Instruction Manual

pLenti6/TR Vector

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Kit Contents and Storage

Shipping/ Storage

The pLenti6/TR Vector Kit is shipped in two boxes as described below. Upon receipt, store as recommended below.

Box	Reagent	Shipping	Storage
1	pLenti6/TR Vector	Room temperature	-20°C
2	Blasticidin	Room temperature	-20°C

Contents

The pLenti6/TR Vector Kit includes the following reagents. **Store at -20°C.**

Reagent	Composition	Amount
pLenti6/TR Vector	Lyophilized in TE Buffer, pH 8.0	20 µg
Blasticidin	Powder	50 mg

Accessory Products

Accessory Products

Blasticidin and other products suitable for use with the pLenti6/TR Vector Kit are available separately from Invitrogen. Ordering information is provided below. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 26).

Note: Some reagents are available in other sizes.

Item	Amount	Catalog no.
Blasticidin	50 mg	R210-01
ViraPower™ Packaging Mix	3 x 195 μg	K4975-00
293FT Cell Line	3 x 10^6 cells, frozen	R700-07
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
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 μl	C7373-03

Invitrogen Expression Systems

The pLenti6/TR vector is intended for use in generating stable cell lines expressing the Tet repressor. These TetR-expressing cell lines may be used as hosts for expression constructs that facilitate tetracycline-regulated expression of a gene or shRNA of interest from Invitrogen's ViraPower™ T-REx™ Lentiviral Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, respectively. Ordering information for these two systems is provided below.

Item	Amount	Catalog no.
ViraPower™ T-REx™ Lentiviral Expression System	20 constructions	K4965-00
BLOCK-iT™ Inducible H1 Lentiviral RNAi System	20 constructions	K4925-00

Introduction

Overview

Introduction

pLenti6/TR is an 8.4 kb lentiviral-based vector that facilitates high-level expression of the tetracycline (Tet) repressor under the control of the human cytomegalovirus (CMV) immediate early promoter. The vector also contains elements that allow packaging of the construct into virions and the Blasticidin resistance marker for selection of stable cell lines. pLenti6/TR is designed for use with the ViraPower™ T-REx™ Lentiviral Expression System (Catalog nos. K4965-00 and K4967-00) and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Catalog no. K4925-00) available from Invitrogen.

Lenti6/TR lentivirus may be transduced into dividing or non-dividing mammalian cells to facilitate high-level stable or transient expression of the Tet repressor. Tetracycline-regulated expression of a gene or short hairpin RNA (shRNA) of interest may then be tested by transducing the appropriate inducible lentiviral expression construct into host cells expressing the Tet repressor.

For more information about the ViraPower™ Lentiviral Technology, ViraPower™ T-REx™ Lentiviral Expression System, and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, see below and pages 2-3. For a brief description about how tetracycline regulation works in these Systems, see page 7.

ViraPower™ Lentiviral Technology

The ViraPower™ Lentiviral Technology facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene or RNA to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. Based on the lentikat™ system developed by Cell Genesys (Dull *et al.*, 1998), the ViraPower™ Lentiviral Technology possesses features which enhance its biosafety while allowing high-level expression in a wider range of cell types than traditional retroviral systems. For more information about the biosafety features of the System, see page 8.

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ViraPower™ T-REx™ Lentiviral Expression System

The ViraPower™ T-REx™ Lentiviral Expression System combines Invitrogen's ViraPower™ Lentiviral and T-REx™ technologies to facilitate highly efficient, *in vitro* or *in vivo* tetracycline-regulated expression of a target gene of interest in dividing or non-dividing mammalian cells using a replication-incompetent lentivirus. The System includes:

- The pLenti6/TR vector containing the *TetR* gene for constitutive, high-level expression of the Tet repressor under the control of a CMV promoter (see page 5 for more information).
- The pLenti4/TO/V5-DEST Gateway® destination vector into which the gene of interest is cloned. The vector contains elements to allow packaging of the construct into virions and tetracycline-regulated expression of the gene of interest.
- The ViraPower™ Packaging Mix and the 293FT Cell Line to facilitate production of replication-incompetent lentiviral stocks from any pLenti-based construct.
- Tetracycline to induce expression of the gene of interest.

For more information about the Gateway® Technology, see page 4. For more information about the ViraPower™ T-REx™ Lentiviral Expression System, refer to the ViraPower™ T-REx™ Lentiviral Expression System manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 26).

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BLOCK-iT™ Inducible H1 Lentiviral RNAi System

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System combines Invitrogen's BLOCK-iT™ RNAi and ViraPower™ T-REx™ Lentiviral technologies to facilitate lentiviral-based, tetracycline-regulated expression of a short hairpin RNA (shRNA) of interest in dividing or non-dividing mammalian cells for RNA interference (RNAi) analysis. The System includes:

- The BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit which includes the pENTR™/H1/TO vector for production of a Gateway® entry clone containing elements required for tetracycline-regulated expression of a double-stranded oligonucleotide encoding an shRNA of interest in mammalian cells. This H1/TO RNAi cassette (*i.e.* human H1/TO promoter + double-stranded oligonucleotide + Polymerase III terminator) can be transferred into the pLenti4/BLOCK-iT™-DEST vector in an LR recombination reaction.
- The promoterless pLenti4/BLOCK-iT™-DEST Gateway® destination vector into which the H1/TO RNAi cassette is transferred. The vector contains elements to allow packaging of the construct into virions and the Zeocin™ resistance gene for selection of stable cell lines.
- The pLenti6/TR vector containing the *TetR* gene for constitutive, high-level expression of the Tet repressor under the control of a CMV promoter (see page 5 for more information).
- The ViraPower™ Packaging Mix and the 293FT Cell Line to facilitate production of replication-incompetent lentiviral stocks from any pLenti-based construct.
- Tetracycline to induce expression of the gene of interest.

For more information about the Gateway® Technology, see the next page. For more information about the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, refer to the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 26).

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Gateway® Technology

Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems. In the ViraPower™ T-REx™ Lentiviral Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System, the gene or double-stranded oligonucleotide of interest, respectively, is cloned into an appropriate Gateway®-adapted entry vector to generate an entry clone. The resulting entry clone is then used in an LR recombination reaction with the appropriate Gateway® destination vector (*e.g.* pLenti4/TO/V5-DEST or pLenti4/BLOCK-iT™-DEST) to generate an expression clone containing the gene or H1/TO RNAi cassette of interest, respectively.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology manual which is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 26).

Overview, continued

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. For more information about the biosafety features of the vector, see page 8.
- 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 gene 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*

For a map of pLenti6/TR, see the **Appendix**, page 21.

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TetR Gene in pLenti6/TR

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 and 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 Hillen and Berens, 1994.

Producing Lentivirus

To use pLenti6/TR to generate a TetR-expressing cell line, you will need to produce a lentiviral stock. To produce a lentiviral stock, you will cotransfect the ViraPower™ Packaging Mix and the pLenti6/TR vector into 293FT producer cells to produce replication-incompetent lentivirus. The Lenti6/TR lentivirus can then be transduced into the mammalian cell line of interest and Blasticidin selection can be performed to select for stable cell lines.

For more information about the ViraPower™ Packaging Mix and the 293FT Cell Line, see page 19.

How Lentivirus Works

Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis and Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher and Wong-Staal, 2000; Luciw, 1996). Once the lentiviral construct has integrated into the genome, the *TetR* gene is expressed.

VSV Envelope Glycoprotein

Most retroviral vectors are limited in their usefulness as delivery vehicles by their restricted tropism and generally low titers. In the ViraPower™ and BLOCK-iT™ Lentiviral Systems, 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).

How Tetracycline Regulation Works

Tetracycline Regulation

The ViraPower™ T-REx™ Lentiviral Expression System and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System use regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994; Hillen *et al.*, 1983) to allow tetracycline-regulated expression of a gene or shRNA of interest, respectively from an appropriate inducible lentiviral construct. 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 gene of interest (Yao *et al.*, 1998). In each system, expression of the molecule of interest is repressed in the absence of tetracycline and induced in its presence (Yao *et al.*, 1998).

In each system, expression of the gene or shRNA of interest from the pLenti4/TO/V5-DEST or pLenti4/BLOCK-iT™-DEST lentiviral construct, respectively is controlled by a hybrid promoter containing 2 copies of the *tet* operator 2 ($TetO_2$) sequence. Each 19-nucleotide $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor.

Mechanism of Repression/ Derepression

In the absence of tetracycline, the Tet repressor (expressed from pLenti6/TR) forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence (Hillen and Berens, 1994) in the promoter of the pLenti4/TO/V5-DEST or pLenti4/BLOCK-iT™-DEST expression construct. The 2 $TetO_2$ sites in the promoter of each construct serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor. Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of your gene or 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 gene or shRNA of interest.

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. The association constant, K_A, of tetracycline for the Tet repressor is 3×10^9 M^{-1} (Hillen and Berens, 1994).

Biosafety Features

Introduction

The lentiviral and packaging vectors supplied in the ViraPower™ T-REx™ Lentiviral Expression System and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System are third-generation vectors based on lentiviral vectors developed by Dull et al., 1998. This third-generation HIV-1-based 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 Invitrogen's Lentiviral Systems

Invitrogen's ViraPower™ T-REx™ and BLOCK-iT™ Inducible H1 RNAi Lentiviral Systems include the following key safety features:

- All pLenti-based 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 plasmids and the pLenti-based plasmid). 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).

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Biosafety Features, continued

Biosafety Features of Invitrogen's Lentiviral Systems, continued

- 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 each pLenti-based vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

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Biosafety Features, continued

Biosafety Level 2

Despite the inclusion of the safety features discussed in this section, the lentivirus produced with the ViraPower™ T-REx™ Lentiviral Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi 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 either System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when creating lentivirus carrying potentially harmful or toxic genes (*e.g.* activated oncogenes) or shRNA molecules targeting human tumor suppressor genes.

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories", 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded from the Web at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.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 ViraPower™ T-REx™ Lentiviral Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System.

Methods

General Considerations to Use pLenti6/TR

Introduction

This section provides general guidelines and discusses the options available to use the pLenti6/TR vector to express Tet repressor in a mammalian cell line of interest.

Options to Use pLenti6/TR

Several options are available to use pLenti6/TR to obtain transient or stable expression of the Tet repressor. Choose the option that best fits your needs.

Option	Procedure	Benefit
1	Produce a lentiviral stock of the pLenti6/TR construct, then co-transduce the Lenti6/TR and the inducible Lenti-DEST-based lentiviral construct into the mammalian cell line of choice.	Perform tetracycline-regulated expression experiments with a single transduction. **Note:** Suitable for dividing and non-dividing mammalian cell lines.
2	Produce a lentiviral stock of the pLenti6/TR construct, then transduce the mammalian cell line of choice with the Lenti6/TR lentivirus and use Blasticidin selection to generate a stable cell line. Use this TetR-expressing cell line as the host for the inducible Lenti-DEST-based lentiviral construct.	Perform tetracycline-regulated experiments with multiple constructs using a cell line that consistently expresses the same amount of Tet repressor.
3	Transfect the pLenti6/TR plasmid into mammalian cells and use Blasticidin selection to generate a stable cell line. Use this TetR-expressing cell line as the host for the inducible Lenti-DEST-based lentiviral construct.	Perform tetracycline-regulated experiments in a dividing mammalian cell line **only**.

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General Considerations to Use pLenti6/TR, continued

Resuspending the Plasmid

The pLenti6/TR plasmid is supplied lyophilized in TE Buffer, pH 8.0. To use, resuspend the DNA in 20 µl of sterile water to prepare a 1 µg/µl stock solution. Store stock solution at -20°C.

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

E. coli Host

For optimal results, we recommend using Stbl3™ *E. coli* for transformation, as this strain is particularly well-suited for cloning unstable DNA such as lentiviral DNA containing direct repeats. Other *E. coli* strains such as TOP10 or DH5α are suitable for transformation but may give rise to some transformants containing unwanted recombinants (*i.e.* plasmids where recombination has occurred between the 5′ and 3′ LTRs). To reduce the frequency of this occurrence, select for TOP10 and DH5α transformants on selective plates containing ampicillin **and** Blasticidin.

Propagating the pLenti6/TR Plasmid

The pLenti6/TR vector contains the ampicillin and Blasticidin resistance genes to facilitate selection of the plasmid in *E. coli*. To propagate and maintain the plasmid:

1. Use the 1 µg/µl pLenti6/TR stock solution (see above) to transform a *rec*A, *end*A *E. coli* strain such as Stbl3™, TOP10, or DH5α. Use 10 ng of plasmid for transformation.
2. Depending on the *E. coli* strain transformed, select transformants on plates containing the following:

If you are transforming…	Then use…
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

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

Guidelines for Transfection

Introduction

To generate a stable cell line expressing Tet repressor from pLenti6/TR, you may either:

- Transfect the pLenti6/TR plasmid directly into a dividing mammalian cell line and use Blasticidin to select for a stable cell line **OR**
- Cotransfect the pLenti6/TR plasmid and the ViraPower™ Packaging Mix into the 293FT producer cell line to produce a lentiviral stock, then transduce the resulting lentiviral stock into any mammalian cell line of choice and use Blasticidin selection to generate a stable cell line

General guidelines are provided in this section to perform transfection. We suggest reading through this section before beginning. For instructions to generate a stable cell line via plasmid transfection, see page 16. For guidelines to produce a lentiviral stock, see page 18.

Plasmid Preparation

Once resuspended, the pLenti6/TR supplied in the kit is ready-to-use for transfection.

If you are propagating the pLenti6/TR plasmid, note that you must use purified plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol or sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) available from Invitrogen or CsCl gradient centrifugation.

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Methods of Transfection

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For established cell lines (*e.g.* 293, COS, A549), consult original references or the supplier of your cell line for the optimal method of transfection. Choose the method and reagent that provides the highest efficiency transfection in your mammalian cell line. We recommend using Invitrogen's Lipofectamine™ 2000 Reagent for transfection (see below).

Important: If you are producing a lentiviral stock, you **must** use Lipofectamine™ 2000 for transfection.

Lipofectamine™ 2000

For high-efficiency transfection in a broad range of mammalian cell lines, we recommend using the cationic lipid-based Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen (Ciccarone *et al.*, 1999). Using Lipofectamine™ 2000 to transfect plasmid DNA into eukaryotic cells offers the following advantages:

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

For more information on Lipofectamine™ 2000 Reagent, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 26).

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Guidelines for Transfection, continued

Determining Blasticidin Sensitivity for Your Cell Line

The pLenti6/TR vector contains the Blasticidin resistance gene to allow generation of stable cell lines using Blasticidin. Before you can generate a stable cell line, you must determine the minimum concentration of Blasticidin required to kill your untransfected mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 2-10 μg/ml Blasticidin are sufficient to kill most untransfected mammalian cell lines. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line. For instructions to prepare and handle Blasticidin, see the **Appendix**, page 24.

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 Blasticidin.
3. Replenish the selective media every 3-4 days and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10-14 days after addition of antibiotic.

Generating a Stable Cell Line Via Plasmid Transfection

Introduction

This section provides general guidelines to transfect the pLenti6/TR plasmid into the mammalian cell line of interest and to use Blasticidin selection to generate a stable cell line.

Important

Because tetracycline-regulated expression in the ViraPower™ T-REx™ Lentiviral Expression System and the BLOCK-iT™ Inducible H1 Lentiviral RNAi System is based on a repression/derepression mechanism, the amount of Tet repressor expressed in the host cell line from the Lenti6/TR lentiviral construct will determine the level of transcriptional repression of the $TetO_2$ sequences in the inducible lentiviral construct. **When generating stable TetR-expressing cell lines, note that Tet repressor levels need to be sufficiently high to suitably repress basal level transcription of the gene or shRNA of interest, as appropriate.**

Materials Needed

To transfect the pLenti6/TR plasmid into mammalian cells, you will need to have the following materials on hand:

- Mammalian cell line of interest (make sure that cells are healthy and > 90% viable before beginning)
- pLenti6/TR plasmid (1 µg/µl stock solution)
- Transfection reagent of choice (*e.g.* Lipofectamine™ 2000)
- 10 mg/ml Blasticidin
- Appropriate growth medium for your cells
- Appropriate tissue culture dishes and supplies

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Generating a Stable Cell Line Via Plasmid Transfection, continued

Guidelines for Transfection and Selection

Follow these guidelines to transfect the pLenti6/TR plasmid into mammalian cells and to select for stable cell lines using Blasticidin. **Important: Do not** use these guidelines if you are preparing a lentiviral stock. See the next section for guidelines to produce a lentiviral stock.

1. One day before transfection, plate cells at a density recommended by the manufacturer of the transfection reagent you are using.
2. On the day of transfection (Day 1), transfect pLenti6/TR plasmid into cells following the recommendations of the manufacturer of your transfection reagent.
3. Six hours after transfection, remove the medium and replace with fresh growth medium. Incubate the cells overnight at 37°C.
4. The following day (Day 2), remove the medium and replace with fresh complete medium containing the appropriate concentration of Blasticidin.
5. Replace medium with fresh medium containing Blasticidin every 3-4 days until Blasticidin-resistant colonies can be identified (generally 10-14 days after selection).
6. Pick at least 10 Blasticidin-resistant colonies. Screen to determine the amount of Tet repressor expressed. Select clones that express the highest levels of Tet repressor to use as hosts for your inducible expression construct.

Detecting TetR Protein

To detect Tet repressor expression, we recommend performing Western blot analysis using an Anti-Tet repressor antibody (MoBiTec, Göttingen, Germany, Catalog no. TET01).

Maintaining TetR-Expressing Cell Lines

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 the cell line in medium containing Blasticidin
- Freeze and store vials of early passage cells

Guidelines to Produce a Lentiviral Stock

Introduction

This section provides general guidelines to produce a lentiviral stock from pLenti6/TR. Once produced, you may:

- Transduce the Lenti6/TR lentivirus into the mammalian cells of choice and use Blasticidin to select for a stable cell line. Use the resulting TetR-expressing cell line as the host for your inducible lentiviral construct.
- Co-transduce the Lenti6/TR lentiviral construct and the inducible lentiviral construct into the mammalian cells of choice and perform transient or stable expression, as appropriate.

For detailed instructions and protocols to produce a lentiviral stock and transduce mammalian cells, refer to the ViraPower™ T-REx™ Lentiviral Expression System manual or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual. Both manuals are available for downloading from our Web site or by contacting Technical Service.

Experimental Outline

To generate a stable TetR-expressing cell line using lentivirus, you will need to do the following:

1. Cotransfect the pLenti6/TR construct and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
2. Titer the lentiviral stock.
3. Transduce the Lenti6/TR lentivirus into the mammalian cells of choice at an appropriate multiplicity of infection (MOI).
4. Use Blasticidin to select for stably transduced cells.
5. Pick Blasticidin-resistant clones and screen for clones that express the highest levels of Tet repressor (see **Important Note** on page 16).

For more information about the ViraPower™ Packaging Mix and the 293FT Cell Line, see the next page.

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Guidelines to Produce a Lentiviral Stock, continued

ViraPower™ Packaging Mix

The ViraPower™ Packaging Mix (Catalog no. K4975-00) contains an optimized mixture of 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 facilitate viral packaging of the pLenti6/TR construct following cotransfection into 293FT producer cells. For more information about the ViraPower™ Packaging Mix and each packaging plasmid, refer to the ViraPower™ T-REx™ Lentiviral Expression System or BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual.

293FT Cell Line

The human 293FT Cell Line (Catalog no. R700-07) facilitates optimal production of lentivirus (Naldini *et al.*, 1996) and is derived from the 293F cell line. The 293FT Cell Line stably expresses the SV40 large T antigen under the control of the human CMV promoter and must be maintained in medium containing Geneticin®. For more information about the 293FT Cell Line including instructions to culture and maintain the cells, refer to the 293FT Cell Line manual, which is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 26).

continued on next page

Guidelines to Produce a Lentiviral Stock, continued

Materials Needed

You will need the following reagents to produce a lentiviral stock from pLenti6/TR:

- pLenti6/TR plasmid (1 µg/µl stock solution)
- ViraPower™ Packaging Mix (Catalog no. K4975-00)
- 293FT cells cultured in complete growth medium (see the 293FT Cell Line manual for details; Catalog no. R700-07)
- Lipofectamine™ 2000 transfection reagent (Catalog no. 11668-027)
- Opti-MEM® I Reduced Serum Medium (recommended for optimal DNA-Lipofectamine™ 2000 complex formation; Catalog no. 31985-062)
- Appropriate growth medium
- Appropriate tissue culture dishes and supplies

For details about the appropriate growth medium to use for lentiviral production, refer to the ViraPower™ T-REx™ Lentiviral Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual.

Producing Lentiviral Stocks

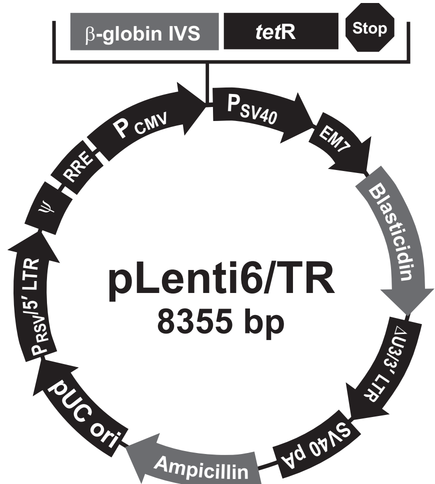
For detailed guidelines and instructions to produce a Lenti6/TR lentiviral stock, titer the lentivirus, and transduce mammalian cells, refer to the ViraPower™ T-REx™ Lentiviral Expression System or the BLOCK-iT™ Inducible H1 Lentiviral RNAi System manual.

Appendix

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 on our Web site (www.invitrogen.com) or by contacting Technical Service (see page 26).**



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

Map and Features of pLenti6/TR, continued

Features of the Vector

The pLenti6/TR (8355 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.*

continued on next page

Map and Features of pLenti6/TR, continued

Features of the Vector, continued

Feature	Benefit
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*.

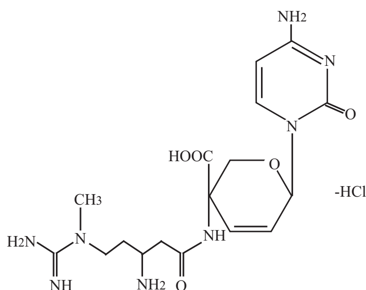
Blasticidin

Blasticidin

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.

continued on next page

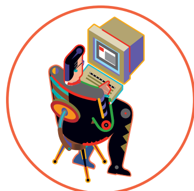
Preparing and Storing Stock Solutions

Blasticidin may be obtained separately from Invitrogen (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should be 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 store the thawed stock solution at +4°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at +4°C for up to 2 weeks.

Technical Service

World Wide Web



Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
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Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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Purchaser Notification

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continued on next page

Purchaser Notification, continued

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Product Qualification

pLenti6/TR

The structure of the pLenti6/TR vector is verified by restriction enzyme digestion.

Blasticidin

Blasticidin is lot-qualified by performing a kill curve on Blasticidin-sensitive and resistant mammalian cell lines. Blasticidin-sensitive cells should be killed at all concentrations tested (2.5-10 μg/ml) within 10 days after addition of Blasticidin.

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