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BLOCK-iT™ Lentiviral RNAi Expression System

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

Cat. nos. K4943-00 and K4944-00

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Manual part no. 25-0677

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User Manual

Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Cat. no.
BLOCK-iT™ Lentiviral RNAi Gateway® Vector Kit	K4943-00
BLOCK-iT™ Lentiviral RNAi Expression System	K4944-00

Intended Use

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

Kit Components

The BLOCK-iT™ Lentiviral RNAi Kits include the following components. For a detailed description of the contents of each component, see pages v-vii. 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 293FT Cell Line, see the 293FT Cell Line manual.

Components	Cat. no.	
	K4943-00	K4944-00
pLenti6/BLOCK-iT™-DEST Gateway® Vector Kit	✓	✓
Gateway® LR Clonase® II Enzyme Mix		✓
One Shot® Stbl3™ Chemically Competent *E. coli*	✓	✓
ViraPower™ Bsd Lentiviral Support Kit		✓
293FT Cell Line		✓
BLOCK-iT™ U6 RNAi Entry Vector Kit		✓

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Kit Contents and Storage, Continued

Shipping and Storage

The BLOCK-iT™ Lentiviral RNAi Kits are shipped as described below. Upon receipt, store each item as detailed below. For more 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 BLOCK-iT™ Lentiviral RNAi Gateway® Vector Kit includes Box 1 and Box 3 **only**.

Box	Component	Shipping	Storage
1	pLenti6/BLOCK-iT™-DEST Gateway® Vector Kit	Room temperature	–20°C
2	Gateway® LR Clonase® II Enzyme Mix	Dry ice	–20°C
3	One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C
4–5	ViraPower™ Bsd Lentiviral Support Kit	ViraPower™ Packaging Mix and Lipofectamine® 2000: Blue ice Blasticidin: Room temperature	ViraPower™ Packaging Mix and Blasticidin: –20°C Lipofectamine® 2000: 4°C **(do not freeze)**
6	293FT Cell Line	Dry ice	Liquid nitrogen
7–8	BLOCK-iT™ U6 RNAi Entry Vector Kit	Dry ice	U6 RNAi Entry Vector Reagents: –20°C One Shot® TOP10 Chemically Competent *E. coli*: –80°C

pLenti6/BLOCK-iT™-DEST Vector Kit

The following vectors are included with the pLenti6/BLOCK-iT™-DEST Gateway® Vector Kit (Box 1). **Store the vectors at –20°C.**

Vector	Composition	Amount
pLenti6/BLOCK-iT™-DEST	40 µL of vector at 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pLenti6-GW/U6-lamin^{shRNA} Control Plasmid	20 µL of vector at 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg

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Kit Contents and Storage, Continued

Gateway® 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/mL 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	150 µL

Note: The pENTR™-gus control included with the Gateway® LR Clonase® II Enzyme Mix may be used as a positive control for the LR recombination reaction **only** (see page 17). Do not use the resulting expression clone to produce lentivirus for expression purposes as the pLenti6/BLOCK-iT™-DEST vector does not contain a eukaryotic promoter and the *gus* gene will not be expressed in mammalian cells.

One Shot® Stbl3™ Chemically Competent *E. coli*

The following reagents are included with the One Shot® Stbl3™ Chemically Competent *E. coli* kit (Box 3). Transformation efficiency is ≥ 1×10^8 cfu/µg plasmid DNA. **Store Box 3 at –80°C.**

Reagent	Composition	Amount
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL
S.O.C. Medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL
Stbl3™ Cells	--	21 × 50 µL

Genotype of Stbl3™ Cells

F^- *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ^- *leu mtl*-1

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ViraPower™ Bsd Lentiviral Support Kit Contents

The following reagents are included with the ViraPower™ Bsd Lentiviral Support Kit (Boxes 4 and 5). Store the ViraPower™ Packaging Mix and Blasticidin at −20°C. Store Lipofectamine® 2000 Reagent at 4°C.

Important: Do not freeze Lipofectamine® 2000 Reagent.

Reagent	Composition	Amount
ViraPower™ Packaging Mix	Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids, 1 µg/µL in TE, pH 8.0	195 µg
Lipofectamine® 2000	Proprietary	0.75 mL
Blasticidin	Powder	50 mg

***TE buffer, pH 8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

293FT Cell Line

The BLOCK-iT™ Lentiviral RNAi Expression System includes the 293FT Cell Line (Box 5) for producing lentiviral stocks. The 293FT Cell Line is supplied as one vial containing 3×10^6 frozen cells in 1 mL of Freezing Medium. **Upon receipt, store in liquid nitrogen.**

For instructions to thaw, culture, and maintain the 293FT Cell Line, see the 293FT Cell Line manual.

BLOCK-iT™ U6 RNAi Entry Vector Kit

The BLOCK-iT™ Lentiviral 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 (Box 6)
- One Shot® TOP10 Chemically Competent *E. coli* (Box 7)

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.

Introduction

System Summary

Description of the System

The BLOCK-iT™ Lentiviral RNAi Expression System combines BLOCK-iT™ RNAi and ViraPower™ Lentiviral technologies to facilitate creation of a replication-incompetent lentivirus that 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 to express 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 lentiviral expression plasmid (see below) using Gateway® Technology.
- A promoterless pLenti6/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 and the Blasticidin resistance marker for selection of stably transduced cell lines.
- Components of the ViraPower™ Lentiviral System for production of a replication-incompetent lentivirus that stably expresses the shRNA of interest from the U6 RNAi cassette in both dividing and non-dividing mammalian cells.

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

Advantages of the BLOCK-iT™ Lentiviral RNAi Expression System

Use of the BLOCK-iT™ Lentiviral RNAi Expression System to facilitate lentiviral-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 recombination of the U6 RNAi cassette from the pENTR™/U6 vector into the pLenti6/BLOCK-iT™-DEST vector.
- Generates a replication-incompetent lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential RNAi applications beyond those of other traditional retroviral systems (Naldini, 1998).
- Efficiently delivers the shRNA of interest to mammalian cells in culture or *in vivo.*
- Provides stable, long-term expression of the shRNA of interest beyond that offered by traditional adenoviral-based systems.
- Produces a pseudotyped virus with a broadened host range (Yee, 1999).
- Includes multiple features designed to enhance the biosafety of the system.

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System Summary, Continued

The BLOCK-iT™ RNAi Technology

A variety of BLOCK-iT™ RNAi products are available to facilitate RNAi analysis in mammalian and invertebrate systems. The BLOCK-iT™ U6 RNAi Entry Vector Kit supplied with the BLOCK-iT™ Lentiviral 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 Stealth™ RNAi, short interfering RNA (siRNA), diced siRNA (d-siRNA) or double-stranded RNA (dsRNA) for RNAi analysis in mammalian cells or invertebrate organisms. 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 Support (see page 55).

The ViraPower™ Lentiviral Technology

The ViraPower™ Lentiviral 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 lentivirus. Based on the lentikat™ system developed by Cell Genesys (Dull *et al.*, 1998), the ViraPower™ Lentiviral Technology possesses features which enhance its biosafety while allowing high-level expression in a wider range of cell types than traditional retroviral systems. The key components of the ViraPower™ Lentiviral Expression System include:

- A pLenti-based expression vector (*e.g.*, pLenti6/BLOCK-iT™-DEST) for cloning a DNA sequence of interest. This vector contains elements required to allow packaging of the expression construct into virions and an antibiotic resistance marker to allow selection of stably transduced cell lines. For more information, see page 6.
- The ViraPower™ Packaging Mix, an optimized mixture of the three packaging plasmids required for production of the lentivirus.
- An optimized 293FT producer cell line to facilitate optimal production of virus.

For more information about the biosafety features of the System, see page 10.

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 a DNA sequence of interest into multiple vector systems. To express an shRNA of interest in mammalian cells using the BLOCK-iT™ Lentiviral RNAi Expression System and Gateway® Technology, simply:

1. Clone a double-stranded oligonucleotide encoding an 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 pLenti6/BLOCK-iT™-DEST vector.
3. Use your expression clone to produce a lentiviral construct.
4. Transduce the lentiviral construct into mammalian cells to express the shRNA of interest. Select for stably transduced cells, if desired.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual which is available at www.invitrogen.com or by contacting Technical Support (see page 55).

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System Summary, Continued

Purpose of this Manual

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

1. Use the pLenti6/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. Co-transfect the pLenti6/BLOCK-iT™-DEST expression construct and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
3. Titer the lentiviral stock.
4. Transduce the lentiviral construct into mammalian cells and perform "transient" RNAi analysis or
5. Generate a stably transduced cell line, if desired.

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 293FT producer cell line, refer to the 293FT Cell Line manual. Both of these manuals are supplied with the BLOCK-iT™ Lentiviral RNAi Expression System, but are also available at www.invitrogen.com or by contacting Technical Support (see page 55).

Note

The One Shot® Stbl3™ Chemically Competent *E. coli*, Gateway® LR Clonase® II Enzyme Mix, and Lipofectamine® 2000 Reagent included in the BLOCK-iT™ Lentiviral RNAi Expression System are available separately and are supplied with individual documentation detailing general use of the product. **For instructions to use these products specifically with the BLOCK-iT™ Lentiviral RNAi Kits, follow the recommended protocols in this manual.**

Important

The BLOCK-iT™ Lentiviral RNAi Expression System is designed to help you create a lentivirus to deliver and 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 toward those users who are familiar with the principles of retrovirus biology and gene silencing. We highly recommend that users possess a working knowledge of viral and tissue culture techniques, lipid-mediated transfection, and the RNAi pathway. For more information about the following topics, refer to these published references:

- Retrovirus biology and the retroviral replication cycle: see Buchschacher and Wong-Staal, 2000 and Luciw, 1996.
- Retroviral and lentiviral vectors: see Naldini, 1999, Naldini, 1998, and Yee, 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 *et al.*, 2002.

Where to Go For More Information

For more information about any of the BLOCK-iT™ RNAi products and other reference materials relating to RNAi, refer to the RNAi Central application portal at www.invitrogen.com/rnai.

The BLOCK-iT™ Lentiviral RNAi Expression System

Components of the System

The BLOCK-iT™ Lentiviral RNAi Expression System facilitates highly efficient, *in vitro* or *in vivo* delivery of an shRNA of interest to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus, 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 mammalian cells for transient RNAi analysis or used to transfer the U6 RNAi cassette into the pLenti6/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 pLenti6/BLOCK-iT™-DEST expression vector into which the U6 RNAi cassette will be cloned. The vector also contains the elements required to allow packaging of the expression construct into virions (*e.g.*, 5′ and 3′ LTRs, ψ packaging signal) and a selectable marker to allow generation of stable cell lines. For more information about the pLenti6/BLOCK-iT™-DEST vector, see page 6 and pages 44–45.
- The ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see the **Appendix**, pages 47–52.
- An optimized 293FT producer cell line that stably expresses the SV40 large T antigen under the control of the human CMV promoter and facilitates optimal production of virus. For more information about the 293FT Cell Line, refer to the 293FT Cell Line manual.

You will co-transfect the ViraPower™ Packaging Mix and the pLenti6/BLOCK-iT™-DEST expression construct containing the U6 RNAi cassette into 293FT cells to produce a replication-incompetent lentivirus, which can then be transduced into a mammalian cell line of interest. Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). Following integration into the genome, the shRNA of interest is constitutively expressed, allowing you to perform transient RNAi analysis or use Blasticidin selection to generate a stable cell line for long-term knockdown studies.

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VSV Envelope Glycoprotein

Most retroviral vectors are limited in their usefulness as delivery vehicles by their restricted tropism and generally low titers. In the BLOCK-iT™ Lentiviral RNAi Expression System, this limitation has been overcome by use of the G glycoprotein gene from Vesicular Stomatitis Virus (VSV-G) as a pseudotyping envelope, thus allowing production of a high titer lentivirus with a significantly broadened host cell range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).

pLenti6-GW/U6-lamin^{shRNA} Control

The BLOCK-iT™ Lentiviral RNAi Kits also include the pLenti6-GW/U6-lamin^{shRNA} plasmid for use as a positive control for lentivirus production. Once generated, the control lentiviral 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 & Worman, 1993). Lamin A/C is a structural component of the nuclear envelope and has been shown to be non-essential in development (Harborth *et al.*, 2001).

Note

Use of the pLenti6-GW/U6-lamin^{shRNA} lentiviral construct for RNAi analysis is limited by the following factors:

- Not all mammalian cell lines express the lamin A/C gene, and the control lentiviral 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 using the control lentiviral construct 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 using the control lentiviral construct include CHO-S, K562, and MDCK.

- The shRNA produced from the control lentiviral 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 how effective this particular shRNA is for facilitating knockdown of the lamin A/C gene across species. A non human-derived cell line that has been used successfully in a knockdown experiment using the control lentiviral construct is COS-7.

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Features of the pLenti6/BLOCK-iT™-DEST Vector

The pLenti6/BLOCK-iT™-DEST vector contains the following elements:

- Rous Sarcoma Virus (RSV) enhancer/promoter for Tat-independent production of viral mRNA in the producer cell line (Dull *et al.*, 1998)
- Modified HIV-1 5′ and 3′ Long Terminal Repeats (LTR) for viral packaging and reverse transcription of the viral mRNA (Dull *et al.*, 1998; Luciw, 1996)

  Note: The U3 region of the 3′ LTR is deleted (U3) and facilitates self-inactivation of the 5′ LTR after transduction to enhance the biosafety of the vector (Dull *et al.*, 1998)
- HIV-1 psi (Ψ) packaging sequence for viral packaging (Luciw, 1996)
- HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989)
- 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
- Blasticidin resistance gene (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) for selection in *E. coli* and mammalian cells
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

Important

Note that the pLenti6/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 pLenti6/BLOCK-iT™-DEST after LR recombination. For more information about the features of the U6 RNAi cassette, see page 9.

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 & Macino, 1997; Cogoni & Macino, 1999; Romano & 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 & 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 & 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 these reviews (Bosher & Labouesse, 2000; Dykxhoorn *et al.*, 2003; Hannon, 2002; Plasterk & 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).

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Using shRNA for RNAi Analysis, Continued

Structural Features of shRNA

Exogenous short hairpin RNA can be transcribed by RNA Polymerase III (Paule & 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 & 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 & White, 2000; White, 1998).

Using a Vector-Based System to Express shRNA

A limitation of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is the transient nature of siRNA. The Gateway®-adapted pENTR™/U6 vector (supplied in the BLOCK-iT™ U6 RNAi Entry Vector Kit) addresses this limitation by facilitating 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 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 pLenti6/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.

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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 pLenti6/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 & Brown, 1981)

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

Human U6 Promoter

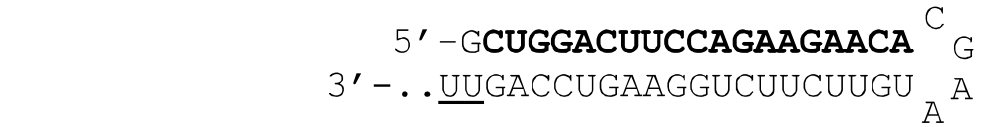
Expression of the shRNA of interest from pLenti6/BLOCK-iT™-DEST (or pENTR™/U6) is controlled by the human U6 promoter. The endogenous U6 promoter normally controls expression of the U6 RNA, a small nuclear RNA (snRNA) involved in splicing, and has been well-characterized (Kunkel *et al.*, 1986; Kunkel & Pederson, 1988; Paule & 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 - all elements required to control expression of the shRNA are located upstream of the transcription start site (Paule & White, 2000)

Structure of the shRNA

Once you have used the BLOCK-iT™ Lentiviral RNAi Expression System to generate a lentiviral construct containing the U6 RNAi cassette, you will transduce the lentivirus 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 pLenti6-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 lentiviral and packaging vectors supplied in the BLOCK-iT™ Lentiviral RNAi Expression System are third-generation vectors based on lentiviral vectors developed by Dull *et al.*, 1998. This third-generation lentiviral system includes a significant number of safety features designed to enhance its biosafety and to minimize its relation to the wild-type, human HIV-1 virus. These safety features are discussed below.

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

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

- The pLenti6/BLOCK-iT™-DEST expression vector contains a deletion in the 3′ LTR (ΔU3) that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yee *et al.*, 1987; Yu *et al.*, 1986; Zufferey *et al.*, 1998). Once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome.
- The number of genes from HIV-1 that are used in the system has been reduced to three (*i.e. gag*, *pol*, and *rev*).
- The VSV-G gene from Vesicular Stomatitis Virus is used in place of the HIV-1 envelope (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
- Genes encoding the structural and viral genome packaging components are separated onto four plasmids. All four plasmids have been engineered not to contain any regions of homology with each other to prevent undesirable recombination events that could lead to the generation of a replication-competent virus (Dull *et al.*, 1998).
- Although the three packaging plasmids allow expression *in trans* of proteins required to produce viral progeny (*e.g.* gal, pol, rev, env) in the 293FT producer cell line, none of them contain LTRs or the Ψ packaging sequence. This means that none of the HIV-1 structural genes are actually present in the packaged viral genome, and thus, are never expressed in the transduced target cell. No new replication-competent virus can be produced.
- The lentiviral particles produced in this system are replication-incompetent and only carry the gene of interest. No other viral species are produced.
- Expression of the *gag* and *pol* genes from pLP1 has been rendered Rev-dependent by virtue of the HIV-1 RRE in the gag/pol mRNA transcript. Addition of the RRE prevents gag and pol expression in the absence of Rev (Dull *et al.*, 1998).
- A constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the pLenti6/BLOCK-iT™-DEST expression vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

Continued on next page

Biosafety Features of the System, Continued

Biosafety Level 2

Despite the inclusion of the safety features discussed on the previous page, the lentivirus 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 lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when creating lentivirus carrying potential harmful or toxic genes (*e.g.*, activated oncogenes).

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at the following address:

http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

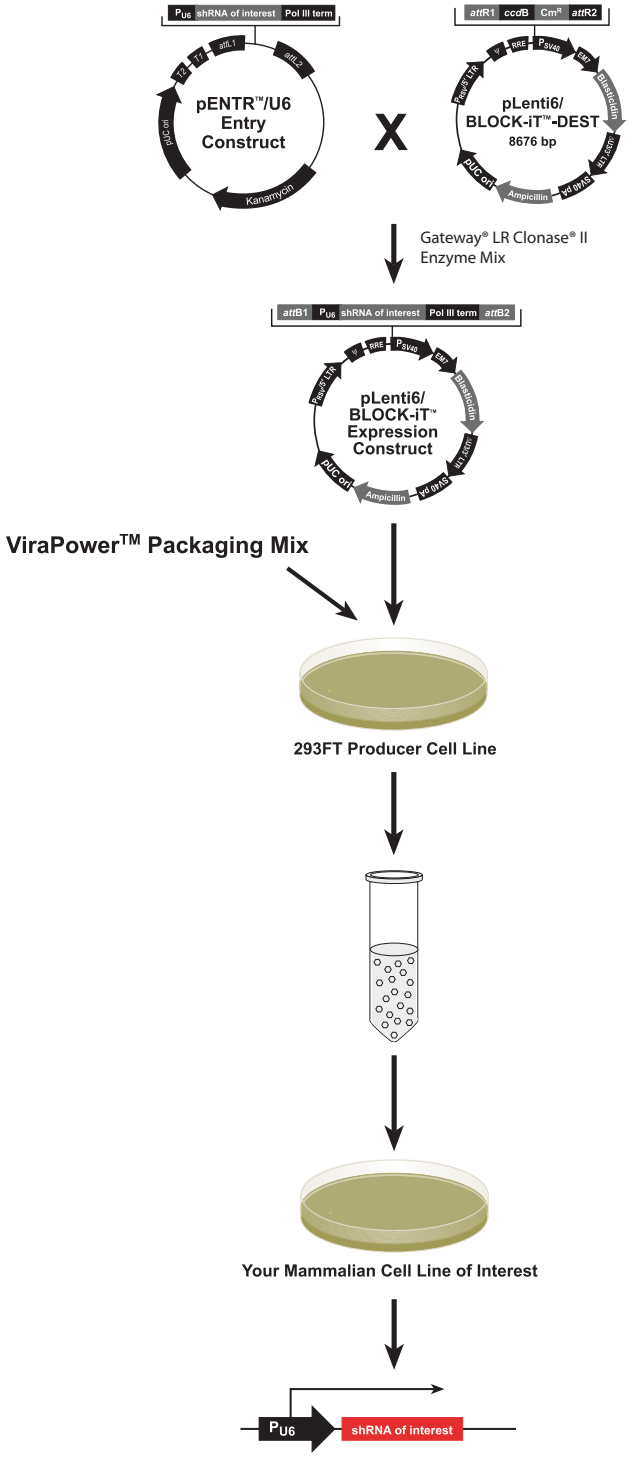
Important

Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of lentiviruses may vary at individual institutions, consult the health and safety guidelines and/or officers at your institution prior to use of the BLOCK-iT™ Lentiviral RNAi Expression System.

Experimental Outline

Flow Chart

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



1. Perform an LR recombination reaction between the pENTR™/U6 entry construct and pLenti6/ BLOCK-iT™-DEST to generate the pLenti6/BLOCK-iT™ expression construct.
2. Cotransfect the 293FT producer cell line with your pLenti6/ BLOCK-iT™ expression construct and the optimized packaging mix.
3. Harvest viral supernatant and determine the titer.
4. Add the viral supernatant to your mammalian cell line of interest. Select for stably transduced cells using blasticidin, if desired.
5. Assay for knockdown of the target gene.

Methods

Generating an Entry Clone

Introduction

To express your shRNA of interest from pLenti6/BLOCK-iT™-DEST, first 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 & 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 the BLOCK-iT™ Lentiviral RNAi Expression System, but is also available separately (see page 54 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 BLOCK-iT™ Lentiviral RNAi Expression System but is also available at www.invitrogen.com or by calling Technical Support (see page 55).

Creating Expression Clones

Introduction

After you have generated an entry clone, you are ready to perform the LR recombination reaction using your pENTR™/U6 entry construct and the pLenti6/BLOCK-iT™-DEST vector to generate an expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the sections entitled **Performing the LR Recombination Reaction** (pages 16–17) and **Transforming One Shot® Stbl3™ Competent *E. coli*** (pages 18–19) 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 pLenti6/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 18).
3. Select for expression clones (see the next page for a diagram of the recombination region of expression clones in pLenti6/BLOCK-iT™-DEST).

Important

The pLenti6/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 LR recombination, further testing has found that linearization of pLenti6/BLOCK-iT™-DEST is **not** required to obtain optimal results for any downstream application.

Propagating the Destination Vectors

To propagate and maintain the pLenti6/BLOCK-iT™-DEST vector, use 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (see page 54). The One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *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 to 100 µg/mL ampicillin and 15 to 30 µg/mL chloramphenicol.

Note: Do not use general *E. coli* cloning strains including Stbl3™, TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects.

Continued on next page

Recombination Region of pLenti6/BLOCK-iT™-DEST

The recombination region of the expression clone resulting from pLenti6/BLOCK-iT™-DEST × pENTR™/U6 entry clone is shown below.

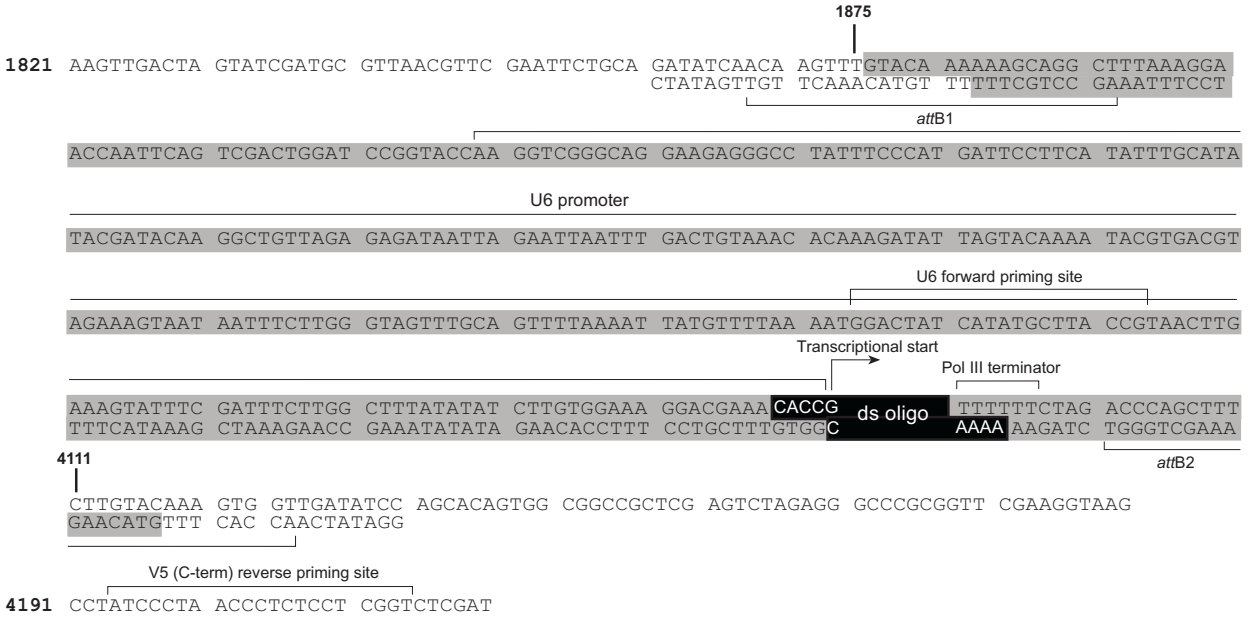
Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the pENTR™/U6 entry clone into the pLenti6/BLOCK-IT™-DEST vector by recombination. Non-shaded regions are derived from the pLenti6/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 1,875 and 4,111 of the pLenti6/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 pLenti6/BLOCK-iT™-DEST vector. We recommend including a negative control (no Gateway® LR Clonase® II) in your experiment to help evaluate results.

Recommended *E. coli* Host

For optimal results, use Stbl3™ *E. coli* for transformation as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA, which contains direct repeats. One Shot® Stbl3™ Chemically Competent *E. coli* are included in the kit for transformation. For instructions, see **Transforming One Shot® Stbl3™ Competent *E. coli***, page 18. Note that transformants containing unwanted recombinants (see **Note** below) are **not** obtained when Stbl3™ *E. coli* are used for transformation.

Note

You may transform the LR recombination reaction into other *rec*A, *end*A *E. coli* strains including TOP10 and DH5α™, if desired. Note however, that these strains are not as well-suited for cloning unstable DNA, and may give rise to a low percentage (< 5%) of transformants containing unwanted recombinants (*i.e.* plasmids where recombination has occurred between the 5′ and 3′ LTRs) when selected on plates containing only ampicillin. **These events occur less frequently when selection is performed using both ampicillin (100 µg/mL) and Blasticidin (50 µg/mL).** Note also that transformed *E. coli* grow more slowly in LB media containing ampicillin and Blasticidin, and may require slightly longer incubation times to obtain visible colonies. For more information about Blasticidin, see the **Appendix**, page 43.

Tip: When using TOP10 *E. coli* for transformation, we have observed that transformants containing a plasmid that has recombined between the 5′ and 3′ LTRs (*i.e.* unwanted recombinants) generally give rise to larger colonies than those containing an intact plasmid. **Select small colonies for analysis.**

Important

Do not transform the LR recombination reaction 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.

Gateway® LR Clonase® II Enzyme Mix

Gateway® LR Clonase® II enzyme mix is supplied with the BLOCK-iT™ Lentiviral RNAi Expression System and is also available separately (see page 54). The Gateway® 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 17 to perform the LR recombination reaction using Gateway® LR Clonase® II enzyme mix.

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

Continued on next page

Performing the LR Recombination Reaction, Continued

Positive Control

The pENTR™-gus plasmid is included with the Gateway® LR Clonase® II enzyme mix for use as a positive control for the LR recombination reaction. Use pENTR™-gus in your LR recombination reaction to verify the efficiency of the LR reaction. However, the resulting expression clone cannot be used as an expression control because neither the pLenti6/BLOCK-iT™-DEST vector nor pENTR™-gus include a eukaryotic promoter to control expression of the *gus* gene in mammalian cells.

Materials Needed

- Purified plasmid DNA of your pENTR™/U6 entry clone (50–150 ng/µL in TE Buffer)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), see page 54
- Sterile 0.5 mL microcentrifuge tubes

Components supplied with the kits

- pLenti6/BLOCK-iT™-DEST vector (150 ng/µL in TE Buffer, pH 8.0)

Components supplied with the BLOCK-iT™ Lentiviral RNAi Expression System only

- pENTR™-gus control
- Gateway® LR Clonase® II enzyme mix (store at –20°C until immediately before use)
- 2 µg/µL Proteinase K solution (thaw and keep on ice until use)

Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR reaction between the pENTR™/U6 entry clone and the pLenti6/BLOCK-iT™-DEST vector. If you want to include a negative control, set up a separate reaction but omit the Gateway® 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
pLenti6/BLOCK-iT™-DEST vector (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

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

   Reminder: Return Gateway® LR Clonase® II enzyme mix to –20°C immediately after use.
5. Incubate the reaction at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µL Proteinase K (2 µg/µL) to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® Stbl3™ Competent *E. coli***, next page.

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

Transforming One Shot® Stbl3™ Competent *E. coli*

Introduction

Follow the instructions in this section to transform the LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli* (Box 3) included with the kit. The transformation efficiency of One Shot® Stbl3™ Chemically Competent *E. coli* is 1×10^8 cfu/µg plasmid DNA.

Materials Needed

- LR recombination reaction (from Step 7, previous page)
- LB Medium (if performing the pUC19 control transformation)
- 42°C water bath
- LB plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- 37°C shaking and non-shaking incubator

Components supplied with the kits

- One Shot® Stbl3™ Chemically Competent *E. coli* (Box 3; one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (Box 3; warm to room temperature)
- pUC19 positive control (if desired to verify the transformation efficiency; Box 3)

One Shot® Stbl3™ Transformation Procedure

Use this procedure to transform the LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli*.

1. Thaw, on ice, one vial of One Shot® Stbl3™ chemically competent cells for each transformation.
2. Add 2 to 3 µL of the LR recombination reaction (from Step 7, page 17) into a vial of One Shot® Stbl3™ cells and mix gently. **Do not mix by pipetting up and down.** For the pUC19 control, add 10 pg (1 µL) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C water bath and place them on ice for 2 minutes.
6. Add 250 µL of pre-warmed S.O.C. Medium to each vial.
7. Cap the vial(s) tightly and shake horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 25–100 µL of the transformation mix on a pre-warmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium (*e.g.*, add 100 µL of the transformation mix to 900 µL of LB Medium) and plate 25–100 µL.
9. Store the remaining transformation mix at 4°C. Plate out additional cells the next day, if desired.

Continued on next page

Expected Results

When using One Shot® Stbl3™ Chemically Competent cells for transformation, the LR recombination reaction should result in greater than 5,000 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 chloramphenicol-sensitive **and** ampicillin- and Blasticidin-resistant. Transformants containing a plasmid with a mutated *ccdB* gene will be chloramphenicol-, ampicillin-, and Blasticidin-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.

Sequencing

Sequencing the expression construct is not required as transfer of the U6 RNAi cassette from pENTR™/U6 into the pLenti6/BLOCK-iT™-DEST vector preserves the orientation of the cassette. However, if you wish to sequence your pLenti6/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.

Primer	Sequence
U6 Forward	5′-GGACTATCATATGCTTACCG-3′
V5(C-term) Reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Note: For information about a convenient custom primer synthesis service go to www.invitrogen.com or call Technical Support (see page 55).

Maintaining the Expression Clone

Once you have generated your expression clone, maintain and propagate the expression clone in LB medium containing 100 µg/mL ampicillin. Addition of Blasticidin is not required.

Producing Lentivirus in 293FT Cells

Introduction

Before creating a stably transduced cell line expressing your shRNA of interest, first produce a lentiviral stock (containing the packaged pLenti6/BLOCK-iT™-DEST expression construct) by co-transfecting the optimized ViraPower™ Packaging Mix and your pLenti6/BLOCK-iT™-DEST expression construct into the 293FT Producer Cell Line.

Plasmid Preparation

After generating your expression clone, you **must** isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating lentiviral plasmid DNA using the PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit (see page 54). Resuspend the purified pLenti6/BLOCK-iT™-DEST expression plasmid in sterile water or TE Buffer, pH 8.0, to a final concentration ranging from 0.1–3.0 µg/µL. You will need 3 µg of the expression plasmid for each transfection.

Important: Do not use mini-prep plasmid DNA for transfection.

Materials Needed

- pLenti6/BLOCK-iT™-DEST expression construct (0.1–3.0 µg/µL in sterile water or TE Buffer, pH 8.0)
- 293FT cells cultured in the appropriate medium (*i.e.* D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1% penicillin/streptomycin)

  Note: D-MEM already contains 4 mM L-glutamine, which is enough to support cell growth of the 293FT Cell Line. However, since L-glutamine slowly decays over time, supplement the medium with 2 mM L-glutamine. 293FT cells grow well in 6 mM L-glutamine, but higher concentrations of L-glutamine may reduce growth.
- Opti-MEM® I Reduced Serum Medium (pre-warmed; see page 54)
- Fetal bovine serum (FBS; see page 54)
- Complete growth medium containing sodium pyruvate (*i.e.* D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate)

  Note: MEM Sodium Pyruvate provides an extra energy source for the cells and is available separately, see page 54. See note above for L-glutamine concentration.
- Sterile, 10 cm tissue culture plates (one each for the lentiviral construct, positive control, and negative control)
- Sterile, tissue culture supplies
- 5 and 15 mL sterile, capped, conical tubes
- Cryovials

Continued on next page

Producing Lentivirus in 293FT Cells, Continued

Materials Supplied with the Kits

Components supplied with the kits

- pLenti6-GW/U6-lamin^{shRNA} positive control vector

Components supplied with the BLOCK-iT™ Lentiviral RNAi Expression System only

- ViraPower™ Packaging Mix
- Lipofectamine® 2000 transfection reagent (store at 4°C and mix gently before use)

293FT Cell Line

The human 293FT Cell Line is supplied with the BLOCK-iT™ Lentiviral RNAi Expression System to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo and must be maintained in medium containing Geneticin®. For more information about pCMVSPORT6TAg.neo and how to culture and maintain 293FT cells, refer to the 293FT Cell Line manual. This manual is supplied with the BLOCK-iT™ Lentiviral RNAi Expression System, but is also available at www.invitrogen.com or by calling Technical Support (see page 55).

Note: The 293FT Cell Line is also available separately, see page 54.

The health of your 293FT cells at the time of transfection has a critical effect on the success of lentivirus production. Use of "unhealthy" cells can negatively affect the transfection efficiency, resulting in production of a low titer lentiviral stock. For optimal lentivirus production (*i.e.* producing lentiviral stocks with the expected titers), follow the guidelines below to culture 293FT cells before use in transfection:

- Make sure that cells are greater than 90% viable.
- Subculture and maintain cells as recommended in the 293FT Cell Line manual. Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 20 passages.

ViraPower™ Packaging Mix

The pLP1, pLP2, pLP/VSVG plasmids are provided in an optimized mixture to facilitate viral packaging of your pLenti6/BLOCK-iT™-DEST expression vector following cotransfection into 293FT producer cells. The amount of the packaging mix (195 µg) and Lipofectamine® 2000 Reagent (0.75 mL) supplied in the BLOCK-iT™ Lentiviral RNAi Expression System is sufficient to perform 20 cotransfections in 10 cm plates using the recommended protocol on page 24.

Note: ViraPower™ Packaging Mix is available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit, see page 54.

Continued on next page

Lipofectamine® 2000

The Lipofectamine® 2000 reagent supplied with the BLOCK-iT™ Lentiviral RNAi Expression System (Ciccarone *et al.*, 1999) is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids into eukaryotic cells. Using Lipofectamine® 2000 to transfect 293FT cells offers the following advantages:

- Provides the highest transfection efficiency in 293FT 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 is not required, although complexes can be removed after 4–6 hours without loss of activity

Note: Lipofectamine® 2000 is available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit, see page 54 for ordering information.

Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine® 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium (see page 54 for ordering information). For more information about Opti-MEM® I, go to www.invitrogen.com or call Technical Support (see page 55).

Positive Control

The pLenti6-GW/U6-lamin^{shRNA} plasmid is included with the BLOCK-iT™ Lentiviral RNAi Kits as a control for lentivirus production. We recommend including the positive control vector in your cotransfection experiment to generate a control lentiviral stock. Once generated, the control lentivirus 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.

Continued on next page

Recommended Transfection Conditions

We produce lentiviral stocks in 293FT cells using the **optimized** transfection conditions shown below. The amount of lentivirus produced using these recommended conditions (at a titer of 1×10^5 to 1×10^7 transducing units (TU)/mL) is generally sufficient to transduce 1×10^6 to 1×10^8 cells at a multiplicity of infection (MOI) = 1.

Condition	Amount
Tissue culture plate size	10 cm (one per lentiviral construct)
Number of 293FT cells to transfect	6×10^6 cells (see **Recommendation** on page 21 to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix	9 µg (9 µL of 1 µg/µL stock)
Amount of pLenti6/BLOCK-iT™-DEST expression plasmid	3 µg
Amount of Lipofectamine® 2000 Reagent to use	36 µL

Note: You may produce lentiviral stocks using other tissue culture formats, but keep in mind that optimization will be necessary to obtain the expected titers.

The recommended procedure to co-transfect 293FT cells differs from the traditional Lipofectamine® 2000 transfection procedure in that you will:

- First prepare DNA:Lipofectamine® 2000 complexes and add them to plates containing growth media, then
- Add the 293FT cells to the media containing DNA:Lipofectamine® 2000 complexes and allow the cells to attach and transfect overnight (see next page).

Using this procedure, we consistently obtain lentiviral stocks with titers that are **3 to 4-fold higher** than lentiviral stocks generated using the traditional Lipofectamine® 2000 transfection procedure (*i.e.* plating cells first followed by transfection with DNA:Lipofectamine® 2000 complexes). You may use the traditional Lipofectamine® 2000 transfection procedure, if desired, but keep in mind that the viral titer obtained may be lower (see **Alternative Transfection Procedure**, page 25).

Continued on next page

Transfection Procedure

Follow the procedure below to cotransfect 293FT cells. Include a negative control (no DNA, no Lipofectamine® 2000) in your experiment to help evaluate results. You will need 6×10^6 293FT cells for each sample.

1. **For each transfection sample,** prepare DNA-Lipofectamine® 2000 complexes as follows:
   a. In a sterile 5 mL tube, dilute 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti6/BLOCK-iT™-DEST expression plasmid DNA (12 µg total) in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently.
   b. In a separate sterile 5 mL tube, mix Lipofectamine® 2000 gently before use, then dilute 36 µL in 1.5 mL 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.
2. While DNA-lipid complexes are forming, trypsinize and count the 293FT cells. Resuspend the cells at a density of 1.2×10^6 cells/mL in growth medium containing serum (or Opti-MEM® I Medium containing serum).
3. Add the DNA-Lipofectamine® 2000 complexes to a 10 cm tissue culture plate containing 5 mL of growth medium containing serum (or Opti-MEM® I Medium containing serum). **Do not add antibiotics to the medium.**
4. Add 5 mL of the 293FT cell suspension (6×10^6 total cells) to the plate containing media and DNA-Lipofectamine® 2000 complexes and 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 media containing the DNA-Lipofectamine® 2000 complexes and replace with complete culture medium containing sodium pyruvate (see page 20).

   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of multinucleated syncitia. This morphological change is normal and does not affect production of the lentivirus.
6. Harvest virus-containing supernatants 48–72 hours posttransfection by removing medium to a 15 mL sterile, capped, conical tube.

   Note: Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours posttransfection.

   Caution: Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see pages 11 and 28 for more information).
7. Centrifuge at 3000 rpm for 5 minutes at 4°C to pellet cell debris. Perform filtration step, if desired (see **Note** on the next page).
8. Pipet viral supernatants into cryovials in 1 mL aliquots. Store viral stocks at −80°C. Proceed to **Titering Your Lentiviral Stock**, page 26.

Continued on next page

Alternative Transfection Procedure

An alternative transfection procedure is provided below to cotransfect 293FT cells. Note that use of this procedure generally results in production of lentiviral stocks with a slightly lower titer that those produced when using the recommended **Transfection Procedure**, previous page.

1. The day before transfection, plate 293FT cells in a 10 cm tissue culture plate such that they will be 90–95% confluent on the day of transfection (*i.e.* 6×10^6 cells in 10 mL of growth medium containing serum).
2. On the day of transfection, remove the culture medium from the 293FT cells and replace with 5 mL of growth medium containing serum (or Opti-MEM® I Medium containing serum). **Do not include antibiotics in the medium.**
3. Prepare DNA-Lipofectamine® 2000 complexes as instructed in the recommended **Transfection Procedure**, Step 1, previous page.
4. Add the DNA-Lipofectamine® 2000 complexes dropwise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO_2 incubator.

Follow Steps 5–8 as instructed in the recommended **Transfection Procedure**, previous page.

Note

If you plan to use your lentiviral construct for *in vivo* applications, filter your viral supernatant through a sterile, 0.45 µm low protein binding filter after the low-speed centrifugation step (see Step 7, previous page) to remove any remaining cellular debris. We recommend using Millex®-HV 0.45 µm PVDF filters (Millipore, Cat. no. SLHV033RB) for filtration.

If you wish to concentrate your viral stock to obtain a higher titer, perform the filtration step first before concentrating your viral stock.

Long-Term Storage

Place lentiviral stocks at −80°C for long-term storage. Repeated freezing and thawing is not recommended as it may result in loss of viral titer. When stored properly, viral stocks of an appropriate titer should be suitable for use for up to one year. After long-term storage, re-titer your viral stocks before transducing your mammalian cell line of interest.

Scaling Up Virus Production

It is possible to scale up the cotransfection experiment to produce a larger volume of lentivirus, if desired. For example, we have scaled up the cotransfection experiment from a 10 cm plate to a T-175 cm^2 flask and harvested up to 30 mL of viral supernatant. If you wish to scale up your cotransfection, remember that you will need to increase the number of cells plated and the amounts of DNA, Lipofectamine® 2000, and medium used in proportion to the difference in surface area of the culture vessel.

Titering Your Lentiviral Stock

Introduction

Before transducing your mammalian cell line and expressing your shRNA for RNAi analysis, we recommend determining the titer of your lentiviral stock. While this procedure is not required for some applications, it is necessary if:

- You wish to control the number of integrated copies of the lentivirus
- You wish to generate reproducible gene knockdown results

Experimental Outline

To determine the titer of a lentiviral stock, you will:

1. Prepare 10-fold serial dilutions of your lentiviral stock
2. Transduce the different dilutions of lentivirus into the mammalian cell line of your choice in the presence of Polybrene®
3. Select for stably transduced cells using Blasticidin
4. Stain and count the number of Blasticidin-resistant colonies in each dilution

Factors Affecting Viral Titer

A number of factors can influence lentiviral titers including:

- The characteristics of the cell line used for titering (see below).
- The age of your lentiviral stock. Viral titers may decrease with long-term storage at −80°C. If your lentiviral stock has been stored for longer than 6 months, titer or re-titer your lentiviral stock prior to use in an RNAi experiment.
- Number of freeze/thaw cycles. Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- Improper storage of your lentiviral stock. Lentiviral stocks should be aliquotted and stored at −80°C (see page 25 for recommended storage conditions).

Selecting a Cell Line

You may titer your lentiviral stock using any mammalian cell line of choice. Generally, we recommend using the same mammalian cell line to titer your lentiviral stock as you will use to perform your expression studies. However, in some instances, you may wish to use a different cell line to titer your lentivirus (*e.g.*, if you are performing RNAi studies in a non-dividing cell line or a primary cell line). In these cases, we recommend that you choose a cell line with the following characteristics to titer your lentivirus:

- Grows as an adherent cell line
- Easy to handle
- Exhibits a doubling time in the range of 18–25 hours
- Non-migratory

We generally use the HT1080 human fibrosarcoma cell line (ATCC, Cat. no. CCL-121) for titering purposes.

Important: You may use other cell lines, including HeLa and NIH/3T3, to titer your lentivirus. However, note that the titer obtained when using HeLa cells or NIH/3T3 cells is approximately **10-fold lower** than the titer obtained when using HT1080 cells.

Continued on next page

Titering Your Lentiviral Stock, Continued

Note

The titer of a lentiviral construct may vary depending on which cell line is chosen (see **Factors Affecting Viral Titer,** previous page). If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line.

Antibiotic Selection

The pLenti6/BLOCK-iT™-DEST expression construct contains the Blasticidin resistance gene (*bsd*) (Kimura *et al.*, 1994) to allow for Blasticidin selection (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) of mammalian cells that have stably transduced the lentiviral construct.

If you are using the BLOCK-iT™ Lentiviral RNAi Expression System, Blasticidin is supplied with the kit. Blasticidin is also available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 54 for ordering information).

Preparing Blasticidin

For more information about how to prepare and handle Blasticidin, refer to the **Appendix**, page 43.

Determining Antibiotic Sensitivity

To select for stably transduced cells using Blasticidin, first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 2–10 µg/mL Blasticidin are sufficient to kill most untransduced mammalian cell lines. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate cells at approximately 25% confluence. Prepare a set of 6 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Blasticidin (*e.g.*, 0, 2, 4, 6, 8, 10 µg/mL Blasticidin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10–14 days after addition of antibiotic.

Using Polybrene® During Transduction

Transduction of lentivirus into mammalian cells may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene®). For best results, we recommend performing transduction in the presence of Polybrene®. Note however, that some cells are sensitive to Polybrene® (*e.g.*, primary neurons). Before performing any transduction experiments, you may want to test your cell line for sensitivity to Polybrene®. If your cells are sensitive to Polybrene® (*e.g.*, exhibit toxicity or phenotypic changes), do not add Polybrene® during transduction. In this case, cells should still be successfully transduced.

Continued on next page

Titering Your Lentiviral Stock, Continued

Preparing and Storing Polybrene®

Follow the instructions below to prepare Polybrene® (Sigma-Aldrich, Cat. no. H9268):

1. Prepare a 6 mg/mL stock solution in deionized, sterile water.
2. Filter-sterilize and dispense 1 mL aliquots into sterile microcentrifuge tubes.
3. Store at –20°C for long-term storage. Stock solutions may be stored at –20°C for up to 1 year. Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

   Note: The working stock may be stored at 4°C for up to 2 weeks.

Materials Needed

- Your pLenti6/ BLOCK-iT™ lentiviral stocks (store at –80°C until use)
- Adherent mammalian cell line (HT1080 human fibrosarcoma or other)
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- 6-well tissue culture plates
- Crystal violet (Sigma-Aldrich®, Cat. no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
- Phosphate-Buffered Saline (PBS; page 54)

Components supplied with the BLOCK-iT™ Lentiviral RNAi Expression System

- Blasticidin (10 mg/mL stock) for selection

Remember that you will be working with media containing infectious virus. Follow the recommended Federal and institutional 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 and dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

Continued on next page

Titering Your Lentiviral Stock, Continued

Transduction and Titering Procedure

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of your choice. You will use **at least** one 6-well plate for every lentiviral stock to be titered (one mock well plus five dilutions).

Note: If you have generated a lentiviral stock of the pLenti6-GW/U6-lamin^{shRNA} control construct, we recommend titering this stock as well.

1. The day before transduction (Day 1), trypsinize and count the cells, plating them in a 6-well plate such that they will be 30–50% confluent at the time of transduction. Incubate cells at 37°C overnight.

   Example: When using HT1080 cells, we usually plate 2×10^5 cells per well in a 6-well plate.

2. On the day of transduction (Day 2), thaw your lentiviral stock and prepare 10-fold serial dilutions ranging from 10^{-2} to 10^{-6}. For each dilution, dilute the lentiviral construct into complete culture medium to a final volume of 1 mL. **DO NOT** vortex.

   Note: You may prepare a wider range of serial dilutions (10^{-2} to 10^{-8}), if desired.

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. Add Polybrene® (if desired) to each well to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.
5. The following day (Day 3), remove the media containing virus and replace with 2 mL of complete culture medium.
6. The following day (Day 4), remove the medium and replace with complete culture medium containing the appropriate amount of Blasticidin to select for stably transduced cells.
7. Replace medium with fresh medium containing Blasticidin every 3–4 days.
8. After 10–12 days of selection (day 14–16), you should see no live cells in the mock well and discrete Blasticidin-resistant colonies in one or more of the dilution wells. Remove the medium and wash the cells twice with PBS.
9. Add crystal violet solution (1 mL for 6-well dish; 5 mL for 10 cm plate) and incubate for 10 minutes at room temperature.
10. Remove the crystal violet stain and wash the cells with PBS. Repeat wash.
11. Count the blue-stained colonies and determine the titer of your lentiviral stock.

Expected Results

When titering pLenti6/BLOCK-iT™ lentiviral stocks using HT1080 cells, we generally obtain titers ranging from 5×10^5 to 2×10^7 transducing units (TU)/mL. For an example of expected results obtained from a typical titering experiment, see the next page.

Note: If the titer of your lentiviral stock is less than 1×10^5 TU/mL, we recommend producing a new lentiviral stock. See page 26 and the **Troubleshooting** section, page 38 for more tips and guidelines to optimize the viral yield.

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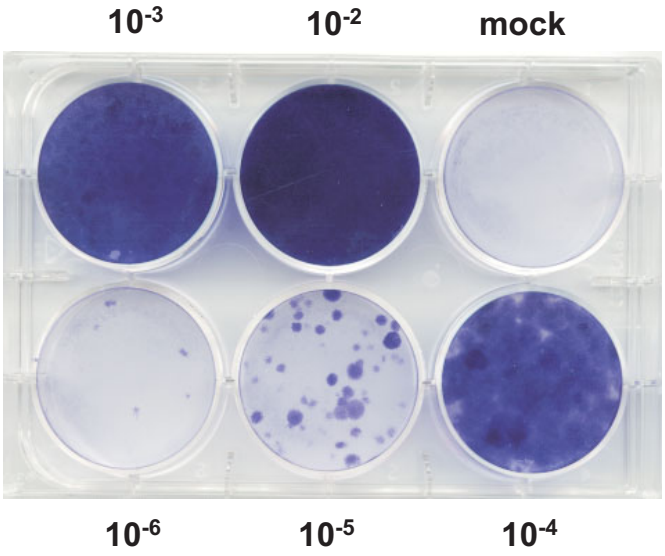
Titering Your Lentiviral Stock, Continued

Example of Expected Results

In this experiment, a pLenti6 lentiviral stock was generated using the protocol on page 24. HT1080 cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^{-2} to 10^{-6} dilutions) or untransduced (mock) following the protocol on page 29. Forty-eight hours post-transduction, the cells were placed under Blasticidin selection (10 µg/mL). After 10 days of selection, the cells were stained with crystal violet (see plate below), and colonies were counted.



In the plate above, the colony counts were:

- Mock: no colonies
- 10^{-2} dilution: confluent; undeterminable
- 10^{-3} dilution: confluent; undeterminable
- 10^{-4} dilution: confluent; undeterminable
- 10^{-5} dilution: 46
- 10^{-6} dilution: 5

Thus, the titer of this lentiviral stock is 4.8×10^6 TU/mL (*i.e.* average of 46×10^5 and 5×10^6).

Transduction and Analysis

Introduction

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

Reminder: Remember that your lentiviral construct contains a deletion in the 3′ LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. Once integrated into the genome, the lentivirus can no longer produce packageable virus.

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 vs. Stable Expression

After transducing your lentiviral construct into the mammalian cell line of your choice, you may assay for target gene knockdown in the following ways:

- Pool a heterogeneous population of cells and test for gene knockdown directly after transduction (*i.e.* "transient" RNAi analysis). Note that you must wait for a minimum of 48–72 hours after transduction before harvesting your cells to allow expressed shRNA molecules to accumulate in transduced cells.
- Select for stably transduced cells using Blasticidin. This requires a minimum of 10–12 days after transduction, but allows generation of clonal cell lines that stably express the shRNA of interest.

Determining Antibiotic Sensitivity for Your Cell Line

Before selecting for stably transduced cells, first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). For guidelines to perform a kill curve experiment, see page 27. If you titered your lentiviral construct in the same mammalian cell line that you are using to generate a stable cell line, then you may use the same concentration of Blasticidin for selection that you used for titering.

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 lentiviral 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 the number of integration events and as a result, expression. Typically, shRNA expression levels increase as the MOI increases.

Continued on next page

Transduction and Analysis, Continued

Determining the Optimal MOI

A number of factors can influence the 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 lentiviral construct into the mammalian cell line of choice for the first time, we recommend using a range of MOIs (*e.g.,* 0, 1, 5, 10, 50) to determine the MOI required to obtain the optimal degree of target gene knockdown.

Note

In general, non-dividing cell types transduce lentiviral constructs less efficiently than actively dividing cell lines. If you are transducing your lentiviral 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 control pLenti6-GW/U6-lamin^{shRNA} lentiviral construct, you may use this lentiviral stock as a negative control for the RNAi response in any mammalian cell line. In addition, you may use this lentiviral 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**, page 5).

Note: If your cell line expresses lamin A/C, you may detect the protein using Western blot analysis (see page 34).

Important

Remember that viral supernatants are generated by harvesting spent media containing virus from the 293FT 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 cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their transducibility. If the titer of your lentiviral stock is relatively low (less than 5×10^5 TU/mL) and your experiment requires that you use a large volume of viral supernatant (*e.g.,* a relatively high MOI), you may wish to concentrate your virus before proceeding to transduction. For details and guidelines to concentrate your virus, refer to published reference sources (Yee, 1999).

Continued on next page

Transduction and Analysis, Continued

Materials Needed

- Your titered lentiviral stock (store at −80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- Appropriately sized tissue culture plates for your application

Components supplied with the BLOCK-iT™ Lentiviral RNAi Expression System

- 10 mg/mL Blasticidin stock (used if selecting for stably transduced cells)

Transduction Procedure

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

1. Plate cells 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 lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.

   Note: If you are transducing cells with undiluted viral stock and are concerned about possible toxicity or growth effects caused by overnight incubation, it is possible to incubate cells for as little as 6 hours prior to changing medium.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium.
6. The following day (Day 3), perform one of the following:
   - Harvest the cells and assay for inhibition of your target gene if you are performing transient expression experiments. If you wish to assay the cells at a later time, you may continue to culture the cells or replate them into larger-sized tissue culture formats as necessary.
   - Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin to select for stably transduced cells. Proceed to Step 7.
7. Replace medium with fresh medium containing Blasticidin every 3–4 days until Blasticidin-resistant colonies can be identified (generally 10–12 days after selection).
8. Pick at least 5 Blasticidin-resistant colonies (see **Note** on the next page) and expand each clone to assay for knockdown of the target gene.

Continued on next page

Note

Integration of the lentivirus into the genome is random. The influence of the surrounding genomic sequences at the integration site may affect target gene knockdown from different Blasticidin-resistant clones. Test at least 5 Blasticidin-resistant clones and select the clone that provides the optimal degree of gene knockdown for further studies.

Performing RNAi Analysis

You may use any method as appropriate to assay for knockdown of your target gene including functional analysis, immunofluorescence, western blot, qRT-PCR with the appropriate LUX™ primers, or real-time qRT-PCR using TaqMan® products. For more information about LUX™ primers or TaqMan® products, see www.invitrogen.com.

Expected Results

When performing RNAi studies using pLenti6/BLOCK-iT™ lentiviral 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 31. Note that 100% gene knockdown is generally not observed, but > 80% is possible with optimized conditions

For an example of results obtained from RNAi experiments using the pLenti6-GW/U6-lamin^{shRNA} lentiviral construct, see the next page.

Assaying for Lamin A/C Expression

If you perform RNAi analysis using the pLenti6-GW/U6-lamin^{shRNA} control lentiviral stock, you may assay for lamin A/C expression and knockdown using Western blot. We use an Anti-Lamin A/C Antibody (BD Biosciences, Cat. no. 612162) to detect lamin A/C expression.

Examples of Expected Results

Introduction

This section provides examples of results obtained from two RNAi experiments performed using the pLenti6-GW/U6-lamin^{shRNA} control lentiviral construct.

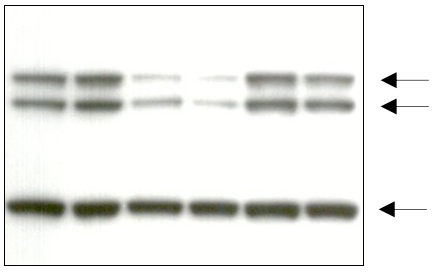
Example 1: Knockdown of Lamin A/C in HeLa Cells

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 pLenti6/BLOCK-iT™-DEST vector using the LR recombination reaction to generate the pLenti6-GW/U6-lamin^{shRNA} and pLenti6-GW/U6-luc^{shRNA} expression constructs. Lentiviral stocks were generated and titered in HT1080 cells following the protocols in this manual (see pages 20–29).

HeLa cells plated in a 12-well plate were transduced with each lentiviral construct at an MOI of 100. Cell lysates were prepared from one set of wells 48 hours (*i.e.* 2 days) after transduction. The transduced cells in the second set of wells were replated into a 6-well plate, then cultured for an additional 72 hours. Cell lysates were prepared 120 hours (*i.e.* 5 days) after transduction. Equivalent amounts of cell lysate were analyzed by Western blot using an Anti-Lamin A/C Antibody (1:1,000 dilution, BD Biosciences, Cat. no. 612162) and an Anti-β-Actin Antibody (1:5,000 dilution, Abcam, Cat. no. ab6276).

Results:

- Only the lamin A/C-specific shRNA (Lanes 3 and 4) inhibits expression of the lamin A/C gene, while no lamin A/C knockdown is observed with the luciferase shRNA (Lanes 5 and 6).
- A greater degree of lamin A/C knockdown is observed 5 days after transduction (> 80%) when compared to 2 days.
- 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).



Lane 1. Untransduced – Day 2

Lane 2. Untransduced – Day 5

Lane 3. Lenti6-GW/U6-lamin^{shRNA} construct – Day 2

Lane 4. Lenti6-GW/U6-lamin^{shRNA} construct – Day 5

Lane 5. Lenti6-GW/U6-luc^{shRNA} construct – Day 2

Lane 6. Lenti6-GW/U6-luc^{shRNA} construct – Day 5

Continued on next page

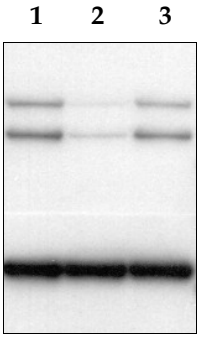
Examples of Expected Results, Continued

Example 2: Knockdown of Lamin A/C in COS-7 Cells

In this experiment, the pLenti6-GW/U6-lamin^{shRNA} and pLenti6-GW/U6-luc^{shRNA} lentiviral constructs described in Example 1 were used to transduce COS-7 (African Green monkey kidney) cells plated in a 12-well plate at an MOI of 50. Cell lysates were prepared 48 hours after transduction and equivalent amounts of cell lysate were analyzed by Western blot using an Anti-Lamin A/C Antibody (1:1,000 dilution, BD Biosciences, Cat. no. 612162) and an Anti-β-Actin Antibody (1:5,000 dilution, Abcam, Cat. no. ab6276).

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 (Lane 3).
- The lamin A/C shRNA expressed from pLenti6-GW/U6-lamin^{shRNA} is active in a non human-derived cell line.



Lane 1. Untransduced
Lane 2. Lenti6-GW/U6-lamin^{shRNA} construct
Lane 3. Lenti6-GW/U6-luc^{shRNA} construct

Troubleshooting

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.
	Didn't use the suggested amount of Gateway® LR Clonase® II enzyme mix or Gateway® LR Clonase® II enzyme mix was inactive	• Make sure to store the Gateway® LR Clonase® II enzyme mix at −20°C or −80°C. • Do not thaw the Gateway® LR Clonase® II enzyme mix more than 10 times. • Use the recommended amount of Gateway® LR Clonase® II enzyme mix (see page 17). • Test another aliquot of the Gateway® LR Clonase® II enzyme mix.
	Not enough LR reaction transformed	Transform 2–3 µL of the LR reaction into One Shot® Stbl3™ Chemically Competent *E. coli*.
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C with shaking before plating.
	Too much entry clone DNA used in the LR reaction	Use 50–150 ng of the entry clone in the LR reaction.
Different sized colonies (*i.e.* large and small) appear when using TOP10 *E. coli* for transformation	Some transformants contain plasmids in which unwanted recombination has occurred between 5′ and 3′ LTRs	• Select for transformants on LB plates containing **both** 100 µg/mL ampicillin and 50 µg/mL Blasticidin. • Use the One Shot® Stbl3™ Chemically Competent *E. coli* supplied with the kit for transformation. Stbl3™ *E. coli* are recommended for cloning unstable DNA including lentiviral DNA containing direct repeats and generally do not give rise to unwanted recombinants.

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LR Reaction and Transformation, Continued

Problem	Reason	Solution
Few or no colonies obtained from the transformation control	Competent cells stored incorrectly	• Store the One Shot® Stbl3™ Chemically Competent *E. coli* at −80°C. • Thaw a vial of One Shot® cells on ice immediately before use.
	After addition of DNA, competent cells mixed by pipetting up and down	After adding DNA, mix competent cells gently. **Do not mix by pipetting up and down.**

Generating the Lentiviral Stock

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

Problem	Reason	Solution
Low viral titer	Low transfection efficiency: • Used poor quality expression construct plasmid DNA (*i.e.* DNA from a mini-prep) • Unhealthy 293FT cells; cells exhibit low viability • Cells transfected in media containing antibiotics (*i.e.* Geneticin®) • Plasmid DNA:transfection reagent ratio incorrect • 293FT cells plated too sparsely	• **Do not** use plasmid DNA from a mini-prep for transfection. Use the PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit to prepare plasmid DNA. • Use healthy 293FT cells under passage 20; do not overgrow. • **Do not** add Geneticin® to media during transfection as this reduces transfection efficiency and causes cell death. • Use a DNA (in µg):Lipofectamine® 2000 (in µL) ratio ranging from 1:2 to 1:3. • Plate cells such that they are 90–95% confluent at the time of transfection OR use the recommended transfection protocol (*i.e.* add cells to media containing DNA:lipid complexes; see page 24).
	Transfected cells not cultured in media containing sodium pyruvate	One day after transfection, remove media containing DNA:lipid complexes and replace with complete media containing sodium pyruvate. Sodium pyruvate provides an extra energy source for the cells.
	Lipofectamine® 2000 Reagent handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion before use. **Do not vortex.**

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Generating the Lentiviral Stock, Continued

Problem	Reason	Solution
Low viral titer, Continued	Viral supernatant harvested too early	Viral supernatants can generally be collected 48–72 hours posttransfection. If many cells are still attached to the plate and look healthy at this point, wait an additional 24 hours before harvesting the viral supernatant.
	Viral supernatant too dilute	Concentrate virus using any method of choice (Yee, 1999).
	Viral supernatant frozen and thawed multiple times	**Do not** freeze/thaw viral supernatant more than 3 times.
	Poor choice of titering cell line	Use HT1080 cells or another adherent cell line with the characteristics discussed on page 26.
	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.
	Polybrene® not included during titering procedure	Transduce the lentiviral construct into cells in the presence of Polybrene®.
No colonies obtained upon titering	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve experiment. Use the minimum Blasticidin concentration required to kill your untransduced cell line.
	Viral stocks stored incorrectly	Aliquot and store stocks at −80°C. Do not freeze/thaw more than 3 times.
	Polybrene® not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene®.
Titer indeterminable; cells confluent	Too little Blasticidin used for selection	Increase amount of Blasticidin used for selection.
	Viral supernatant not diluted sufficiently	Titer lentivirus using a wider range of 10-fold serial dilutions (*e.g.*, 10^{-2} to 10^{-8}).

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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
Low levels of gene knockdown observed	Low transduction efficiency: • Polybrene® not included during transduction • Non-dividing cell type used	• Transduce the lentiviral construct into cells in the presence of Polybrene®. • Transduce your lentiviral construct into cells using a higher MOI.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
	Cells harvested and assayed 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 before assaying for gene knockdown or place cells under Blasticidin selection. **Note:** Placing cells under Blasticidin selection can improve gene knockdown results by killing untransduced cells.
	Target gene is important for cell viability	Make sure that your target gene is not essential for cell viability or growth.
	Viral stocks not titered	Titer the lentiviral stock using the procedure on page 29 before use.
	Viral stock stored incorrectly	• Aliquot and store stocks at −80°C. • Do not freeze/thaw more than 3 times. • If stored for longer than 6 months, re-titer stock before use.
	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 pLenti6/BLOCK-iT™-DEST vector and proceed to generate lentivirus. **Note:** Generally, transient transfection greatly overexpresses shRNA, so moderately active pENTR™/U6 entry clones may be less active when expressed from a lentiviral construct.

Continued on next page

Troubleshooting, Continued

Transduction and RNAi Analysis, Continued

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 pLenti6/BLOCK-iT™-DEST vector and proceed to generate lentivirus.
	Viral stocks stored incorrectly	Aliquot and store stocks at −80°C. Do not freeze/thaw more than 3 times.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
Cytotoxic effects observed after transduction	Target gene is essential for cell viability	Make sure that your target gene is not essential for cell viability or growth.
	Large volume of viral supernatant used for transduction	• Remove the "spent" media containing virus and replace with fresh, complete media. • Concentrate the virus (Yee, 1999).
	Polybrene® used during transduction	Verify the sensitivity of your cells to Polybrene®. If cells are sensitive, omit the Polybrene® during transduction.
	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve. Use the minimum Blasticidin concentration required to kill your untransduced cell line.
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 pLenti6-GW/U6-lamin^{shRNA} control lentivirus	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 pLenti6-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

LB (Luria-Bertani) Medium

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 Liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 Liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic, if desired.
4. Store at 4°C.

LB Plates Containing Ampicillin and Blasticidin

Follow the instructions below to prepare LB agar plates containing ampicillin and Blasticidin.

Important Note: The stability of Blasticidin may be affected by high temperature; therefore, we **do not** recommend adding Blasticidin to warm LB agar. Let LB agar cool to room temperature before adding Blasticidin.

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add ampicillin to a final concentration of 100 µg/mL and pour into 10 cm plates.
4. Let harden, then spread 50 µg/mL Blasticidin on each plate.
5. Invert and store at 4°C, in the dark. Plates containing Blasticidin may be stored at 4°C for up to 2 weeks.

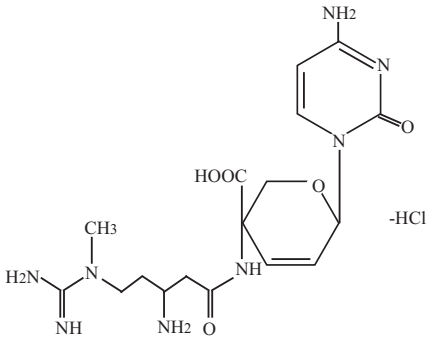
Blasticidin

Description

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseo-chromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells. Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *BSD* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and a laboratory coat when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

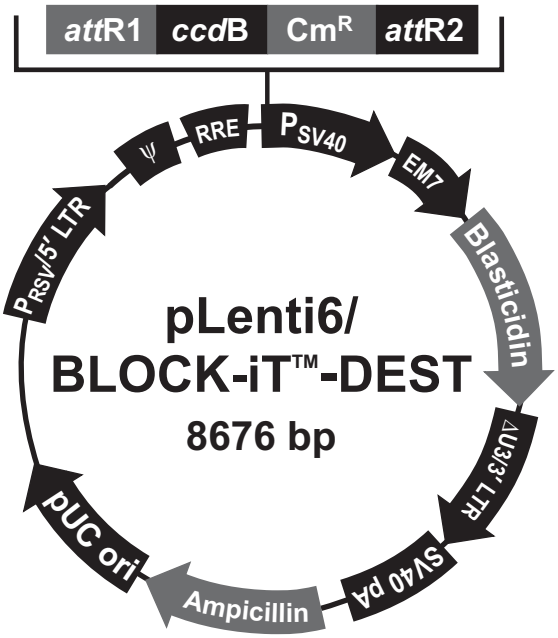
Blasticidin may be obtained in 50 mg aliquots (see page 54 for ordering information).

- Blasticidin is soluble in water and acetic acid.
- Prepare a stock solution of 5 to 10 mg/mL Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use and freeze at −20°C for long-term storage or store at 4°C for short term storage.
- Aqueous stock solutions are stable for 1 week at 4°C and 6–8 weeks at −20°C.
- pH of the aqueous solution should not exceed 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Map and Features of pLenti6/BLOCK-iT™-DEST

Map of pLenti6/BLOCK-iT™-DEST

The map below shows the elements of pLenti6/BLOCK-iT™-DEST. DNA from the entry clone replaces the region between bases 1,875 and 4,111. **The sequence for pLenti6/BLOCK-iT™-DEST is available at www.invitrogen.com or by contacting Technical Support (see page 55).**



Comments for pLenti6/BLOCK-iT™-DEST
8676 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
*att*R1 site: bases 1868-1992
*ccd*B gene: bases 2421-2726 (C)
Chloramphenicol resistance gene (Cm^R): bases 3068-3727 (C)
*att*R2 site: bases 4008-4132
SV40 early promoter and origin: bases 4281-4590
EM7 promoter: bases 4645-4711
Blasticidin resistance gene: bases 4712-5110
ΔU3/3′ LTR: bases 5196-5430
    ΔU3: bases 5196-5249
    3′ LTR: bases 5250-5430
SV40 polyadenylation signal: bases 5502-5636
bla promoter: bases 6492-6590
Ampicillin (*bla*) resistance gene: bases 6591-7451
pUC origin: bases 7596-8269

(C) = complementary strand

Continued on next page

Map and Features of pLenti6/BLOCK-iT™-DEST, Continued

Features of the Vector

The pLenti6/BLOCK-iT™-DEST (8,676 bp) vector contains the following elements. All features have been functionally tested.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
*ccd*B gene	Permits negative selection of the plasmid.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).
ΔU3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.

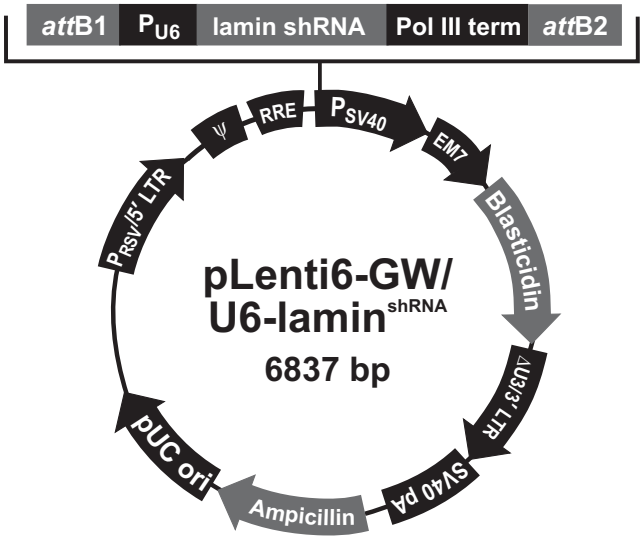
Map of pLenti6-GW/U6-lamin^{shRNA}

Description

pLenti6-GW/U6-lamin^{shRNA} is a 6,837 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 pLenti6/BLOCK-iT™-DEST vector using the Gateway® LR recombination reaction to generate the pLenti6-GW/U6-lamin^{shRNA} vector.

Map of pLenti6-GW/U6-lamin^{shRNA}

The map below shows the elements of pLenti6-GW/U6-lamin^{shRNA}. **The sequence of the vector is available at www.invitrogen.com or by calling Technical Support (see page 55).**



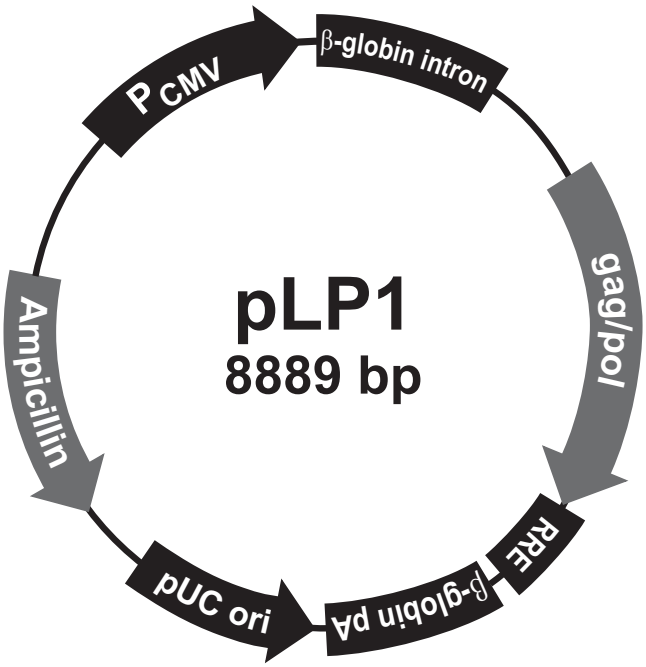
Comments for pLenti6-GW/U6-lamin^{shRNA}
6837 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
RSV promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
*att*B1 site: bases 1891-1915
U6 promoter: bases 1952-2215
Lamin A/C shRNA: bases 2216-2258
Pol III terminator: bases 2259-2264
*att*B2 site: bases 2269-2293 (C)
SV40 early promoter and origin: bases 2442-2751
EM7 promoter: bases 2806-2872
Blasticidin resistance gene: bases 2873-3271
ΔU3/3′ LTR: bases 3357-3591
ΔU3: bases 3357-3410
3′ LTR: bases 3411-3591
SV40 polyadenylation signal: bases 3663-3797
bla promoter: bases 4653-4751
Ampicillin (*bla*) resistance gene: bases 4752-5612
pUC origin: bases 5757-6430

Map and Features of pLP1

pLP1 Map

The figure below shows the features of the pLP1 vector. Note that the *gag* and *pol* genes are initially expressed as a gag/pol fusion protein, which is self-cleaved by the viral protease into individual Gag and Pol polyproteins. The sequence of pLP1 is available at **www.invitrogen.com** or by contacting Technical Support (see page 55).



Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
    TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
    gag coding sequence: bases 1355-2857
    gag/pol frameshift: base 2650
    pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

C=complementary strand

Continued on next page

Map and Features of pLP1, Continued

Features of pLP1

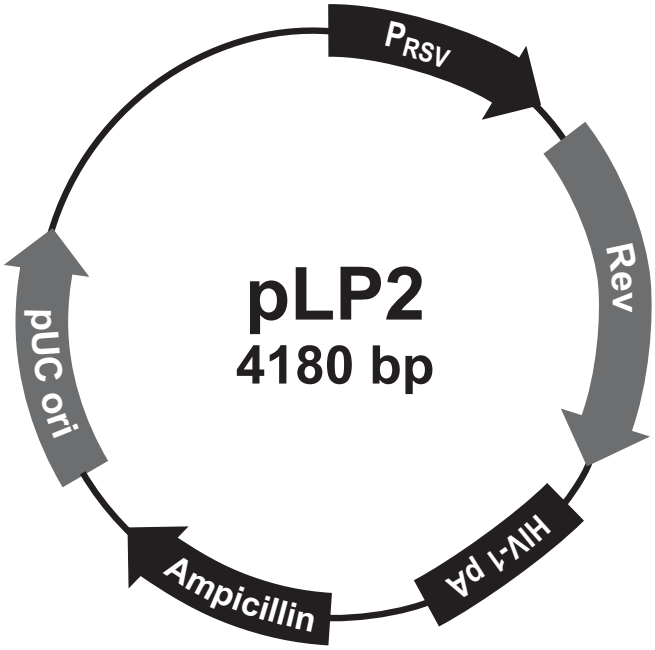
pLP1 (8,889 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) promoter	Permits high-level expression of the HIV-1 *gag* and *pol* genes in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the *gag* and *pol* genes in mammalian cells.
HIV-1 *gag* coding sequence	Encodes the viral core proteins required for forming the structure of the lentivirus (Luciw, 1996).
HIV-1 *pol* coding sequence	Encodes the viral replication enzymes required for replication and integration of the lentivirus (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent expression of the *gag* and *pol* genes.
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli.*
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli.*

Map and Features of pLP2

pLP2 Map

The figure below shows the features of the pLP2 vector. The sequence of pLP2 is available at **www.invitrogen.com** or by contacting Technical Support (see page 55).



Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

Continued on next page

Map and Features of pLP2, Continued

Features of pLP2

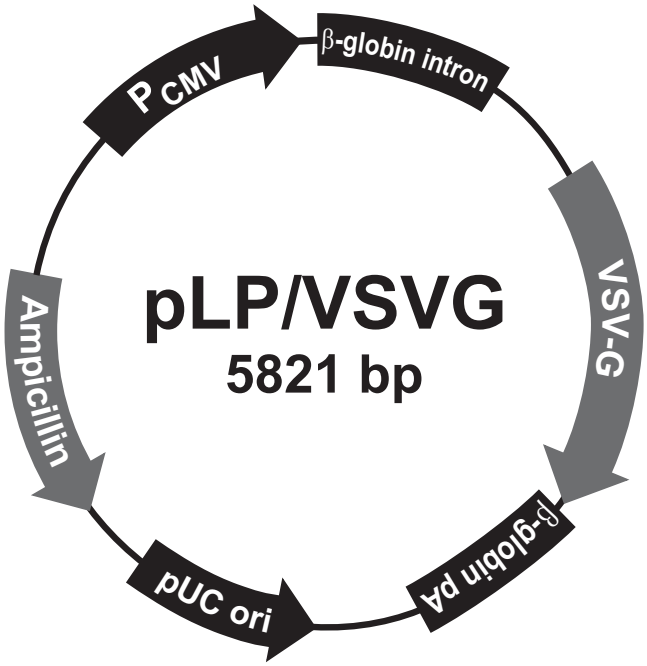
pLP2 (4,180 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
RSV enhancer/promoter	Permits high-level expression of the *rev* gene (Gorman *et al.*, 1982).
HIV-1 Rev ORF	Encodes the Rev protein that interacts with the RRE on pLP1 and on the pLenti6/BLOCK-iT™-DEST expression vector to induce Gag and Pol expression, which promotes the nuclear export of the unspliced viral RNA for packaging into viral particles.
HIV-1 LTR polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli.*
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli.*

Map and Features of pLP/VSVG

pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. The sequence of pLP/VSVG is available at www.invitrogen.com or by contacting Technical Support (see page 55).



Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

C=complementary strand

Continued on next page

Map and Features of pLP/VSVG

Features of pLP/VSVG

pLP/VSVG (5,821 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human CMV promoter	Permits high-level expression of the VSV-G gene in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the VSV-G gene in mammalian cells.
VSV G glycoprotein (VSV-G)	Encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped retrovirus with a broad host range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

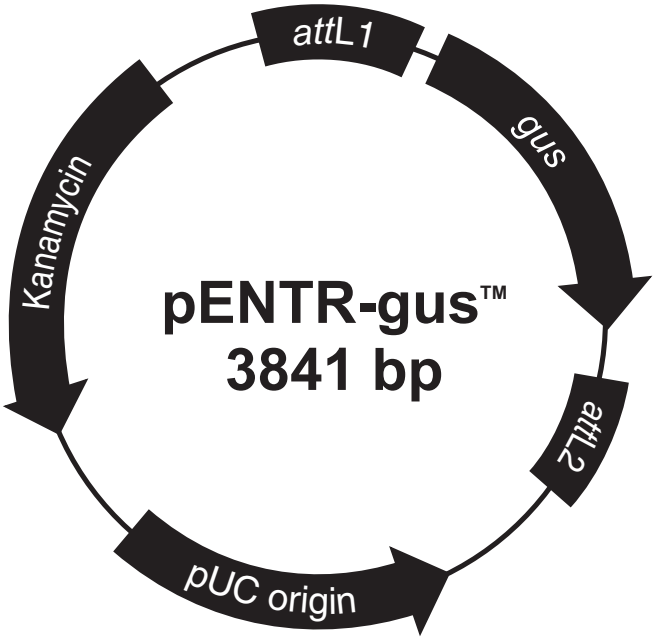
Map of pENTR™-gus

Description

pENTR™-gus is a 3,841 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 at www.invitrogen.com or by contacting Technical Support (see page 55).

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The sequence for pENTR™-gus is available** at www.invitrogen.com or by contacting Technical Support (see page 55).



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

Additional Products

Accessory Products

Many of the reagents supplied in the BLOCK-iT™ Lentiviral RNAi Kits as well as other products suitable for use with the kits are available separately. Ordering information is provided below. For more information, go to www.invitrogen.com or contact Technical Support (see page 55).

Product	Amount	Cat. no.
BLOCK-iT™ U6 RNAi Entry Vector Kit	20 constructions	K4945-00
Gateway® LR Clonase® II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µL	C7373-03
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 transformations	A10460
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970-00
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975-00
Lipofectamine® 2000 Reagent	0.75 mL 1.5 mL	11668-027 11668-019
Opti-MEM® I Reduced Serum Medium	100 mL 500 mL	31985-062 31985-070
Blasticidin	50 mg	R210-01
293FT Cell Line	3 × 10^6 cells	R700-07
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL 1 L	10010-023 10010-031
Ampicillin	200 mg	11593-027
TE, pH 8.0	500 mL	AM9849
PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit	25 reactions	K2100-04
Fetal Bovine Serum (FBS), Certified	500 mL	16000-044
MEM Sodium Pyruvate Solution (100X)	100 mL	11360-070

BLOCK-iT™ RNAi Accessory Products

Other BLOCK-iT™ RNAi products are available to facilitate RNAi analysis. 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, go to www.invitrogen.com or call Technical Support (see page 55).

Product	Amount	Cat. no.
BLOCK-iT™ RNAi TOPO® Transcription Kit	10 reactions	K3500-01
BLOCK-iT™ Dicer RNAi Transfection Kit	150 transfections	K3600-01
BLOCK-iT™ Complete Dicer RNAi Kit	150 transfections	K3650-01

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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

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E-mail: eurotech@invitrogen.com

SDS

Safety Data Sheets (SDSs) are available on our website at www.invitrogen.com/sds.

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The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Continued on next page

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Gateway® Clone Distribution Policy

Introduction

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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.

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Additional Terms and Conditions

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