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ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit

Gateway®-adapted promoterless lentiviral vectors for cloning and high-level expression in dividing and non-dividing mammalian cells

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User Manual

Contents

Kit Contents and Storage

System Components

The ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit includes pLenti6.4/R4R2/V5-DEST Kit, pENTR™5′-TOPO® TA Cloning® Kit, One Shot® Stbl3™ Chemically Competent *E. coli*, One Shot® TOP10 Chemically Competent *E. coli*, and pENTR Gus positive control plasmid.

For a detailed description of the contents of each component, see pages v–vi. For a detailed description of the contents of the pENTR™5′-TOPO® TA Cloning® Kit, see the pENTR™5′-TOPO® TA Cloning® Kit manual (part no. 25-0744).

Shipping/Storage

The ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit components are shipped as described below. Upon receipt, store each item as detailed below.

Component	Shipping	Storage
pLenti6.4/R4R2/V5-DEST Kit	Dry ice	–20°C
pENTR™5′-TOPO® TA Cloning® Kit	Dry ice	pENTR™5′-TOPO® Reagents: –20°C One Shot® TOP10 Chemically Competent *E. coli*: –80°C
One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C
One Shot® TOP10 Chemically Competent *E. coli*	Dry ice	–80°C
pENTR™ Gus Positive Control	Blue ice	–20°C

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pLenti6.4/R4R2/V5-DEST Kit

The following vectors are included in the pLenti6.4/R4R2/V5-DEST Kit. **Store at –20°C.**

Vector	Composition	Amount
pLenti6.4/R4R2/V5-DEST	40 µL vector at 150 ng/µL in TE Buffer, pH 8.0*	6 µg
pLenti6.4/CMV/V5-MSGW/*lacZ*	20 µL vector at 0.5 µg/µL in TE Buffer, pH 8.0	10 µg
pENTR™5′/EF1αP	20 µL vector at 0.5 µg/µL in TE Buffer, pH 8.0	10 µg
pENTR™ 5′/CMVp	20 µL vector at 0.5 µg/µL in TE Buffer, pH 8.0	10 µg

***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* cells. Transformation efficiency is ≥ 1×10^8 cfu/µg plasmid DNA. **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 8.0	50 µL

Genotype of Stbl3™ *E. coli*

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+.

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pENTR™5′-TOPO® TA Cloning® Kit

The ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit includes the pENTR™5′-TOPO® TA Cloning® Kit to facilitate production of an *att*L4 and *att*R1-flanked entry clone containing your eukaryotic promoter of interest. The pENTR™5′-TOPO® TA Cloning Kit contains:

- pENTR™5′-TOPO® Reagents
- One Shot® TOP10 Chemically Competent *E. coli*

Refer to the pENTR™5′-TOPO® TA Cloning® Kit manual (part no. 25-0744) for a detailed description of the reagents provided with the kit and instructions to produce the entry construct. This manual is also available for downloading at www.invitrogen.com, or by contacting Technical Support (page 38).

One Shot® TOP10 Chemically Competent *E. coli*

The following reagents are included with the One Shot® TOP10 Chemically Competent *E. coli* cells. Transformation efficiency is ≥ 1×10^9 cfu/µg plasmid DNA. **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
TOP10 Cells	--	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL

Genotype of TOP10 *E. coli*

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ$^-$

Introduction

Description of the System

System Overview

The ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit combines Invitrogen's ViraPower™ HiPerform™ Lentiviral and MultiSite Gateway® technologies to facilitate the cloning of gene of interest under the control of a promoter of choice (CMV, EF-1α, or other) for high-level, lentiviral-based expression in dividing or non-dividing mammalian cells. The Kit includes:

- The pENTR™5′-TOPO® TA Cloning Kit containing the pENTR™5′-TOPO® vector for production of an entry clone containing the promoter of interest. The vector is TOPO®- and MultiSite Gateway®-adapted. These adaptations allow TOPO® Cloning of a *Taq* polymerase-amplified PCR product encoding the promoter of interest and easy transfer of the promoter sequence into the pLenti6.4/R4R2/V5-DEST vector. For detailed information about the pENTR™5′-TOPO® vector and instructions for generating an entry clone, refer to the pENTR™5′-TOPO® TA Cloning® Kit manual.

  Important: To generate the pLenti6.4/R4R2/V5-DEST expression construct, you also need to generate an entry clone containing your gene of interest. In this instance, you may use any standard Gateway® entry vector except pENTR™5′-TOPO®.

- The pLenti6.4/R4R2/V5-DEST expression vector into which the promoter and gene of interest will be simultaneously cloned using the MultiSite Gateway® Technology. The pLenti6.4/R4R2/V5-DEST vector also contains the elements required for packaging of the expression construct into virions (5′ and 3′ LTRs, ψ packaging signal) and the Blasticidin resistance marker to allow generation of stable cell lines. For more information about the pLenti6.4/R4R2/V5-DEST vector, see pages 30–31.

For more information about the ViraPower™ HiPerform™ Lentiviral and the MultiSite Gateway® Technologies, see pages 2–4.

Advantages of the System

- Allows production of a lentiviral construct that facilitates expression of a gene of interest under the control of a promoter of choice.
- Generates replication-incompetent lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential applications beyond those of traditional retroviral systems (Naldini, 1998).
- Efficiently delivers the gene of interest to mammalian cells in culture or *in vivo* (Dull *et al.*, 1998).
- Provides stable, long-term, high-level expression of a target gene beyond that offered by adenoviral-based systems (Dull *et al.*, 1998; Naldini *et al.*, 1996) (see **HiPerform™ Technology**, page 2).
- Produces a pseudotyped virus with a broad host range (Yee *et al.*, 1994) (see **VSV Envelope Glycoprotein**, below).
- Includes a MultiSite Gateway®-adapted expression vector for easy, simultaneous, recombination-based cloning of multiple DNA fragments in a defined order and orientation (see **MultiSite Gateway® Technology**, page 4).
- Includes multiple features designed to enhance the biosafety of the system.

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Description of the System, Continued

ViraPower™ Lentiviral Technology

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.

How Lentivirus Works

After the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). Once the lentiviral construct integrates into the genome, you may assay for transient expression of your recombinant protein or use antibiotic selection to generate a stable cell line for long-term expression studies.

VSV Envelope Glycoprotein

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

HiPerform™ Technology

The promoterless pLenti6.4/R4R2/V5-DEST lentiviral expression vector, included in the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit, contains 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. It increases the nuclear export of the transcript and enhances 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.

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Features of the pLenti6.4/R4R2/V5-DEST Vector

- 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)
- Option for using the human CMV promoter for high-level, constitutive expression of your gene of interest, the non-viral human EF-1α promoter for lower but more ubiquitous expression in primary cells and *in vivo* with lower risk of promoter shut down, or your own promoter of interest cloned into the pENTR™5′/TOPO® vector
- Two recombination sites, *att*R2 and *att*R4 for recombinational cloning of the gene of interest from an entry clone using MultiSite Gateway® Technology
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- The *ccd*B gene located between the *att*R sites for negative selection
- C-terminal V5 epitope for detecting 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*

The MultiSite Gateway® Technology

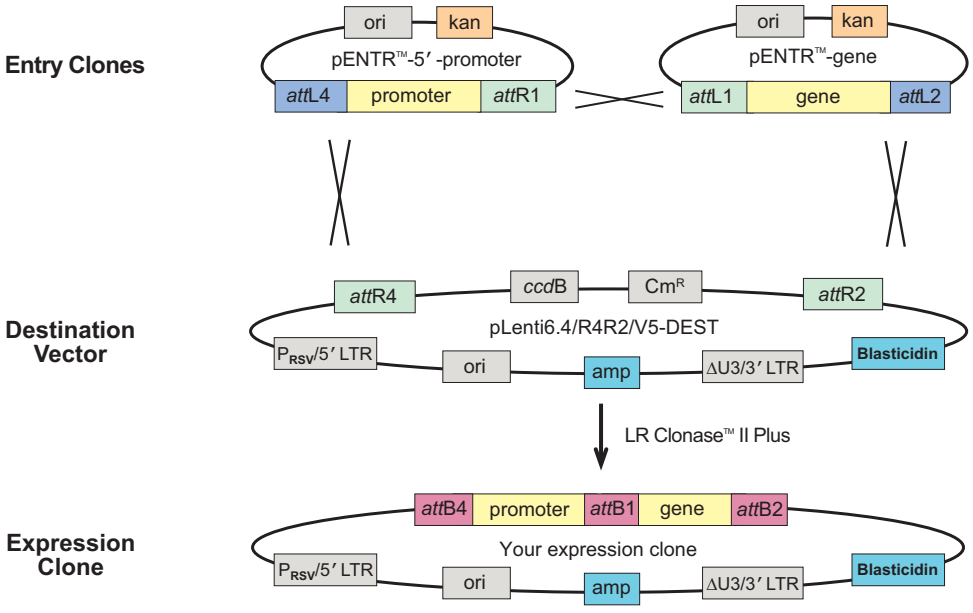
MultiSite Gateway® Technology

The Gateway® Technology is a universal cloning system that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989). It provides a rapid and highly efficient way for moving a single DNA sequence of interest into multiple vector systems. The MultiSite Gateway® Technology uses modifications of the Gateway® Technology to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation, and facilitates the creation of an expression construct that expresses a gene of interest from a promoter of choice.

In the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit, the MultiSite Gateway® Technology facilitates recombinational cloning of two DNA fragments encoding a promoter and gene of choice into the pLenti6.4/R4R2/V5-DEST lentiviral destination vector. To generate your lentiviral expression clone:

1. TOPO® Clone the promoter of choice into the pENTR™5′-TOPO® vector containing *att*L4 and *att*R1 recombination sites to create a pENTR™5′-promoter entry clone. The pENTR™5′-TOPO® vector and manual are included in this kit.
2. Clone the gene of interest into any standard Gateway® entry vector containing *att*L1 and *att*L2 recombination sites to create a pENTR™-gene entry clone.
3. Use the two entry clones in a single MultiSite Gateway® LR recombination reaction with the pLenti6.4/R4R2/V5-DEST vector containing *att*R4 and *att*R2 recombination sites to create your expression clone of interest.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual (part no. 25-0749). This manual is available for downloading at www.invitrogen.com or by contacting Technical Support (see page 38).



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The MultiSite Gateway® Technology, Continued

att Sites

In the Gateway® Technology, recombinational cloning is mediated via optimized *att* sites. To accommodate simultaneous recombinational cloning of multiple DNA fragments in the MultiSite Gateway® Technology, these *att* sites have been further modified and optimized. Modifications include alterations to both the sequence and length of the *att* sites, resulting in the creation of "new" *att* sites exhibiting enhanced specificities and the improved efficiency required to permit cloning of multiple DNA fragments in a single reaction. In the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit, the entry and destination vectors contain the following *att* sites (see the figure on the previous page):

- pENTR™ 5′-TOPO® containing your promoter of interest: *att*L4 and *att*R1
- Entry vector containing your gene of interest: *att*L1 and *att*L2
- pLenti6.4/R4R2/V5-DEST lentiviral destination vector: *att*R4 and *att*R2

To facilitate proper generation of a lentiviral expression construct, only this combination of entry clones and destination vector may be used in the MultiSite Gateway® LR recombination reaction.

Important

Note that the *att* sites used in MultiSite Gateway®-adapted vectors have been optimized to improve specificity and efficiency of the MultiSite Gateway® LR recombination reaction, and may vary in size and sequence from those used in the Gateway® Technology.

LR Clonase™ II Plus Enzyme Mix

The MultiSite Gateway® LR recombination reaction is catalyzed by LR Clonase™ II Plus enzyme mix (available separately from Invitrogen or as part of the ViraPower™ HiPerform™ Promoterless Gateway® Expression System, see page 38). LR Clonase™ II Plus enzyme mix facilitates efficient recombinational cloning of multiple DNA fragments, but is also suitable for use in standard Gateway® LR reactions. Note, however, that standard LR Clonase™ enzyme mix **is not suitable** for use in the MultiSite Gateway® LR recombination reaction.

Note

Recombination between *att*R and *att*L sites generates *att*B sites (see figure on the previous page) in the lentiviral expression vector. We have shown that the presence of *att*B sites within the expression cassette does not affect gene expression.

Biosafety Features of the System

Introduction

The lentiviral vectors supplied in the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit 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 described below.

Biosafety Features of the ViraPower™ Promoterless Lentiviral System

The ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit includes the following key safety features:

- The pLenti6.4/R4R2/V5-DEST 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 (i.e., three packaging plasmids and pLenti6.4/R4R2/V5-DEST). 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.
- A constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the pLenti6.4/R4R2/V5-DEST vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

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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 safety officer(s) at your institution prior to using the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit.

Purpose of the Manual

Purpose of the Manual

This manual provides an overview of the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit and provides instructions and guidelines for:

1. Generating entry clones containing the promoter and gene of interest, one in pENTR™5′-TOPO® and the second in any Gateway® entry vector.
2. Using the pLenti6.4/R4R2/V5-DEST vector and two entry clones containing the promoter and gene of interest in a MultiSite Gateway® LR recombination reaction to generate an expression clone.

Other Manuals

- For detailed instructions to generate the entry clone containing the promoter of interest, refer to the pENTR™5′-TOPO® TA Cloning Kit manual.
- For instructions to generate the entry clone containing the gene of interest, refer to the manual for the entry vector you select.
- For detailed instructions on producing lentivirus in 293FT cells, tittering your lentiviral stock, and transducing a mammalian cell line of choice, refer to the ViraPower™ HiPerform™ Promoterless Gateway® Expression System manual.
- For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual.

All manuals are available for downloading at www.invitrogen.com or by contacting Technical Support (see page 40).

Important

The ViraPower™ HiPerform™ Promoterless Gateway® Expression System is designed to help you create a lentivirus to deliver and express a gene of interest from a promoter of choice in mammalian cells. Although the system has been designed to help you express your recombinant protein of interest in the simplest, most direct fashion, use of the system is geared towards those users who are familiar with the principles of retrovirus biology and retroviral vectors. In addition, we highly recommend that users possess a working knowledge of:

- Viral and tissue culture techniques
- Gateway® Technology and site-specific recombination

For more information about these topics, refer to the following published reviews:

- Retrovirus biology and the retroviral replication cycle: see Buchschacher and Wong-Staal (2000) and Luciw (1996).
- Retroviral and lentiviral vectors: see Naldini (1999), Naldini (1998), and Yee (1999)
- Gateway® Technology and site-specific recombination: see Hartley *et al.* (2000) and Landy (1989)

Methods

Generating Entry Clones

Introduction

Before you can generate an expression construct in pLenti6.4/R4R2/V5-DEST, you first need to generate the following entry clones:

- **An *att*L4 and *att*R1-flanked entry clone containing your eukaryotic promoter of interest.** To generate this entry clone, you **must** use the pENTR™5′-TOPO® entry vector and the pENTR™5′-TOPO® TA Cloning® Kit supplied with the ViraPower™ Promoterless Lentiviral Gateway® Kit. See below for more information.

  Note: You may also use a pENTR™5′ Promoter Clone (see page 12) or any other *att*L4- and *att*R1-flanked entry clone containing your promoter of interest.

- **An *att*L1 and *att*L2-flanked entry clone containing your gene of interest.** To generate this entry clone, you may use any traditional Gateway® entry vector or obtain an Ultimate™ ORF Clone available from Invitrogen. See page 10 for more information.

General guidelines are provided in this section to help you generate the appropriate entry clones. For detailed instructions, refer to the manual for the entry vector you are using.

Note

The ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit and ViraPower™ HiPerform™ Promoterless Gateway® Expression System also include pENTR™5′/CMVp and pENTR™5′/EF1αp plasmids (i.e., pENTR™5′ Promoter Clones) for easy cloning of the CMV or the EF-1 promoter, respectively. See **Using the pENTR™5′ Promoter Clone**, page 12, for more information.

Generating an Entry Clone Containing a Promoter of Interest

The pENTR™5′-TOPO® TA Cloning® Kit containing the pENTR™5′-TOPO® vector is supplied with the ViraPower™ HiPerform™ Promoterless Gateway® Expression System to facilitate generation of an *att*L4 and *att*R1-flanked entry clone containing your eukaryotic promoter of interest. Note that you **must** use the pENTR™5′-TOPO® vector in this application; other Gateway® entry vectors are not suitable. To generate an entry clone using pENTR™5′-TOPO®:

1. Use *Taq* polymerase to produce a PCR product encoding your eukaryotic promoter of interest.
2. TOPO® Clone the PCR product into pENTR™5′-TOPO® using a 5-minute bench-top ligation.
3. Transform the TOPO® Cloning reaction into chemically competent *E. coli* supplied with the kit and select for entry clones.

For instructions and protocols, refer to the pENTR™5′-TOPO® TA Cloning® Kit manual. This manual is supplied with the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit, but is also available for downloading from our website at www.invitrogen.com or by contacting Technical Support (see page 40).

Note: The pENTR™5′-TOPO® TA Cloning® kit is also available separately from Invitrogen (see page 38).

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Important

pLenti6.4/R4R2/V5-DEST has a limited cloning size of 4 to 4.5. For efficient packaging to occur, **the combined size of your promoter + gene of interest must not exceed 4 to 4.5 kb.** Inserts larger than 4.5 kb reduce packaging efficiency, resulting in lower lentiviral titers.

Promoter Sequence Considerations

Consider the following when cloning your eukaryotic promoter sequence:

- Make sure that your DNA fragment contains all promoter and enhancer sequences (e.g., TATA box, transcription factor binding sites) necessary to regulate expression of the downstream gene of interest (following MultiSite Gateway® LR recombination).
- Make sure that your promoter sequence contains a transcription initiation site.
- Make sure that your promoter sequence does **not** contain an ATG translation initiation codon.

Generating an Entry Clone Containing a Gene of Interest

To generate an *att*L1 and *att*L2-flanked entry clone containing the gene of interest, you may use any Gateway® entry vector available from Invitrogen except pENTR™5′-TOPO®. For fast and easy generation of an entry clone using TOPO® Cloning, we recommend using the pENTR™/D-TOPO® entry vector. Other TOPO®-adapted entry vectors are also available (see page 39).

Once you have selected an entry vector, refer to the manual for the specific entry vector you are using for instructions to construct an entry clone. All entry vector manuals are available for downloading from www.invitrogen.com or by contacting Technical Support (see page 40).

Note: When used in a MultiSite Gateway® LR recombination reaction with a pENTR™5′-TOPO® entry clone and pLenti6.4/R4R2/V5-DEST, entry clones generated in pCR®8/GW/TOPO® recombine less efficiently, resulting in slightly fewer total colonies. If you want to maximize the number of MultiSite Gateway® LR recombinants obtained, we suggest generating *att*L1 and *att*L2-containing entry clones in pENTR™/D-TOPO®.

To express a human gene of interest in pLenti6.4/R4R2/V5-DEST, you may use an Ultimate™ Human ORF (hORF) Clone available from Invitrogen. The Ultimate™ hORF Clones are fully sequenced clones and are provided in an *att*L1 and *att*L2-flanked Gateway® entry vector. They are ready to use in a recombination reaction with a pENTR™5′-TOPO® entry clone and the pLenti6.4/R4R2/V5-DEST vector. For more information about the Ultimate™ hORF Clones available, refer to www.invitrogen.com/clones or contact Technical Support (see page 40).

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.

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ORF Sequence Considerations

pLenti6.4/R4R2/V5-DEST allows fusion of your gene of interest to a C-terminal tag. When generating your entry clone, your gene of interest 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

  (G/A)NNATG**G**.

  Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.
- Be in frame with the C-terminal tag after recombination with pLenti6.4/R4R2/V5-DEST.
- **NOT** contain a stop codon.

Using the pENTR™ 5' Promoter Clone

pENTR™5′ Promoter Clones

If you prefer to use the CMV or the EF-1α promoter for expressing your gene of interest, you may use the pENTR™5′/CMVp and pENTR™5′/EF1αp plasmids (i.e., pENTR™5′ Promoter Clones), respectively, for easy cloning of promoter sequences. This section provides information on using the pENTR™5′ promoter clones.

Note: You may also use the pENTR™5′ Promoter Clones with any *att*L1 and *att*L2-flanked entry clone in your MultiSite Gateway® LR recombination reaction to verify the efficiency of the reaction.

CMV Promoter

pENTR™5′/CMVp contains the human CMV immediate early promoter to allow high-level, constitutive expression of the gene of interest in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). Although highly active in most mammalian cell lines, activity of the viral promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both.

EF-1α Promoter

pENTR™5′/EF1αp contains the elongation factor 1 α-subunit promoter (EF-1α) for high-level expression across a broad range of species and cell types (Goldman *et al.*, 1996; Mizushima & Nagata, 1990). The EF-1α promoter is expressed in a wide range of mammalian cell types, including those where the CMV promoter expression is absent or inconsistent.

Which Promoter to Use

pENTR™5′/CMVp carries the CMV promoter and is suitable for use in most cell line applications. pENTR™5′/EF1αp contains the EF-1α promoter and may be more appropriate for long-term gene expression in certain mouse cell lines, stem cells, primary cells, and for *in vivo* use.

Features of the pENTR™5' Promoter Clones

- Choice of human CMV immediate early promoter or EF-1α promoter
- *att*L4 and *att*R1 sites to allow two-fragment or three-fragment recombination with appropriate entry clone(s) and a MultiSite Gateway® destination vector to generate an expression construct
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

For a map and features of each pENTR™5′ Promoter Clone, see pages 34–37.

Cloning your own Promoter

To clone your own promoter, you need the pENTR™5′/TOPO™ TA Cloning Kit (see page 38 for ordering information). For details on how to clone your own promoter, refer to the pENTR™5′/TOPO™ TA Cloning Kit manual, which is available for downloading at www.invitrogen.com, or by contacting Technical Support (page 40).

Guidelines for Generating Expression Clones

Introduction

After you have generated separate entry clones containing your promoter and gene of interest, perform the MultiSite Gateway® LR recombination reaction to simultaneously transfer the two DNA fragments into the pLenti6.4/R4R2/V5-DEST vector to create an expression clone with the following structure:

***att*B4-promoter-*att*B1-gene of interest-*att*B2**

To ensure that you obtain the best possible results, we recommend that you read this section and the sections entitled **Performing the MultiSite Gateway® LR Recombination Reaction** (pages 16–19) and **Transforming One Shot® Stbl3™ Competent *E. coli*** (pages 20–23) before beginning.

Experimental Outline

To generate an expression clone:

1. Perform a MultiSite Gateway® LR recombination reaction using the appropriate entry clones and pLenti6.4/R4R2/V5-DEST (see below).
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones

For an illustration of the recombination region of pLenti6.4/R4R2/V5-DEST expression clone, refer to page 15.

Substrates for the MultiSite Gateway® LR Recombination Reaction

To perform the two-fragment MultiSite Gateway® LR recombination reaction, you **must** have the substrates listed below.

- *att*L4 and *att*R1-flanked entry clone containing the promoter of interest (i.e., your pENTR™5′-TOPO® construct containing your promoter of interest)

  Note: You may also use a pENTR™5′ Promoter Clone (see previous page) or any other *att*L4 and *att*R1-flanked entry clone containing your promoter of interest.
- *att*L1 and *att*L2-flanked entry clone containing the gene of interest
- *att*R4 and *att*R2-flanked pLenti6.4/R4R2/V5-DEST vector

Keep in mind the following:

- You **cannot** successfully create a two-fragment expression clone using the MultiSite Gateway® LR recombination reaction if you have any combination of *att*-flanked entry clones other than the ones listed above.
- You **must** use the pLenti6.4/R4R2/V5-DEST destination vector for this reaction. Other pLenti-based destination vectors or Gateway® destination vectors **cannot** be used.

Important

For optimal results, we recommend performing the MultiSite Gateway® LR recombination reaction using:

- Supercoiled entry clones
- Supercoiled pLenti6.4/R4R2/V5-DEST

Continued on next page

Guidelines for Generating Expression Clones, Continued

Plasmid Preparation

Once you have generated your entry clones, prepare purified plasmid DNA from each entry clone to use in the MultiSite Gateway® LR recombination reaction. You may use any method of choice to isolate plasmid DNA. We recommend using Invitrogen's PureLink™ HiPure Plasmid Midiprep Kit (see page 38).

Resuspend the purified plasmid DNA in sterile water or TE Buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a final concentration of 150 ng/µL.

Propagating the Destination Vector

For propagating and maintaining the pLenti6.4/R4R2/V5-DEST vector prior to cloning your gene of interest, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells from Invitrogen (see page 38). 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.

Note: Do not use general *E. coli* cloning strains including Stbl3™, TOP10, or DH5 ™ for propagation and maintenance as these strains are sensitive to CcdB effects.

Genotype of *ccd*B Survival™ 2 T1^R

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhuA::IS2*

Guidelines to Propagate the Destination Vector

Follow the guidelines below when using *ccd*B Survival™ 2 T1^R *E. coli* to propagate the pLenti6.4/R4R2/V5-DEST plasmid:

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

Continued on next page

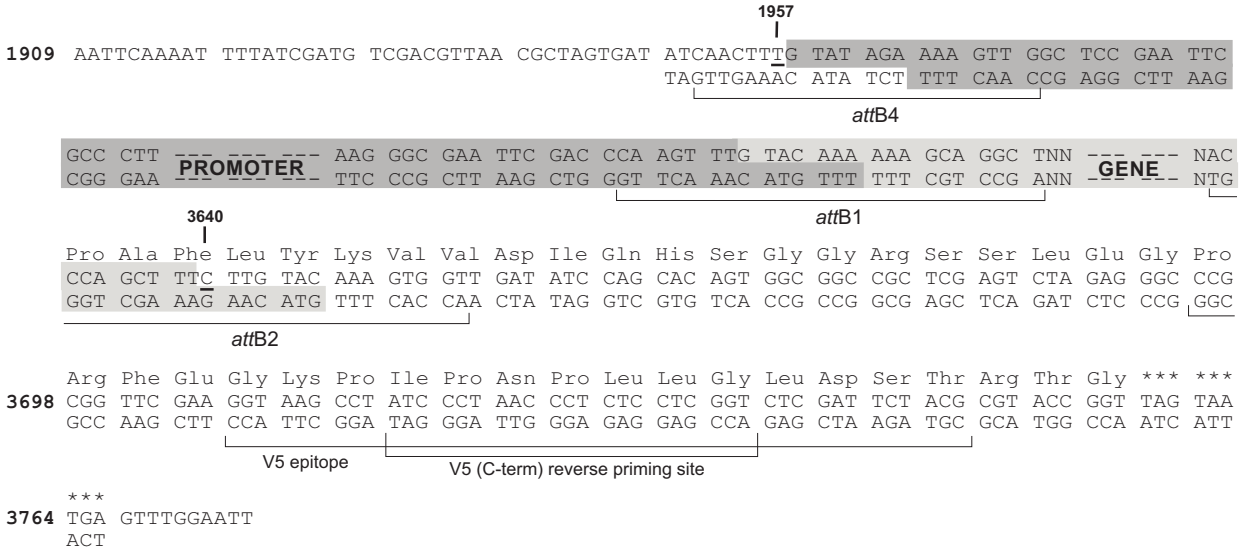
Guidelines for Generating Expression Clones, Continued

Recombination Region of the Expression Clone

The recombination region of the expression clone resulting from *att*L4-promoter-*att*R1 entry clone × *att*L1-gene-*att*L2 entry clone × pLenti6.4/R4R2/V5-DEST is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the two entry clones into the pLenti6.4/R4R2/V5-DEST vector by recombination. Note that the sequences comprising the *att*B1 site are entirely supplied by the entry clones. Non-shaded regions are derived from the pLenti6.4/R4R2/V5-DEST vector.
- Bases 1957 and 3640 of the pLenti6.4/R4R2/V5-DEST sequence are marked.



Performing the MultiSite Gateway® LR Recombination Reaction

Introduction

Follow the guidelines and instructions in this section to perform the MultiSite Gateway® LR recombination reaction using the appropriate entry clones and the pLenti6.4/R4R2/V5-DEST vector. We recommend including a negative control (no LR Clonase™ II Plus) in your experiment to help you evaluate your results.

Determining How Much DNA to Use in the Reaction

For optimal efficiency, we recommend using the following amounts of plasmid DNA (i.e., entry clones and destination vector) in a 10 µL MultiSite Gateway® LR recombination reaction:

- An equimolar amount of each plasmid
- 10 fmoles of **each** entry clone and 20 fmoles of pLenti6.4/R4R2/V5-DEST

Converting Femtomoles (fmoles) to Nanograms (ng)

Use the following formula to convert femtomoles (fmoles) of DNA to nanograms (ng) of DNA:

$$ng = (x\ fmoles)(N)(\frac{660\ fg}{fmoles})(\frac{1\ ng}{10^6\ fg})$$

where x is the number of fmoles and N is the size of the DNA in bp.

Example

20 fmoles of the pLenti6.4/R4R2/V5-DEST vector (8,922 bp) in ng is:

$$(20\ fmoles)(8{,}922\ bp)(\frac{660\ fg}{fmoles})(\frac{1\ ng}{10^6\ fg}) = 117.8\ ng$$

Continued on next page

Performing the MultiSite Gateway® LR Recombination Reaction, Continued

Recommended *E. coli* Host

For optimal results, we recommend using Stbl3™ *E. coli* for transformation after the LR recombination reaction. 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 20. Note that transformants containing unwanted recombinants are obtained less frequently when Stbl3™ *E. coli* are used for transformation (see **Note** below).

Other *E. coli* Hosts

You may transform the LR recombination reaction into other *rec*A, *end*A *E. coli* strains including TOP10 and DH5α™, if desired. Note however, that these strains are not as well-suited for cloning unstable DNA, and may give rise to a low percentage (< 5%) of transformants containing unwanted recombinants (i.e., plasmids where recombination has occurred between the 5′ and 3′ LTRs) when selected on plates containing only ampicillin. If you wish to use TOP10 or DH5α™ cells for transformation, follow the guidelines below to reduce the frequency of obtaining unwanted recombinants:

- **Select for transformants using 100 µg/mL ampicillin and 50 µg/mL Blasticidin.** Note that transformed *E. coli* grow more slowly in LB media containing two selection agents, and may require slightly longer incubation times to obtain visible colonies. For a recipe to prepare LB agar plates containing ampicillin and Blasticidin, see page 27. For more information about Blasticidin, see page 28.
- **Select small colonies for analysis** as transformants containing a plasmid that has recombined between the 5′ and 3′ LTRs (i.e., unwanted recombinants) generally give rise to larger colonies than those containing an intact plasmid.

Important

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

Continued on next page

Performing the MultiSite Gateway® LR Recombination Reaction, Continued

Materials Needed

- 10 fmoles of the purified plasmid DNA of your *att*L4 and *att*R1-flanked entry clone containing your promoter of interest or 10 fmoles of the control plasmid pENTR™5′/CMVp or pENTR™ 5′/EF1αp (if desired)
- 10 fmoles of purified plasmid DNA of your *att*L1 and *att*L2-flanked entry clone containing your gene of interest
- 20 fmoles of pLenti6.4/R4R2/V5-DEST vector
- LR Clonase™ II Plus enzyme mix (keep at –80°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 LR Clonase™ II Plus enzyme mix; thaw and keep on ice until use)
- Sterile 0.5 mL microcentrifuge tubes

Important

You **must** use the LR Clonase™ II Plus enzyme mix for this application. Do not use the standard LR Clonase™ or LR Clonase™ II enzyme mix.

LR Clonase™ II Plus enzyme mix is supplied with the ViraPower™ HiPerform™ Promoterless Gateway® Expression System Kit, but not with the Vector Kit. The enzyme mix is also available separately from Invitrogen. See page 38 for ordering information.

Positive Control Entry Clone

The pENTR™ Gus positive control plasmid is included in the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit for use as a positive control for LR recombination and expression. Using the pENTR™ Gus entry clone in an LR recombination reaction with any pDEST™ vector allows you to generate an expression clone containing the β-glucuronidase (*gus*) gene. For the vector map of pENTR™ Gus positive control plasmid, see page 33.

You may also use the pENTR™5′/CMVp or the pENTR™ 5′/EF1αp plasmids (i.e., the pENTR™5′ Promoter Clones) as a positive control for the MultiSite Gateway® LR recombination reaction. pENTR™5′/CMVp and pENTR™ 5′/EF1αp plasmids are entry clones containing the human CMV and EF-1α promoters, respectively. Use these entry clones with any *att*L1 and *att*L2-flanked entry clone in your MultiSite Gateway® LR recombination reaction to verify the efficiency of the reaction. For more information on pENTR™5′/CMVp and pENTR™ 5′/EF1αp, see page 12. For vector maps of these plasmids, see pages 34 and 36, respectively.

Continued on next page

Performing the MultiSite Gateway® LR Recombination Reaction, Continued

Setting Up the MultiSite Gateway® LR Recombination Reaction

Follow this procedure to perform the MultiSite Gateway® LR recombination reaction between your entry clones and the pLenti6.4/R4R2/V5-DEST vector. To include a negative control, set up a separate reaction in which you omit the LR Clonase™ II Plus enzyme mix.

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

Component	Sample	Negative Control	Positive Control
pENTR™5′-promoter entry clone (10 fmol)	1–7 µL	1–7 µL	–
pENTR™-gene entry clone (10 fmol)	1–7 µL	1–7 µL	1–6 µL
pENTR™5′-positive control (10 fmol)	–	–	1 µL
pLenti6.4/R4R2/V5-DEST (20 fmol)	1 µL	1 µL	1 µL
TE Buffer, pH 8.0	to 8 µL	to 8 µL	to 8 µL

2. Remove the LR Clonase™ II Plus enzyme mix from –80°C and thaw on ice (~2 minutes).
3. Vortex the LR Clonase™ II Plus enzyme mix briefly twice (2 seconds each time).
4. Add 2 µL of LR Clonase™ II Plus enzyme mix to the sample and positive control vials. **Do not** add LR Clonase™ II Plus enzyme mix to the negative control vial. Mix well by pipetting up and down.
   Note: Return LR Clonase™ II Plus enzyme mix to –80°C immediately after use.
5. Incubate the reaction at room temperature (20–25°C) from 16 hours to overnight.
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 MultiSite Gateway® 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 MultiSite Gateway® 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

- MultiSite Gateway® 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)
- S.O.C. Medium (supplied with the kit; warm to room temperature)
- pUC19 positive control (if desired to verify the transformation efficiency; supplied with the kit)
- LB Medium (if performing the pUC19 control transformation)
- 42°C water bath
- LB plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- 37°C shaking and non-shaking incubator

One Shot® Stbl3™ Transformation Procedure

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

1. Thaw on ice one vial of One Shot® Stbl3™ chemically competent cells for each transformation.
2. Add 2 µL of the MultiSite Gateway® 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.** Add 10 pg (1 µL) of the pUC19 control into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C water bath and place on ice for 2 minutes.
6. Add 250 µL of pre-warmed S.O.C. Medium to each vial.
7. Cap the vial(s) tightly and shake horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 25–100 µL of the transformation mix on a pre-warmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium (e.g., add 100 µL of the transformation mix to 900 µL of LB Medium) and plate 25–100 µL.
9. Store the remaining transformation mix at 4°C. Plate out additional cells the next day.

Continued on next page

What You Should See

Using the entire MultiSite Gateway® LR reaction to transform *E. coli* cells with a transformation efficiency of 1×10^9 cfu/µg and plating the entire transformation mixture results in approximately 1,000 to 5,000 colonies.

Note: MultiSite Gateway® LR recombination reaction using a pCR®8/GW/TOPO® entry clone results in fewer colonies.

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB medium containing 100 µg/mL ampicillin.
2. Isolate plasmid DNA using any method of choice.
3. Analyze the plasmids by restriction analysis to confirm the presence and orientation of your inserts (promoter + gene).

Note: 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.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives.

- True expression clones are chloramphenicol-sensitive **and** ampicillin- and Blasticidin-resistant.
- Transformants containing a plasmid with a mutated *ccdB* gene are chloramphenicol-, ampicillin-, and Blasticidin-resistant.

To check your putative expression clone, test for growth on LB plates containing 30 µg/mL chloramphenicol. A true expression clone does not grow in the presence of chloramphenicol.

Continued on next page

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants kill the cells, and salt interferes with lipid complexing, decreasing transfection efficiency.

When isolating plasmid DNA from *E. coli* strains (such as Stbl3™) that are wild type for endonuclease 1 (*end*A1+) using commercially available kits, ensure that the Lysis or Resuspension Buffer contains 10 mM EDTA. EDTA inactivates endonucleases and prevents DNA nicking and vector degradation. Alternatively, follow the instructions for purifying *end*A1+ *E. coli* strains included in the plasmid purification kits.

Resuspend the purified pLenti6.4/R4R2/V5-DEST expression plasmid containing your promoter + gene of interest in sterile water or TE Buffer, pH 8.0 to a final concentration ranging from 0.1–3.0 µg/mL. Each transfection requires 3 µg of the expression plasmid.

Important

Do **not** use mini-prep plasmid DNA for lentivirus production. We recommend preparing lentiviral plasmid DNA using the PureLink™ HiPure Plasmid MidiPrep kit which contains 10 mM EDTA in the Resuspension Buffer (see page 38 for ordering information).

Sequencing

Sequencing the expression construct is not required, because the transfer of the promoter and gene of interest from the entry vectors into the pLenti6.4/R4R2/V5-DEST vector preserves the orientation and reading frame. However, to confirm that your gene of interest is in frame with the C-terminal tag in pLenti6.4/R4R2/V5-DEST, you may sequence your expression construct. We recommend using the following primers for sequencing. Refer to the diagram on page 15 for the location of the primer binding site in the vector.

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 (see page 40).

Primer	Sequence
CMV Forward (for CMVp)	5′-CGCAAATGGGCGGTAGGCGTG-3′
T7 Promoter (for EF-1αp)	5′-TAATACGACTCACTATAGGA-3′
V5(C-term) Reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Maintaining the Expression Clone

Once 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.

Continued on next page

Verifying Expression of Recombinant Protein

Before generating a lentiviral stock of the pLenti6.4/R4R2/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 the recombinant protein. Follow the guidelines below:

- 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 (see below).
- Follow the manufacturer's instructions for the transfection reagent you are using to perform plasmid transfection.

Lipofectamine™ 2000

The Lipofectamine™ 2000 Reagent (Ciccarone *et al.*, 1999) is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids into eukaryotic cells. Using Lipofectamine™ 2000 to transfect 293FT cells offers the following advantages:

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

Note: Lipofectamine™ 2000, included in the ViraPower™ HiPerform™ Promoterless Gateway® Expression System, is also available separately from Invitrogen (see page 38 for ordering information).

Next Steps

Experimental Outline

After you have generated and purified the pLenti6.4/R4R2/V5-DEST expression construct containing your promoter + gene of interest, follow the experimental outline below to express your protein of interest.

1. Co-transfect the pLenti-based expression vector and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
2. Titer the lentiviral stock.
3. Use the lentiviral stock to transduce your mammalian cell line of choice.
4. Assay for "transient" expression of your recombinant protein, or
5. Generate a stably transduced cell line.

For detailed instructions, refer to the ViraPower™ HiPerform™ Promoterless Gateway® Expression System manual. For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual.

These manuals are supplied with the ViraPower™ HiPerform™ Promoterless Gateway® Expression System, and are also available for downloading at www.invitrogen.com or by contacting Technical Support (page 40).

Troubleshooting

MultiSite Gateway® LR Reaction

The table below lists some potential problems and possible solutions that may help you troubleshoot the MultiSite Gateway® LR recombination reaction. To troubleshoot experimental steps involved in generating your entry clones, refer to the pENTR™5′-TOPO® TA Cloning® Kit manual and the manual for the specific entry vector you have used.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing 100 µg/mL ampicillin.
	Recombination reaction was not treated with proteinase K	Treat reactions with proteinase K before transformation.
	Used incorrect *att* sites for the reaction	Use the appropriate entry clones (i.e., *att*L4 and *att*R1-flanked entry clone and *att*L1 and *att*L2-flanked entry clone) and pLenti6/R4R2/V5-DEST for the MultiSite Gateway® LR reaction (see page 9 for details about suitable entry vectors to use to generate entry clones).
	LR Clonase™ II Plus enzyme mix is inactive or did not use the suggested amount of LR Clonase™ II Plus enzyme mix	• Store the LR Clonase™ II Plus enzyme mix at –80°C for long term storage (> 6 months) • Do not freeze/thaw the LR Clonase™ II Plus enzyme mix more than 10 times. • Use the recommended amount of LR Clonase™ II Plus enzyme mix (see page 19).
	Used standard LR Clonase™ enzyme mix rather than the LR Clonase™ II Plus enzyme mix	Use the LR Clonase™ II Plus enzyme mix for the MultiSite Gateway® LR reaction. **Do not** use other LR Clonase™ enzyme mixes.
	Used too much DNA in the MultiSite Gateway® LR reaction	• Use a molar ratio of 1:1:2 for the amount of each entry clone and the destination vector (see page 16). • Do not exceed 1 µg of total DNA in the reaction.
	MultiSite Gateway® LR reaction not incubated for sufficient time	Incubate the MultiSite Gateway® LR reaction at 25°C for 16 hours or overnight.

Continued on next page

MultiSite Gateway® LR Reaction, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Insufficient amount of *E. coli* transformed or plated	Transform 2 µL of the reaction; plate 50 µL or 100 µL.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C with shaking before plating.
Different sized colonies (i.e., large and small) appear when using TOP10 or DH5α *E. coli* for transformation	Some transformants contain plasmids in which unwanted recombination has occurred between 5′ and 3′ LTRs	• Select for transformants on LB plates containing **both** 100 µg/mL ampicillin and 50 µg/mL Blasticidin. • Use the One Shot® Stbl3™ Chemically Competent *E. coli* supplied with the kit for transformation. Stbl3™ *E. coli* are recommended for cloning unstable DNA including lentiviral DNA containing direct repeats and generally give rise to fewer unwanted recombinants.
Few or no colonies obtained from the transformation control	Competent cells stored incorrectly	Store competent cells at –80°C.
	After addition of DNA, competent cells mixed by pipetting up and down	After adding the DNA, mix competent cells gently. **Do not** mix by pipetting up and down.

Appendix

Recipes

LB (Luria-Bertani) Medium

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

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

LB Plates Containing Ampicillin and Blasticidin

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

Important: High temperatures may affect the stability of Blasticidin. **Do not** add Blasticidin to warm LB agar. Let LB agar cool to room temperature before adding Blasticidin.

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

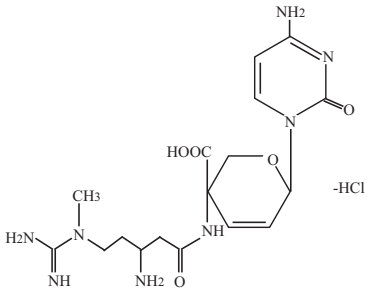
Blasticidin

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.

Preparing and Storing Stock Solutions

Blasticidin is also available separately from Invitrogen in 50 mg aliquots. Blasticidin is soluble in water. Use sterile water 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.

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Determining Blasticidin Sensitivity

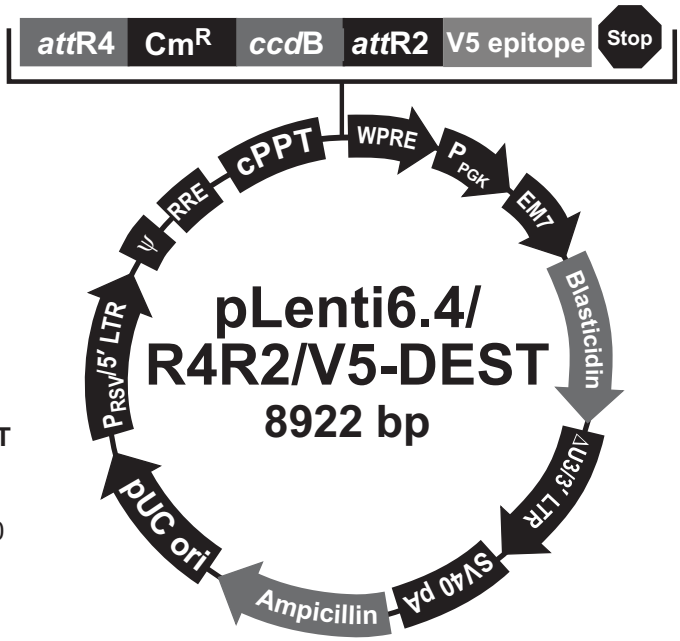
To select for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (i.e., perform a kill curve experiment). Typically, concentrations ranging from 2–10 µg/mL Blasticidin are sufficient to kill 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 cells at approximately 25% confluence. Prepare a set of 7 plates. Allow cells to adhere overnight.
2. The next day, substitute 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.

Map and Features of pLenti6.4/R4R2/V5-DEST

Map of pLenti6.4/R4R2/V5-DEST

The map below shows the elements of pLenti6.4/R4R2/V5-DEST. DNA from the entry clone replaces the region between bases 2447 and 4130. **The complete sequence for pLenti6.4/R4R2/V5-DEST is available at www.invitrogen.com or by contacting Technical Support (see page 40).**



Comments for pLenti6.4/R4R2/V5-DEST
8922 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
*att*R4 site: bases 1951-2075
Chloramphenicol resistance gene (Cm^R): bases 2801-3460
*ccd*B gene: bases 3184-3489
*att*R2 site: bases 3530-3654
V5 epitope: bases 3707-3748
WPRE: bases 3767-4364
PGK promoter: bases 4374-4880
EM7 promoter: bases 4891-4957
Blasticidin resistance gene: bases 4958-5356
ΔU3/3′ LTR: bases 5442-5676
    ΔU3: bases 5442-5495
    3′ LTR: bases 5496-5676
SV40 polyadenylation signal: bases 5748-5879
bla promoter: bases 6738-6836
Ampicillin (*bla*) resistance gene: bases 6837-7697
pUC origin: bases 7842-8515

Continued on next page

Map and Features of pLenti6.4/R4R2/V5-DEST, Continued

Features of the Vector

The pLenti6.4/R4R2/V5-DEST (8,922 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001)
*att*R4 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the miR RNA and promoter of interest from Gateway® entry clones (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991).
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey et al., 1998)
mPGK promoter	Allows expression of the selection marker in a broad variety of mammalian cells..
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).
ΔU3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.

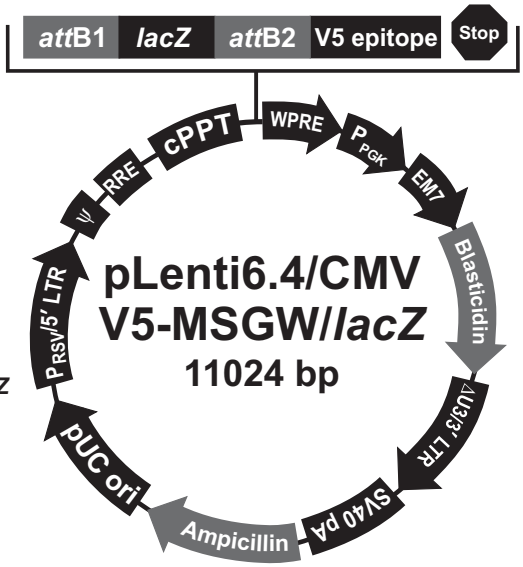
Map of pLenti6.4/CMV/V5-MSGW/*lacZ*

Description

pLenti6.4/CMV/V5-MSGW/*lacZ* is an 11,024 bp control vector expressing β-galactosidase. It was generated using the MultiSite Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene, an entry clone containing the CMV promoter, and pLenti6.4/R4R2/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

Map of pLenti6.4/CMV/ V5-MSGW/*lacZ*

The map below shows the elements of pLenti6.4/CMV/V5-MSGW/*lacZ*. **The complete sequence of the vector is available at www.invitrogen.com or by contacting Technical Support (see page 40).**



Comments for pLenti6.4/CMV/V5-MSGW/*lacZ*
11024 nucleotides

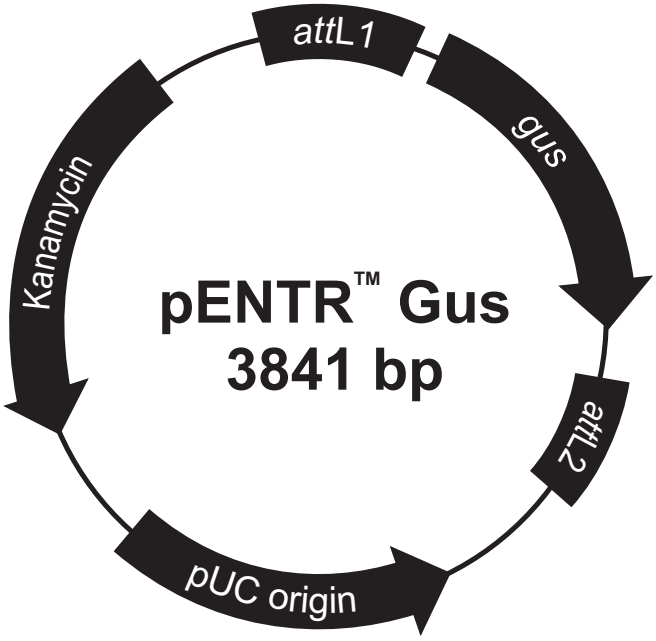
RSV/5′ LTR hybrid promoter: bases 1-410
RSV promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
cPPT: bases 1801-1923
CMV promoter: bases 1979-2563
att B1 site: bases 2611-2635
lacZ ORF: bases 2656-5717
att B2 site: bases 5732-5756
V5 epitope: bases 5809-5850
WPRE: bases 5869-6466
PGK promoter: bases 6476-6982
EM7 promoter: bases 6993-7059
Blasticidin resistance gene: bases 7060-7458
ΔU3/3′ LTR: bases 7544-7778
ΔU3: bases 7544-7597
3′ LTR: bases 7598-7778
SV40 polyadenylation signal: bases 7860-7981
bla promoter: bases 8840-8938
Ampicillin (*bla*) resistance gene: bases 8939-9799
pUC origin: bases 9944-10617

Continued on next page

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 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 40).**



Comments for pENTR™ Gus
3841 nucleotides

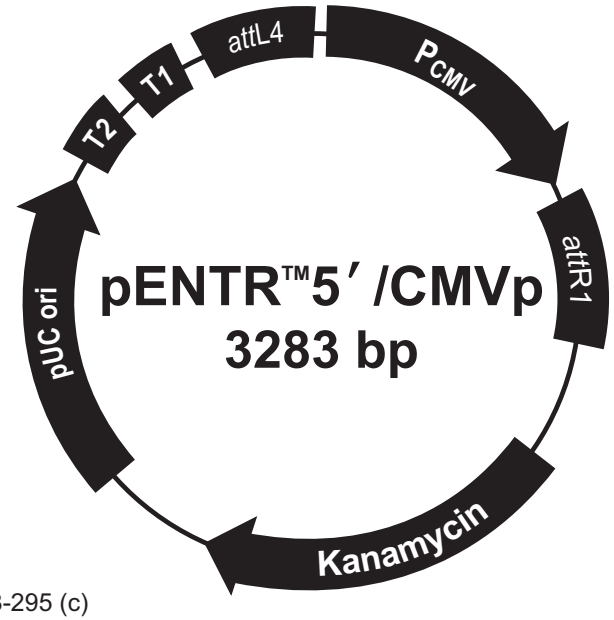
*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Map and Features of pENTR™5'/CMVp

Map of pENTER™5'/CMVp

The map below shows the elements of the pENTR™5′/CMVp vector. **The complete sequence of pENTR™5′/CMVp is available for downloading at www.invitrogen.com or by contacting Technical Support (page 40).**



Comments for pENTR™5′/CMVp
3283 nucleotides

*rrn*B T2 transcription terminator: bases 268-295 (c)
*rrn*B T1 transcription terminator: bases 427-470 (c)
M13 forward (-20) priming site: bases 537-552
*att*L4: bases 592-688
GW1 priming site: bases 630-654
CMV promoter: bases 696-1280
*att*R1: bases 1329-1452
GW3 priming site: bases 1359-1388
M13 reverse priming site: bases 1548-1564
Kanamycin resistance gene: 1677-2486
pUC origin: bases 2607-3280

(c) = complementary strand

Continued on next page

Map and Features of pENTR™5'/CMVp, Continued

Features of the Vector

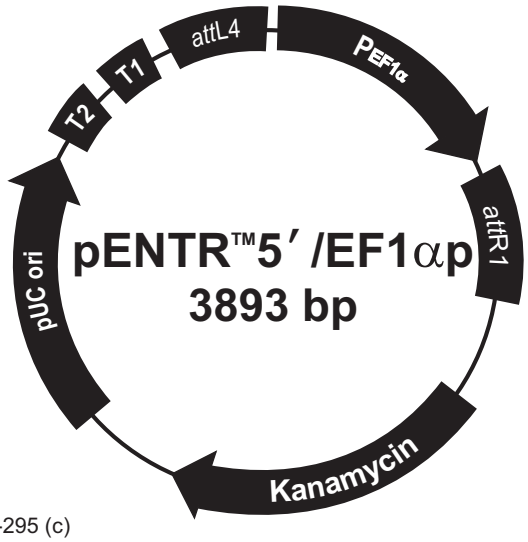
The pENTR™5'/CMVp (3,283 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

Feature	Benefit
rrnB T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (–20) priming site	Allows sequencing of the insert.
GW1 priming site	Allows sequencing of the insert.
attL4 and attR1 sites	Bacteriophage λ-derived recombination sequences that have been optimized to allow recombinational cloning of a DNA fragment in the entry construct with a suitable MultiSite Gateway® destination vector in conjunction with any attL1 and attL2-flanked entry clone (Landy, 1989).
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits high-level, constitutive expression of the gene of interest (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987).
GW3 priming site	Allows sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli.*
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Map and Features of pENTR™5'/EF1αp

Map of pENTER™5'/EF1αp

The map below shows the elements of the pENTR™ 5′/EF1αp vector. **The complete sequence of pENTR™ 5′/EF1αp is available for downloading at www.invitrogen.com or by contacting Technical Support (page 40).**



Comments for pENTR™5′/EF1αp
3893 nucleotides

*rrn*B T2 transcription terminator: bases 268-295 (c)
*rrn*B T1 transcription terminator: bases 427-470 (c)
M13 forward (-20) priming site: bases 537-552
*att*L4: bases 592-688
GW1 priming site: bases 630-654
EF1α promoter: bases 704-1893
*att*R1: bases 1939-2062
GW3 priming site: bases 1969-1998
M13 reverse priming site: bases 2158-2174
Kanamycin resistance gene: 2287-3096
pUC origin: bases 3217-3890

(c) = complementary strand

Continued on next page

Map and Features of pENTR™5'/EF1αp, Continued

Features of the Vector

The pENTR™5'/EF1αp (3,893 bp) vector contains the following elements. All features have been functionally tested and the vector is fully sequenced.

Feature	Benefit
rrnB T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (–20) priming site	Allows sequencing of the insert.
GW1 priming site	Allows sequencing of the insert.
attL4 and attR1 sites	Bacteriophage λ-derived recombination sequences that have been optimized to allow recombinational cloning of a DNA fragment in the entry construct with a suitable MultiSite Gateway® destination vector in conjunction with any attL1 and attL2-flanked entry clone (Landy, 1989).
Elongation Factor 1 promoter (EF-1αp)	Encodes the EF-1α enzyme that catalyzes the GTP-dependent binding of aminoacyl-tRNA to ribosomes and is expressed in almost all kinds of mammalian cells.
GW3 priming site	Allows sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Accessory Products

Additional Products

Many of the reagents supplied in the ViraPower™ HiPerform™ Promoterless Gateway® Vector Kit as well as other products suitable for use with the kits are available separately from Invitrogen. Ordering information is provided below. For more information, refer to www.invitrogen.com or contact Technical Support (see page 40).

Product	Amount	Cat. no.
ViraPower™ HiPerform™ Promoterless Gateway® Expression System	1 kit	A11145
pENTR™5′-TOPO® TA Cloning Kit	20 reactions	K591-20
Gateway® LR Clonase™ II Plus Enzyme Mix	20 reactions 100 reactions	12538-020 12538-100
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µL	C7373-03
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli*	10 reactions	A10460
Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector	20 µg	V370-06
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
Ampicillin	5 g	Q100-16
Blasticidin S HCl	50 mg	R210-01
Geneticin®, liquid	20 mL 100 mL	10131-035 10131-027
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
Fetal Bovine Serum (FBS), Certified	500 mL	16000-044
β-Gal Assay Kit	80 mL	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
FluoReporter® *lacZ*/Galactosidase Quantitation Kit	1 kit	F-2905

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Gateway® entry vectors

To generate an *att*L1 and *att*L2-flanked entry clone containing the gene of interest, you may use any Gateway® entry vector available from Invitrogen except pENTR™5′-TOPO®. For fast and easy generation of an entry clone using TOPO® Cloning, we recommend using the pENTR™/D-TOPO® entry vector. Other TOPO®-adapted entry vectors are also available (see below).

Entry Vector Kit	Cat. No.
pENTR™/D-TOPO® Cloning Kit	K2400-20
pCR®8/GW/TOPO® TA Cloning Kit*	
with One Shot® TOP10 Chemically Competent E. coli	K2500-20
with One Shot® Mach1™-T1R Chemically Competent E. coli	K2520-20

*When used in a MultiSite Gateway® LR recombination reaction with a pENTR™5′-TOPO® entry clone and pLenti6.4/R4R2/V5-DEST, entry clones generated in pCR®8/GW/TOPO® recombine less efficiently, resulting in slightly fewer total colonies. If you want to maximize the number of MultiSite Gateway® LR recombinants obtained, we suggest generating *att*L1 and *att*L2-containing entry clones in pENTR™/D-TOPO®.

Detecting Recombinant Protein

You may detect expression of your recombinant protein using an antibody to the V5 epitope. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step chemiluminescent or colorimetric detection. A fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments. The amount of antibody supplied is sufficient for 25 western blots.

Item	Quantity	Cat. No.
Anti-V5 Antibody	50 µL	R960-25
Anti-V5-HRP Antibody	50 µL	R961-25
Anti-V5-AP Antibody	125 µL	R962-25
Anti-V5-FITC Antibody	50 µL	R963-25

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 web site at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product and is searchable by product lot number, which is printed on each box. CofAs are .available on our website at www.invitrogen.com/support.

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Purchaser Notification

Introduction

Use of the ViraPower™ HiPerform™ Promoterless Gateway® Expression System is covered under a number of different licenses including those detailed below.

Information for European Customers

The 293FT Cell Line is genetically modified and carries the pUC-derived plasmid, pCMVSPORT6TAg.neo. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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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 45.

Continued on next page

Limited Use Label License No. 27: Lipofectamine™ 2000

Use of this product in conjunction with methods for the introduction of RNA molecules into cells may require licenses to one or more patents or patent applications. Users of these products should determine if any licenses are required.

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.

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The Lentiviral Technology (based upon the lentikat™ system) is licensed from Cell Genesys, Inc., under U.S. Patent Nos. 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.

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Limited Use Label License No. 304: Improved Transfection Reagent

This product is covered by U.S. Pat. No. 7,145,039, 7,166,745, 7,173,154.

Continued on next page

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

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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.

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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**