invitrogen™

ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit

Gateway®-adapted lentiviral vectors for cloning and regulated high-level expression in mammalian cells

Cat. no. A11144

Revision date: 8 June 2010
Manual part no. A11226

MAN0001706

User Manual

Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Cat. no.
ViraPower™ HiPerform™ T-REx™ Gateway® Expression System	A11141
ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit	A11144

Kit Components

The following table shows the components associated with the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit. For a detailed description of the contents of each component, see next page. Upon receipt, store each component as detailed below.

Component	Shipping	Storage
ViraPower™ HiPerform™ T-REx™ Gateway® Vector Module: • Vectors • Tetracycline	Dry ice	 –20°C –20°C (**protected from light**)
pENTR™ Gus Positive Control	Blue ice	–20°C
One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C

Continued on next page

ViraPower™ HiPerform™ T-REx™ Gateway® Vector Module

The following reagents are included with the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit. Store the vectors at –20°C. Store the tetracycline at –20°C, protected from light.

Reagent	Composition	Amount
pLenti6.3/TO/V5-DEST	40 µL of vector at 150 ng/µL in TE Buffer, pH 8.0*	6 µg
pLenti3.3/TR	40 µL of vector at 0.5 µg/µL in TE Buffer, pH 8.0	20 µg
pLenti6.3/TO/V5-GW/*lac*Z	20 µL of vector at 0.5 µg/µL in TE Buffer, pH 8.0	10 µg
pFastBac™ Gus control plasmid	20 µL of vector at 50 ng/µL in TE Buffer, pH 8.0	1 µg
Tetracycline	10 mg/mL in water	1 mL

***TE Buffer, pH8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

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

The following reagents are included with the One Shot® Stbl3™ Chemically Competent *E. coli* kit. Transformation efficiency is ≥ 1×10^8 cfu/µg plasmid DNA. There are enough reagents for 20 reactions. Store 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	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

Note: This strain is *end*A1+

Introduction

Description of the System

Introduction

The ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit combines Invitrogen's ViraPower™ HiPerform™ and T-REx™ Lentiviral technologies with the Gateway® Cloning technology to facilitate the generation of lentiviral-based, regulated, high-level expression of a target gene in dividing or non-dividing mammalian cells. The kit includes two pLenti-based expression vectors, one to inducibly express the gene of interest and one to express the Tet repressor, and a control vector.

ViraPower™ HiPerform™ T-REx™ 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 pages 6–7.

The HiPerform™ Technology uses two genetic elements (WPRE and cPPT) that enhance viral titer and expression in certain cell types. The **WPRE** (Woodchuck Posttranscriptional Regulatory Element) from the woodchuck hepatitis virus, is placed directly downstream of the gene of interest, thereby increasing the nuclear export of the transcript and enhancing transgene expression (Mastroyiannopoulos *et al.*, 2005; Zufferey *et al.*, 1998). The **cPPT** (Polypurine Tract) from the HIV-1 integrase gene, increases the copy number of lentivirus integrating into the host genome (Park, 2001) and allows for a two-fold increase in viral titer. WPRE and cPPT together produce at least a four-fold increase in protein expression in most cell types, compared to other vectors that do not contain these elements.

The T-REx™ Technology facilitates tetracycline-regulated expression of a gene of interest in mammalian cells through the use of regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen & Berens, 1994; Hillen *et al.*, 1983). Tetracycline regulation in the T-REx™ 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).

The T-REx™ Technology uses an inducible expression construct containing the gene of interest under a hybrid promoter consisting of the human cytomegalovirus (CMV) promoter and two tetracycline operator 2 ($TetO_2$) sites and a regulatory expression construct that facilitates high-level, constitutive expression of the Tet repressor (TetR). When the inducible expression construct and the regulatory expression construct are present in the same mammalian cell, expression of the gene of interest is repressed in the absence of tetracycline and induced in its presence (Yao *et al.*, 1998).

Continued on next page

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 gene of interest into multiple vector systems. To express your gene of interest in mammalian cells using the Gateway® Technology, simply:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone. For more information on Gateway® entry vectors, refer to www.invitrogen.com or contact Technical Support (page 30).
2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway® destination vector (i.e., pLenti6.3/TO/V5-DEST).
3. Use your expression clone in the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System (see below).

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual. This manual is available from our website (www.invitrogen.com) or by contacting Technical Support (page 30).

Components of the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit

The ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit includes two pLenti-based expression vectors, one to inducibly express the gene of interest and one to express the Tet repressor. The kit also includes a control vector.

- The pLenti6.3/TO/V5-DEST destination vector that is adapted for use with the Gateway® Technology into which the gene of interest is cloned. The tetracycline-regulated, hybrid CMV/TO promoter controls the expression of the cloned gene. This destination vector also contains elements that allow packaging of the expression construct into virions, and the Blasticidin resistance marker for selection of stably transduced cell lines.
- The pLenti3.3/TR repressor plasmid that constitutively expresses high levels of the tetracycline (Tet) repressor under the control of a CMV promoter. This plasmid also contains elements that allow viral packaging and the Neomycin resistance marker for selection of stably transduced cell lines.
- The the pLenti6.3/TO/V5-GW/*lacZ* plasmid for use as a positive control for lentivirus production and expression. Once generated, the control lentiviral construct can be packaged and transduced into mammalian cell lines to express a C-terminal, V5 epitope-tagged, β-galactosidase fusion protein.

Continued on next page

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System

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

The System contains the optimized 293FT producer cell line to facilitate optimal virus production, the ViraPower™ Packaging Mix containing an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG to supply helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus, and the Lipofectamine™ 2000 reagent for high-efficiency transfection of 293FT producer cell line.

For more information about the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System, refer to the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System manual (part no. A11182), which is available for downloading at www.invitrogen.com or by contacting Technical Support (page 30).

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System is available separately from Invitrogen. See page 29 for ordering information.

Features of the pLenti6.3/TO/V5-DEST Vector

The pLenti6.3/TO/V5-DEST destination vector contains the following elements. For a map of the vector, see **Appendix**, page 22.

- 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)
- Polypurine Tract from HIV (cPPT) for increased viral titer (Park, 2001)
- Hybrid CMV/TO promoter consisting of the human CMV promoter and tetracycline operator 2 ($TetO_2$) sites for high-level, tetracycline-regulated expression of the gene of interest (see the next page for more information about the Tet operator sequences)
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV/TO promoter for recombinational cloning of the gene of interest from an entry clone
- 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
- C-terminal V5 epitope for detection of the recombinant protein of interest (Southern *et al.*, 1991)
- Woodchuck Posttranscriptional Regulatory Element (WPRE) for increased transgene expression (Zufferey *et al.*, 1998)
- Blasticidin (*bsd*) resistance gene for selecting stably transduced mammalian cell lines (Kimura *et al.*, 1994)
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

Promoter Driving Blasticidin

The pLenti6.3/TO/V5-DEST vector contains the SV40 promoter to drive mammalian expression of the Blasticidin selection marker. In some mammalian cell types, the activity of viral promoters such as SV40 may become significantly reduced over time due to promoter silencing from methylation (Curradi *et al.*, 2002) or histone deacetylation (Rietveld *et al.*, 2002).

Continued on next page

Control Vector

The ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit also include the pLenti6.3/TO/V5-GW/*lacZ* plasmid for use as a positive control for lentivirus production and expression. Once generated, the control lentiviral construct can be packaged and transduced into mammalian cell lines to express a C-terminal, V5 epitope-tagged β-galactosidase fusion protein. For more information about the vector, see the **Appendix**, page 27.

Features of the pLenti3.3/TR Vector

The pLenti3.3/TR vector contains the following elements. For a map of the vector, see **Appendix**, page 25.

- 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)
- Polypurine Tract from HIV (cPPT) for increased viral titer (Park, 2001)
- 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)
- Woodchuck Posttranscriptional Regulatory Element (WPRE) for increased transgene expression (Zufferey *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*

TetR Gene

The *TetR* gene in pLenti3.3/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.

Biosafety Features of the System

Introduction

The ViraPower™ HiPerform™ T-REx™ Gateway® Expression System is based on lentiviral vectors developed by Dull *et al.*, 1998 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 ViraPower™ HiPerform™ T-REx™ Gateway® Lentiviral System

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

Continued on next page

Biosafety Features of the System, Continued

Biosafety Level 2

Despite the inclusion of the safety features discussed on the previous page, the lentivirus produced with this System can still pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when creating lentivirus carrying potential harmful or toxic genes (e.g., activated oncogenes).

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition, published by the Centers for Disease Control (CDC). You can download this document from the following address:

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 ViraPower™ HiPerform™ T-REx™ Gateway® Expression System.

Methods

Generating an Entry Clone

Introduction

To recombine your DNA sequence of interest into pLenti6.3/TO/V5-DEST, first generate an entry clone containing the DNA sequence of interest. Many entry vectors are available from Invitrogen to facilitate generation of entry clones After you have selected an entry vector, refer to the manual for the specific entry vector you are using for instructions on constructing an entry clone. All entry vector manuals are available for downloading at www.invitrogen.com or by contacting Technical Support (page 30).

To express a human gene of interest in pLenti6.3/TO/V5-DEST, you may use an Ultimate™ Human Open Reading Frame (hORF) Clone available from Invitrogen. The Ultimate™ hORF Clones are fully sequenced clones provided in a Gateway® entry vector that is ready to use in a recombination reaction with pLenti6.3/TO/V5-DEST. For more information about the Ultimate™ hORF Clones available, refer to www.invitrogen.com/clones or contact Technical Support (page 30).

Note: Each Ultimate™ hORF Clone contains a stop codon. To express the C-terminal V5 tag, remove the stop codon from hORF using site directed mutagenesis.

Points to Consider Before Recombining into pLenti6.3/TO/V5-DEST

The pLenti6.3/TO/V5-DEST vector allows the fusion of your gene of interest to a C-terminal tag. Remember that your gene of interest in the entry clone must:

- Contain an ATG initiation codon in the context of a Kozak translation initiation sequence for proper initiation of translation in mammalian cells (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position 4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

  (G/A)NN<u>ATG</u>**G**

- Be in frame with the C-terminal tag after recombination
- **NOT** contain a stop codon if you want to express a fusion protein

Refer to page 10 for a diagram of the recombination region of the pLenti6.3/TO/V5-DEST vector.

Creating Expression Clones

Introduction

After you have generated an entry clone, perform the LR recombination reaction to transfer the gene of interest into the pLenti6.3/TO/V5-DEST vector to create your expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the sections entitled **Performing the LR Recombination Reaction** (page 11) and **Transforming One Shot® Stbl3™ Competent *E. coli*** (page 13) before beginning.

Experimental Outline

To generate an expression clone:

1. Perform an LR recombination reaction using the *att*L–containing entry clone and the *att*R–containing pLenti6.3/TO/V5-DEST Gateway® Vector.
   Note: The entry clone and the destination vector should be supercoiled (see **Important** below).
2. Use the reaction mixture to transform One Shot® Stbl3™ Competent *E. coli* (page 13).
3. Analyze expression clones using restriction enzyme analysis and sequencing.

Important

The pLenti6.3/TO/V5-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual has previously recommended using a linearized destination vector for more efficient LR recombination, further testing at Invitrogen has found that linearization of pLenti6.3/TO/V5-DEST is **not** required to obtain optimal results for any downstream application.

Propagating the Destination Vector

If you wish to propagate and maintain the pLenti6.3/TO/V5-DEST vector, we recommend using One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli*. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

- To maintain integrity of the vector, select for transformants in LB media containing 50–100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol.
- Due to the potential for re-arrangement of lentiviral vectors caused by recombination between the 5′ and 3′ LTRs (i.e., unwanted recombinants), we recommend analyzing 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.

One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli* are available separately from Invitrogen (see page 29 for ordering information).

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

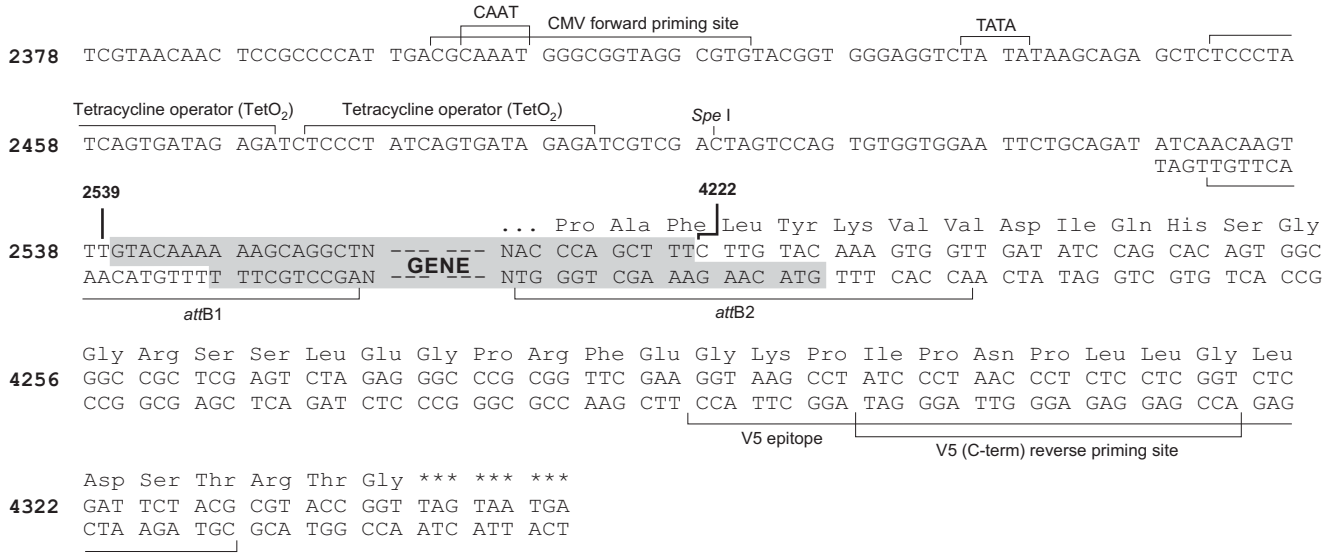
Continued on next page

Recombination Region of pLenti6.3/TO/V5-DEST

The Gateway® recombination region of the expression clone resulting from pLenti6.3/TO/V5-DEST × entry clone is shown below. For a map and features of the pLenti6.3/TO/V5-DEST vector, see the **Appendix**, pages 22–24.

Features of the Gateway® Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pLenti6.3/TO/V5-DEST vector by recombination.
- Non-shaded regions are derived from the pLenti6.3/TO/V5-DEST Gateway® vector.
- DNA from the entry clone replaces the region between bases 2,539 and 4,222 on the pLenti6.3/TO/V5-DEST vector. Bases 2,539 and 4,222 of the pLenti6.3/TO/V5-DEST sequence are marked below.



Performing the LR Recombination Reaction

Introduction

Follow the guidelines and instructions in this section to perform the LR recombination reaction using your entry clone containing the gene of interest and the pLenti6.3/TO/V5-DEST vector. We recommend including a negative control (**no** LR Clonase™ II) in your experiment to help you evaluate your results.

Recommended *E. coli* Host

For optimal results, we recommend using Stbl3™ *E. coli* for transformation as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing 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 13. Transformants containing unwanted recombinants are generally **not** obtained when Stbl3™ *E. coli* are used for transformation.

Important

Do not transform the LR recombination reaction into *E. coli* strains that contain the F′ episome (e.g., TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Gateway® LR Clonase™ II Plus Enzyme Mix

To catalyze the LR recombination reaction, you will use Gateway® LR Clonase™ II Plus Enzyme Mix, which is available separately from Invitrogen (page 29). The Gateway® LR Clonase™ II Plus Enzyme Mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in Gateway® LR Clonase™ II Plus Enzyme Mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol under **Setting up the LR Recombination Reaction** (next page) to perform the LR recombination reaction using Gateway® LR Clonase™ II Plus Enzyme Mix.

Positive Control for LR Reaction

The pENTR™ Gus plasmid is included with the Gateway® LR Clonase™ II Plus Enzyme Mix for use as a positive control for the LR recombination reaction. You may use this entry clone in your LR recombination reaction to verify the efficiency of the LR reaction. For a map of pENTR™ Gus, see the **Appendix**, page 28.

Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/µL in TE, pH 8.0)
- pLenti6.3/TO/V5-DEST vector (supplied with the kit)
- Gateway® LR Clonase™ II Plus Enzyme Mix (available separately, page 29; keep at –20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µL Proteinase K solution (supplied with the Gateway® LR Clonase™ II Plus Enzyme Mix; thaw and keep on ice until use)

Continued on next page

Setting up the LR Recombination Reaction

Follow the procedure below to perform the LR recombination reaction between your entry clone and the pLenti6.3/TO/V5-DEST vector. If you want to include a negative control, set up a separate reaction but omit the Gateway® LR Clonase™ II Plus Enzyme Mix.

1. Add the following components to 1.5 mL microcentrifuge tubes at room temperature and mix.

Component	Sample	Control
Entry clone (50–150 ng/reaction)	1–7 µL	–
pENTR™ Gus	–	1 µL
Destination vector (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	to 8 µL	6 µL

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

   Reminder: Return Gateway® LR Clonase™ II Plus 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*,** next page.

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

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* included with the kit. The transformation efficiency of One Shot® Stbl3™ Chemically Competent *E. coli* is ≥ 1×10^8 cfu/µg plasmid DNA.

Materials Needed

- LR recombination reaction (from Step 7, previous page)
- One Shot® Stbl3™ Chemically Competent *E. coli* (supplied with the kit, one vial per transformation; thaw on ice immediately before use)
- LB Medium pre–warmed to 37°C
  Note: You may use S.O.C. Medium provided with the kit in place of LB Medium for cell recovery
- pUC19 positive control (if desired to verify the transformation efficiency; supplied with the kit)
- 42°C water bath
- LB Amp 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

One Shot® Stbl3™ Transformation Procedure

1. Thaw on ice, one vial of One Shot® Stbl3™ chemically competent cells for each transformation.
2. Add 2–3 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 30 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. Transfer the cells gently to a sterile, 15-mL tube containing 1mL of pre-warmed LB Medium
7. Cap the tube(s) tightly and shake them horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 100 µL of the transformation mix on a pre-warmed LB–Amp plate and incubate overnight at 37°C. 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.
10. Proceed to **Analyzing Transformants**, next page.

Analyzing Transformants

Introduction

We recommend analyzing the transformants using restriction digestion and sequencing or PCR as described below. This allows you to confirm the presence of the insert as well as ensure the absence of any aberrant lentiviral vector recombination between the LTRs.

After verifying the correct clones, use the miniprep DNA to retransform Stbl3™ *E. coli*, and re-isolate plasmid DNA for transfection and lentivirus production. Plasmid DNA for transfection into 293FT cells must be very clean and free from contaminants and salts, and should be isolated by midiprep.

Note

Do **not** use PCR screening of clones in place of restriction analysis. For example, clones that contain both correct and aberrantly recombined DNA may look positive by PCR but may not be optimal for lentivirus production.

Analyzing Transformants

To analyze transformants:

1. Pick 10–20 ampicillin-resistant colonies from plating the transformation mix. Culture cells overnight.
2. Isolate plasmid DNA for each colony using a **miniprep** kit (e.g., PureLink™ HQ Mini Plasmid Purification Kit; see **Important**, below).
3. Analyze the plasmids by restriction analysis to confirm the presence and orientation of your insert as well as the integrity of the vector.
4. *Optional:* Sequence the plasmids or perform PCR to determine that your gene of interest is in frame with the C-terminal V5 epitope tag.
5. Re-transform One Shot® Stbl3™ Chemically Competent *E. coli* separately with the validated clones.
6. Inoculate LB–ampicillin with a fresh colony and grow to generate a starter culture.
7. Inoculate the starter culture into LB–ampicillin and grow.
8. Isolate plasmid DNA using a **midiprep** kit for lentivirus production (e.g., PureLink™ MidiPrep Kits; see **Important**, below).

Important

Stbl3™ *E. coli* is wild type for endonuclease 1 (*end*A1+). When performing plasmid DNA isolation with commercially available kits, ensure that Solution I of the Lysis buffer (often called Resuspension Buffer) contains 10 mM EDTA to inactivate the endonuclease to avoid DNA nicking and vector degradation. Alternatively, follow the instructions included in the plasmid purification kits for *end*A1+ *E. coli* strains.

We recommend using the PureLink™ HQ Mini Plasmid Purification Kit and preparing lentiviral plasmid DNA using PureLink™ MidiPrep Kits (page 29).

Continued on next page

Analyzing Transformants, Continued

Materials Needed

You will need the following materials:

- LB medium containing 100 µg/mL ampicillin
- PureLink™ HQ Mini Plasmid Purification Kit (page 29) or equivalent
- Appropriate restriction enzymes
- E-Gels® 1.2% agarose gels (page 29) or equivalent

Screening Colonies by Miniprep

For each transformation:

1. Pick 10–20 colonies from plates obtained after plating the transformation mix (Step 8, page 13). Culture colonies overnight in LB medium containing 100 µg/mL ampicillin.
2. Isolate plasmid DNA using PureLink™ HQ Mini Plasmid Purification Kit or equivalent (see **Important**, previous page).

   Note: The typical yield of pLenti DNA with PureLink™ HQ Mini Plasmid Purification Kit is ~5–7 µg, which is lower than the average DNA yield using this purification kit.
3. Perform restriction digestion on plasmid DNA, then analyze the digested DNA on 0.8% or 1.2% agarose gels to confirm the correct clones.

Restriction Digestion

To confirm that no rearrangement in the LTR regions of the plasmid has taken place, perform restriction digestion using a combination of *Afl* II and *Xho* I.

Afl II sites are present in both LTRs. The *Xho* I site is present in the plasmid backbone at the 3′ end of the insert. Assuming there are no *Afl* II or *Xho* I sites in the insert, 3 DNA fragments are generated from the *Afl* II + *Xho* I digest. Any unexpected DNA fragments are a result of LTR recombination.

If *Afl* II and/or *Xho* I sites are present in the insert, you can use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert. The complete restriction enzyme maps of vectors are available at www.invitrogen.com.

What You Should See

Depending on the restriction sites you are using, determine the number and size of bands you should obtain from your digestion. Agarose gel analysis should show the correct digestion pattern indicating proper recombination into the lentiviral vector. Additional or unexpected bands indicate aberrant recombination of the lentiviral vector.

Continued on next page

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a primer such as the V5 (C-term) reverse primer (see below for sequence) and a primer that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel since mispriming or contaminating template may produce artifacts.

Materials Needed:

AccuPrime™ *Pfx* SuperMix (page 29)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µL of AccuPrime™ *Pfx* SuperMix into a 0.5 mL microcentrifuge tube. Add 1 µL each of the forward and reverse PCR primer.
2. Pick 10–20 colonies that have been analyzed by restriction digest with *Afl* II and *Xho* I (see previous page) and resuspend them individually in 50 µL of SuperMix containing PCR primers.
   Note: Remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate the nucleases.
4. Amplify for 20–30 cycles.
5. For the final extension, incubate the reaction at 72°C for 10 minutes. Store the PCR products at 4°C.
6. Visualize PCR products by agarose gel electrophoresis.

Sequencing

You may sequence your expression construct to confirm that your gene is cloned in the correct orientation and in frame with the V5 epitope. We recommend using the following primers to help you sequence your expression construct. Refer to the diagram on page 10 for the location of the primer binding sites in pLenti6.3/TO/V5-DEST.

Note: For your convenience, Invitrogen has a custom primer synthesis service. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (page 30).

Primer	Sequence
CMV forward primer	5′–CGCAAATGGGCGGTAGGCGTG–3′
V5 (C–term) reverse primer	5′–ACCGAGGAGAGGGTTAGGGAT–3′

Continued on next page

Isolating Lentiviral Plasmid DNA

This protocol provides general steps for re-transforming Stbl3™ *E. coli* and isolating plasmid DNA for lentivirus production. pLenti plasmid DNA midipreps often have lower yields; therefore, use 100 mL of culture volume for each DNA midiprep.

1. Dilute 1 µL of miniprep plasmid DNA from a positive clone 1:500 in TE.
2. Use 1 µL of this diluted DNA to retransform into One Shot® Stbl3™ Chemically Competent Cells as described on page 13.
3. Plate approximately one-tenth of the transformation on LB plates containing 100 µg/mL ampicillin and incubate at 37°C overnight.
4. Pick 1 colony and culture in 2–3 mL LB medium containing 100 µg/mL ampicillin for 6–8 hours at 37°C to obtain a starter culture.
5. Inoculate the entire volume of the starter culture into LB medium containing 100 µg/mL ampicillin and culture at 37°C overnight.
   Note: Use 100 mL culture volume for large scale or midiprep isolation of DNA.
6. Isolate the plasmid DNA using the PureLink™ MidiPrep Kit (see **Important**, page 14).
7. Perform restriction analysis (see page 15) to confirm the presence of the insert.
8. Use the purified plasmid DNA from the positive clone for producing the lentivirus and to check protein expression (optional, see below).
   Note: A typical DNA yield is ~300–400 µg with an O.D. $_{260/280}$ ratio of between 1.8 and 2.1.

Maintaining the Expression Clone

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

Optional: Verifying Expression of Recombinant Protein

Before generating a lentiviral stock of your pLenti6.3/TO/V5-DEST expression construct, you may verify that the construct expresses the gene of interest by transfecting the plasmid directly into mammalian cells and assaying for your recombinant protein. To verify expression of your gene of interest:

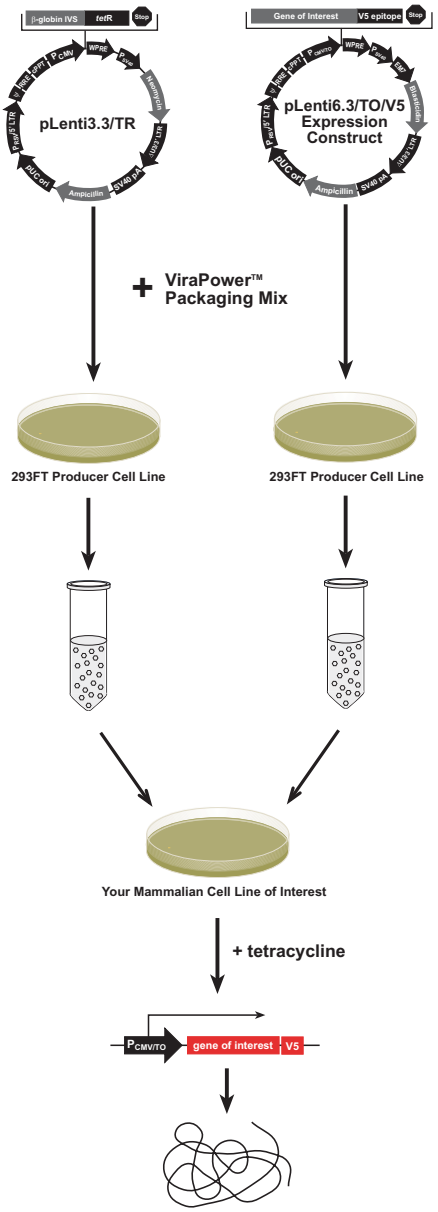
- Use an easy-to-transfect, dividing mammalian cell line (e.g., HEK 293 or COS-7).
- Use a transfection reagent that allows high-efficiency transfection. We recommend using Lipofectamine™ 2000 Reagent (available separately from Invitrogen, see page 29 for ordering information).
  Note: Lipofectamine™ 2000 is also supplied with the ViraPower™ HiPerform™ T-REx™ Gateway® Expression System.
- 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.

Expression and Analysis

Introduction

After you have obtained purified plasmid DNA of your pLenti6.3/TO/V5-DEST expression construct, you are ready to use Invitrogen's ViraPower™ HiPerform™ Lentiviral Expression System to produce a viral stock. Use the viral stock to transduce your mammalian cell line of choice to express your recombinant protein. The diagram below describes the general steps required to express your gene of interest using the ViraPower™ HiPerform™ Lentiviral Expression System.

Refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual (part no. A11182) for detailed instructions to perform the steps described below. TheViraPower™ HiPerform™ Lentiviral Expression System manual is available for downloading at www.invitrogen.com or by contacting Technical Support (page 30).



1. Generate the pLenti6.3/TO/V5 expression construct containing your gene of interest.

2. Cotransfect the 293FT producer cell line with pLenti3.3/TR or your pLenti6.3/TO/V5 expression construct and the optimized Packaging Mix.

3. Harvest viral supernatant and determine the titer.

4a. Add the Lenti3.3/TR viral supernatant to your mammalian cell line and use Neomycin selection to generate a ViraPower™ T-REx™ cell line. Add the Lenti6.3/TO/V5 viral supernatant to the ViraPower™ T-REx™ cells, and use Blasticidin to select for stably transduced cells, if desired.

OR

4b. Co-transduce your mammalian cell line with the Lenti3.3/TR and Lenti6.3/TO/V5 viral supernatants. Select for stably transduced cells, if desired.

5. Add tetracycline. Assay for recombinant protein of interest.

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 the solution on liquid cycle for 20 minutes. Allow the solution to cool to ~55°C and add antibiotic, if desired.
4. Store at 4°C.

LB Plates Containing Ampicillin

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

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave the solution on liquid cycle for 20 minutes.
3. After autoclaving, cool the solution to ~55°C, add ampicillin to a final concentration of 100 µg/mL, and pour into 10 cm plates.
4. Let the plates harden, then invert, and store at 4°C in the dark.

Blasticidin

Description

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

Handling Blasticidin

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

Preparing and Storing Stock Solutions

- Blasticidin 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.
- You may store the medium containing Blasticidin at 4°C for up to 2 weeks.

Determining Blasticidin Sensitivity

Since you will be selecting for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (i.e., perform a kill curve experiment). Typically, concentrations ranging from 2–10 µg/mL Blasticidin are sufficient to kill most untransduced mammalian cell lines. We recommend that you test a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate the cells at approximately 25% confluence. Prepare a set of 7 plates. Allow the cells to adhere overnight.
2. The next day, substitute the culture medium with medium containing varying concentrations of 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 Blasticidin that kills the cells within 10–14 days after addition of antibiotic.

Geneticin®

Geneticin® (G-418)

The pLenti3.3/TR vector contains the neomycin resistance gene which confers resistance to the antibiotic Geneticin® (also known as G-418 sulfate). Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial gene (APH), derived from Tn*5*, results in detoxification of Geneticin® (Southern & Berg, 1982).

Note: Geneticin® is also available separately from Invitrogen (see page 29 for ordering information).

Geneticin® is harmful. It may cause sensitization by skin contact, and is irritating to eyes and skin. In case of contact with eyes, rinse eyes immediately with plenty of water, and seek medical advice. Avoid contact with skin and eyes. Wear suitable protective clothing and gloves when handling Geneticin® and Geneticin®-containing solutions.

Preparing and Storing Geneticin®

Follow the instructions provided with Geneticin® to prepare your working stock solution. Geneticin® in powder form should be stored at room temperature and at 4°C as a solution. The stability of Geneticin® is guaranteed for two years, if stored properly.

Determining Geneticin® Sensitivity

The amount of Geneticin® required to be present in culture media to select for resistant cells varies with a number of factors including cell type. Although the development work with this kit utilized 293FT cells and 500 µg/mL Geneticin®, we recommend that you re-evaluate the optimal concentration whenever experimental conditions are altered (including use of Geneticin® from a different lot). Note that Geneticin® in powder form has only 75% of the potency of Geneticin® available in liquid form.

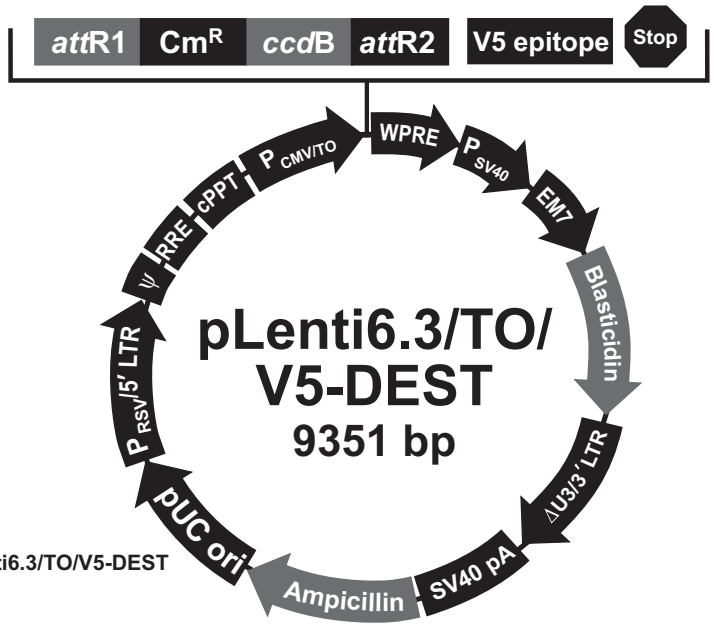
1. Plate or split a confluent plate so that the cells are approximately 25% confluent. Prepare a set of 7 plates. Allow the cells to adhere overnight.
2. The next day, substitute the culture medium with medium containing varying concentrations of Geneticin® (0, 50, 100, 250, 500, 750, and 1000 µg/mL Geneticin®).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Note the percentage of surviving cells at regular intervals to determine the appropriate concentration of Geneticin® that kills the cells within 1–2 weeks after addition of Geneticin®.

Note: Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take up to two weeks of growth in selective medium.

Map and Features of pLenti6.3/TO/V5-DEST

pLenti6.3/TO/V5-DEST Map

The map below shows the elements of pLenti6.3/TO/V5-DEST. DNA from the entry clone replaces the region between bases 2,539 and 4,222. **The complete sequence for pLenti6.3/TO/V5-DEST is available at www.invitrogen.com or by contacting Technical Support (see page 30).**



Comments for pLenti6.3/TO/V5-DEST
9351 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
cPPT: bases 1801-1923
CMV/TO promoter: bases 1937-2491
- CMV promoter: bases 1937-2436
- TATA box: bases 2436-2442
- Tetracycline operator (2X $TetO_2$) sequences: bases 2452-2491

*att*R1 site: bases 2532-2656
Chloramphenicol resistance gene (Cm^R): bases 2765-3424
*ccd*B gene: bases 3766-4071
*att*R2 site: bases 4112-4236
V5 epitope: bases 4289-4330
WPRE: bases 4349-4946
SV40 promoter: bases 4957-5265
EM7 promoter: bases 5320-5386
Blasticidin resistance gene: bases 5387-5785
ΔU3/3′ LTR: bases 5871-6105
- ΔU3: bases 5871-5924
- 3′ LTR: bases 5925-6105

SV40 polyadenylation signal: bases 6177-6308
bla promoter: bases 7167-7265
Ampicillin (*bla*) resistance gene: bases 7266-8126
pUC origin: bases 8271-8944

Continued on next page

Map and Features of pLenti6.3/TO/V5-DEST, Continued

Features of pLenti6.3/TO/V5-DEST

pLenti6.3/TO/V5-DEST (9,351 bp) contains the following elements. 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).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001).
CMV/TO promoter	Hybrid promoter consisting of the human cytomegalovirus promoter (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987) and two tandem tetracycline operator (O_2) sequences for high-level, inducible expression of the gene of interest. The tetracycline operator sequences serve as binding sites for Tet repressor homodimers (Hillen & Berens, 1994).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
V5 epitope	Allows detection of the recombinant fusion protein using the Anti-V5 Antibodies (Southern *et al.*, 1991).
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey *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).

Continued on next page

Map and Features of pLenti6.3/TO/V5-DEST, Continued

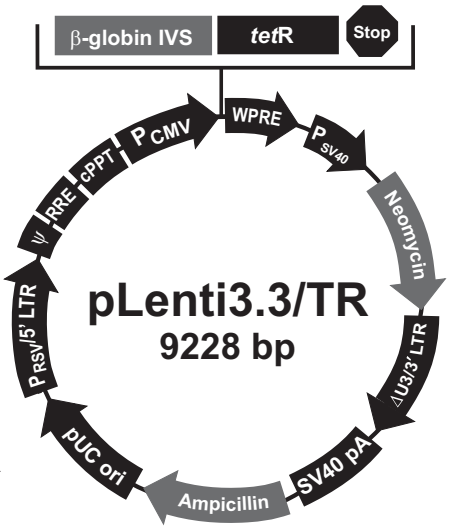
Features of pLenti6.3/TO/V5-DEST, continued

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 and Features of pLenti3.3/TR

pLenti3.3/TR Map

The map below shows the elements of pLenti3.3/TR. **The complete sequence for pLenti3.3/TR is available at www.invitrogen.com or by contacting Technical Support (see page 30).**



Comments for pLenti3.3/TR
9228 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
cPPT: bases 1801-1923
CMV promoter: bases 1935-2519
Rabbit β-globin intron II (IVS): bases 2567-3139
*tet*R gene: bases 3223-3870
WPRE: bases 3892-4489
SV40 promoter: bases 4500-4808
Neomycin resistance gene: bases 4883-5677
ΔU3/3' LTR: bases 5748-5982
ΔU3: bases 5748-5801
3′ LTR: bases 5802-5982
SV40 polyadenylation signal: bases 6054-6185
bla promoter: bases 7044-7142
Ampicillin (*bla*) resistance gene: bases 7143-8003
pUC origin: bases 8148-8821

TetR Gene

The *TetR* gene in pLenti3.3/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 pLenti3.3/TR, Continued

Features of pLenti3.3/TR

pLenti3.3/TR (9,228 bp) contains the following elements. Features have been functionally tested.

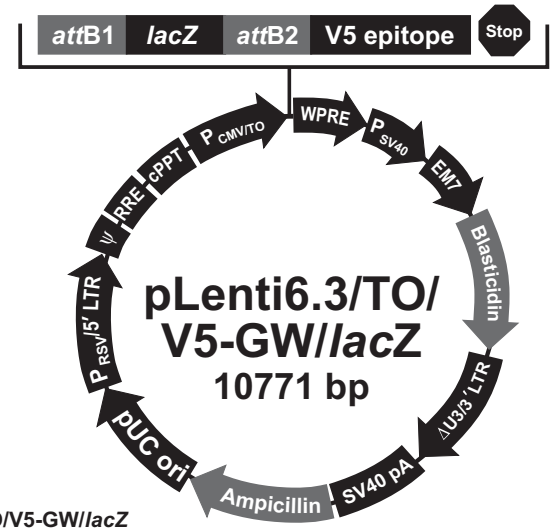
Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Allows viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector 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).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001)
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).
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey *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.*
Neomycin resistance gene	Allows selection of stably transduced mammalian cell lines.
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 pLenti6.3/TO/V5-GW/*lacZ*

pLenti6.3/TO/V5-GW/*lacZ* Map

pLenti6.3/TO/V5-GW/*lacZ* is a 10,771 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and pLenti6.3/TO/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

The map below shows the elements of pLenti6.3/TO/V5-GW/*lacZ*. **The complete sequence for pLenti6.3/TO/V5-GW/*lacZ* is available at www.invitrogen.com or by contacting Technical Support (see page 30).**



Comments for pLenti6.3/TO/V5-GW/*lacZ*
10771 nucleotides

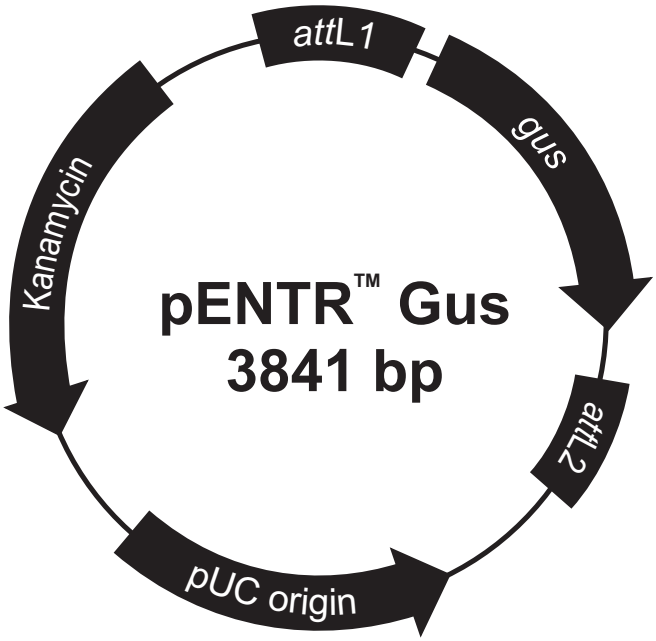
RSV enhancer/promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
cPPT: bases 1801-1923
CMV/TO promoter: bases 1937-2442
  CMV promoter: bases 1937-2435
  TATA box: bases 2436-2442
Tetracycline operator (2X $TetO_2$) sequences: bases 2452-2491
*att*B1 site: bases 2533-2553
lacZ ORF: bases 2559-5630
*att*B2 site: bases 5644-5656
V5 epitope: bases 5709-5750
WPRE: bases 5769-6366
SV40 early promoter and origin: bases 6377-6685
EM7 promoter: bases 6740-6806
Blasticidin resistance gene: bases 6807-7205
ΔU3/HIV-1 3′ LTR: bases 7291-7525
  ΔU3: bases 7291-7344
  Truncated HIV-1 3′ LTR: bases 7345-7525
SV40 polyadenylation signal: bases 7597-7728
bla promoter: bases 8587-8685
Ampicillin (*bla*) resistance gene: bases 8686-9546
pUC origin: bases 9691-10364

Map of pENTR™ Gus

pENTR™ Gus Map

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 for downloading from our website at www.invitrogen.com or by contacting Technical Support (see page 30).

The map below shows the elements of pENTR™ Gus. **The complete sequence for pENTR™ Gus is available at www.invitrogen.com or by contacting Technical Support (see page 30).**



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

Accessory Products

Additional Products

The products listed below may be used with the ViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit. For more information, visit our website at www.invitrogen.com or contact Technical Support (page 30).

Item	Quantity	Cat. no.
ViraPower™ HiPerform™ T-REx™ Gateway® Expression System	1 kit	A11141
ViraPower™ Lentiviral Support Kit	20 reactions	K4970-00
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975-00
293FT Cells	3×10^6 cells	R700-07
PureLink™ HiPure Plasmid Midiprep Kit	25 reactions 50 reactions	K2100-04 K2100-05
PureLink™ Quick96 Plasmid Kit	4 × 96 preps 24 × 96 preps	K2110-04 K2110-24
PureLink™ HQ Plasmid Miniprep Kit	100 reactions	K2100-01
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
Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector	20 µg	V370-06
Lipofectamine™ 2000 Reagent	0.75 mL 1.5 mL	11668-027 11668-019
Ampicillin	5 g	Q100-16
Blasticidin S HCl	50 mg	R210-01
Geneticin®, liquid	20 mL 100 mL	10131-035 10131-027
LR Clonase™ II Plus Enzyme Mix	20 reactions	12538-120
AccuPrime™ *Pfx* SuperMix	200 reactions	12344-040
E-Gel® 1.2% Starter Pak (6 gels + Powerbase™)	1 kit	G6000-01
E-Gel® 1.2% 18 Pak	18 gels	G5018-01
β-Gal Antiserum, rabbit IgG fraction	500 µL	A11132
β-Gal Assay Kit	1 kit	K1455-01
β-Gal Staining Kit	1 kit	K1465-01

Technical Support

Web Resources

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

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

Contact Us

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

Corporate Headquarters:
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: techsupport@invitrogen.com

Japanese Headquarters:
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: 44 (0) 141 814 6100
Tech Fax: 44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

MSDS

MSDSs (Material Safety Data Sheets) are available on our website at www.invitrogen.com/msds.

Certificate of Analysis

Product qualification is described in the Certificate of Analysis (CofA), available on our website by product lot number at www.invitrogen.com/cofa.

Limited Warranty

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

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

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

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

Purchaser Notification

Introduction

Use of theViraPower™ HiPerform™ T-REx™ Gateway® Vector Kit is covered under a number of different licenses including those detailed below.

Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For products that are subject to multiple limited use label licenses, the terms of the most restrictive limited use label license shall control. Life Technologies Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Life Technologies Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information about purchasing a license to use this product or the technology embedded in it for any use other than for research use please contact Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008 or outlicensing@lifetech.com

Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 34.

Continued on next page

Purchaser Notification, Continued

Limited Use Label License No. 51: Blasticidin and the Blasticidin Selection Marker

Blasticidin and the blasticidin resistance gene (*bsd*) are the subject of U.S. Patent No.5,527,701 sold under patent license for research purposes only. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. email: outlicensing@invitrogen.com

Limited Use Label License No. 108: Lentiviral Technology

The Lentiviral Technology (based upon the lentikat™ system) is licensed from Cell Genesys, Inc., under U.S. Patent Nos. 5,686,279; 5,834,256; 5,858,740; 5,994,136; 6,013,516; 6,051,427; 6,165,782 and 6,218,187 and corresponding patents and applications in other countries for internal research purposes only. Use of this technology for gene therapy applications or bioprocessing other than for non-human research use requires a license from Cell Genesys (Cell Genesys, Inc. 342 Lakeside Drive, Foster City, California 94404). The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity), including non-gene therapy research and target validation applications in laboratory animals.

Limited Use Label License No. 109: Retroviral Helper Lines

Retroviral helper cell lines are licensed from Wisconsin Alumni Research Foundation, under U.S. Patents and corresponding patents and applications in other countries for internal research purposes only. Use of these cell lines for Commercial Purposes requires a license from Life Technologies.

Limited Use Label License No. 308: WPRE Element in Lentiviral Vectors

This product contains the Woodchuck Post-transcriptional Regulatory Element ("WPRE") which is the subject of intellectual property owned by The Salk Institute for Biological Studies, and licensed to Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; and/or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. In addition, any use of WPRE outside of this product or the product's authorized use requires a separate license from the Salk Institute. Invitrogen will not assert a claim against the buyer of infringement of patents owned by Invitrogen and claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product or for a Commercial Purpose. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. email: outlicensing@invitrogen.com., or The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, Attn.: Office of Technology Management, Phone: (858) 453-4100 extension 1275, Fax: (858) 546-8093.

continued on next page

Purchaser Notification, Continued

Limited Use Label License No. 309: cPPT (DNA flap) Element	This product and its use are the subject of one or more of patents: US 6,682,907 and/or other pending U.S. and foreign patent applications licensed to Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation and Institut Pasteur will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to the DNA-Flap technology for purposes other than research, contact the Transfer of Technology Office, Institut Pasteur, 28 rue du Docteur Roux, 75 724 Paris Cedex 15 (www.pasteur.fr)
Limited Use Label License No. 317: LentiVector® Technology	This product is licensed under U.S. Pat. Nos. 5,817,491; 5,591,624; 5,716,832; 6,312,682; 6,669,936; 6,235,522; 6,924,123 and foreign equivalents from Oxford BioMedica (UK) Ltd., Oxford, UK, and is provided for use in academic and commercial in vitro and in vivo research for elucidating gene function, and for validating potential gene products and pathways for drug discovery and development, but excludes any use of LentiVector® technology for: creating transgenic birds for the purpose of producing useful or valuable proteins in the eggs of such transgenic birds, the delivery of gene therapies, and for commercial production of therapeutic, diagnostic or other commercial products not intended for research use where such products do not consist of or incorporate a lentiviral vector. Information about licenses for commercial uses excluded under this license is available from Oxford BioMedica (UK), Ltd., Medawar Centre, Oxford Science Park, Oxford OX4 4GA UK enquiries@oxfordbiomedica.co.uk or BioMedica Inc., 11622 EI Camino Real #100, San Diego CA 92130-2049 USA. LentiVector is a registered US and European Community trademark of Oxford BioMedica plc.
Limited Use Label License No. 345: Gateway® Vectors	This product or one or more vectors made using this product is the subject of U.S. Patent No. 5,888,732 owned by Life Technologies Corporation.

Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989) Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530

Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J.-K. (1993) Vesicular Stomatitis Virus G Glycoprotein Pseudotyped Retroviral Vectors: Concentration to a Very High Titer and Efficient Gene Transfer into Mammalian and Nonmammalian Cells. Proc. Natl. Acad. Sci. USA *90*, 8033-8037

Curradi, M., Izzo, A., Badaracco, G., and Landsberger, N. (2002) Molecular Mechanisms of Gene Silencing Mediated by DNA Methylation. Mol. Cell. Biol. *22*, 3157-3173

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. *72*, 8463-8471

Emi, N., Friedmann, T., and Yee, J.-K. (1991) Pseudotype Formation of Murine Leukemia Virus with the G Protein of Vesicular Stomatitis Virus. J. Virol. *65*, 1202-1207

Hillen, W., and Berens, C. (1994) Mechanisms Underlying Expression of Tn*10* Encoded Tetracycline Resistance. Annu. Rev. Microbiol. *48*, 345-369

Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K., and Meier, I. (1983) Control of Expression of the Tn*10*-encoded Tetracycline Resistance Genes: Equilibrium and Kinetic Investigations of the Regulatory Reactions. J. Mol. Biol. *169*, 707-721

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991) Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233

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

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994) Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA *1219*, 653-659

Kjems, J., Brown, M., Chang, D. D., and Sharp, P. A. (1991) Structural Analysis of the Interaction Between the Human Immunodeficiency Virus Rev Protein and the Rev Response Element. Proc. Natl. Acad. Sci. USA *88*, 683-687

Kozak, M. (1987) An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148

Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305

Kozak, M. (1991) An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903

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

Luciw, P. A. (1996) in *Fields Virology* (Fields, B. N., Knipe, D. M., Howley, P. M., Chanock, R. M., Melnick, J. L., Monath, T. P., Roizman, B., and Straus, S. E., eds), 3rd Ed., pp. 1881-1975, Lippincott-Raven Publishers, Philadelphia, PA

Continued on next page

Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V., and Cullen, B. R. (1989) The HIV-1 Rev Trans-activator Acts Through a Structured Target Sequence to Activate Nuclear Export of Unspliced Viral mRNA. Nature *338*, 254-257

Mastroyiannopoulos, N. P., Feldman, M. L., Uney, J. B., Mahadevan, M. S., and Phylactou, L. A. (2005) Woodchuck post-transcriptional element induces nuclear export of myotonic dystrophy 3' untranslated region transcripts. EMBO Rep *6*, 458-463

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987) Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129

Park, F., and Kay, MA. (2001) Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression *in vitro* and *in vivo*. Mol Ther. 4(3). 164-173

Postle, K., Nguyen, T. T., and Bertrand, K. P. (1984) Nucleotide Sequence of the Repressor Gene of the Tn*10* Tetracycline Resistance Determinant. Nuc. Acids Res. *12*, 4849-4863

Rietveld, L. E., Caldenhoven, E., and Stunnenberg, H. G. (2002) In vivo Repression of an Erythroid-Specific Gene by Distinct Corepressor Complexes. EMBO J. *21*, 1389-1397

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991) Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557

Southern, P. J., and Berg, P. (1982) Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene Under Control of the SV40 Early Region Promoter. J. Molec. Appl. Gen. *1*, 327-339

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958) Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5

van Ooyen, A., van den Berg, J., Mantei, N., and Weissmann, C. (1979) Comparison of Total Sequence of a Cloned Rabbit Beta-globin gene and its Flanking Regions With a Homologous Mouse Sequence. Science *206*, 337-344

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965) Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem (Tokyo) *57*, 667-677

Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C., and Eriksson, E. (1998) Tetracycline Repressor, tetR, Rather than the tetR-Mammalian Cell Transcription Factor Fusion Derivatives, Regulates Inducible Gene Expression in Mammalian Cells. Hum. Gene Ther. *9*, 1939-1950

Yee, J.-K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C., and Friedmann, T. (1994) A General Method for the Generation of High-Titer, Pantropic Retroviral Vectors: Highly Efficient Infection of Primary Hepatocytes. Proc. Natl. Acad. Sci. USA *91*, 9564-9568

Yee, J. K., Moores, J. C., Jolly, D. J., Wolff, J. A., Respess, J. G., and Friedmann, T. (1987) Gene Expression from Transcriptionally Disabled Retroviral Vectors. Proc. Natl. Acad. Sci. USA *84*, 5197-5201

Yu, S. F., Ruden, T. v., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., Anderson, W. F., Wagner, E. F., and Gilboa, E. (1986) Self-Inactivating Retroviral Vectors Designed for Transfer of Whole Genes into Mammalian Cells. Proc. Natl. Acad. Sci. USA *83*, 3194-3198

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998) Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. J. Virol. 72. 9873-9880

.

invitrogen™

Corporate Headquarters
Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech_support@invitrogen.com

For country-specific contact information, visit our web site at **www.invitrogen.com**