mix Lipofectamine™ 2000 gently before use, then dilute 3 µl in 250 µl of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine™ 2000. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
4. Add the DNA-Lipofectamine™ 2000 complexes dropwise to each well. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO_2 incubator.
5. The next day, remove the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with complete culture medium (*i.e.* D-MEM containing 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin).
6. 48 hours post-transfection, trypsinize cells and transfer the contents of each well to a sterile 10 cm tissue culture plate containing 10 ml of complete culture medium.

   Caution: Remember that you are working with infectious virus at this stage and in all subsequent procedures. Follow the recommended guidelines for working with BL-2 organisms (see page 10 for more information).
7. Replace culture medium with fresh, complete culture medium every 2-3 days until visible regions of cytopathic effect (CPE) are observed (typically 7-10 days post-transfection). For an example, see the next page.
8. Replenish culture medium and allow infections to proceed until approximately 80% CPE is observed (typically 10-13 days post-transfection).
9. Harvest adenovirus-containing cells by squirting cells off the plate with a 10 ml tissue culture pipette. Transfer cells and media to a sterile, 15 ml, capped tube. Proceed to **Preparing a Crude Viral Lysate**, page 25.

continued on next page

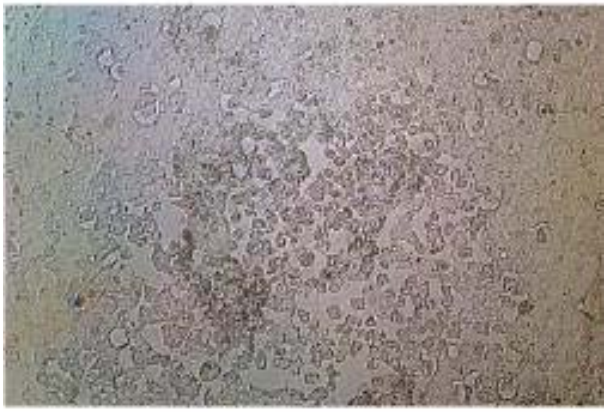
Producing Adenovirus in 293A Cells, continued

Example of CPE

In this example, *Pac* I-digested pAd-DEST™-based plasmid was transfected into 293A cells using the recommended protocol on the previous page. The photographs show transfected cells as they undergo CPE.

Day 4-6 post-transfection

At this early stage, cells producing adenovirus first appear as patches of rounding, dying cells.



Day 6-8 post-transfection

As the infection proceeds, cells containing viral particles lyse and infect neighboring cells. A plaque begins to form.

Day 8-10 post-transfection

At this late stage, infected neighboring cells lyse, forming a plaque that is clearly visible.

continued on next page

Producing Adenovirus in 293A Cells, continued

Preparing a Crude Viral Lysate

After you have harvested adenovirus-containing cells and media, you will use several freeze/thaw cycles followed by centrifugation to prepare a crude viral lysate. The freeze/thaw cycles cause the cells to lyse and allow release of intracellular viral particles.

1. Place the tube containing harvested cells and media from **Transfection Procedure**, Step 9, page 23 at -80°C for 30 minutes. Remove tube and place in a 37°C water bath for 15 minutes to thaw. Repeat the freezing and thawing steps twice.

   Note: Do not incubate samples at 37°C for longer than 15 minutes.
2. Centrifuge the cell lysate in a table-top centrifuge at 3000 rpm for 15 minutes at room temperature to pellet the cell debris.
3. Transfer the supernatant containing viral particles to cryovials in 1 ml aliquots. Store the viral stocks at -80°C.

What to Do Next

Once you have prepared a crude viral stock, you may:

- Amplify the viral stock by infecting 293A cells (see the next section for details). This procedure is recommended to obtain the highest viral titers and optimal results in your transduction studies.
- Determine the titer (see pages 29-32 for instructions).
- Use this viral stock to transduce your mammalian cells of interest to verify the functionality of your adenoviral construct in preliminary expression experiments (see pages 33-35 for more information).

Long-Term Storage

Place viral stocks at -80°C for long-term storage. Because adenovirus is non-enveloped, viral stocks remain relatively stable and some freezing and thawing of the viral stocks is acceptable. We **do not** recommend freezing and thawing viral stocks more than 10 times as loss of viral titer can occur. When stored properly, viral stocks of an appropriate titer should be suitable for use for up to one year. After long-term storage, we recommend re-titering your viral stocks before use.

Amplifying Your Adenoviral Stock

Introduction

Once you have created a crude viral stock, you can use this stock to infect 293A cells to generate a higher titer viral stock (*i.e.* amplify the virus). The titer of the initial viral stock obtained from transfecting 293A cells generally ranges from 1×10^7 to 1×10^8 plaque-forming units (pfu)/ml. Amplification allows production of a viral stock with a titer ranging from 1×10^8 to 1×10^9 pfu/ml and is generally recommended. Guidelines and protocols are provided in this section to amplify the recombinant adenovirus using 293A cells plated in a 10 cm dish. Larger-scale amplification is possible (see page 28).

Note: Other 293 cell lines or cell lines expressing the E1 proteins are suitable.

Remember that you will be working with infectious virus. Follow the recommended Federal guidelines for working with BL-2 organisms.

- Perform all manipulations within a certified biosafety cabinet.
- Treat media containing virus with bleach.
- Treat used pipets, pipette tips, and other tissue culture supplies with bleach or dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

Note

We have not observed wild-type RCA contamination in small-scale (*i.e.* 3×10^6 293A cells plated in a 10 cm dish) adenoviral amplification using the protocol on page 27. However, if you plan to perform large-scale amplification of virus, we recommend screening for wild-type RCA contamination. Note that even in large-scale preparations, contamination of adenoviral stocks with wild-type RCA is a rare event. For more information, see page 11.

Materials Needed

You should have the following materials on hand before beginning:

- Crude adenoviral stock of your pAd/BLOCK-iT™-DEST construct (from **Preparing a Crude Viral Lysate**, Step 3, page 25)

  Note: If you have produced an adenoviral stock of the pAd-GW/U6-lamin^{shRNA} construct, we recommend amplifying this viral stock as well.
- 293A cells cultured in the appropriate medium (see the 293A Cell Line manual for details)
- Sterile 10 cm tissue culture plates
- Sterile, tissue culture supplies
- 15 ml sterile, capped, conical tubes
- Table-top centrifuge
- Water bath (set to 37°C)
- Cryovials

continued on next page

Amplifying Your Adenoviral Stock, continued

Infection Conditions

To amplify the adenoviral stock, we typically infect 293A cells using the following conditions:

Condition	Amount
Tissue culture plate size	10 cm (one per adenoviral construct)
Number of 293A cells to infect	3 x 10^6 cells
Amount of crude adenoviral stock to use	100 µl (see **Note** below)

Note

We generally infect a 10 cm plate of 293A cells with 100 µl of untitered crude viral stock. Assuming a viral titer of 1 x 10^7 to 1 x 10^8 pfu/ml, this generally allows us to harvest the desired number adenovirus-containing cells 2-3 days after infection. You may vary the volume of crude viral stock used to infect cells, if desired. We have used up to 1 ml of crude viral stock.

If you have determined the titer of your crude viral stock, we recommend infecting 293A cells at a multiplicity of infection (MOI) = 3 to 5.

Amplification Procedure

Follow the procedure below to amplify your adenoviral stock using 293A cells. Make sure that your 293A cells are healthy at the time of plating.

1. The day before infection, trypsinize and count the 293A cells, plating them at 3 x 10^6 cells per 10 cm plate. Plate cells in 10 ml of normal growth medium containing serum.
2. On the day of infection, verify that the cells are at 80-90% confluence before proceeding. Add the desired amount of crude adenoviral stock (*e.g.* 100 µl) to the cells. Swirl the plate gently to mix.
3. Incubate the cells at 37°C in a CO_2 incubator and allow infection to proceed until 80-90% of the cells have rounded up and are floating or lightly attached to the tissue culture dish (typically 2-3 days post-infection). This indicates that cells are loaded with adenoviral particles.

   Note: If you have used less than 100 µl of crude viral stock or a lower titer stock for infection, you may need to perform a longer incubation.
4. Harvest adenovirus-containing cells by squirting cells off the plate with a 10 ml tissue culture pipette. Transfer cells and media to a sterile, 15 ml, capped tube.
5. Place the tube containing harvested cells and virus at -80°C for 30 minutes. Remove tube and place in a 37°C water bath for 15 minutes to thaw. Repeat the freezing and thawing steps twice.
6. Centrifuge the cell lysate in a table-top centrifuge at 3000 rpm for 15 minutes at room temperature to pellet the cell debris.
7. Transfer the supernatant containing viral particles to cryovials in 1 ml aliquots. Store the viral stocks at -80°C. For long-term storage, store as described on page 25. Proceed to **Titering Your Adenoviral Stock**, next section.

continued on next page

Amplifying Your Adenoviral Stock, continued

Scale-Up

The amplification procedure is easily scalable to any size tissue culture dish or roller bottle. If you wish to scale up the amplification, remember that you will need to increase the number of cells and amount of crude viral stock and medium used in proportion to the difference in surface area of the culture vessel.

Important Reminder: Remember to screen for the presence of wild-type RCA contamination in your amplified stock. Refer to published references for suitable screening protocols (Dion *et al.*, 1996; Zhang *et al.*, 1995).

Titering Your Adenoviral Stock

Introduction

Before proceeding to transduce the mammalian cell line of interest and express your shRNA of interest, we highly recommend determining the titer of your adenoviral stock. While this procedure is not required for some applications, it is necessary if:

- You wish to control the number of adenoviral particles introduced to each cell
- You wish to generate reproducible gene knockdown results

Guidelines and protocols are provided in this section.

Experimental Outline

To determine the titer of an adenoviral stock, you will:

1. Plate 293A cells in 6-well tissue culture plates.
2. Prepare 10-fold serial dilutions of your adenoviral stock.
3. Infect 293A cells overnight with serial dilutions of adenoviral stock.
4. Perform a plaque assay by overlaying the infected 293A cells with an agarose/plaquing media solution. Allow 8-12 days for plaques to form.
5. Stain and count the number of plaques in each dilution

Factors Affecting Viral Titer

A number of factors can influence viral titers including:

- The characteristics of the cell line used for titering (see below for more information).
- The age of your adenoviral stock. Viral titers may decrease with long-term storage at -80°C. If your adenoviral stock has been stored for 6 months to 1 year, we recommend titering or re-titering your adenoviral stock prior to use in an RNAi experiment.
- Number of freeze/thaw cycles. A limited number of freeze/thaw cycles is acceptable, but viral titers can decrease with more than 10 freeze/thaw cycles.
- Improper storage of your adenoviral stock. Adenoviral stocks should be aliquotted and stored at -80°C (see page 25 for recommended storage conditions).

Selecting a Cell Line

We recommend using the 293A cell line to titer your adenoviral stock. Other cell lines are suitable. If you wish to use another cell line, choose one with the following characteristics:

- **Must** express the E1 proteins
- Grows as an adherent cell line
- Easy to handle
- Exhibits a doubling time in the range of 18-25 hours
- Non-migratory

continued on next page

Titering Your Adenoviral Stock, continued

Note

The titer of an adenoviral construct may vary depending on which cell line is chosen. If you have more than one adenoviral construct, we recommend that you titer all of the adenoviral constructs using the same mammalian cell line.

Materials Needed

To determine the titer of your adenoviral stock, you should have the following materials on hand before beginning:

- Your pAd/BLOCK-iT™ adenoviral stock (store at -80°C until use)
- 293A Cell Line or other appropriate mammalian cell line of choice (see previous page)
- Complete culture medium for your cell line
- 6-well tissue culture plates
- 4% agarose (see **Recipes**, page 42; equilibrate to 65°C before use)
- Plaquing media (*i.e.* normal growth medium containing 2% FBS; equilibrate to 37°C before use)
- 5 mg/ml MTT solution or other appropriate reagent for staining (see **Recipes**, page 42; see below for alternatives)

Staining Reagents

We recommend using the vital dye, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue (MTT) as a staining reagent to help visualize plaques. Other vital stains including Neutral Red (Sigma, Catalog no. N7005) are suitable. If you wish to use Neutral Red, prepare a 1% solution (100X stock solution) in water and store at +4°C.

continued on next page

Titering Your Adenoviral Stock, continued

Titering Procedure

Follow the procedure below to determine the titer of your adenoviral stock using the 293A Cell Line or other appropriate cell line. You will use **at least** one 6-well plate for every adenoviral stock to be titered (six dilutions or one mock well and five dilutions). **Note:** If you have generated an adenoviral stock of the pAd-GW/U6-lamin^{shRNA} positive expression control, we recommend titering this stock as well.

1. The day before infection (Day 1), trypsinize and count the cells, plating them such that they will be 80-90% confluent at the time of infection. Incubate cells at 37°C overnight.

   Example: When using 293A cells, we generally plate 1 x 10^6 cells per well in a 6-well plate.

2. On the day of infection (Day 2), thaw your adenoviral stock and prepare 10-fold serial dilutions ranging from 10^{-4} to 10^{-9}. For each dilution, dilute the adenoviral stock into complete culture medium to a final volume of 1 ml. **Do not** vortex.
3. Remove the culture medium from the cells. Mix each dilution gently by inversion and add to one well of cells (total volume = 1 ml).
4. Swirl the plate gently to disperse the media. Incubate at 37°C overnight.
5. The following day (Day 3), remove the media containing virus and gently overlay the cells with 2 ml of agarose overlay solution per well. Prepare the agarose overlay solution (enough to overlay one 6-well plate at a time) as described below:
   a. For one 6-well plate (2 ml overlay per well), gently mix 12 ml of pre-warmed (at 37°C) plaquing media and 1.2 ml of pre-warmed (at 65°C) 4% agarose. Avoid formation of bubbles.
   b. Apply the overlay to the cells by gently pipetting the overlay down the side of each aspirated well. Work quickly to prevent premature solidification.
   c. Place the 6-well plate in a level tissue-culture hood at room temperature for 15 minutes or until the agarose overlay solidifies. Return the plate to a 37°C humidified CO_2 incubator.
6. 3-4 days following the initial overlay (Day 6-7), gently overlay the cells with an additional 1 ml of agarose overlay solution per well. Prepare the agarose overlay solution as described in Step 5. Allow the agarose overlay to solidify before returning the plate to a 37°C humidified CO_2 incubator.
7. Monitor the plates until plaques are visible (generally 8-12 days post-infection = Day 10-14). For each well, gently layer the 5 mg/ml MTT solution (1/10 the volume of the agarose overlay) on top of the solidified agar to stain. Make sure the MTT solution is evenly distributed over the entire surface of the well.

   Example: If each well contains 3 ml of agarose overlay, use 300 µl of 5 mg/ml MTT.

8. Incubate plates for 3 hours at 37°C.
9. Count the plaques and determine the titer of your adenoviral stock.

continued on next page

Titering Your Adenoviral Stock, continued

What You Should See

When titering pAd/BLOCK-iT™ amplified, adenoviral stocks using 293A cells, we generally obtain titers ranging from 1 x 10^8 to 1 x 10^9 pfu/ml. Adenoviral stocks with titers in this range are generally suitable for use in most applications.

Note: If the titer of your adenoviral stock is less than 1 x 10^7 pfu/ml, we recommend producing a new adenoviral stock. See page 29 and the **Troubleshooting** section, page 37 for more tips and guidelines to optimize your viral yield.

Concentrating Virus

For some applications, viral titers higher than 1 x 10^9 pfu/ml may be desired. It is possible to concentrate adenoviral stocks using a variety of methods (*e.g.* CsCl purification; Engelhardt *et al.*, 1993) without significantly affecting their transducibility. Use of these methods allows generation of adenoviral stocks with titers as high as 1 x 10^{11} pfu/ml.

Transduction and Analysis

Introduction

Once you have generated an adenoviral stock with a suitable titer, you are ready to transduce the adenoviral construct into the mammalian cell line of choice to express the shRNA of interest and perform RNAi analysis. Guidelines are provided below.

Factors Affecting Gene Knockdown Levels

A number of factors can influence the degree to which expression of your gene of interest is reduced (*i.e.* gene knockdown) in an RNAi experiment including:

- Transduction efficiency
- MOI used to transduce cells
- Transcription rate of the target gene of interest
- Stability of the target protein
- Growth characteristics of your mammalian cell line
- Activity of your shRNA in transient transfections

Take these factors into account when designing your transduction and RNAi experiments.

Transient Expression

The pAd/BLOCK-iT™ adenoviral construct is replication-incompetent and does not integrate into the host genome. Therefore, once transduced into the mammalian cells of choice, your shRNA of interest will be expressed only as long as the viral genome is present. The adenovirus terminal protein (TP) covalently binds to the ends of the viral DNA, and helps to stabilize the viral genome in the nucleus (Russell, 2000).

In actively dividing cells, the adenovirus genome is gradually diluted out as cell division occurs, resulting in an overall decrease in shRNA expression over time (*i.e.* target protein levels generally return to background levels within 1-2 weeks after transduction). In non-dividing cells or animal tissues, shRNA expression is generally more stable and can persist for longer periods of time following transduction.

Note

In actively dividing cells (*i.e.* doubling time of approximately 24 hours), we have found that target gene knockdown may be detectable within 48 hours of transduction, with maximal knockdown levels observed at 72-120 hours (3-5 days) post-transduction. Target protein levels generally start to rise 6 days or later after transduction as the adenovirus genome is diluted out. In cell lines that exhibit longer doubling times or non-dividing cell lines, high levels of shRNA expression may persist for a longer time.

If you are transducing the adenoviral construct into your mammalian cell line for the first time, we recommend performing a time course experiment to determine the conditions for optimal target gene knockdown.

continued on next page

Multiplicity of Infection (MOI)

To obtain optimal expression of your shRNA of interest and therefore, the highest degree of target gene knockdown, you will need to transduce the adenoviral construct into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of virus particles per cell and generally correlates with expression. Typically, shRNA expression levels increase as the MOI increases.

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell line (*e.g.* non-dividing vs. dividing cell type; see **Note** below), its transduction efficiency, and the nature of your target gene of interest. If you are transducing your adenoviral construct into the mammalian cell line of choice for the first time, we recommend using a range of MOIs (*e.g.* 0, 0.5, 1, 2, 5, 10, 20, 50) to determine the MOI required to obtain the optimal degree of target gene knockdown.

Note

In general, adenoviral constructs transduce non-dividing cell types less efficiently than actively dividing cell lines. If you are transducing your adenoviral construct into a non-dividing cell type, you may need to increase the MOI to achieve an optimal degree of target gene knockdown.

Positive Control

If you have generated the pAd-GW/U6-lamin^{shRNA} control adenoviral construct, we recommend using the adenoviral stock as a negative control for the RNAi response in any mammalian cell line. In addition, you may use this adenoviral construct as a positive control to help you determine the optimal MOI and verify the RNAi response **in some cell lines**. To use the construct as a positive control, remember that you must use a cell line that expresses the lamin A/C gene (see **Note** on page 5).

Note: If your cell line expresses lamin A/C, you may detect the protein using Western blot analysis (see page 35).

Important

Remember that viral supernatants are generated by lysing cells containing virus into spent media harvested from the 293A producer cells. Spent media lacks nutrients and may contain some toxic waste products. If you are using a large volume of viral supernatant to transduce your mammalian cell line (*e.g.* 1 ml of viral supernatant per well in a 6-well plate), note that growth characteristics or morphology of the target cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

continued on next page

Transduction and Analysis, continued

Materials to Have on Hand

You should have the following materials on hand before beginning:

- Your amplified, titered adenoviral stock (store at -80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- Appropriately sized tissue culture plates for your application

Transduction Procedure

Follow the procedure below to transduce the mammalian cell line of choice with your adenoviral construct.

1. Plate your mammalian cells of choice in complete media as appropriate for your application. When determining the density at which to plate cells, remember to take into account the length of time cells will be cultured prior to performing RNAi analysis (*e.g.* 48 hours vs. 120 hours).
2. On the day of transduction (Day 1), thaw your adenoviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. **Do not** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells. Swirl the plate gently to disperse the medium. Incubate at 37°C overnight.
4. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium.
5. Harvest the cells on the desired day (*e.g.* 5 days post transduction) and assay for knockdown of the target gene.

Performing RNAi Analysis

You may use any method as appropriate to assay for knockdown of your target gene including functional analysis, immunofluorescence, western blot, or qRT-PCR with the appropriate LUX™ primers. For more information about LUX™ primers, see our Web site (www.invitrogen.com/lux).

What You Should See

When performing RNAi studies using pAd/BLOCK-iT™ adenoviral constructs, we generally observe inhibition of gene expression within 48 to 120 hours after transduction. The degree of gene knockdown depends on the time of assay, stability of the protein of interest, and on the other factors listed on page 33. Note that 100% gene knockdown is generally not observed, but > 80% is possible with optimized conditions.

For an example of results obtained from an RNAi experiment using the pAd-GW/U6-lamin^{shRNA} adenoviral construct, see the next page.

Assaying for Lamin A/C Expression

If you perform RNAi analysis using the pAd-GW/U6-lamin^{shRNA} control adenoviral construct, you may assay for lamin A/C expression and knockdown by Western blot. We use an Anti-Lamin A/C Antibody (BD Biosciences, Catalog no. 612162) to detect lamin A/C expression.

Example of Expected Results

Introduction

This section provides an example of results obtained from an RNAi experiment performed using the pAd-GW/U6-lamin^{shRNA} control adenoviral construct.

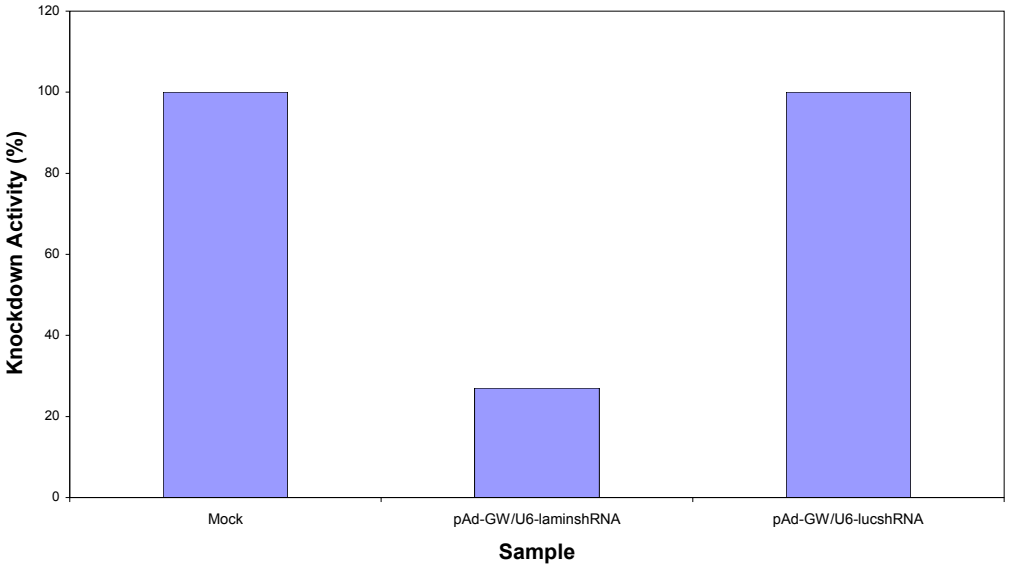
Example: Knockdown of Lamin A/C in HeLa Cells

In this experiment, double-stranded oligonucleotides targeting the endogenous lamin A/C gene and the luciferase reporter gene were generated and cloned into pENTR™/U6 using the BLOCK-iT™ U6 RNAi Entry Vector Kit. The U6-lamin and U6-luciferase (U6-luc) RNAi cassettes were transferred into the pAd/BLOCK-iT™-DEST vector using the LR recombination reaction to generate the pAd-GW/U6-lamin^{shRNA} and pAd-GW/U6-luc^{shRNA} expression constructs. Adenoviral stocks were generated, amplified, and titered following the protocols in this manual (see pages 20-32).

HeLa cells plated in a 12-well plate were transduced with each adenoviral construct at an MOI of 10. Cell lysates were prepared 120 hours (*i.e.* 5 days) after transduction, and equivalent amounts of cell lysate were analyzed by Western blot using an Anti-Lamin A/C Antibody (1:1000 dilution, BD Biosciences, Catalog no. 612162) and an Anti-β-Actin Antibody (1:5000 dilution, Abcam, Catalog no. ab6276). The Western blot was scanned and quantitated by densitometry. Knockdown activity was calculated by setting the level of lamin A/C expression in the mock transduction to 100%.

Results:

- Only the lamin A/C-specific shRNA (lane 2) inhibits expression of the lamin A/C gene, while no lamin A/C knockdown is observed with the luciferase shRNA (lanes 3).
- The degree of lamin A/C gene blocking achieved using the lamin shRNA is similar to that achieved with the well-characterized, chemically synthesized siRNA (Elbashir *et al.*, 2001; Harborth *et al.*, 2001).



Troubleshooting

Introduction

Review the information in this section to troubleshoot your adenoviral expression experiments.

LR Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the LR recombination and transformation procedures.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing 100 µg/ml ampicillin.
	LR recombination reaction not treated with proteinase K	Treat reaction with proteinase K before transformation.
	pAd/BLOCK-iT™-DEST plasmid DNA was sheared	Use care when handling the pAd/BLOCK-iT™-DEST plasmid DNA. Do not perform excessive manipulations (*e.g.* vortex or pipet the solution vigorously) that may shear the DNA.
	Didn't use the suggested amount of LR Clonase™ II enzyme mix or LR Clonase™ II enzyme mix was inactive	• Make sure to store the LR Clonase™ II enzyme mix at -20°C. • Do not freeze/thaw the LR Clonase™ II enzyme mix more than 10 times. • Use the recommended amount of LR Clonase™ II enzyme mix (see page 18). • Test another aliquot of the LR Clonase™ II enzyme mix.
	Not enough LR reaction transformed	Transform 2-3 µl of the LR reaction into the appropriate competent *E. coli* strain. Use *E. coli* cells with a transformation efficiency of > 1 x 10^8 cfu/µg.
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
	Too much entry clone DNA used in the LR reaction	Use 50-150 ng of the entry clone in the LR reaction.
High background in the negative control sample (no LR Clonase™ II)	LR reaction transformed into an *E. coli* strain containing the F′ episome and the *ccd*A gene	Use an *E. coli* strain that does not contain the F′ episome for transformation (*e.g.* TOP10, DH5α-T1^R).

continued on next page

Troubleshooting, continued

LR Reaction and Transformation, continued

Problem	Reason	Solution
High background in the negative control sample (no LR Clonase™ II), continued	Deletions (full or partial) of the *ccd*B gene from the pAd/BLOCK-iT™-DEST vector	The pAd/BLOCK-iT™-DEST vector is provided in solution and is ready-to-use in an LR reaction. However, if you have propagated the pAd/BLOCK-iT™-DEST vector: • Be sure to propagate the vector in media containing 100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol to maintain the integrity of the vector. • Prepare plasmid DNA from one or more colonies and verify the integrity of the vector before use.

Generating the Adenoviral Stock

The table below lists some potential problems and possible solutions that may help you troubleshoot your transfection, amplification, and titering experiments.

Problem	Reason	Solution
Low viral titer	Low transfection efficiency: • Incomplete *Pac* I digestion or digested DNA contaminated with phenol, ethanol, or salts • pAd/BLOCK-iT™-DEST expression clone plasmid DNA was sheared • Unhealthy 293A cells; cells exhibit low viability • 293A cells plated too sparsely on the day before transfection • Plasmid DNA:transfection reagent ratio incorrect	 • Repeat the *Pac* I digestion. Make sure purified DNA is not contaminated with phenol, ethanol, or salts. • Use care when handling plasmid DNA. Avoid performing excessive manipulations that may shear the DNA. • Use healthy 293A cells; do not overgrow. • Cells should be 90-95% confluent at the time of transfection. • Optimize such that plasmid DNA (in µg):Lipofectamine™ 2000 (in µl) ratio ranges from 1:2 to 1:3. If you are using another transfection reagent, optimize according to the manufacturer's recommendations.
	Viral supernatant too dilute	Concentrate virus using CsCl purification (Engelhardt *et al.*, 1993) or any method of choice.
	Viral supernatant frozen and thawed multiple times	**Do not** freeze/thaw viral supernatant more than 10 times.

continued on next page

Troubleshooting, continued

Generating the Adenoviral Stock, continued

Problem	Reason	Solution
Low viral titer, continued	Target gene is essential for cell viability	Make sure that your target gene is not essential for cell viability or growth by performing a transient transfection with the entry construct containing the shRNA of interest.
No plaques obtained upon titering	Viral stocks stored incorrectly	Aliquot and store stocks at -80°C. Do not freeze/thaw more than 10 times.
	Incorrect titering cell line used	Use the 293A cell line or any cell line with the characteristics discussed on page 29.
	Agarose overlay incorrectly prepared	Make sure that the agarose is not too hot before addition to the cells; hot agarose will kill the cells.
Titer indeterminable; cells completely lysed	Viral supernatant not diluted sufficiently	Titer adenovirus using 10-fold serial dilutions ranging from 10^{-4} to 10^{-9}.

Transduction and RNAi Analysis

The table below lists some potential problems and possible solutions that may help you troubleshoot your transduction and knockdown experiment.

Problem	Reason	Solution
No gene knockdown observed	shRNA with no activity chosen	Select a different target region. If possible, screen shRNA first by transient transfection of the pENTR™/U6 construct to verify its activity, then perform LR recombination with the pAd/BLOCK-iT™-DEST vector and proceed to generate adenovirus.
	Viral stocks stored incorrectly	Aliquot and store stocks at -80°C. Do not freeze/thaw more than 10 times.
	Gene of interest contains a *Pac* I site	Perform mutagenesis to change or remove the *Pac* I site.
	MOI too low	Transduce your adenoviral construct into cells using a higher MOI.
Low levels of gene knockdown observed	Low transduction efficiency: • Mammalian cells were not healthy • Non-dividing cell type used	• Make sure that your cells are healthy before transduction. • Transduce your adenoviral construct into cells using a higher MOI.
	MOI too low	Transduce your adenoviral construct into cells using a higher MOI.

continued on next page

Troubleshooting, continued

Transduction and RNAi Analysis, continued

Problem	Reason	Solution
Low levels of gene knockdown observed, continued	Low viral titer	Amplify the adenoviral stock using the procedure on page 27.
	shRNA with weak activity chosen	Select a different target region. If possible, screen shRNA first by transient transfection of the pENTR™/U6 construct to verify its activity, then perform LR recombination with the pAd/BLOCK-iT™-DEST vector and proceed to generate adenovirus. **Note:** Generally, transient transfection greatly overexpresses shRNA, so moderately active pENTR™/U6 entry clones may be less active when expressed from an adenoviral construct.
	Adenoviral stock contaminated with RCA	• Screen for RCA contamination (Dion *et al.*, 1996; Zhang *et al.*, 1995). • Prepare a new adenoviral stock or plaque purify to isolate recombinant adenovirus.
	Cells harvested too soon after transduction	Do not harvest cells until at least 48-72 hours after transduction to allow expressed shRNA to accumulate in transduced cells. If low levels of knockdown are observed at 48 hours, culture cells for a longer period of time (*e.g.* 96-120 hours) before assaying for gene knockdown.
	Cells harvested too long after transduction	For actively dividing cells, assay for maximal levels of gene knockdown within 5 days of transduction.
	Target gene is important for cell viability	Make sure that your target gene is not essential for cell viability or growth.
Persistent toxicity in target cells	Too much crude viral stock used	• Reduce the amount crude viral stock used for transduction **or** dilute the crude viral stock. • Amplify the adenoviral stock. • Concentrate the crude viral stock.
	Wild-type RCA contamination	Screen for RCA contamination (Dion *et al.*, 1996; Zhang *et al.*, 1995). Plaque purify to isolate recombinant adenovirus or prepare a new adenoviral stock.

continued on next page

Troubleshooting, continued

Transduction and RNAi Analysis, continued

Problem	Reason	Solution
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a different target region.
No gene knockdown observed when cells are transduced with the pAd-GW/U6-lamin^{shRNA} control adenovirus	Used a cell line that does not express the lamin A/C gene	Use a cell line that expresses the lamin A/C gene (*e.g.* A549, HeLa, HEK 293, HT1080, COS-7).
	Used a cell line that expresses the lamin A/C gene, but does not share 100% homology with the shRNA sequence	Use a human cell line that expresses the lamin A/C gene (*e.g.* A549, HeLa, HEK 293, HT1080) or use COS-7 cells. **Note:** The pAd-GW/U6-lamin^{shRNA} control expresses an shRNA targeted to the human lamin A/C gene. If you are using a non-human cell line, the lamin A/C gene may contain a polymorphism in the target region that renders the shRNA inactive.

Appendix

Recipes

4% Agarose

Follow the procedure below to prepare a 4% Agarose solution.

Materials Needed:

Ultra Pure Agarose (Invitrogen, Catalog no. 15510-027)

Deionized, sterile water

Protocol:

1. Prepare a 4% stock solution in deionized, sterile water.
2. Autoclave at 121°C for 20 minutes to sterilize.
3. Equilibrate to 65°C in a water bath and use immediately or store at room temperature indefinitely. If you store the agarose solution at room temperature, you will need to melt the agarose before use. Microwave the agarose to melt, then equilibrate to 65°C in a water bath before use.

5 mg/ml MTT

Follow the procedure below to prepare a 5 mg/ml MTT solution.

Materials Needed:

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue (MTT; Sigma, Catalog no. M2128)

Phosphate-Buffered Saline (PBS; Invitrogen, Catalog no. 10010-023)

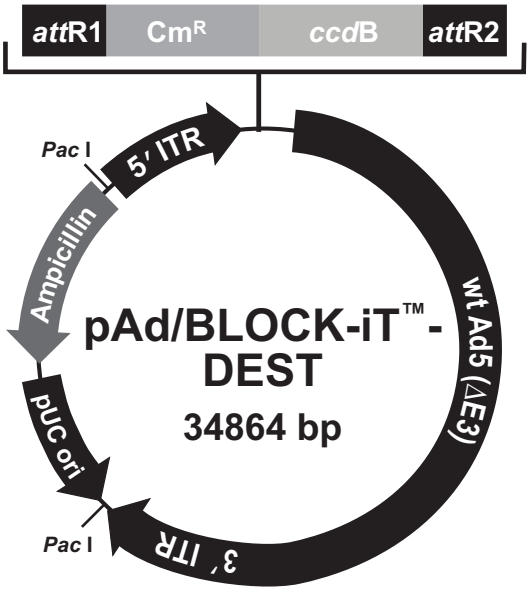
Protocol:

1. Prepare a 5 mg/ml stock solution in PBS.
2. Filter-sterilize and dispense 5 ml aliquots into sterile, conical tubes.
3. Store at +4°C for up to 6 months.

Map and Features of pAd/BLOCK-iT™-DEST

Map of pAd/BLOCK-iT™-DEST

The map below shows the elements of pAd/BLOCK-iT™-DEST. DNA from the entry clone replaces the region between bases 519 and 2202. **The complete sequence for pAd/BLOCK-iT™-DEST is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).**



Comments for pAd/BLOCK-iT™-DEST 34864 nucleotides

Human Ad5 sequences (wt 1-458; includes 5′ L-ITR and packaging signal): 1-458
pAd forward priming site: bases 361-384
*att*R1 site: bases 512-636
Chloramphenicol resistance gene (Cm^R): bases 745-1404
*ccd*B gene: bases 1746-2051
*att*R2 site: bases 2092-2216
Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3′ R-ITR): bases 2234-32782
pAd reverse priming site: bases 2237-2260
pUC origin: bases 32959-33620 (C)
Ampicillin (*bla*) resistance gene: bases 33746-34606 (C)
bla promoter: bases 34607-34705 (C)
Pac I restriction sites: bases 32788 and 34862

(C) = complementary strand

continued on next page

Map and Features of pAd/BLOCK-iT™-DEST, continued

Features of the Vector

The pAd/BLOCK-iT™-DEST vector (34864 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human adenovirus type 5 sequences (corresponds to wild-type 1-458 and 3513-35935 sequence) **Note:** The E1 and E3 regions are deleted.	Encodes all elements (except E1 and E3 proteins) required to produce replication-incompetent adenovirus (Russell, 2000) including: • Left and right ITRs • Encapsidation signal for packaging • E2 and E4 regions • Late genes
pAd forward priming site	Allows sequencing of the insert.
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the DNA sequence of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
pAd reverse priming site	Allows sequencing of the insert in the anti-sense orientation.
pUC origin	Allows high-copy replication and maintenance in *E. coli*.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
Pac I restriction sites (positions 32788 and 34862)	Allows exposure of the left and right ITRs required for viral replication and packaging.

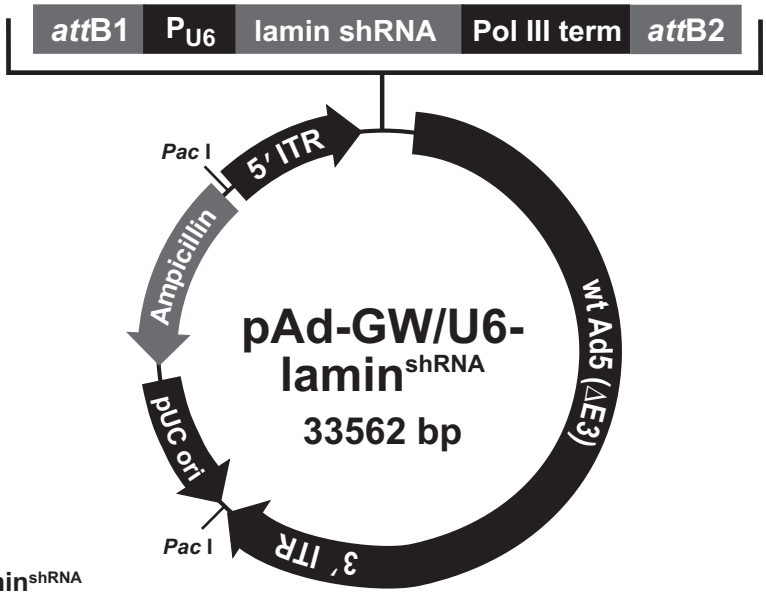
Map of pAd-GW/U6-lamin^{shRNA}

Description

pAd-GW/U6-lamin^{shRNA} is a 33562 bp control vector expressing an shRNA targeting the lamin A/C gene. A double-stranded oligonucleotide encoding the lamin shRNA was cloned into the pENTR™/U6 vector using the reagents supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit to generate an entry construct containing the U6-lamin^{shRNA} RNAi cassette. The U6-lamin^{shRNA} RNAi cassette was transferred into the pAd/BLOCK-iT™-DEST vector using the Gateway® LR recombination reaction to generate the pAd-GW/U6-lamin^{shRNA} vector.

Map of pAd-GW/U6-lamin^{shRNA}

The map below shows the elements of pAd-GW/U6-lamin^{shRNA}. **The complete sequence of the vector is available from our Web site (www.invitrogen.com) or by calling Technical Service (see page 47).**



Comments for pAd-GW/U6-lamin^{shRNA}
33562 nucleotides

Human Ad5 sequences (wt 1-458; includes 5′ L-ITR and packaging signal): 1-458
pAd forward priming site: bases 361-384
*att*B1 site: bases 512-536
U6 promoter: bases 573-836
Lamin A/C shRNA: bases 837-879
Pol III terminator: bases 880-885
*att*B2 site: bases 890-914
Human Ad5 sequences (wt 3513-35935; E3 region deleted, includes 3′ R-ITR): bases 932-30931
pAd reverse priming site: bases 935-958
pUC origin: bases 31657-32318 (C)
Ampicillin (*bla*) resistance gene: bases 32444-33304 (C)
bla promoter: bases 33305-33403 (C)
Pac I restriction sites: bases 31486 and 33560

(C) = complementary strand

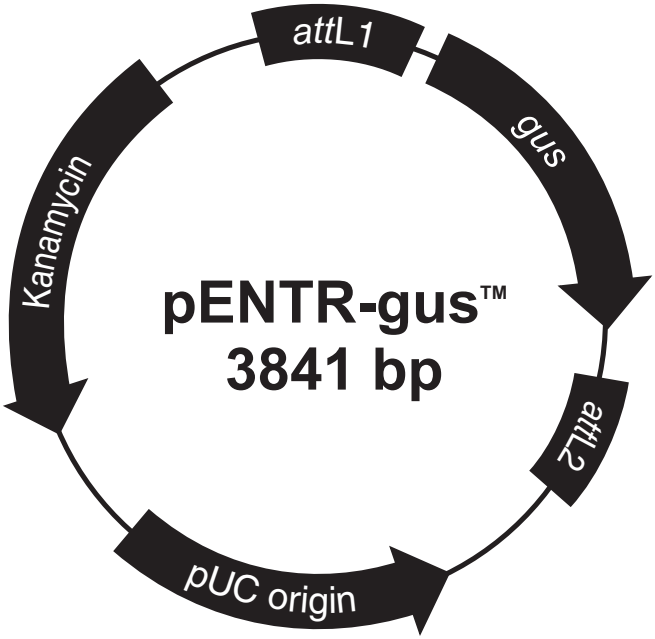
Map of pENTR™-gus

Description

pENTR™-gus is a 3841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991). The *gus* gene was amplified using PCR primers containing *att*B recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR201™ to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from our Web site or by contacting Technical Service.

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The complete sequence for pENTR™-gus is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 47).**



Comments for pENTR-gus™
3841 nucleotides

*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our web page (www.invitrogen.com).

Corporate Headquarters:

Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
USA

Tel: 1 760 603 7200

Tel (Toll Free): 1 800 955 6288

Fax: 1 760 602 6500

E-mail: tech_service@invitrogen.com

Japanese Headquarters:

Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park
Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi

Tel: 81 3 3663 7972

Fax: 81 3 3663 8242

E-mail: jpinfo@invitrogen.com

European Headquarters:

Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK

Tel: +44 (0) 141 814 6100

Tech Fax: +44 (0) 141 814 6117

E-mail: eurotech@invitrogen.com

MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

continued on next page

Technical Service, continued

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

Use of the BLOCK-iT™ Adenoviral RNAi Expression Kits is covered under a number of different licenses including those detailed below.

Limited Use Label License No. 19: Gateway® Cloning Products

This product and its use is the subject of one or more of U.S. Patent Nos. 5,888,732, 6,143,557, 6,171,861, 6,270,969, and 6,277,608 and/or other pending U.S. and foreign patent applications owned by Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Invitrogen Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase™ purchased from Invitrogen Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Invitrogen under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200.

Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 52.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 23: GUS Control Vector

The GUS positive control vector in these products is claimed in patents and patent applications (See U.S. Patent No. 5,599,670 and Great Britain Patent No. 2,197,653) licensed to Invitrogen by Cambia Biosystems, L.L.C. ("CBL"). Use of the GUS gene is restricted to use as a positive control. Any other use may require a license from CBL.

Limited Use Label License No. 116: Adenoviral Technology Products

This product is covered by United States Patent No. 6,136,594, under which Invitrogen has been granted a limited right to provide products for research purposes. Your use of this product constitutes your agreement to use this product for internal research purposes only and not for any clinical, therapeutic, prophylactic, diagnostic or production use. If you do not agree to be bound by these terms, return the unopened container(s) to Invitrogen for a full refund.

Limited Use Label License No. 173: Inhibition of Gene Expression by Double-Stranded RNA

This product may be covered by one or more of U.S. Patent No. 6,506,559 and/or foreign equivalents, and is sold under license to Invitrogen Corporation by the Carnegie Institution of Washington, 1530 P Street, N.W. Washington, DC 20005. A separate license from the Carnegie Institution of Washington may be required to use this product.

Limited Use Label License No. 177: *In vivo* Oligonucleotide Generator

This product is for non-clinical research use only. It is not to be used for commercial purposes. Use of this product to produce products for sale or for diagnostic, therapeutic or high throughput drug discovery purposes (the screening of more than 10,000 compounds per day) is prohibited. In order to obtain a license to use this product for these commercial purposes, contact The Regents of the University of California. This product or the use of this product is covered by U.S. Patent No. 5,624,803 owned by The Regents of the University of California.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 179: Gateway® RNAi Vectors

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Use of this product in conjunction with methods for the introduction of RNA molecules into cells may require licenses to one or more patents or patent applications. Users of these products should determine if any licenses are required. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned by Invitrogen and claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Information for European Customers

The 293A Cell Line is genetically modified and carries adenovirus type 5 sequences. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

Product Qualification

Introduction	This section describes the criteria used to qualify the components of the BLOCK-iT™ Adenoviral RNAi Expression Kits.
Vectors	The structure of each vector is verified by restriction enzyme digestion. In addition, the functionality of the pAd/BLOCK-iT™-DEST vector is qualified in a recombination assay using Gateway® LR Clonase™ II enzyme mix. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.
LR Clonase™ II Enzyme Mix	Gateway® LR Clonase™ II enzyme mix is functionally tested in a one hour recombination reaction followed by a transformation assay.
293A Cell Line	Each lot of cells is tested for cell growth and viability post-recovery from cryopreservation. The flat morphology is verified by visual inspection. Master Cell Banks are screened for viruses, mycoplasma, and sterility.
Adenovirus Production	Using the reagents provided in the kit, the pAd-GW/U6-lamin^{shRNA} plasmid is transfected into 293A cells using the protocol on page 23. Cells are harvested 10 days post-transfection, and a crude viral lysate is prepared using the protocol on page 25. The crude viral lysate is used to infect 293A cells, cells are harvested 3 days post-infection, and viral supernatant is prepared and titered. The pAd-GW/U6-lamin^{shRNA} adenoviral construct must demonstrate a titer of greater than 1×10^8 pfu/ml.
BLOCK-iT™ U6 RNAi Entry Vector Reagents	Refer to the BLOCK-iT™ U6 RNAi Entry Vector Kit manual for a detailed description of the criteria used to qualify the components of the BLOCK-iT™ U6 RNAi Entry Vector Kit.

References

Ambros, V. (2001). MicroRNAs: Tiny Regulators with Great Potential. Cell *107*, 823-826.

Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H., and Vance, V. B. (1998). A Viral Suppressor of Gene Silencing in Plants. Proc. Natl. Acad. Sci. USA *95*, 13079-13084.

Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a Common Receptor for Coxsackie B Viruses and Adenoviruses 2 and 5. Science *275*, 1320-1323.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference. Nature *409*, 363-366.

Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. (1994). An Efficient and Flexible System for Construction of Adenovirus Vectors with Insertions or Deletions in Early Regions 1 and 3. Proc. Natl. Acad. Sci. USA *91*, 8802-8806.

Bogenhagen, D. F., and Brown, D. D. (1981). Nucleotide Sequences in Xenopus 5S DNA Required for Transcription Termination. Cell *24*, 261-270.

Bosher, J. M., and Labouesse, M. (2000). RNA Interference: Genetic Wand and Genetic Watchdog. Nature Cell Biol. *2*, E31-E36.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science *296*, 550-553.

Carrington, J. C., and Ambros, V. (2003). Role of MicroRNAs in Plant and Animal Development. Science *301*, 336-338.

Ciccarone, V., Chu, Y., Schifferli, K., Pichet, J.-P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. (1999). Lipofectamine™ 2000 Reagent for Rapid, Efficient Transfection of Eukaryotic Cells. Focus *21*, 54-55.

Cogoni, C., and Macino, G. (1999). Gene Silencing in *Neurospora crassa* Requires a Protein Homologous to RNA-Dependent RNA Polymerase. Nature *399*, 166-169.

Cogoni, C., and Macino, G. (1997). Isolation of Quelling-Defective (qde) Mutants Impaired in Posttranscriptional Transgene-Induced Gene Silencing in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA *94*, 10233-10238.

Cogoni, C., Romano, N., and Macino, G. (1994). Suppression of Gene Expression by Homologous Transgenes. Antonie Van Leeuwenhoek *65*, 205-209.

Dion, L. D., Fang, J., and R.I. Garver, J. (1996). Supernatant Rescue Assay vs. Polymerase Chain Reaction for Detection of Wild Type Adenovirus-Contaminating Recombinant Adenovirus Stocks. J. Virol. Methods *56*, 99-107.

Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A. (2003). Killing the Messenger: Short RNAs that Silence Gene Expression. Nat. Rev. Mol. Cell Biol. *4*, 457-467.

continued on next page

References, continued

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-Nucleotide RNAs Mediate RNA Interference in Cultured Mammalian Cells. Nature *411*, 494-498.

Engelhardt, J. F., Yang, Y., Stratford-Perricaudet, L. D., Allen, E. D., Kozarsky, K., Perricaudet, M., Yankaskas, J. R., and Wilson, J. M. (1993). Direct Gene Transfer of Human CFTR Into Human Bronchial Epithelia of Xenografts with E1-Deleted Adenoviruses. Nature Genetics *4*, 27-34.

Fallaux, F. J., Bout, A., Velde, I. V. d., Wollenberg, D. J. V. d., Hehir, K. M., Keegan, J., Auger, C., Cramer, S. J., Ormondt, H. V., Eb, A. J. V. d., Valerio, D., and Hoeben, R. C. (1998). New Helper Cells and Matched Early Region 1-Deleted Adenovirus Vectors Prevent Generation of Replication-Competent Adenoviruses. Hum. Gene Ther. *9*, 1909-1917.

Fallaux, F. J., Kranenburg, O., Cramer, S. J., Houweling, A., Ormondt, H. V., Hoeben, R. C., and Eb, A. J. V. d. (1996). Characterization of 911: A New Helper Cell Line for the Titration and Propagation of Early Region 1-Deleted Adenoviral Vectors. Hum. Gene Ther. *7*, 215-222.

Fisher, D. Z., Chaudhary, N., and Blobel, G. (1986). cDNA Sequencing of Nuclear Lamins A and C Reveals Primary and Secondary Structural Homology to Intermediate Filament Proteins. Proc. Natl. Acad. Sci. USA *83*, 6450-6454.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. J. Gen. Virol. *36*, 59-74.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G., and Mello, C. C. (2001). Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs That Control *C. elegans* Developmental Timing. Cell *106*, 23-34.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-Directed Nuclease Mediates Genetic Interference in *Caenorhabditis elegans*. Nature *404*, 293-296.

Hannon, G. J. (2002). RNA Interference. Nature *418*, 244-251.

Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T., and Weber, K. (2001). Identification of Essential Genes in Cultured Mammalian Cells Using Small Interfering RNAs. J. Cell Science *114*, 4557-4565.

Hitt, M. M., Parks, R. J., and Graham, F. L. (1999) Structure and Genetic Organization of Adenovirus Vectors. In *The Development of Human Gene Therapy*, T. Friedmann, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 61-86.

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T., and Zamore, P. D. (2001). A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the let-7 Small Temporal RNA. Science *293*, 811-813.

Jones, A. L., Thomas, C. L., and Maule, A. J. (1998). *De novo* Methylation and Co-Suppression Induced by a Cytoplasmically Replicating Plant RNA Virus. EMBO J. *17*, 6385-6393.

Kertbundit, S., Greve, H. d., Deboeck, F., Montagu, M. V., and Hernalsteens, J. P. (1991). *In vivo* Random β-glucuronidase Gene Fusions in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *88*, 5212-5216.

continued on next page

References, continued

Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001). Dicer Functions in RNA Interference and in Synthesis of Small RNA Involved in Developmental Timing in *C. elegans*. Genes Dev. *15*, 2654-2659.

Kozarsky, K. F., and Wilson, J. M. (1993). Gene Therapy: Adenovirus Vectors. Curr. Opin. Genet. Dev. *3*, 499-503.

Krougliak, V., and Graham, F. L. (1995). Development of Cell Lines Capable of Complementing E1, E4, and Protein IX Defective Adenovirus Type 5 Mutants. Hum. Gene Ther. *6*, 1575-1586.

Kunkel, G. R., Maser, R. L., Calvet, J. P., and Pederson, T. (1986). U6 Small Nuclear RNA is Transcribed by RNA Polymerase III. Proc. Natl. Acad. Sci. USA *83*, 8575-8579.

Kunkel, G. R., and Pederson, T. (1988). Upstream Elements Required for Efficient Transcription of a Human U6 RNA Gene Resemble Those of U1 and U2 Genes Even Though a Different Polymerase is Used. Genes Dev. *2*, 196-204.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The *C. elegans* Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*. Cell *75*, 843-854.

Li, W. X., and Ding, S. W. (2001). Viral Suppressors of RNA Silencing. Curr. Opin. Biotechnol. *12*, 150-154.

Lin, F., and Worman, H. J. (1993). Structural Organization of the Human Gene Encoding Nuclear Lamin A and Nuclear Lamin C. J. Biol. Chem. *268*, 16321-16326.

Lochmuller, H., Jani, A., Huard, J., Prescott, S., Simoneau, M., Massie, B., Karpati, G., and Acsadi, G. (1994). Emergence of Early Region 1-Containing Replication-Competent Adenovirus in Stocks of Replication-Defective Adenovirus Recombinants (Delta E1 + Delta E3) During Multiple Passages in 293 Cells. Hum. Gene Ther. *5*, 1485-1491.

McManus, M. T., and Sharp, P. A. (2002). Gene Silencing in Mammals by Small Interfering RNAs. Nature Rev. Genet. *3*, 737-747.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chalcone Synthase Gene into *Petunia* Results in Reversible Co-Suppression of Homologous Genes *in trans*. Plant Cell *2*, 279-289.

Nykanen, A., Haley, B., and Zamore, P. D. (2001). ATP Requirements and Small Interfering RNA Structure in the RNA Interference Pathway. Cell *107*, 309-321.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002). Short Hairpin RNAs (shRNAs) Induce Sequence-Specific Silencing in Mammalian Cells. Genes Dev. *16*, 948-958.

Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002). Effective Expression of Small Interfering RNA in Human Cells. Nat. Biotechnol. *20*, 505-508.

continued on next page

References, continued

Paule, M. R., and White, R. J. (2000). Transcription by RNA Polymerases I and III. Nuc. Acids Res. *28*, 1283-1298.

Plasterk, R. H. A., and Ketting, R. F. (2000). The Silence of the Genes. Curr. Opin. Genet. Dev. *10*, 562-567.

Romano, N., and Macino, G. (1992). Quelling: Transient Inactivation of Gene Expression in *Neurospora crassa* by Transformation with Homologous Sequences. Mol. Microbiol. *6*, 3343-3353.

Russell, W. C. (2000). Update on Adenovirus and its Vectors. J. Gen. Virol. *81*, 2573-2604.

Smith, C. J., Watson, C. F., Bird, C. R., Ray, J., Schuch, W., and Grierson, D. (1990). Expression of a Truncated Tomato Polygalacturonase Gene Inhibits Expression of the Endogenous Gene in Transgenic Plants. Mol. Gen. Genet. *224*, 477-481.

Sui, G., Soohoo, C., Affar, E. B., Gay, F., Shi, Y., Forrester, W. C., and Shi, Y. (2002). A DNA Vector-Based RNAi Technology to Suppress Gene Expression in Mammalian Cells. Proc. Natl. Acad. Sci. USA *99*, 5515-5520.

van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N., and Stuitje, A. R. (1990). Flavonoid Genes in *Petunia*: Addition of a Limited Number of Gene Copies May Lead to a Suppression of Gene Expression. Plant Cell *2*, 291-299.

Voinnet, O., Pinto, Y. M., and Baulcombe, D. C. (1999). Suppression of Gene Silencing: A General Strategy Used by Diverse DNA and RNA Viruses of Plants. Proc. Natl. Acad. Sci. USA *96*, 14147-14152.

White, R. J. (1998). RNA Polymerase III Transcription (New York, NY: Springer-Verlag).

Wivel, N. A. (1999) Adenoviral Vectors. In *The Development of Human Gene Therapy*, T. Friedmann, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 87-110.

Yu, J. Y., DeRuiter, S. L., and Turner, D. L. (2002). RNA Interference by Expression of Short-interfering RNAs and Hairpin RNAs in Mammalian Cells. Proc. Natl. Acad. Sci. USA *99*, 6047-6052.

Zamore, P. D. (2001). RNA Interference: Listening to the Sound of Silence. Nat. Struct. Biol. *8*, 746-750.

Zhang, W. W., Koch, P. E., and Roth, J. A. (1995). Detection of Wild-Type Contamination in a Recombinant Adenoviral Preparation by PCR. BioTechniques *18*, 444-447.