USER GUIDE

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BLOCK-iT™ Inducible H1 Lentiviral RNAi System

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

Catalog numbers K4925-00 and V488-20

Revision date 4 January 2012
Publication Part number 250722

MAN0000452

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Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Cat. no.
BLOCK-iT™ Inducible H1 Lentiviral RNAi System	K4925-00
BLOCK-iT™ Lentiviral RNAi Zeo Gateway® Vector Kit	V488-20

Kit Components

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System and the BLOCK-iT™ Lentiviral RNAi Zeo Gateway® Vector Kit include the following components. For a detailed description of the contents of each component, see pages vi-viii. For a detailed description of the contents of the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit and how to use the reagents supplied, see the BLOCK-iT™ Inducible H1 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.	
	V488-20	K4925-00
pLenti4/BLOCK-iT™-DEST Gateway® Vector Kit	✓	✓
Gateway® LR Clonase® II Enzyme Mix		✓
One Shot® Stbl3™ Chemically Competent *E. coli*	✓	✓
pLenti6/TR Vector		✓
Blasticidin		✓
ViraPower™ Zeo Lentiviral Support Kit		✓
293FT Cell Line		✓
BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit		✓

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Shipping and Storage

The BLOCK-iT™ Inducible H1 Lentiviral RNAi Kits are shipped as described below. Upon receipt, store each item as indicated below. For more detailed information about the reagents supplied in the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, refer to the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual.

Note: The BLOCK-iT™ Lentiviral RNAi Zeo Gateway® Vector Kit includes **Boxes 1 and 3 only.**

Box	Component	Shipping	Storage
1	pLenti4/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	pLenti6/TR Vector	Room temperature	–20°C
5	Blasticidin	Room temperature	–20°C
6	ViraPower™ Zeo Lentiviral Support Kit	Blue ice	ViraPower™ Packaging Mix: –20°C Zeocin™: –20°C, protected from light Lipofectamine® 2000: 4°C **(do not freeze)**
7	293FT Cell Line	Dry ice	Liquid nitrogen
8–9	BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit	Dry ice	Inducible H1 RNAi Entry Vector Reagents: –20°C Tetracycline: –20°C, protected from light One Shot® TOP10 Chemically Competent *E. coli*: –80°C

Vectors

The following vectors are included with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Boxes 1 and 4). **Store the vectors at –20°C.**

Important: The BLOCK-iT™ Lentiviral RNAi Zeo Gateway® Vector Kit **does not** include the pLenti6/TR vector.

Vector	Composition	Amount
pLenti4/BLOCK-iT™-DEST	40 µL of vector at 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pLenti4-GW/H1/TO-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
pLenti6/TR	40 µL of vector at 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg

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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/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µL
pENTR™-gus Positive Control	50 ng/µL in TE buffer, pH 8.0	20 µL

Note: The pENTR™-gus control included with the Gateway® LR Clonase® II Enzyme Mix may be used as a positive control for the LR recombination reaction **only** (see page 24). Do not use the resulting expression clone to produce lentivirus for expression purposes as the pLenti4/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
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
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	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 table below lists the reagents included with the Blasticidin (Box 5) and ViraPower™ Zeo Lentiviral Support Kit (Box 6). Store as follows:

- ViraPower™ Packaging Mix and Blasticidin: –20°C
- Zeocin™: –20°C, protected from light
- Lipofectamine® 2000 Reagent: 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 Reagent	Proprietary	0.75 mL
Zeocin™	100 mg/mL in sterile, deionized water	125 mg
Blasticidin	Powder	50 mg

293FT Cell Line

The BLOCK-iT™ Inducible H1 Lentiviral RNAi Expression System includes the 293FT Cell Line (Box 7) 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™ Inducible H1 RNAi Entry Vector Kit

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System includes the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit to facilitate production of a Gateway® entry construct containing an H1/TO RNAi cassette for tetracycline-regulated expression of your short hairpin RNA (shRNA) of interest. The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit contains:

- Inducible H1 RNAi Entry Vector Reagents and Tetracycline (Box 8)
- One Shot® TOP10 Chemically Competent *E. coli* (Box 9)

Refer to the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual for a detailed description of the reagents provided with the kit and instructions to produce the Gateway® entry construct.

Product Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

Introduction

System Summary

Description of the System

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System combines BLOCK-iT™ RNAi and ViraPower™ T-REx™ Lentiviral technologies to facilitate creation of a replication-incompetent lentivirus that delivers an inducible short hairpin RNA (shRNA) of interest to dividing or non-dividing mammalian cells for RNA interference (RNAi) analysis. The System includes:

- The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit for production of an entry clone that contains elements required for tetracycline-regulated expression of a double-stranded oligonucleotide (ds oligo) encoding an shRNA of interest in mammalian cells (i.e. human H1/TO promoter and RNA Polymerase III (Pol III) terminator). The entry vector containing this H1/TO RNAi cassette (H1/TO promoter + ds oligo + Pol III terminator) is used to transfer the H1/TO RNAi cassette into the lentiviral expression plasmid (see page 3) using Gateway® Technology.
- A promoterless pLenti4/BLOCK-iT™-DEST destination vector into which the H1/TO RNAi cassette of interest is transferred. This expression plasmid contains elements that allow packaging of the construct into virions and the Zeocin™ resistance marker for selection of stably transduced cell lines.
- The pLenti6/TR vector that constitutively expresses high levels of the tetracycline (Tet) repressor. 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™ T-REx™ Lentiviral System for producing a replication-incompetent lentivirus that transiently or stably expresses the shRNA of interest (after tetracycline induction) or Tet repressor in both dividing and non-dividing mammalian cells.

For more information about the BLOCK-iT™ RNAi Technology, ViraPower™ T-REx™ Lentiviral Technology, and Gateway® Technology, see pages 2–3.

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System Summary, continued

Advantages of the BLOCK-iT™ Inducible H1 Lentiviral RNAi System

Using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System to facilitate lentiviral-based delivery of regulated shRNA to mammalian cells provides the following advantages:

- The pENTR™/H1/TO 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, tetracycline-regulated expression cassette (i.e. H1/TO RNAi cassette) for use in RNAi analysis.
- The vectors in the System are Gateway®-adapted for easy transfer of the H1/TO RNAi cassette from the pENTR™/H1/TO vector into the pLenti4/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 or the Tet repressor to mammalian cells in culture or *in vivo.* Expression of the shRNA of interest can be regulated by tetracycline.
- Includes a lentiviral construct expressing the Tet repressor to facilitate generation of a stable TetR-expressing cell line for rapid screening of multiple expression constructs.
- 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.

The BLOCK-iT™ RNAi Technology

A large selection of BLOCK-iT™ RNAi products is available to facilitate RNAi analysis in mammalian and invertebrate systems. The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System uses a vector-based approach to allow efficient generation of H1/TO RNAi cassettes for regulated expression of shRNA molecules in mammalian cells. For constitutive expression of shRNA molecules in mammalian cells, use the BLOCK-iT™ U6 RNAi Entry Vector Kit or the BLOCK-iT™ Lentiviral RNAi Expression System. Other BLOCK-iT™ RNAi products are available to facilitate production and delivery of synthetic short interfering RNA (siRNA), diced siRNA (d-siRNA) or double-stranded RNA (dsRNA) for RNAi analysis in mammalian cells or invertebrate organisms, as appropriate. For more information about any of the BLOCK-iT™ RNAi products and other RNAi resources, see the RNAi Central application portal at **www.lifetechnologies.com/rnai**.

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System Summary, continued

The ViraPower™ Lentiviral Technology

The ViraPower™ T-REx™ Lentiviral Technology combines ViraPower™ Lentiviral and T-REx™ technologies to facilitate highly efficient, tetracycline-regulated, *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™ T-REx™ Lentiviral Technology possesses features which enhance its biosafety while allowing high-level, regulated expression in a wider range of cell types than traditional retroviral systems. The main components of the ViraPower™ T-REx™ Lentiviral Technology include:

- A pLenti-based expression vector into which the DNA sequence of interest will be cloned. 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.
- The pLenti6/TR expression vector for high-level, constitutive expression of the Tet repressor under the control of a CMV promoter. This vector also contains elements to allow packaging of the construct into virions and the Blasticidin resistance marker for selection of stably transduced cell lines.
- The ViraPower™ Packaging Mix, an optimized mixture of the three packaging plasmids required for production of the lentivirus.
- An optimized 293FT cell line to facilitate optimal production of virus.

For more information about these components, see page 6. For more information about the biosafety features of the System, see page 16.

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™ Inducible H1 Lentiviral RNAi System and Gateway® Technology, simply:

1. Clone a double-stranded oligonucleotide encoding an shRNA of interest into the pENTR™/H1/TO 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™/H1/TO entry clone and the pLenti4/BLOCK-iT™-DEST vector.
3. Use your expression clone and the reagents supplied in the kit to produce lentivirus.
4. Transduce the lentiviral construct into TetR-expressing mammalian cells and add tetracycline to induce expression of 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.lifetechnologies.com** or by contacting Technical Support (see page 79).

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System Summary, continued

Purpose of this Manual

This manual provides an overview of the BLOCK-iT™ Inducible H1 Lentiviral RNAi System and provides instructions and guidelines to:

1. Use the pLenti4/BLOCK-iT™-DEST vector and a pENTR™/H1/TO entry clone in an LR recombination reaction to generate an expression clone containing the H1/TO RNAi cassette of interest.
2. Co-transfect the pLenti4/BLOCK-iT™-DEST expression construct (or the pLenti6/TR vector) and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
3. Titer the lentiviral stock.
4. Co-transduce the Lenti4/BLOCK-iT™-DEST and Lenti6/TR lentiviral constructs into mammalian cells and add tetracycline to perform "transient" RNAi analysis. Alternatively, co-transduce the two lentiviral constructs into mammalian cells and select for dual stable cell lines using Zeocin™ and Blasticidin. Add tetracycline to induce expression of the shRNA of interest for RNAi analysis.
5. Transduce the Lenti6/TR lentiviral construct into mammalian cells and use Blasticidin to select for a stable TetR-expressing cell line. Use this cell line as the host for the Lenti4/BLOCK-iT™-DEST lentiviral construct. Add tetracycline to induce expression of the shRNA of interest for RNAi analysis.

For details and instructions to generate a pENTR™/H1/TO entry clone containing the H1/TO RNAi cassette, refer to the BLOCK-iT™ Inducible H1 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™ Inducible H1 Lentiviral RNAi System, but are also available at **www.lifetechnologies.com** or by contacting Technical Support (see page 79).

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™ Inducible H1 Lentiviral RNAi System, follow the recommended protocols in this manual.**

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Important

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System is designed to help you create a lentivirus to deliver and inducibly express an shRNA of interest in mammalian cells for RNAi analysis. Although the system has been designed to help you express an shRNA of interest in the simplest, most direct fashion, use of the system is geared towards 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 regulated expression of shRNA in mammalian cells: see Brummelkamp et al., 2002, Czauderna et al., 2003, Matsukura et al., 2003, McManus and Sharp, 2002, and McManus 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.lifetechnologies.com/rnai**.

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System

Components of the System

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System facilitates highly efficient, tetracycline-regulated, *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™ Inducible H1 RNAi Entry Vector Kit containing the pENTR™/H1/TO 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 H1/TO RNAi cassette (i.e. human H1/TO promoter + double-stranded oligonucleotide + Polymerase III terminator) may be directly transfected into mammalian cells for RNAi analysis or used to transfer the H1/TO RNAi cassette into the pLenti4/BLOCK-iT™-DEST expression plasmid (see below) using Gateway® Technology. For more information about the H1/TO RNAi cassette, see page 12. For detailed information about the pENTR™/H1/TO vector and instructions to generate an entry clone, refer to the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual.
- The pLenti4/BLOCK-iT™-DEST expression vector into which the H1/TO 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 the Zeocin™ resistance marker to allow generation of stable cell lines. For more information about the pLenti4/BLOCK-iT™-DEST vector, see page 8 and pages 65–66.
- The pLenti6/TR vector containing the *TetR* gene for high-level constitutive expression of the Tet repressor under the control of the human CMV promoter. The vector also contains elements to allow viral packaging and the Blasticidin resistance marker to allow generation of stable cell lines. For more information about the pLenti6/TR vector, see page 9 and pages 67–68.
- 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 70–75.
- 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.
- Tetracycline, the inducing agent for regulated expression of the shRNA of interest.

Co-transfect the ViraPower™ Packaging Mix and the pLenti4/BLOCK-iT™-DEST construct containing the H1/TO RNAi cassette or pLenti6/TR into 293FT cells to produce two replication-incompetent lentiviral stocks, which can then be transduced into the mammalian cell line of interest.

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The BLOCK-iT™ Inducible H1 Lentiviral RNAi System, continued

How Lentivirus Works

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). Once the lentiviral construct has integrated into the genome, the shRNA of interest or the Tet repressor is expressed, constitutively for the Tet repressor and only after addition of tetracycline for the shRNA of interest. For more information about how tetracycline regulation works in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, see pages 14–15.

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).

BLOCK-iT™ Lentiviral Vectors

Introduction

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System includes two pLenti-based expression vectors, one to inducibly express the shRNA of interest and one to express the Tet repressor. The kits also include a control vector. This section provides a brief overview of these expression vectors.

Features of the pLenti4/BLOCK-iT™-DEST Vector

The pLenti4/BLOCK-iT™-DEST destination 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 (ΔU3) 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 H1/TO RNAi cassette from the pENTR™/H1/TO 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
- Zeocin™ resistance gene for selection in *E. coli* and mammalian cells (Drocourt et al., 1990; Mulsant et al., 1988)
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

Important

Note that the pLenti4/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 H1/TO RNAi cassette that is transferred from the pENTR™/H1/TO entry clone into pLenti4/BLOCK-iT™-DEST after LR recombination. For more information about the features of the H1/TO RNAi cassette, see page 12.

pLenti4-GW/H1/TO-lamin^{shRNA} Control

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System includes the pLenti4-GW/H1/TO-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** on the next page), where it expresses an shRNA targeted to the human lamin A/C gene following tetracycline addition (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 tissue culture systems (Harborth et al., 2001).

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Note

Use of the pLenti4-GW/H1/TO-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 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 whether this particular shRNA is able to facilitate knockdown of the lamin A/C gene in non human-derived cell lines. A non human-derived cell line that has been used successfully in a knockdown experiment using the control lentiviral construct is COS-7.

Features of the pLenti6/TR Vector

The pLenti6/TR 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 to enhance the biosafety of the vector.
- 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)
- Human CMV promoter for high-level, constitutive expression of the *TetR* gene
- Rabbit β-globin intron II sequence for enhanced expression of the *TetR* gene in cultured cells (van Ooyen et al., 1979)
- *TetR* gene encoding the Tet repressor to repress transcription of your shRNA in the absence of tetracycline (Postle et al., 1984; Yao et al., 1998)
- 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*

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 recent 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; McManus 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

Use of siRNA (diced siRNA or synthetic siRNA) for RNAi analysis in mammalian cells is limited by their transient nature. To address this limitation, a number of groups have developed vector-based systems to facilitate expression of siRNA and shRNA in mammalian cells (Brummelkamp et al., 2002; McManus et al., 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). The Gateway®-adapted pENTR™/H1/TO vector (supplied in the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit) facilitates 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. H1/TO RNAi cassette; see the next page). The resulting pENTR™/H1/TO entry construct may be introduced into dividing mammalian cells for transient or stable, tetracycline-regulated expression of the shRNA of interest and initial RNAi screening, if desired. Once initial screening is complete, the H1/TO RNAi cassette may then be easily and efficiently transferred into the pLenti4/BLOCK-iT™-DEST vector by LR recombination for use in other RNAi applications (e.g., regulated expression of shRNA in hard-to-transfect or non-dividing mammalian cells).

For more information about the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit, its components, and how to generate the pENTR™/H1/TO construct, refer to the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual.

Continued on next page

Features of the H1/TO RNAi Cassette

The H1/TO RNAi cassette contains all of the elements required to facilitate RNA Polymerase III-controlled expression of an shRNA of interest from pLenti4/BLOCK-iT™-DEST (or pENTR™/H1/TO) including a:

- Modified human H1 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 H1/TO RNAi cassette.

Note: The H1/TO RNAi cassette in pENTR™/H1/TO is flanked by *att* sites to allow easy transfer of the cassette into suitable Gateway® destination vectors (e.g., pLenti4/BLOCK-iT™-DEST).

Human H1 Promoter

Expression of the shRNA of interest from pLenti4/BLOCK-iT™-DEST (or pENTR™/H1/TO) is controlled by the human H1 promoter, which has been modified to include two prokaryotic *tet* operator 2 ($TetO_2$) sequences. The endogenous H1 promoter normally controls expression of H1 RNA, the RNA component of human RNase P involved in tRNA processing (Baer et al., 1990), and has been well-characterized (Hannon et al., 1991; Myslinksi et al., 2001). We and other groups have chosen this particular promoter to control vector-based expression of shRNA molecules in mammalian cells (Brummelkamp et al., 2002; McManus et al., 2002) for the following reasons:

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

For more information about the *tet* operator sequences and how tetracycline regulation works, see pages 14–15.

Continued on next page

Structure of the shRNA

After using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System to generate a lentiviral construct containing the H1/TO RNAi cassette, 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 pLenti4-GW/H1/TO-lamin^{shRNA} construct included with the kit. The 19 bp lamin A/C target sequence is indicated in bold. The underlined bases are derived from the Pol III terminator.

```
    5′-G CUGGACUUCCAGAAGAACA C G
3′-..UU GACCUGAAGGUCUUCUUGU A A
```

Note: The length of the stem and loop may differ depending on how you design the oligo-nucleotides encoding the target sequence. For guidelines to design the oligonucleotides, refer to the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual.

How Tetracycline Regulation Works

Introduction

As described previously, the H1 promoter in the H1/TO RNAi cassette has been modified to include two prokaryotic *tet* operator sequences. The presence of the *tet* operator sequences enables the shRNA of interest to be expressed in a tetracycline-dependent manner. This section describes the *tet* operator sequences in the H1/TO RNAi cassette, and the mechanism of tetracycline regulation in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System.

Tetracycline Regulation and *tet* Operator Sequences

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen & Berens, 1994; Hillen et al., 1983) to allow tetracycline-regulated expression of an shRNA of interest from the pLenti4/BLOCK-iT™-DEST construct following transfer of the H1/TO RNAi cassette from pENTR™/H1/TO. The mechanism of tetracycline regulation in the system is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the shRNA of interest. In the system, expression of the shRNA of interest is repressed in the absence of tetracycline and induced in its presence.

In the BLOCK-iT™ Inducible H1 RNA System, expression of the shRNA of interest from the pLenti4/BLOCK-iT™-DEST construct following transfer of the H1/TO RNAi cassette from pENTR™/H1/TO is controlled by a human H1 promoter into which 2 copies of the 19 nt *tet* operator 2 ($TetO_2$) sequence have been incorporated (i.e. H1/TO promoter). Each 19 nt $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor. Refer to the diagram on page 21 for the location and sequences of the $TetO_2$ sites in the H1/TO promoter.

Mechanism of Repression/ Derepression

In the absence of tetracycline, the Tet repressor (expressed from the pLenti6/TR lentiviral construct) forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence (Hillen & Berens, 1994) in the H1/TO promoter of the pLenti4/BLOCK-iT™-DEST construct following transfer of the H1/TO RNAi cassette from pENTR™/H1/TO. The 2 $TetO_2$ sites in the H1/TO promoter serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor (see figure on the next page). Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of the shRNA of interest. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind the Tet operator. The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription of the shRNA of interest, resulting in target gene knockdown (see figure page 15).

Note: The affinity of the Tet repressor for the *tet* operator is $K_B = 2 \times 10^{11}\ M^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen & Berens, 1994). The association constant, K_A, of tetracycline for the Tet repressor is $3 \times 10^9\ M^{-1}$ (Takahashi et al., 1991).

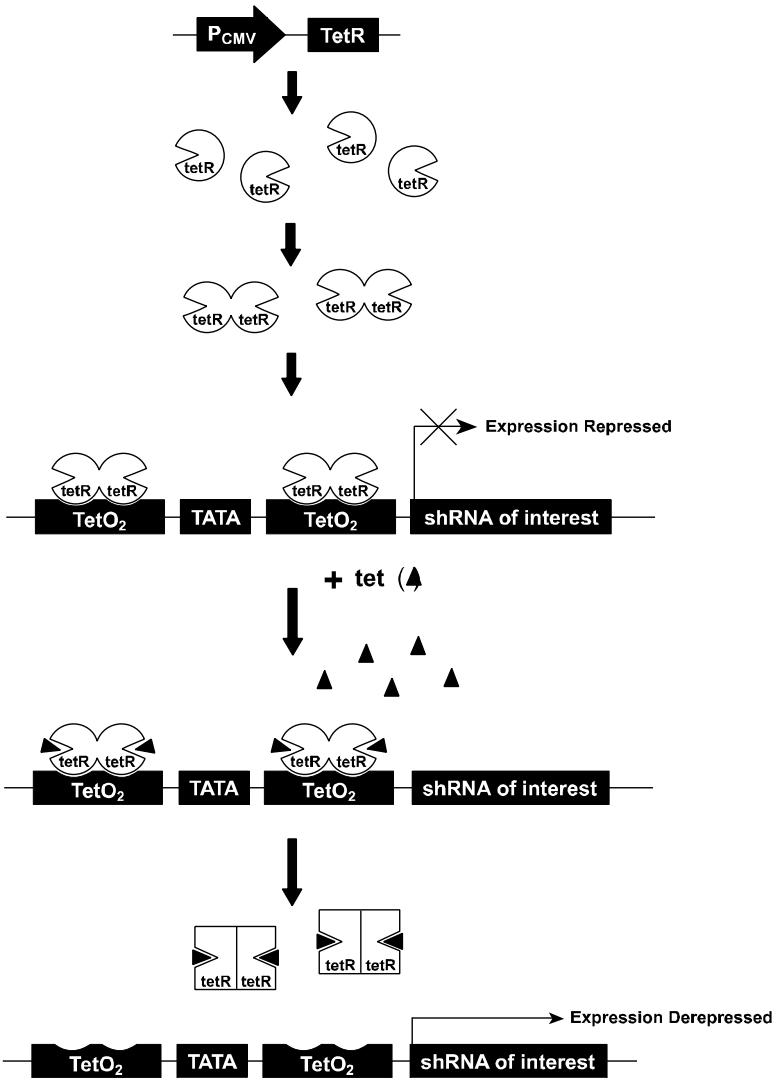
Continued on next page

How Tetracycline Regulation Works, continued

Diagram of Tetracycline Regulation

The figure below illustrates the mechanism of tetracycline-regulated repression and derepression of the shRNA of interest in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System.

1. Transduce the pLenti6/TR lentiviral construct into the mammalian cells of interest. Add Blasticidin to select for a stable TetR-expressing cell line.
2. Transduce the pLenti4/BLOCK-iT™-DEST lentiviral construct into the TetR-expressing cells. Upon transduction, TetR homodimers bind to Tet operator 2 ($TetO_2$) sequences in the pLenti4/BLOCK-iT™-DEST lentiviral construct, repressing transcription of the shRNA of interest.
3. Added tetracycline (tet) binds to tetR homodimers.
4. Binding of tet to tetR homodimers causes a conformational change in tetR, release from the Tet operator sequences, and induction of shRNA transcription.



Biosafety Features of the System

Introduction

The lentiviral and packaging vectors supplied in the BLOCK-iT™ Inducible H1 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™ Inducible H1 Lentiviral RNAi Expression System

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System includes the following key safety features:

- The pLenti4/BLOCK-iT™-DEST and pLenti6/TR vectors contain 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 other components required for packaging the viral genome are separated onto four plasmids (i.e. three packaging plasmid and pLenti4/BLOCK-iT™-DEST or pLenti6/TR). 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 pLenti4/BLOCK-iT™-DEST and pLenti6/TR vectors 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 that express shRNA targeting human genes involved in controlling cell division (e.g., tumor suppressor genes).

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

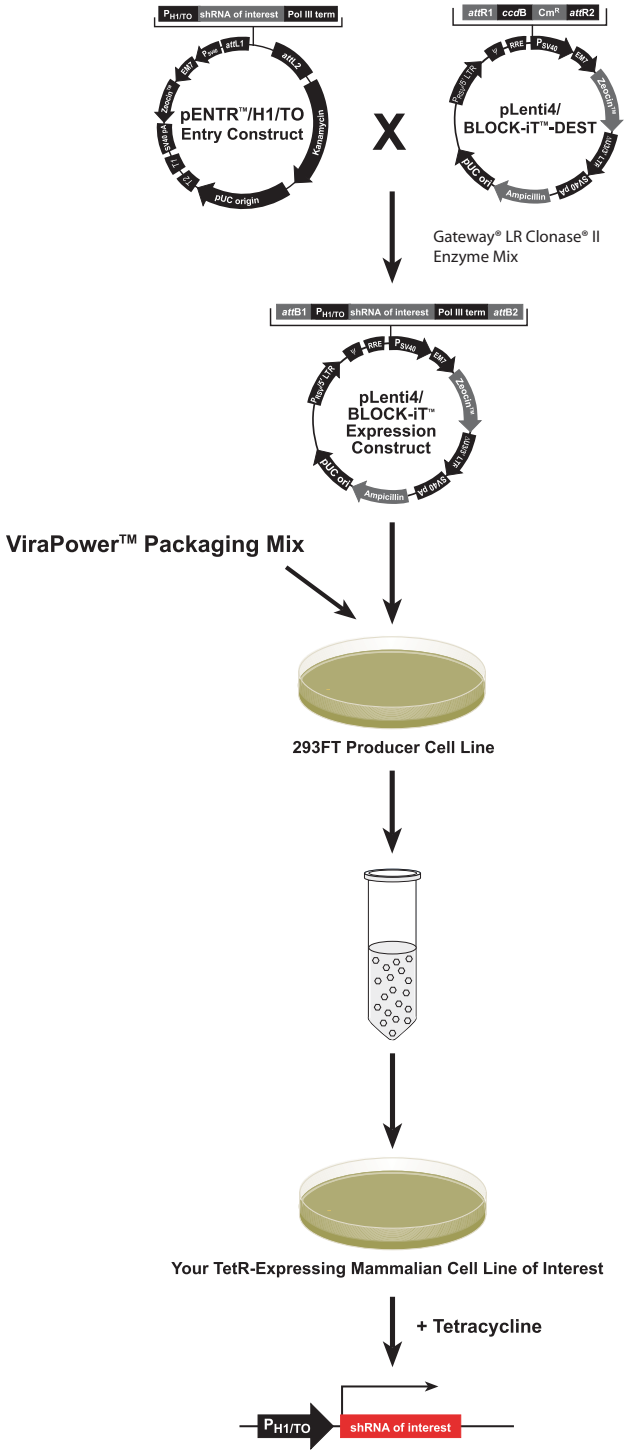
Important

Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of lentiviruses may vary at individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the BLOCK-iT™ Inducible H1 Lentiviral RNAi System.

Experimental Outline

Flow Chart

The diagram below describes the general steps required to express an shRNA of interest using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System.



1. Perform an LR recombination reaction between the pENTR™/H1/TO entry construct and pLenti4/BLOCK-iT™-DEST to generate the pLenti4/BLOCK-iT™ expression construct.
2. Cotransfect the 293FT producer cell line with your pLenti4/ BLOCK-iT™ expression construct and the optimized packaging mix.
3. Harvest viral supernatant and determine the titer.
4. Use pLenti6/TR to generate a TetR-expressing mammalian cell line. Add the Lenti4/BLOCK-iT™ viral supernatant to the TetR-expressing cell line. Select for stably transduced cells using Zeocin™ and Blasticidin, if desired.
5. Add tetracycline and assay for knockdown of the target gene.

Methods

Generating an Entry Clone

Introduction

To express an shRNA of interest from pLenti4/BLOCK-iT™-DEST, first generate an entry clone in the pENTR™/H1/TO vector using the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit. General guidelines are provided (pages 19–21).

Important

To express an shRNA of interest in a tetracycline-regulated manner, use the pENTR™/H1/TO entry vector to generate entry clones containing the shRNA sequence. Although a large selection of Gateway® entry vectors exists to facilitate generation of entry clones, **only** the pENTR™/H1/TO entry vector contains the elements required to facilitate proper regulated expression of shRNA molecules in mammalian cells. These elements include:

- The human H1/TO promoter, an RNA Polymerase III-dependent promoter that facilitates high-level, tetracycline-regulated expression of the shRNA of interest in mammalian cells (Hannon et al., 1991; Myslinksi et al., 2001).
- A Polymerase III (Pol III) terminator for efficient transcription termination of the shRNA molecule.

The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit is supplied with Cat. no. K4925-00, but is also available separately (see page 77 for ordering information).

Using pENTR™/H1/TO

To generate an entry clone in pENTR™/H1/TO:

- Design and synthesize two complementary oligonucleotides encoding an shRNA target sequence according to specified guidelines
- Anneal the oligonucleotides to create a double-stranded oligonucleotide
- Clone the double-stranded oligonucleotide into pENTR™/H1/TO 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™ Inducible H1 RNAi Entry Vector Kit manual. This manual is supplied with Cat. no. K4925-00, but is also available at **www.lifetechnologies.com** or by calling Technical Support (see page 79).

Creating Expression Clones

Introduction

After generating an entry clone, perform the LR recombination reaction using your pENTR™/H1/TO entry construct and pLenti4/BLOCK-iT™-DEST vector to generate an expression clone. To obtain the best possible results, read this section and the sections entitled **Performing the LR Recombination Reaction** (pages 22–24) and **Transforming One Shot® Stbl3™ Competent *E. coli*** (pages 25–26) before beginning.

Experimental Outline

To generate an expression clone:

1. Perform an LR recombination reaction using the *att*L-containing pENTR™/H1/TO entry clone and the *att*R-containing pLenti4/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 22).
3. Select for expression clones (see page 21 for a diagram of the recombination region of expression clones in pLenti4/BLOCK-iT™-DEST).

Important

The pLenti4/BLOCK-iT™-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual previously recommended using a linearized destination vector for more efficient LR recombination, further testing has found that linearization of pLenti4/BLOCK-iT™-DEST is **not** required to obtain optimal results for any downstream application.

Propagating the Destination Vector

To propagate and maintain the pLenti4/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 77). 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. For additional guidelines, see below.

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.

Guidelines for Propagating the Destination Vector

Follow the guidelines below when using One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli* to propagate the pLenti4/BLOCK-iT™-DEST plasmid:

- To maintain the integrity of the vector, select for transformants in media containing 50–100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol.
- Due to the potential for rearrangement of lentiviral vectors caused by recombination between the 5′ and 3′ LTRs (i.e. unwanted recombinants), analyze transformants to verify the integrity of the destination vector before proceeding.
- When propagating transformants, culture bacteria in LB media. **Do not use** "richer" bacterial media as these media tend to give rise to a greater number of unwanted recombinants.

Continued on next page

Recombination Region of pLenti4/BLOCK-iT™-DEST

The recombination region of the expression clone resulting from the pLenti4/BLOCK-iT™-DEST × pENTR™/H1/TO entry clone is shown below.

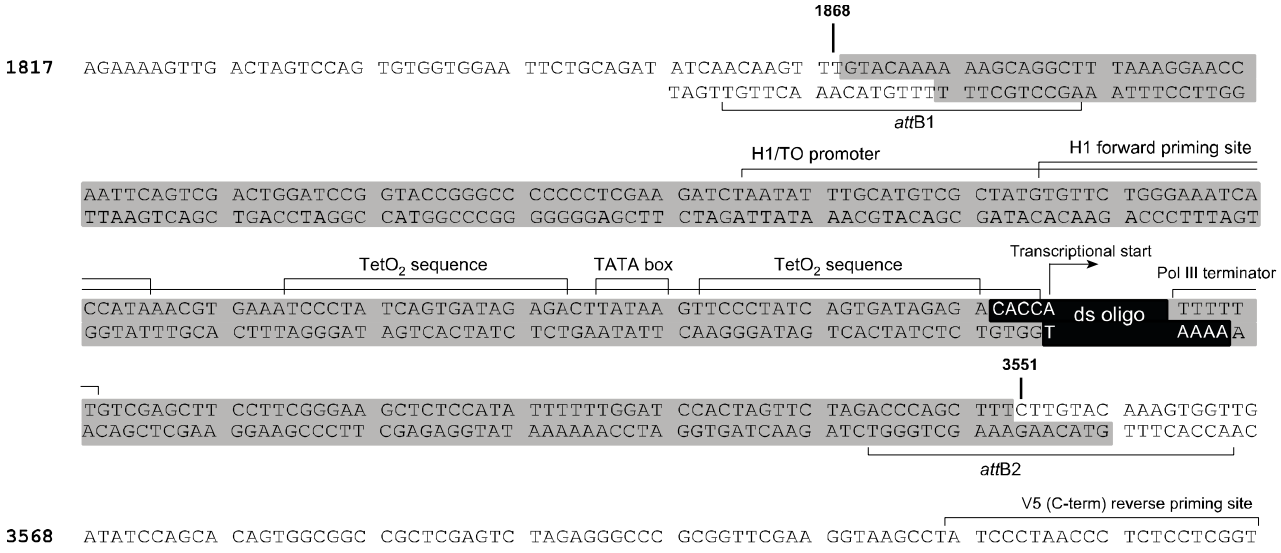
Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the pENTR™/H1/TO entry clone into the pLenti4/BLOCK-iT™-DEST vector by recombination. Non-shaded regions are derived from the pLenti4/BLOCK-iT™-DEST vector.

  Note: The DNA sequences transferred from the pENTR™/H1/TO entry clone consist of an H1/TO RNAi cassette containing the human H1/TO 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 H1/TO promoter sequence.

Bases 1,868 and 3,551 of the pLenti4/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™/H1/TO entry clone and the pLenti4/BLOCK-iT™-DEST vector. Include 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 25. Note that transformants containing unwanted recombinants (see **Note**) are obtained less frequently 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. If you use TOP10 or DH5α™ cells for transformation, follow the guidelines below to reduce the frequency of unwanted recombinants:

- **Select for transformants using Low Salt LB containing both 100 µg/mL ampicillin and 50 µg/mL Zeocin™.** Note that transformed *E. coli* grow more slowly in LB media containing ampicillin and Zeocin™, and may require slightly longer incubation times to obtain visible colonies. For more information about Zeocin™, see the **Appendix**, page 62.
- **Select small colonies for analysis** as 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.

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.

Continued on next page

Performing the LR Recombination Reaction, continued

Gateway® LR Clonase® II Enzyme Mix

Gateway® LR Clonase® II enzyme mix is supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System and is also available separately (see page 77). The Gateway® LR Clonase® II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase Reaction Buffer previously supplied as separate components in Gateway® 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 24 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.

Positive Control for LR Reaction

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 the pENTR™-gus plasmid to verify the efficiency of the LR reaction. However, the resulting expression clone cannot be used as an expression control because neither the pLenti4/BLOCK-iT™-DEST vector nor pENTR™-gus include a eukaryotic promoter to control expression of the *gus* gene in mammalian cells. For a map of pENTR™-gus, see the **Appendix**, page 76.

Materials Needed

- Purified plasmid DNA of your pENTR™/H1/TO entry clone (50–150 ng/µL in TE Buffer, pH 8.0)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), see page 77
- Sterile 0.5 mL microcentrifuge tubes

Components supplied with the kits

- pLenti4/BLOCK-iT™-DEST vector (150 ng/µL in TE Buffer, pH 8.0)

Components supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System only

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

Continued on next page

Performing the LR Recombination Reaction, continued

Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR reaction between the pENTR™/H1/TO entry clone and the pLenti4/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
pLenti4/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 of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® Stbl3™ Competent E. coli**, page 25.

   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 $\geq 1 \times 10^8$ cfu/µg plasmid DNA.

Materials Needed

- LR recombination reaction (from Step 7, from **Setting Up the LR Recombination** Reaction on page 24)
- 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, previous page) 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 Zeocin™-resistant. Transformants containing a plasmid with a mutated *ccdB* gene will be chloramphenicol-, ampicillin-, and Zeocin™-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 H1/TO RNAi cassette from pENTR™/H1/TO into the pLenti4/BLOCK-iT™-DEST vector preserves the orientation of the cassette. However, if you wish to sequence the H1/TO RNAi cassette in pLenti4/BLOCK-iT™-DEST, use the primers shown below. Refer to the diagram on page 21 for the location of the primer binding sites in the expression vector.

Primer	Sequence
H1 Forward	5′-TGTTCTGGGAAATCACCATA-3′
V5(C-term) Reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Note: For information about a convenient custom primer synthesis service go to **www.lifetechnologies.com** or call Technical Support (see page 79).

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 Zeocin™ is not required.

Verifying Expression and Effectiveness of shRNA

Optional: Before generating a lentiviral stock of your pLenti4/BLOCK-iT™-DEST expression construct, you may verify that the construct expresses the shRNA of interest by transfecting the plasmid directly into mammalian cells and assaying for knockdown of the target gene, if desired. Follow the guidelines below:

- Use an easy-to-transfect, dividing mammalian cell line that expresses the target gene.
- Use a transfection reagent that facilitates high-efficiency transfection; we recommend using Lipofectamine® 2000 Reagent (see page 77).

  Note: Lipofectamine® 2000 is supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, but is also available separately (see page 77).
- Follow the manufacturer's instructions for the transfection reagent you are using to perform plasmid transfection. If you are using Lipofectamine® 2000, follow the instructions included with the product.

Note: In the absence of Tet repressor, your shRNA of interest will be constitutively expressed.

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 pLenti4/BLOCK-iT™-DEST expression construct) by co-transfecting the optimized ViraPower™ Packaging Mix and your pLenti4/BLOCK-iT™-DEST expression construct into the 293FT Producer Cell Line.

Lentiviral Stocks

To express your shRNA of interest in a regulated manner, generate lentiviral stocks of the following expression constructs:

- Your pLenti4/BLOCK-iT™-DEST expression construct containing the shRNA of interest
- The pLenti6/TR construct expressing the Tet repressor (see below for more information)

Also generate a lentiviral stock with the pLenti4-GW/H1/TO-lamin^{shRNA} control construct for use as a positive control for lentivirus production and expression, if desired. For more information, see the next page.

Plasmid Preparation

Once you have generated 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. Isolate lentiviral plasmid DNA using the PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit (see page 77 for ordering information).

Resuspend the purified pLenti4/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.

pLenti6/TR

The pLenti6/TR plasmid is included with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, and contains the *TetR* gene and a Blasticidin resistance marker in a pLenti6-based expression vector to allow stable expression of the Tet repressor in any mammalian cell line. To use pLenti6/TR, cotransfect the vector and the ViraPower™ Packaging Mix into 293FT cells to generate a lentiviral stock. Then, transduce the Lenti6/TR lentiviral construct into the mammalian cell line of choice and use Blasticidin selection to generate a stable cell line expressing the Tet repressor. This cell line then becomes the host for your Lenti4/BLOCK-iT™-DEST lentiviral construct. For details about the features of pLenti6/TR, see the **Appendix**, page 67.

The pLenti6/TR plasmid is supplied at 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. You will need 3 µg of the plasmid for transfection.

Note: If you wish to propagate the pLenti6/TR plasmid, see the page 28.

Continued on next page

Positive Control

The pLenti4-GW/H1/TO-lamin^{shRNA} plasmid is included with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System as a control for lentivirus production. Include 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 9) 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.

The pLenti4-GW/H1/TO-lamin^{shRNA} plasmid is supplied at 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. You will need 3 µg of the plasmid for transfection.

Note: If you wish to propagate the pLenti4-GW/H1/TO-lamin^{shRNA} plasmid, see **Propagating the pLenti6/TR and Control Plasmids.**

Propagating the pLenti6/TR and Control Plasmids

To propagate and maintain the pLenti6/TR and pLenti4-GW/H1/TO-lamin^{shRNA} plasmids:

1. Use 10 ng of plasmid to transform a *rec*A, *end*A *E. coli* strain like Stbl3™, TOP10, or DH5α.
2. Select transformants on selective plates containing the following:

If you are selecting for…	Then use…
pLenti6/TR	**Stbl3™:** LB agar plates containing 100 µg/mL ampicillin **TOP10 or DH5α:** LB agar plates containing 100 µg/mL ampicillin and 50 µg/mL Blasticidin
pLenti4-GW/H1/TO-lamin^{shRNA}	**Stbl3™:** LB agar plates containing 100 µg/mL ampicillin **TOP10 or DH5α:** Low Salt LB agar plates containing 100 µg/mL ampicillin and 50 µg/mL Zeocin™ (see page 61 for a recipe)

3. Prepare glycerol stocks of a transformant containing plasmid for long-term storage. Propagate each plasmid in LB containing 100 µg/mL ampicillin.

Continued on next page

Producing Lentivirus in 293FT Cells, continued

Materials Needed

- pLenti4/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 to ensure that the concentration of L-glutamine will not get too low over time due to its slow degradation. 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 77)
- Fetal bovine serum (FBS; see page 77)
- 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 77. 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

Components supplied with the kits:

- pLenti4-GW/H1/TO-lamin^{shRNA} positive control vector, if desired (Box 1; 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Components supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System only:

- pLenti6/TR (Box 4; 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- ViraPower™ Packaging Mix
- Lipofectamine® 2000 transfection reagent (store at 4°C and mix gently before use)

ViraPower™ Packaging Mix

The pLP1, pLP2, pLP/VSVG plasmids are provided in an optimized mixture to facilitate viral packaging of the pLenti4/BLOCK-iT™-DEST, pLenti6/TR, and pLenti4-GW/H1/TO-lamin^{shRNA} expression constructs 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 32.

Note: ViraPower™ Packaging Mix is available separately or as part of the ViraPower™ Zeo Lentiviral Support Kit, see page 77.

Continued on next page

293FT Cell Line

The human 293FT Cell Line is supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi 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® Selective Antibiotic. 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™ Inducible H1 Lentiviral RNAi System, but is also available at **www.lifetechnologies.com** or by calling Technical Support (see page 79).
Note: The 293FT Cell Line is also available separately (see page 77).

The health of your 293FT cells at the time of transfection is critical to 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.

Lipofectamine® 2000

The Lipofectamine® 2000 reagent supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi 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™ Zeo Lentiviral Support Kit, see page 77 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 77 for ordering information). For more information about Opti-MEM® I, go to **www.lifetechnologies.com** or call Technical Support (see page 79).

Continued on next page

Producing Lentivirus in 293FT Cells, continued

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 previous page to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix	9 µg (9 µL of 1 µg/µL stock)
Amount of pLenti4/BLOCK-iT™-DEST or pLenti6/TR expression plasmid	3 µg
Amount of Lipofectamine® 2000 Reagent to use	36 µL

Note: You may produce lentiviral stocks using other tissue culture formats, 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 33).

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, combine 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti-based 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 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 29).

   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 17 and 37 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 page 33).
8. Pipet viral supernatants into cryovials in 1 mL aliquots. Store viral stocks at −80°C. Proceed to **Titering Your Lentiviral Stock**, page 34.

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**, page 32.

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, page 32.
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.
5. Follow Steps 5–8 as instructed in the recommended **Transfection Procedure**, page 32.

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, **Transfection Procedure** page 32) 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 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, 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 proceeding to transduction and expression experiments, we highly recommend determining the titer of your lentiviral stock(s). 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:

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™ cationic polymer
3. Select for stably transduced cells using the appropriate selection agent
4. Stain and count the number of antibiotic-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 the 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 33 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, 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 **Selecting a Cell Line**, previous page). If you have more than one lentiviral construct, titer all of the lentiviral constructs using the same mammalian cell line.

Antibiotic Selection

The pLenti4/BLOCK-iT™-DEST and pLenti4-GW/H1/TO-lamin^{shRNA} expression constructs contain the Zeocin™ resistance gene (Calmels et al., 1991; Drocourt et al., 1990) and the pLenti6/TR construct contains the Blasticidin resistance gene (*bsd*) (Kimura et al., 1994) to allow for Zeocin™ (Mulsant et al., 1988) or Blasticidin selection (Takeuchi et al., 1958; Yamaguchi et al., 1965), respectively, in mammalian cells that have stably transduced the lentiviral construct.

Note: If you are using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, Zeocin™ and Blasticidin are supplied with the kit. Otherwise, Zeocin™ and Blasticidin are available separately (see page 77 for ordering information).

Preparing Zeocin™ or Blasticidin

For more information about how to prepare and handle Zeocin™ and Blasticidin, refer to the **Appendix**, pages 62 and 64, respectively.

Important

Cell density can affect the efficiency of Zeocin™ selection. For the most efficient Zeocin™ selection, make sure that the cells are not greater than 50% confluent at the time of Zeocin™ addition.

Determining Antibiotic Sensitivity

To select for stably transduced cells using Zeocin™ or 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 50 to 1,000 µg/mL Zeocin™ or 2 to 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 6–7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Zeocin™ or Blasticidin, as appropriate.
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Zeocin™ or Blasticidin that kills the cells within 10–14 days after addition of antibiotic.

Continued on next page

Titering Your Lentiviral Stock, continued

Effect of Zeocin™ on Sensitive and Resistant Cells

Zeocin™'s method of killing is quite different from that of other common antibiotics such as Blasticidin, Geneticin® Selective Antibiotic, and hygromycin. **Zeocin™-sensitive cells do not round up and detach from the plate, but may exhibit the following morphological changes:**

- Vast increase in size (similar to the effects of cytomegalovirus infecting permissive cells)
- Abnormal cell shape
- Presence of large empty vesicles in the cytoplasm (breakdown of the endoplasmic reticulum and Golgi apparatus or scaffolding proteins)
- Breakdown of plasma and nuclear membrane (appearance of many holes in these membranes)

Eventually, these "cells" will completely break down and only "strings" of protein will remain.

Zeocin™-resistant cells should continue to divide at regular intervals to form distinct colonies. There should not be any distinct morphological changes in Zeocin™-resistant cells when compared to non-selected cells.

Using Polybrene™ During Transduction

Transduction of lentivirus into mammalian cells may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene™ cationic polymer). For best results, we recommend performing transduction in the presence of Polybrene™ polymer. Note however, that some cells are sensitive to Polybrene™ polymer (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™ polymer (e.g., exhibit toxicity or phenotypic changes), do not add Polybrene™ polymer during transduction. In this case, cells should still be successfully transduced.

Preparing and Storing Polybrene™ solution

Follow the instructions below to prepare Polybrene™ polymer solution (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.

Continued on next page

Titering Your Lentiviral Stock, continued

Materials Needed

- Your pLenti4/ BLOCK-iT™ lentiviral stocks (store at −80°C until use)
- Your Lenti6/TR lentiviral stock (store at −80°C until use)
- Your Lenti4-GW/H1/TO-lamin^{shRNA} lentiviral stock (store at −80°C until use)
- Adherent mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene™ cationic polmer solution, if desired
- 6-well tissue culture plates
- 10 cm tissue culture plates (for Zeocin™ selection only)
- Crystal violet (Sigma-Aldrich®, Cat. no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
- Phosphate-Buffered Saline (PBS; page 77)

Components supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System

- Zeocin™ (100 mg/mL stock) or Blasticidin (10 mg/mL stock), as appropriate 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: Remember to titer the Lenti6/TR and the Lenti4-GW/H1/TO-lamin^{shRNA} lentiviral stocks.

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 the 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™ polymer (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), treat cells as follows:
   - For Zeocin™ selection (Lenti4/BLOCK-iT™-DEST or Lenti4-GW/H1/TO-lamin^{shRNA} stocks), remove the medium and wash the cells once with PBS. For each well of cells, trypsinize the cells and replate the entire amount into one 10 cm plate containing complete culture medium with the appropriate amount of Zeocin™ to select for stably transduced cells.
   - For Blasticidin selection (Lenti6/TR stock), 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 antibiotic every 2–3 days.
8. After 10–12 days of selection (day 14–16), you should see no live cells in the mock well and discrete antibiotic-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.

Continued on next page

Titering Your Lentiviral Stock, continued

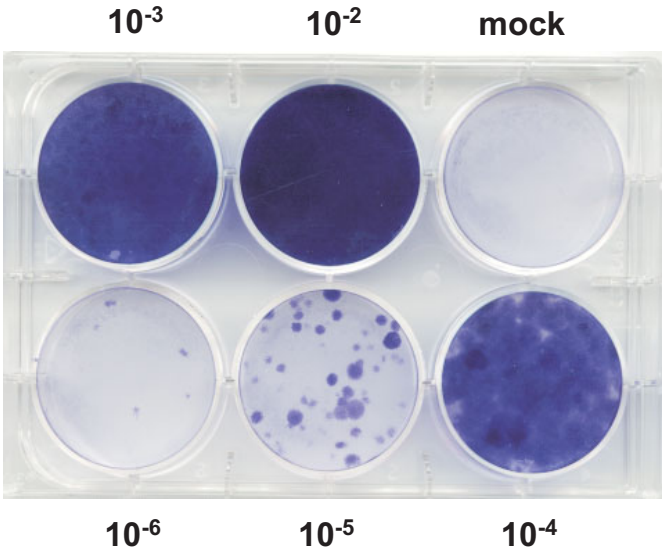
Expected Results

When titering pLenti 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 below.

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 34 and the **Troubleshooting** section, page 54 for more tips and guidelines to optimize the viral yield.

Example of Expected Results

In this experiment, a pLenti6 lentiviral stock was generated using the protocol on page 32. 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 38. 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).

General Considerations for Transduction and Expression

Introduction

After generating lentiviral stocks with suitable titers, you are ready to transduce the lentiviral constructs into the mammalian cell line of choice to inducibly express the shRNA of interest and perform RNAi analysis. General guidelines are provided in this section to help you design transduction and expression experiments.

Reminder: Remember that each 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 to Consider When Designing Transduction and RNAi Experiments

Consider the following factors when designing transduction and RNAi experiments:

- Options available to express your shRNA (see below for more information)
- Whether to express the shRNA transiently or stably (see the next page for more information)
- How much Tet repressor to express in your mammalian cell line (see page 41 for more information)
- How much virus to use for transduction (i.e. MOI; see page 42 for more information)
- Transduction efficiency of your cells
- How much tetracycline to use for induction (see page 43 for more information)
- 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 transduction

shRNA Expression Options

A number of options exist to express your shRNA of interest in the mammalian cell line of choice. Choose the option that best fits your needs.

Option	Procedure	Benefit
1	"Co-transduce" the Lenti6/TR and Lenti4/BLOCK-iT™-DEST lentiviral constructs into mammalian cells (see pages 44–47).	Perform regulated RNAi analysis with a single transduction
2	Transduce your mammalian cell line with the Lenti6/TR lentiviral construct and generate a stable cell line. Use this TetR-expressing cell line as the host for the Lenti4/BLOCK-iT™-DEST lentiviral construct (see pages 48–51).	Perform regulated RNAi analysis with multiple expression constructs using a cell line that consistently expresses the same amount of Tet repressor
3	Transduce your mammalian cell line with the Lenti4/BLOCK-iT™-DEST lentivirus only.	Constitutively express the shRNA of interest

Continued on next page

General Considerations for Transduction and Expression,
continued

For optimal results, generate a stable TetR-expressing cell line, then use this cell line as the host for your Lenti4/BLOCK-iT™-DEST expression construct (i.e. Option 2, previous page). This option is particularly recommended for performing regulated expression experiments with several expression constructs in the same mammalian cell line. For guidelines and instructions to generate a stable TetR-expressing cell line, see **Generating a TetR-Expressing Host Cell Line**, page 48.

Transient vs. Stable Expression

After transducing a 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 target gene knockdown directly after transduction (i.e. "transient" knockdown). Note that you must wait for a minimum of 48–72 hours after transduction and induction (for expression Options 1 and 2, previous page) before harvesting your cells to allow shRNA to be expressed and processed in transduced cells.
- Select for stably transduced cells using Zeocin™. This requires a minimum of 10–12 days after transduction, but allows generation of clonal cell lines that stably express the shRNA of interest. Expression of shRNA will be tetracycline-regulated (for expression Options 1 and 2, page 40) or constitutive (for expression Option 3, page 40).

Determining Antibiotic Sensitivity for Your Cell Line

If you plan to select for stably transduced cells expressing the Lenti6/TR and Lenti4/BLOCK-iT™-DEST lentiviral constructs, first determine the minimum concentration of Blasticidin and Zeocin™, respectively, 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 35. If you titered the Lenti6/TR and Lenti4/BLOCK-iT™-DEST constructs 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 and Zeocin™ for selection that you used for titering.

Expressing Tet Repressor (TetR)

Because tetracycline-regulated expression in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System is based on a repression/derepression mechanism, the amount of Tet repressor that is expressed in the host cell line from the Lenti6/TR lentiviral construct will determine the level of transcriptional repression of the Tet operator sequences in your Lenti4/BLOCK-iT™-DEST lentiviral construct. **Tet repressor levels need to be sufficiently high to suitably repress basal level transcription of the shRNA.** When performing co-transduction experiments, we generally do the following to maximize Tet repressor expression levels:

- Transduce the Lenti6/TR construct into mammalian cells and wait for 24 hours before transducing the Lenti4/BLOCK-iT™-DEST construct to allow time for the Tet repressor protein to be expressed
- Transduce the Lenti6/TR construct into mammalian cells at a higher MOI (see page 45) than the Lenti4/BLOCK-iT™-DEST construct

Continued on next page

General Considerations for Transduction and Expression,
continued

Multiplicity of Infection (MOI)

To obtain optimal expression of Tet repressor or your shRNA of interest, transduce the lentiviral construct into a 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, expression levels increase as the MOI increases.

Determining the Optimal MOI

A number of factors influence optimal MOI including:

- The nature of your mammalian cell line (e.g., non-dividing vs. dividing cell type; see **Note**, below)
- The transduction efficiency of your mammalian cell line
- The nature of your target gene of interest
- The procedure used to express your shRNA (see options on page 40).

If you are transducing the Lenti6/TR and/or your Lenti4/BLOCK-iT™-DEST lentiviral construct into the mammalian cell line of choice for the first time, use a range of MOIs (e.g., 0, 1, 5, 10, 50) to determine the MOI required to obtain the optimal level of target gene knockdown.

Note: Recommended MOIs to use for transduction are provided with each procedure (Expression Options 1 or 2). Use the recommended MOIs as a starting point and optimize as necessary.

Note

In general, lentiviral constructs transduce non-dividing cell types 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 optimal target gene knockdown.

Positive Control

If you have packaged the control Lenti4-GW/H1/TO-lamin^{shRNA} lentiviral construct, you may use this lentiviral construct 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** on page 9).

Note: If your cell line expresses lamin A/C, you may detect the protein using Western blot analysis (see page 47).

Tetracycline

Tetracycline (MW = 444.4) is commonly used as a broad spectrum antibiotic and acts to inhibit translation by blocking polypeptide chain elongation in bacteria. In the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, tetracycline functions as an inducing agent to regulate transcription of the shRNA of interest from the Lenti4/BLOCK-iT™-DEST lentiviral construct. Tetracycline is supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System as a ready-to-use 10 mg/mL stock solution.

Continued on next page

General Considerations for Transduction and Expression,
continued

Using Tetracycline

To induce transcription of the gene of interest in mammalian cells, we generally add tetracycline to a final concentration of 1 µg/mL in complete growth medium. If desired, you may vary the concentration of tetracycline used for induction from 0.001 to 1 µg/mL to modulate expression of the shRNA of interest.

Note: The concentrations of tetracycline used for induction in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System are generally not high enough to be toxic to mammalian cells.

Follow the guidelines below when handling tetracycline.

- Tetracycline is light sensitive. Store the stock solution at −20°C, protected from light. Prepare medium containing tetracycline immediately before use.
- Tetracycline is toxic. Do not ingest solutions containing the drug. If handling the powdered form, do not inhale.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling tetracycline and tetracycline-containing solutions.

Tetracycline in Fetal Bovine Serum

When culturing cells in medium containing fetal bovine serum (FBS), note that many lots of FBS contain tetracycline as FBS is generally isolated from cows that have been fed a diet containing tetracycline. If you culture your mammalian cells in medium containing FBS that is not reduced in tetracycline, you may observe some basal expression of your shRNA (as measured by target gene knockdown) in the absence of tetracycline. We generally culture our mammalian cells in medium containing FBS that may not be reduced in tetracycline, and have observed low basal target gene knockdown in the absence of tetracycline. Depending on your application (e.g., if targeting a gene involved in cell viability), you may wish to culture your cells in tetracycline-tested FBS. Contact Technical Support (see page 79) for more information.

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).

Co-Transduction and Tetracycline-Regulated Expression

Introduction

Guidelines and instructions are provided in this section to co-transduce the Lenti6/TR and Lenti4/BLOCK-iT™-DEST lentiviral constructs into the mammalian cell line of choice, induce shRNA expression with tetracycline, and to assay for target gene knockdown. Use this procedure if you have a single Lenti4/BLOCK-iT™-DEST lentiviral construct and you wish to verify that your shRNA of interest can be inducibly expressed in the mammalian cell line of interest.

If you have multiple Lenti4/BLOCK-iT™-DEST lentiviral constructs, first generate a stable cell line expressing the Tet repressor, and use this cell line as the host for your lentiviral constructs (see **Generating a TetR-Expressing Host Cell Line**, pages 48–51 for details).

Note: If you wish to constitutively express your gene of interest, simply transduce the Lenti4/BLOCK-iT™-DEST construct alone into cells at a suitable MOI.

When performing the co-transduction procedure, we highly recommend using titered Lenti6/TR and Lenti4/ BLOCK-iT™-DEST lentiviral stocks. Optimal expression results are generally obtained (i.e. low basal and high inducible target gene knockdown) when the Lenti6/TR construct is transduced into mammalian cells at a higher MOI than the Lenti4/BLOCK-iT™-DEST construct (see **MOI to Use for Transduction**, next page). Depending on the cell line used and the nature of your target gene of interest, you may need to vary the ratio of Lenti6/TR lentivirus:Lenti4/BLOCK-iT™-DEST lentivirus transduced into host cells to optimize basal and induced shRNA expression levels. This is best accomplished when the titer of each lentiviral stock is known.

Experimental Outline

To express the shRNA of interest using the co-transduction procedure, you will:

1. Transduce the Lenti6/TR lentiviral construct into mammalian cells at a suitable MOI (e.g., MOI = 10).
2. Incubate cells for 24 hours, then transduce the Lenti6/TR-containing cells with the Lenti4/BLOCK-iT™-DEST lentiviral construct at a slightly lower MOI (e.g., MOI = 1–5).
3. Incubate the cells for 24 hours, then remove the medium-containing virus.
4. Incubate the cells for 24 hours, then add tetracycline to induce expression of the shRNA of interest. Alternatively, select for stably transduced cells using Blasticidin and Zeocin™, if desired. Once stable cell lines are generated, you may add tetracycline to induce expression of the shRNA of interest.

Important

When performing the co-transduction procedure, you **must** transduce the Lenti6/TR lentiviral construct into mammalian cells before transducing the Lenti4/BLOCK-iT™-DEST expression construct to enable tetracycline-regulated expression of the shRNA of interest to occur. We generally wait at least 24 hours after transducing the Lenti6/TR construct before transducing the Lenti4/BLOCK-iT™-DEST construct to allow time for the Tet repressor to be expressed.

Continued on next page

Co-Transduction and Tetracycline-Regulated Expression,
continued

MOI to Use for Transduction

You may transduce the Lenti6/TR and Lenti4/BLOCK-iT™-DEST lentiviral constructs into your mammalian cell line at any suitable MOI (see **Determining the Optimal MOI**, page 42). Note however, that to sufficiently repress basal transcription of the shRNA of interest while still obtaining maximal levels of tetracycline-induced expression, transduce the Lenti6/TR construct into cells at a higher MOI than the Lenti4/BLOCK-iT™-DEST construct. **As a starting point, transduce the Lenti6/TR construct into cells at an MOI of 10, and transduce the Lenti4/BLOCK-iT™-DEST construct into cells at an MOI of 1 to 5.** You may optimize basal and tetracycline-induced expression levels by varying the MOI of the Lenti6/TR and/or Lenti4/BLOCK-iT™-DEST lentiviruses transduced.

Materials Needed

- Titered Lenti6/TR lentiviral stock (store at −80°C until use)
- Titered Lenti4/BLOCK-iT™-DEST lentiviral stock (store at −80°C until use)
- Titered Lenti4-GW/H1/TO-lamin^{shRNA} lentiviral stock (if desired, store at −80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene™ solution, if desired
- Appropriately sized tissue culture plates for your application

Components supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System

- 10 mg/mL tetracycline (Box 8; store protected from light)
- 10 mg/mL Blasticidin stock (if selecting for stably transduced Lenti6/TR cells)
- 100 mg/mL Zeocin™ stock (if selecting for stably transduced Lenti4/BLOCK-iT™-DEST cells)

Continued on next page

Co-Transduction and Tetracycline-Regulated Expression,
continued

Co-Transduction Procedure

Follow the procedure below to co-transduce a mammalian cell line of choice with the Lenti6/TR and your Lenti4/BLOCK-iT™-DEST lentiviral constructs to assay for tetracycline-regulated target gene knockdown. Include a negative control (mock transduction) to help evaluate results. If you are selecting for stable cell lines, include two negative control samples, one for Blasticidin selection and the other for Zeocin™ selection.

1. Plate cells in complete growth media as appropriate for your application.
2. On the day of transduction (Day 1), thaw the Lenti6/TR lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 10) 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™ polymer (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.
5. Twenty-four hours following transduction of Lenti6/TR virus (Day 2), thaw the Lenti4/BLOCK-iT™-DEST lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 1 to 5) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **Do not vortex.**
6. Remove the culture medium containing Lenti6/TR virus from the cells. Mix the medium containing Lenti4/BLOCK-iT™-DEST virus gently by pipetting and add to the Lenti6/TR virus-containing cells.
7. Add Polybrene™ polymer (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.
8. Twenty-four hours following transduction of Lenti4/BLOCK-iT™-DEST virus (Day 3), perform one of the following:
   - **Transient knockdown experiments:** Remove the medium containing virus and replace with fresh, complete medium containing 1 µg/mL tetracycline. Incubate the cells at 37°C for 24–48 hours before assaying for target gene knockdown. If you wish to assay the cells at a later time, continue to culture the cells or replate them into larger-sized tissue culture formats as necessary in medium containing tetracycline.
   - **Stable cell lines:** Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin. Incubate the cells at 37°C for 24 hours, then trypsinize and replate cells into a larger-sized tissue culture format in fresh, complete medium containing Blasticidin and Zeocin™. Proceed to Step 9, page 47.

     Example: If transducing cells in a 6-well format, trypsinize and replate cells into a 10 cm tissue culture plate before performing Blasticidin and Zeocin™ selection.

Continued on next page

Co-Transduction Procedure, continued

For stable cell lines only

9. Replace medium with fresh medium containing Blasticidin and Zeocin™ every 2–3 days until Blasticidin- and Zeocin™-resistant colonies can be identified (generally 10–14 days after selection).

   Note: Transducing cells with Lenti6/TR and Lenti4/BLOCK-iT™-DEST lentivirus at a high MOI should result in most of the cells being Blasticidin- and Zeocin™-resistant. In this case, you may not be able to see distinct Blasticidin- and Zeocin™-resistant colonies when performing stable selection. You may also not see many non-transduced cells (i.e. dead cells).

10. Pick at least 10 Blasticidin- and Zeocin™-resistant colonies (see **Note**) and expand each clone. Alternatively, you may pool the heterogeneous population of Blasticidin- and Zeocin™-resistant cells.
11. Induce expression of the shRNA of interest by adding tetracycline to a final concentration of 1 µg/mL. Wait for the appropriate length of time (e.g., 24 to 48 hours) before assaying for target gene knockdown.

Note

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of target gene knockdown from different Blasticidin- and Zeocin™-resistant clones. Test at least 10 Blasticidin- and Zeocin™-resistant clones and select the clone that provides the lowest basal and the highest level of induced target gene knockdown for further studies.

Performing RNAi Analysis

Use any method as appropriate to assay for knockdown of your target gene including functional analysis, immunofluorescence, western blot, or real-time quantitative RT-PCR (qRT-PCR) with the appropriate fluorogenic LUX™ primers. For more information about LUX™ primers or to design LUX™ primers, see **www.lifetechnologies.com/lux**.

Expected Results

When performing RNAi studies using pLenti4/BLOCK-iT™-DEST lentiviral constructs, we generally observe significant 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 40. 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 pLenti4-GW/H1/TO-lamin^{shRNA} lentiviral construct, see page 52.

Assaying for Lamin A/C Expression

If you perform RNAi analysis using the pLenti4-GW/H1/TO-lamin^{shRNA} control lentiviral stock, you may assay for lamin A/C expression and knockdown using Western blot analysis. We use an Anti-Lamin A/C Antibody (BD Biosciences, Cat. no. 612162) to detect lamin A/C expression.

Generating a TetR-Expressing Host Cell Line

Introduction

Once you have performed the co-transduction procedure and established that your Lenti4/BLOCK-iT™-DEST construct can be inducibly expressed, you may wish to establish a stable cell line that constitutively expresses the Tet repressor and inducibly expresses your shRNA of interest. First, create a stable cell line that expresses only the Tet repressor, then use that cell line as the host for your Lenti4/BLOCK-iT™-DEST lentiviral construct.

Several T-REx™ cell lines that stably express the Tet repressor are available (see page 78 for ordering information). If you wish to assay for tetracycline-regulated expression of your gene of interest in 293, HeLa, CHO, or Jurkat cells, you may want to use one of the T-REx™ cell lines as the host for your Lenti4/BLOCK-iT™-DEST lentiviral construct.

Note: The T-REx™ cell lines stably express the Tet repressor from the pcDNA™6/TR expression plasmid. This plasmid is used to generate stable TetR-expressing cell lines in the T-REx™ System. Both pLenti6/TR and pcDNA™6/TR contain the same *TetR* gene. For more information about the T-REx™ cell lines or pcDNA™6/TR, see **www.lifetechnologies.com** or contact Technical Support (see page 79).

Caution: Although suitable for use in inducible gene expression experiments, the T-REx™-HeLa cell line has exhibited fairly significant basal level knockdown of the lamin A/C gene (~20%) after transduction with the Lenti4-GW/H1/TO-lamin^{shRNA} lentivirus. The level of Lamin A/C gene knockdown achieved after tetracycline addition is >90%. If you are performing RNAi analysis of a gene involved in cellular growth control or viability, consider using another T-REx™ cell line or generate your own TetR-expressing cell line.

Important

When generating a stable cell line expressing the Tet repressor, select for clones that express the highest levels of Tet repressor to use as hosts for your inducible Lenti4/BLOCK-iT™-DEST lentiviral construct. Those clones that express the highest levels of Tet repressor should exhibit the most complete repression of basal transcription of your shRNA of interest, resulting in the lowest levels of target gene knockdown in the absence of tetracycline.

Materials Needed

- Titered Lenti6/TR lentiviral stock (store at −80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene™ polymer solution, if desired
- Appropriately sized tissue culture plates for your application

Components supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System:

- 10 mg/mL Blasticidin stock

Continued on next page

Lenti6/TR Transduction Procedure

Follow the procedure below to transduce a mammalian cell line of choice with the Lenti6/TR lentiviral construct and to use Blasticidin selection to generate a stable cell line. Include a negative control (mock transduction) to help evaluate results.

1. Plate cells in complete growth media as appropriate.
2. On the day of transduction (Day 1), thaw the Lenti6/TR lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 10) 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™ polymer (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium.
6. The following day (Day 3), remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin to select for stably transduced cells.
7. Replace medium with fresh medium containing Blasticidin every 2–3 days until Blasticidin-resistant colonies can be identified (generally 10–12 days after selection).

   Note: Transducing cells with Lenti6/TR lentivirus at a high MOI should result in most of the cells being Blasticidin-resistant. In this case, you may not be able to see distinct Blasticidin-resistant colonies when performing stable selection. You may also not see many non-transduced cells (i.e. dead cells).
8. Pick at least 10 Blasticidin-resistant colonies and expand each clone to assay for Tet repressor expression (see next page). Alternatively, you may pool the heterogeneous population of Blasticidin-resistant cells and screen for Tet repressor expression.

Continued on next page

Generating a TetR-Expressing Host Cell Line, continued

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 10 Blasticidin-resistant clones and select the clone that provides the highest level of Tet repressor expression for use as the host for your Lenti4/BLOCK-iT™-DEST construct.

Detecting TetR Expression

To detect Tet repressor expression, perform Western blot analysis using an Anti-Tet repressor antibody (MoBiTec, Göttingen, Germany, Cat. no. TET01).

Maintaining the TetR-Expressing Cell Line

Once you have generated your stable TetR-expressing cell line and have verified that the cells express suitable levels of Tet repressor, we recommend the following:

- Maintain your TetR-expressing cell line in medium containing Blasticidin
- Freeze and store vials of early passage cells

Expressing the shRNA of Interest

To express the shRNA of interest in a tetracycline-regulated manner, use the TetR-expressing cell line as the host for your Lenti4/BLOCK-iT™-DEST lentiviral construct. After transduction, you have two options to express the shRNA of interest:

- You may add tetracycline and assay for transient target gene knockdown

  or

- You may use Zeocin™ to select for a stable cell line, then add tetracycline to assay for target gene knockdown

Choose the option that best fits your needs.

Materials Needed

- Titered Lenti4/BLOCK-iT™-DEST lentiviral stock (store at −80°C until use)
- Your TetR-expressing host cell line cultured in medium containing Blasticidin
- Complete culture medium containing Blasticidin
- 6 mg/mL Polybrene™ polymer solution, if desired
- Appropriately sized tissue culture plates for your application

Components supplied with the BLOCK-iT™ Inducible H1 Lentiviral RNAi System:

- 10 mg/mL tetracycline (Box 8; store protected from light)
- 100 mg/mL Zeocin™ stock (if selecting for stably transduced Lenti4/BLOCK-iT™-DEST cells)

Continued on next page

Lenti4/BLOCK-iT™-DEST Transduction Procedure

Follow the procedure below to transduce your TetR-expressing cells with the Lenti4/BLOCK-iT™-DEST lentiviral construct and to use Zeocin™ to generate a stable cell line, if desired. Include a negative control (mock transduction) to help evaluate results.

1. Plate the TetR-expressing cells in complete growth media as appropriate for your application. If you plan to select for stably transduced cells, plate cells such that they will be 50–60% confluent on the day of transduction.
2. On the day of transduction (Day 1), thaw the Lenti4/BLOCK-iT™-DEST lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI; recommended MOI = 1–5) into fresh complete medium containing Blasticidin. 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™ polymer (if desired) to a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate at 37°C overnight.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete medium containing Blasticidin. Incubate at 37°C overnight.
6. The following day (Day 3), perform one of the following:
   - **Transient knockdown experiments:** Remove the medium containing virus and replace with fresh, complete medium containing 1 µg/mL tetracycline. Incubate the cells at 37°C for 24–48 hours before assaying for target gene knockdown. If you wish to perform the assay at a later time, continue to culture the cells or replate them into larger-sized tissue culture formats as necessary in medium containing tetracycline.
   - **Stable cell lines:** Trypsinize and replate cells into a larger-sized tissue culture format in fresh, complete medium containing Blasticidin and Zeocin™. Proceed to Step 7.

     Example: If transducing cells in a 6-well format, trypsinize and replate cells into a 10 cm tissue culture plate in medium containing Blasticidin and Zeocin™.

For stable cell lines only

7. Replace medium with fresh medium containing Blasticidin and Zeocin™ every 2–3 days until Blasticidin- and Zeocin™-resistant colonies can be identified (generally 10–14 days after selection).
8. Pick at least 10 Blasticidin- and Zeocin™-resistant colonies and expand each clone. Alternatively, you may pool the heterogeneous population of Blasticidin- and Zeocin™-resistant cells.
9. Induce expression of the shRNA of interest by adding tetracycline to a final concentration of 1 µg/mL. Wait for the appropriate length of time (e.g., 24 to 48 hours) before assaying for target gene knockdown.

Examples of Expected Results

Tetracycline-Regulated Knockdown of Lamin A/C in T-REx™-HeLa Cells

In this experiment, double-stranded oligonucleotides targeting the endogenous lamin A/C gene and the *lacZ* reporter gene were generated and cloned into pENTR™/H1/TO using the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit. The H1/TO-lamin and H1/TO-lacZ RNAi cassettes were transferred into the pLenti4/BLOCK-iT™-DEST vector using the LR recombination reaction to generate the pLenti4-GW/H1/TO-lamin^{shRNA} and pLenti4-GW/H1/TO-lacZ^{shRNA} expression constructs. Lentiviral stocks were generated and titered in HT1080 cells following the protocols in this manual (see pages 27–38).

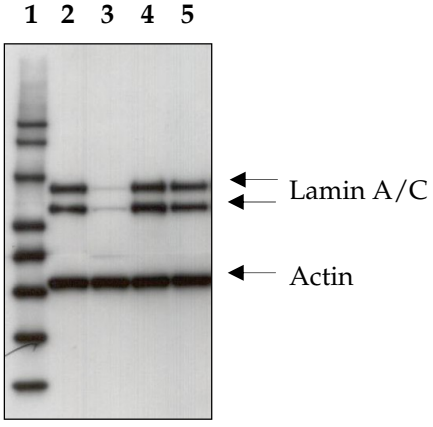
T-REx™-HeLa cells plated in a 12-well plate were transduced with each lentiviral construct at an MOI of 3. Twenty-four hours after transduction, cells were trypsinized, replated, and placed under Zeocin™ (100 µg/mL) and Blasticidin (10 µg/mL) selection for 3 weeks. Zeocin™- and Blasticidin-resistant clonal isolates were plated and treated with 1 µg/mL tetracycline for 6 days. Cell lysates were prepared and equivalent amounts were analyzed by Western blot using an Anti-Lamin A/C Antibody (BD Biosciences, Cat. no. 612162) and an Anti-β-Actin Antibody (Abcam, Cat. no. ab6276).

Results:

- The lamin A/C-specific shRNA inhibits expression of the lamin A/C gene in a tetracycline-regulated manner, while no lamin A/C knockdown is observed with the *lacZ*-specific shRNA. The degree of lamin A/C knockdown observed is >90% after tetracycline addition.

  Note: Some knockdown of Lamin A/C (~20%) is observed in the absence of tetracycline due to promoter leakiness.

- 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. MagicMark™ Western Protein Standard (Cat. no. LC5602)

Lane 2. Lenti4-GW/H1/TO-lamin^{shRNA} construct – Uninduced

Lane 3. Lenti4-GW/H1/TO-lamin^{shRNA} construct – Induced

Lane 4. Lenti4-GW/H1/TO-lacZ^{shRNA} construct – Uninduced

Lane 5. Lenti4-GW/H1/TO-lacZ^{shRNA} construct – Induced

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.

Observation	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 24). • 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 Zeocin™. • 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.

Continued on next page

LR Reaction and Transformation, continued

Observation	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 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 co-transfection and titering experiments.

Observation	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® Selective Antibiotic) • 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® Selective Antibiotic 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 32).
	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.**

Continued on next page

Generating the Lentiviral Stock, continued

Observation	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 34.
	Polybrene™ polymer not included during titering procedure	Transduce the lentiviral construct into cells in the presence of Polybrene™ cationic polymer.
No colonies obtained upon titering	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve experiment. Use the minimum amount of antibiotic 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™ polymer not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene™ cationic polymer.
Titer indeterminable; cells confluent	Too little antibiotic used for selection	Increase amount of antibiotic 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}).

Continued on next page

Transduction and Regulated shRNA Expression

The table below lists some potential problems and possible solutions that may help you troubleshoot your transduction and tetracycline-regulated knockdown experiment. Note that the troubleshooting tips provided in this section are based on the assumption that you are not constitutively expressing the shRNA of interest.

Observation	Reason	Solution
Low levels of gene knockdown observed after tetracycline induction	Low transduction efficiency: • Polybrene™ polymer not included during transduction • Non-dividing cell type used	• Transduce the lentiviral construct into cells in the presence of Polybrene™ polymer. • 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 addition of tetracycline	• Do not assay for target gene knockdown until at least 48–72 hours after tetracycline addition to allow time for shRNA to be expressed and processed. • Place cells under Zeocin™ selection and generate a stable cell line prior to addition of tetracycline. **Note:** Placing cells under Zeocin™ selection can improve gene knockdown results by killing untransduced cells.
	Target gene is important for cell viability	Place cells under Zeocin™ selection and generate a stable cell line prior to addition of tetracycline.
	Viral stocks not titered	Titer the lentiviral stock using the procedure on page 38 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™/H1/TO construct to verify its activity, then perform LR recombination with the pLenti4/BLOCK-iT™-DEST vector and proceed to generate lentivirus. **Note:** Generally, transient transfection causes overexpression of shRNA, so moderately active pENTR™/H1/TO entry clones may be less active when expressed from a lentiviral construct.

Continued on next page

Transduction and Regulated shRNA Expression, continued

Observation	Reason	Solution
No tetracycline-regulated gene knockdown observed	Did not transduce the Lenti4/BLOCK-iT™-DEST lentiviral construct into a Tet repressor-expressing cell line	• Generate a TetR-expressing cell line or obtain a T-REx™ cell line, then use this cell line as the host for the Lenti4/BLOCK-iT™-DEST construct. • Perform the co-transduction procedure (see pages 44–47), making sure that the Lenti6/TR lentivirus is transduced into mammalian cells at least 24 hours before transducing the Lenti4/BLOCK-iT™-DEST lentivirus.
	shRNA with no activity chosen	Select a different target region. If possible, screen shRNA first by transient transfection of the pENTR™/H1/TO construct to verify its activity, then perform LR recombination with the pLenti4/BLOCK-iT™-DEST vector and proceed to generate lentivirus.
	Forgot to add tetracycline	After transducing the Lenti4/BLOCK-iT™-DEST lentivirus, add tetracycline to a final concentration of 1 µg/mL to induce expression of the shRNA of interest. Wait for at least 24 hours before assaying for target gene knockdown.
Cytotoxic effects observed after transduction	Target gene is essential for cell viability	After transducing the Lenti4/BLOCK-iT™-DEST lentivirus, add Zeocin™ to select for a stable cell line. Add tetracycline to induce expression of the shRNA of interest.
	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™ polymer used during transduction	Verify the sensitivity of your cells to Polybrene™ polymer. If cells are sensitive, omit the Polybrene™ polymer during transduction.
	Too much Zeocin™ used for selection	Determine the Zeocin™ sensitivity of your cell line by performing a kill curve. Use the minimum Zeocin™ concentration required to kill your untransduced cell line.

Continued on next page

Transduction and Regulated shRNA Expression, continued

Observation	Reason	Solution
High basal level target gene knockdown observed	Did not transduce the Lenti4/BLOCK-iT™-DEST construct into Tet repressor-expressing cells	Use a TetR-expressing cell line as the host for your Lenti4/BLOCK-iT™-DEST lentiviral construct.
	TetR-expressing cell line expresses insufficient levels of Tet repressor	Transduce the Lenti6/TR lentiviral construct into mammalian cells at a suitable MOI, and perform Blasticidin selection to generate stable cell lines. Screen Blasticidin-resistant clones and choose the clone that exhibits the highest level of Tet repressor expression for use as the host for your Lenti4/BLOCK-iT™-DEST construct.
	Transient co-transduction experiments: • Transduced Lenti6/TR viral construct at too low of an MOI when compared to the expression construct • Did not wait for a sufficient amount of time after transducing the Lenti6/TR viral construct before transducing the Lenti4/BLOCK-iT™-DEST viral construct	• Transduce the Lenti6/TR viral construct into mammalian cells at a higher MOI (e.g., MOI = 10) than the expression construct (e.g., MOI = 1–5). • Transduce mammalian cells with the Lenti6/TR construct, then wait for 24 hours before transducing cells with the Lenti4/BLOCK-iT™-DEST construct.
Stably transduced Lenti4/BLOCK-iT™-DEST cells not selected when Zeocin™ is added	Cells were too dense	Zeocin™ selection works best when cells are less than 50% confluent. After transduction with the Lenti4/BLOCK-iT™-DEST lentiviral construct, trypsinize and replate cells into a larger tissue culture format before performing Zeocin™ selection.
	Cells not selected for long enough	Zeocin™ selection typically takes longer than selection with other antibiotics. When performing Zeocin™ selection, select cells for at least 10–14 days until Zeocin™-resistant colonies are visible.
Non-specific off-target gene knockdown observed	Target sequence contains strong homology to other genes	Select a different target region.

Continued on next page

Transduction and Regulated shRNA Expression, continued

Observation	Reason	Solution
No regulated target gene knockdown observed when cells are transduced with the pLenti4-GW/H1/TO-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 pLenti4-GW/H1/TO-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.

Generating the TetR-Expressing Cell Line

The table below lists some potential problems and possible solutions that may help you troubleshoot the process of generating a stable TetR-expressing cell line using the Lenti6/TR lentiviral construct.

Observation	Reason	Solution
Few Blasticidin-resistant colonies obtained	Low transduction efficiency: • Polybrene™ polymer not included during transduction • MOI too low	• Transduce the lentiviral construct into cells in the presence of Polybrene™ polymer. • Transduce your lentiviral construct into cells using a higher MOI.
	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.
No Blasticidin-resistant colonies obtained	Cells transduced with incorrect lentiviral construct	Transduce cells with the Lenti6/TR lentiviral construct.
	Non-dividing cell type used	Stable cell lines cannot be generated in non-dividing cells. Use the "co-transduction" procedure (see page 46).

Continued on next page

Generating the TetR-Expressing Cell Line, continued

Observation	Reason	Solution
Low levels of Tet repressor expressed	Lenti6/TR construct integrated into an inactive region of the genome	Screen other Blasticidin-resistant colonies. Choose the clone that exhibits the highest level of Tet repressor expression for use as the host for your Lenti4/TO/V5-DEST construct.
	Transduced Lenti6/TR into a mammalian cell line in which the CMV promoter is down-regulated	Use another mammalian cell line for transduction.
Cytotoxic effects observed after transduction	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™ polymer used during transduction	Verify the sensitivity of your cells to Polybrene™ polymer. If cells are sensitive, omit the Polybrene™ polymer during transduction.

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.

Low Salt LB Medium with Zeocin™

1.0% Tryptone
0.5% Yeast Extract
0.5% NaCl

1. Dissolve 10 g tryptone, 5 g yeast extract, and 5 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.5 with 5 M NaOH and bring the volume up to 1 Liter. For plates, add 15 g/L agar before autoclaving.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add Zeocin™ to a final concentration of 50 µg/mL.
4. Store at 4°C in the dark. Plates containing Zeocin™ are stable for 1–2 weeks.

Zeocin™

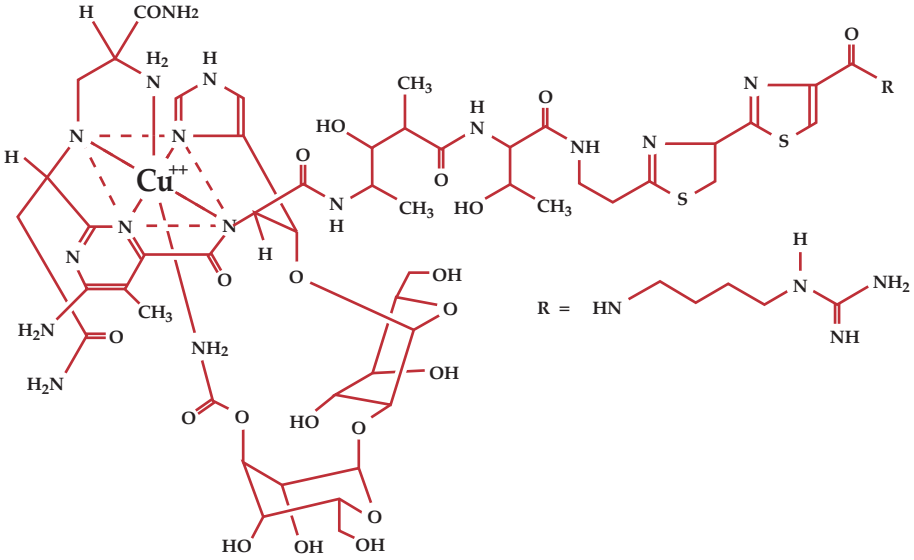
Zeocin™

Zeocin™ belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Antibiotics in this family are broad spectrum antibiotics that act as strong antibacterial and antitumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells (Baron et al., 1992; Drocourt et al., 1990; Mulsant et al., 1988; Perez et al., 1989).

The Zeocin™ resistance protein has been isolated and characterized (Calmels et al., 1991; Drocourt et al., 1990). This protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), is a 13.7 kDa protein that binds Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{55}H_{85}O_{21}N_{20}S_2Cu$-HCl and the molecular weight is 1,527.5. The diagram below shows the structure of Zeocin™.



Applications of Zeocin™

Zeocin™ is used for selection in mammalian cells (Mulsant et al., 1988); plants (Perez et al., 1989); yeast (Baron et al., 1992); and prokaryotes (Drocourt et al., 1990). Suggested concentrations of Zeocin™ for selection in mammalian cell lines and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/mL in **Low Salt LB** medium* (see page 61 for recipe)
Mammalian Cells	50–1,000 µg/mL (varies with cell line)

*Efficient selection requires that the concentration of NaCl be no more than 5 g/L (<90 mM).

Continued on next page

Zeocin™, continued

Handling Zeocin™

- **High salt and acidity or basicity inactivate Zeocin™.** Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see the recipe for **Low Salt LB Medium,** page 61). Note that the pH and salt concentration do not need to be adjusted when preparing tissue culture medium containing Zeocin™.
- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and plates or medium containing drug, in the dark at 4°C. Culture medium containing Zeocin™ may be stored at 4°C protected from exposure to light for up to 1 month.
- **Caution:** wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- **Warning!** Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Preparing and Storing Zeocin™

Zeocin™ is supplied in autoclaved, deionized water in 1.25 mL aliquots at a concentration of 100 mg/mL. The stability of Zeocin™ is guaranteed for six months, if stored at –20°C protected from exposure to light.

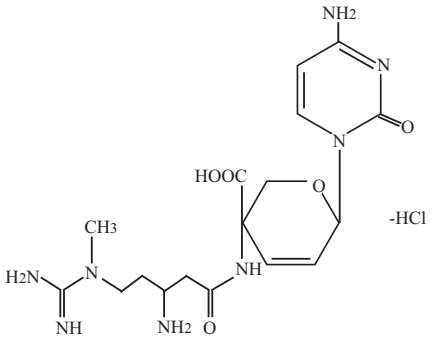
Blasticidin

Description

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi et al., 1958; Yamaguchi et al., 1965). 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 protective clothing (e.g., a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

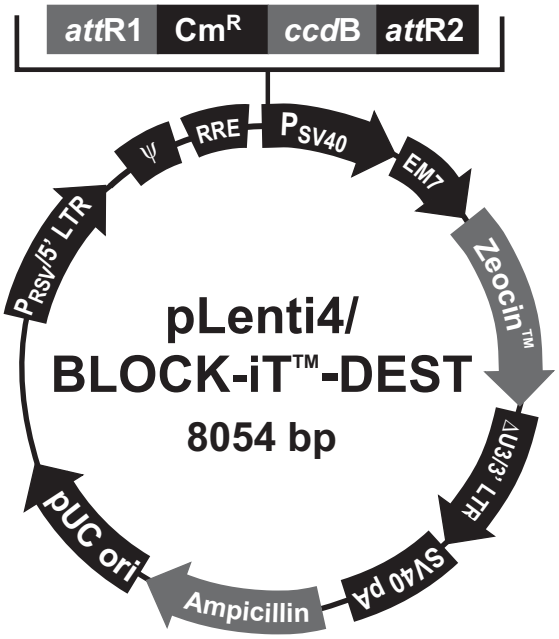
Blasticidin may be obtained in 50 mg aliquots (see page 78 for ordering information).

- 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 pLenti4/BLOCK-iT™-DEST

Map of pLenti4/BLOCK-iT™-DEST

The map below shows the elements of pLenti4/BLOCK-iT™-DEST. DNA from the entry clone replaces the region between bases 1,868 and 3,551. **The sequence for pLenti4/BLOCK-iT™-DEST is available at www.lifetechnologies.com or by contacting Technical Support (see page 79).**



Comments for pLenti4/BLOCK-iT™-DEST
8054 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 1861-1985
Chloramphenicol resistance gene (Cm^R): bases 2094-2753
*ccd*B gene: bases 3095-3400
*att*R2 site: bases 3441-3565
SV40 early promoter and origin: bases 3714-4022
EM7 promoter: bases 4041-4107
Zeocin™ resistance gene: bases 4108-4482
ΔU3/3' LTR: bases 4574-4808
  ΔU3: bases 4574-4627
  3' LTR: bases 4627-4808
SV40 polyadenylation signal: bases 4880-5014
bla promoter: bases 5870-5968
Ampicillin (*bla*) resistance gene: bases 5969-6829
pUC origin: bases 6974-7647

Continued on next page

Map and Features of pLenti4/BLOCK-iT™-DEST, continued

Features of the Vector

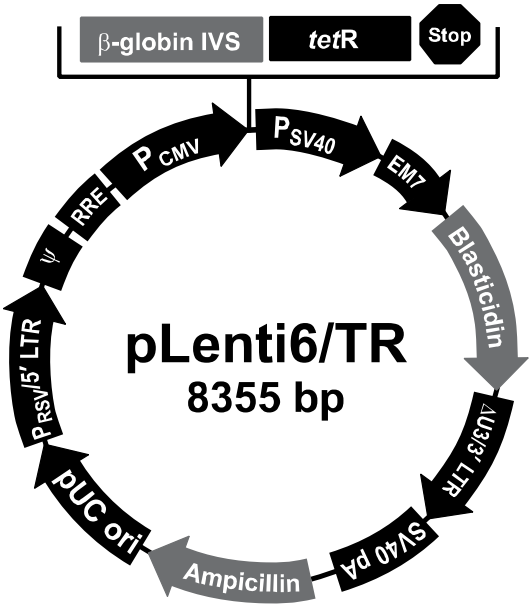
The pLenti4/BLOCK-iT™-DEST (8,054 bp) vector contains the following elements. All features have been functionally tested.

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 H1/TO RNAi cassette 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.*
Zeocin™ resistance (*Sh ble*) gene	Allows selection of stably transduced mammalian cell lines (Drocourt et al., 1990; Mulsant et al., 1988).
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 and Features of pLenti6/TR

Map of pLenti6/TR

The map below shows the elements of pLenti6/TR. **The complete sequence for pLenti6/TR is available at www.lifetechnologies.com or by contacting Technical Support (see page 79).**



Comments for pLenti6/TR
8355 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
CMV promoter: bases 1809-2392
Rabbit β-globin intron II (IVS): bases 2552-3124
*tet*R gene: bases 3208-3855
SV40 early promoter and origin: bases 3960-4269
EM7 promoter: bases 4324-4390
Blasticidin resistance gene: bases 4391-4789
ΔU3/3′ LTR: bases 4875-5109
ΔU3: bases 4875-4928
3′ LTR: bases 4929-5109
SV40 polyadenylation signal: bases 5181-5315
bla promoter: bases 6171-6269
Ampicillin (*bla*) resistance gene: bases 6270-7130
pUC origin: bases 7275-7948

TetR Gene

The *TetR* gene in pLenti6/TR was originally isolated from the Tn*10* transposon which confers resistance to tetracycline in *E. coli* and other enteric bacteria (Postle et al., 1984). The *TetR* gene from Tn*10* encodes a class B Tet repressor and is often referred to as *TetR(B)* in the literature (Hillen & Berens, 1994).

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa. For more information about the Tet repressor and its interaction with the Tet operator, refer to the review by Hillen and Berens, 1994.

Continued on next page

Map and Features of pLenti6/TR, continued

Features of the Vector

The pLenti6/TR (8,355 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	Allows 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)	Allows Rev-dependent nuclear export of unspliced viral mRNA (Kjems et al., 1991; Malim et al., 1989).
CMV promoter	Allows high-level, constitutive expression of the Tet repressor in mammalian cells (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).
Rabbit β-globin intron II (IVS)	Enhances expression of the *TetR* gene in mammalian cells (van Ooyen et al., 1979).
*Tet*R gene	Encodes the Tet repressor that binds to tet operator sequences to repress transcription of the gene of interest in the absence of tetracycline (Postle et al., 1984; Yao et al., 1998).
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	Allows 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	Allows high-copy replication and maintenance in *E. coli*.

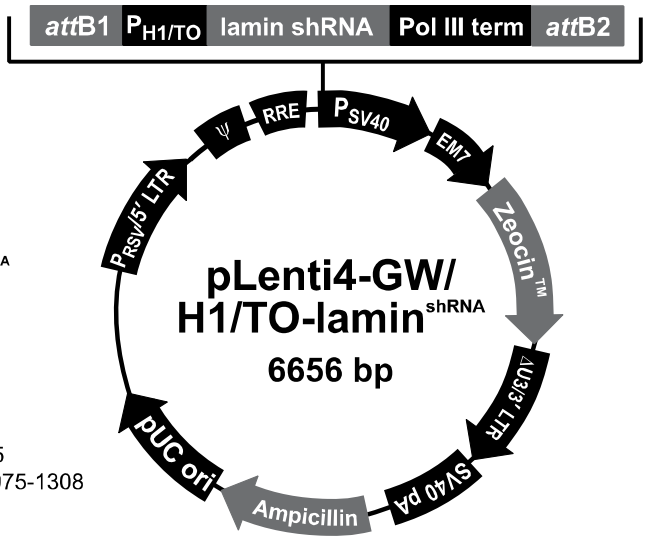
Map of pLenti4-GW/H1/TO-lamin^{shRNA}

Description

pLenti4-GW/H1/TO-lamin^{shRNA} is a 6,656 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™/H1/TO vector using the reagents supplied in the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit to generate an entry construct containing the H1/TO-lamin^{shRNA} RNAi cassette. The H1/TO-lamin^{shRNA} RNAi cassette was transferred into the pLenti4/BLOCK-iT™-DEST vector using the Gateway® LR recombination reaction to generate the pLenti4-GW/H1/TO-lamin^{shRNA} vector. This vector has been fully sequenced.

Map of pLenti4-GW/H1/TO-lamin^{shRNA}

The map below shows the elements of pLenti4-GW/H1/TO-lamin^{shRNA}. **The complete sequence of the vector is available at www.lifetechnologies.com or from Technical Support (see page 79).**



Comments for pLenti4-GW/H1/TO-lamin^{shRNA}
6656 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 1861-1885
H1/TO promoter: bases 1942-2041
- $tetO_2$ site: bases 1991-2009
- TATA box: bases 2012-2016
- $tetO_2$ site: bases 2019-2037

Lamin A/C shRNA: bases 2042-2084
Pol III terminator: bases 2085-2090
*att*B2 site: bases 2143-2167 (C)
SV40 early promoter and origin: bases 2316-2624
EM7 promoter: bases 2643-2709
Zeocin™ resistance gene: bases 2710-3084
ΔU3/3′ LTR: bases 3176-3410
- ΔU3: bases 3176-3229
- 3′ LTR: bases 3229-3410

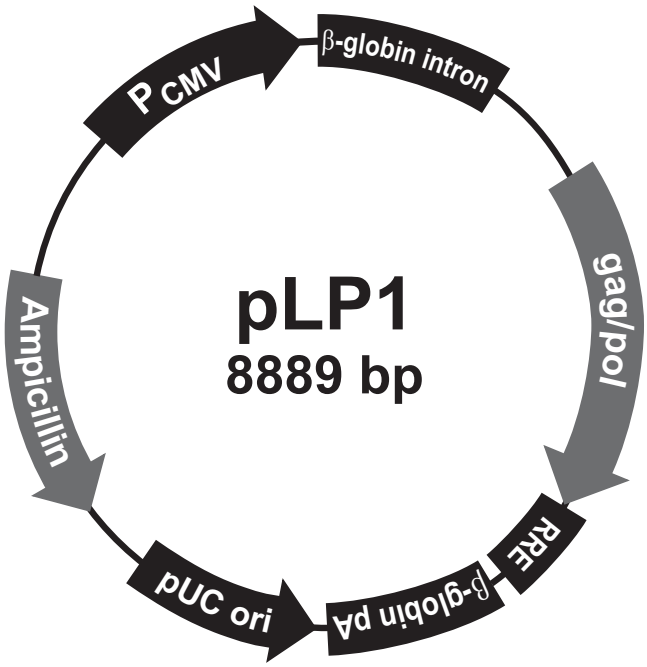
SV40 polyadenylation signal: bases 3482-3616
bla promoter: bases 4472-4570
Ampicillin (*bla*) resistance gene: bases 4571-5431
pUC origin: bases 5576-6249

(C) = complementary strand

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.lifetechnologies.com** or by contacting Technical Support (see page 79).



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

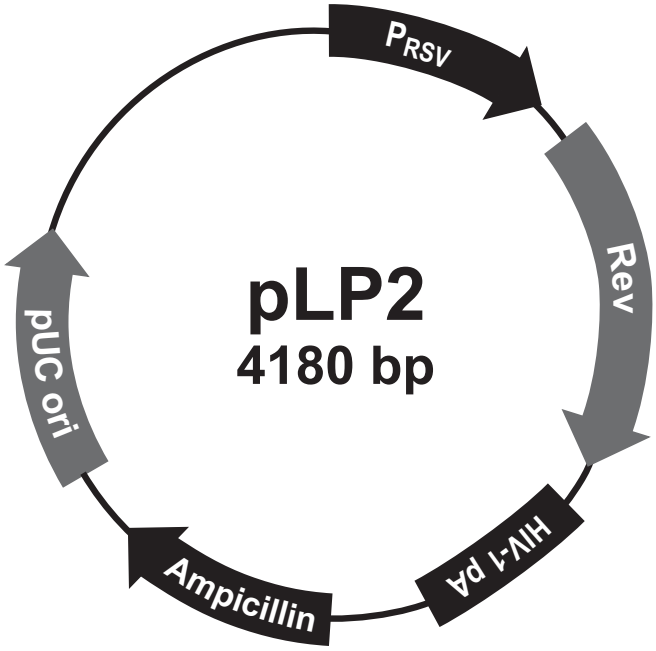
pLP1 (8,889 bp) contains the following elements. All features have been functionally tested.

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.lifetechnologies.com** or by contacting Technical Support (see page 79).



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

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Map and Features of pLP2, Continued

Features of pLP2

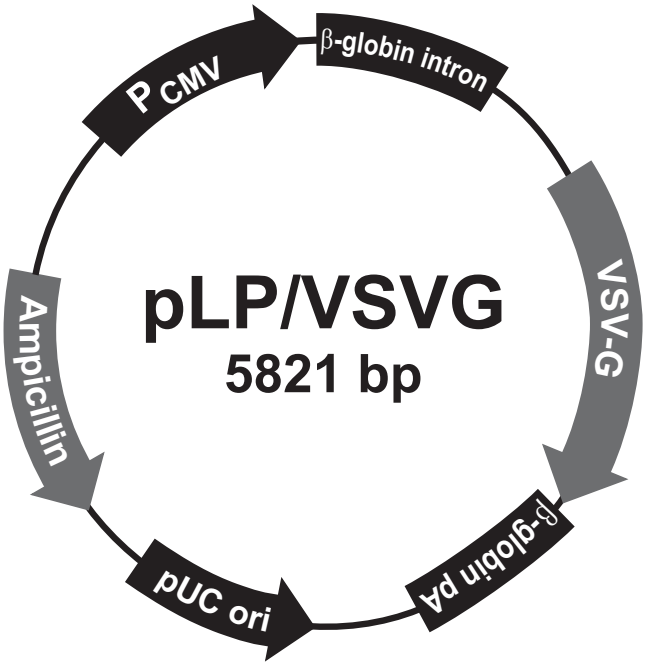
pLP2 (4,180 bp) contains the following elements. All features have been functionally tested.

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.lifetechnologies.com** or by contacting Technical Support (see page 79).



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, continued

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*.

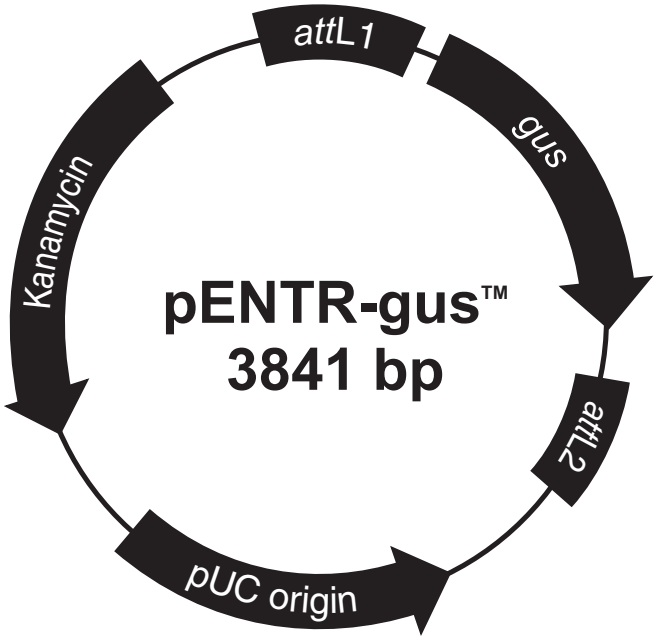
Map of pENTR™-gus

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.lifetechnologies.com** or by contacting Technical Support (see page 79).

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector. **The sequence for pENTR™-gus is available** at **www.lifetechnologies.com** or by contacting Technical Support (see page 79).



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™ Inducible H1 Lentiviral RNAi System 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.lifetechnologies.com** or contact Technical Support (see page 79).

Product	Amount	Cat. no.
BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit	20 constructions	K4920-00
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 *E. coli*	10 transformations	A10460
pLenti6/TR Vector Kit	20 µg	V480-20
ViraPower™ Zeo Lentiviral Support Kit	20 reactions	K4985-00
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
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	11593027
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

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Additional Products, continued

Selection Agents

The table below lists ordering information for the selection agents required for use in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System.

Note: Geneticin® Selective Antibiotic is required for maintenance of the 293FT cells.

Product	Amount	Cat. no.
Blasticidin	50 mg	R210-01
Zeocin™	1 g 5 g	R250-01 R250-05
Geneticin® Selective Antibiotic	1 g 5 g 25 g 20 mL (50 mg/mL) 100 mL (50 mg/mL)	11811-023 11811-031 11811-098 10131-035 10131-027

T-REx™ Cell Lines

A number of cell lines that stably express the Tet repressor from pcDNA™6/TR (TetR-expressing plasmid from the T-REx™ System) are available separately. The cell lines should be maintained in medium containing Blasticidin. For more information about pcDNA™6/TR and the T-REx™ System, see **www.lifetechnologies.com** or contact Technical Support.

Product	Amount	Cat. no.
T-REx™-293 Cell Line	3×10^6 cells, frozen	R710-07
T-REx™-HeLa Cell Line	3×10^6 cells, frozen	R714-07
T-REx™-CHO Cell Line	3×10^6 cells, frozen	R718-07
T-REx™-Jurkat Cell Line	3×10^6 cells, frozen	R722-07
Flp-In™ T-REx™-293 Cell Line	3×10^6 cells, frozen	R780-07

Technical Support

Obtaining Support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**

Certificate of Analysis

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.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Limited Use Label License: ULB ccdB Selection Technology

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Purchaser Notification, continued

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

Life Technologies 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. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway® Expression Clones

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

Additional Terms and Conditions

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

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