invitrogen™
by life technologies™

NativePure™ Lentiviral Expression System

Lentiviral system for expression and purification of N- and C-terminal biotinylated fusion proteins and associated complexes in mammalian cells

Catalog nos. BN3001, BN3004, BN3005, and BN3007

Rev. date: 27 October 2010
Manual part no. 25-0891

MAN0000555

User Manual

Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Cat. no.
NativePure™ Lentiviral Gateway® Vector Kit	BN3001
NativePure™ Lentiviral Expression Kit	BN3004
NativePure™ Lentiviral Affinity Purification Kit	BN3005
NativePure™ Lentiviral Expression and Affinity Purification System	BN3007

Intended Use

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

Kit Components

The following table shows the components associated with the NativePure™ Lentiviral Expression System catalog numbers listed above.

Components	Cat. no.			
	BN3001	BN3004	BN3005	BN3007
NativePure™ Lentiviral Gateway® Vector Kit	✓	✓	✓	✓
ViraPower™ Bsd Lentiviral Support Kit		✓		✓
293FT Cell Line		✓		✓
One Shot® Stbl3™ Chemically Competent *E. coli*		✓		✓
Gateway® LR Clonase® II		✓		✓
NativePure™ Affinity Purification Kit			✓	✓

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Kit Contents and Storage, Continued

Shipping and Storage

NativePure™ Lentiviral Kits are shipped as described below. Upon receipt, store each component as detailed below. All reagents are guaranteed for a minimum of six months if stored properly.

Box	Component	Shipping	Storage
1	NativePure™ Lentiviral Gateway® Vector Kit	Room temperature	–20°C
2	ViraPower™ Bsd Lentiviral Support Kit:		
	• ViraPower™ Packaging Mix	Blue ice	–20°C
	• Lipofectamine® 2000	Blue ice	4°C **(do not freeze)**
	• Blasticidin	Room temperature	–20°C
3	One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C
4	293FT Cell Line	Dry ice	Liquid nitrogen
5	Gateway® LR Clonase® II Enzyme Mix	Dry ice	–20°C
6	NativePure™ Binding and Purification Module:		
	• Streptavidin Agarose	Blue ice	4°C
	• 10% NP40	Blue ice	4°C
	• NativePure™ 5X Lysis/Binding Buffer	Blue ice	4°C
	• NativePure™ 10X TEV Buffer	Blue ice	4°C
	• NativePure™ Columns	Blue ice	4°C
	• NativePure™ Concentrators	Blue ice	4°C
7	NativePure™ AcTEV™ Protease Module:		
	• AcTEV™ Protease	Dry ice	–20°C
	• 100 mM DTT	Dry ice	–20°C

NativePure™ Lentiviral Gateway® Vectors

Each NativePure™ Lentiviral Kit contains the following vectors. **Store the vectors at –20°C.**

Vector	Composition	Amount
pLenti6/capTEV™-NT-DEST1	40 µL of vector at 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pLenti6/capTEV™-CT-DEST	40 µL of vector at 150 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	6 µg
pLenti6/capTEV™-CT-GW/ARPC2	20 µL of vector at 500 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	10 µg

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Kit Contents and Storage, Continued

ViraPower™ Bsd Lentiviral Support Kit Contents

The ViraPower™ Bsd Lentiviral Support Kit includes the following vectors and reagents (Cat. nos. BN3004 and BN3007 only).

Important: Do not freeze Lipofectamine® 2000, store at 4°C

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

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

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

The following reagents are included with the One Shot® Stbl3™ Chemically Competent *E. coli* kit (Cat. nos. BN3004 and BN3007 only). 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™ 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+

293FT Cell Line

The 293FT Cell Line is supplied as one vial containing 3×10^6 frozen cells in 1 mL of Freezing Medium (Cat. nos. BN3004 and BN3007 only). **Upon receipt, store in liquid nitrogen.** For 293FT thawing, culturing, and maintenance instructions, see the 293FT Cell Line manual.

Continued on next page

Gateway® LR Clonase® II Enzyme Mix

The Gateway® LR Clonase® II Enzyme Mix contains the following reagents:

Reagent	Composition	Amount
Gateway® LR Clonase® II Enzyme Mix	Proprietary	40 µL
Proteinase K Solution	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$	40 µL
pENTR™-gus Positive Control	50 ng/µL in TE buffer, pH 8.0	20 µL

Note: The pENTR™-gus control included with the LR Clonase® II Enzyme Mix may be used as a positive control for the LR recombination reaction only.

NativePure™ Binding and Purification Module

The following reagents are included in the NativePure™ Binding and Purification Module (supplied with Cat. nos. BN3005 and BN3007 only). **Store at 4°C.**

Reagent	Composition	Amount
Streptavidin Agarose	10 mL of a 50% slurry containing 5 mL of packed Streptavidin Agarose beads in 0.1 M sodium phosphate, pH 7.5, 0.1 M NaCl, and 2 mM sodium azide	5 mL packed resin
10% NP40	10% (v/v) NP40 in deionized water	8 mL
NativePure™ 5X Lysis/Binding Buffer	0.5 M Tris-HCl, pH 8.0 0.5 M KCl 1 mM EDTA 7.5 mM $MgCl_2$	100 mL
NativePure™ 10X TEV Buffer	0.1 M Tris-HCl, pH 8.0 1.5 M NaCl 5 mM EDTA	40 mL
NativePure™ Columns	Polypropylene columns	10 each
NativePure™ Concentrator	Includes a concentrator fitted with a membrane and a filtration chamber	10 each

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NativePure™ AcTEV™ Protease Module

The following reagents are included in the NativePure™ AcTEV Protease Module (supplied with Cat. nos. BN3005 and BN3007 only). **Store at –20°C.**

Reagent	Composition	Amount
AcTEV™ Protease	10 U/µL AcTEV™ Protease in: 50 mM Tris-HCl, pH 7.5 1 mM EDTA 5 mM DTT 50% (v/v) glycerol 0.1% (w/v) Triton® X-100	400 µL
100 mM DTT	100 mM DTT in deionized water	500 µL

Introduction

System Summary

Description of the System

The NativePure™ Lentiviral Expression System includes NativePure™ Lentiviral Gateway® Vectors and the NativePure™ Affinity Purification Kit. The NativePure™ Lentiviral Vectors allow the creation of a replication-incompetent, HIV-1-based lentivirus with your gene of interest as an N- or C-terminal biotinylated fusion using Gateway® Technology (see page 6 for details on Gateway® Technology).

After lentiviral transduction into a mammalian cell line of choice, the NativePure™ Lentiviral Gateway® Vectors allow *in vivo* biotinylation and expression of the biotin-tagged protein of interest ("bait"). The biotin-tagged recombinant protein "bait" can be used to identify novel proteins that specifically interact with the protein of interest or to test complex formation between proteins or protein domains for which there is a prior reason to expect an interaction.

NativePure™ Lentiviral Expression System

The NativePure™ Lentiviral Gateway® Vectors allow the creation of a lentivirus stock. The number of infectious particles can be determined prior to delivery into mammalian cells by titering, which allows the user to control expression of the *in vivo* biotinylated bait protein of interest.

The NativePure™ Affinity Purification System is based on the TAP (Tandem Affinity Purification) method used to purify native protein complexes (Puig *et al.*, 2001). The purification of native protein complexes requires the use of a high affinity tag that allows rapid affinity purification of the tagged protein and associated protein complexes when present in low concentrations from cells without any prior information on the protein complex. The purified protein complexes are released from the affinity resin using a highly specific protease under native conditions.

The streptavidin agarose included with the NativePure™ Affinity Purification Kit permits the rapid and efficient purification of the bait protein and associated complexes even when present at low concentrations. Analyze the biotin-tagged protein and associated protein complexes by native gel electrophoresis or other techniques such as mass spectrometry.

The NativePure™ Affinity Purification Kit when combined with mass spectrometry provides a novel experimental approach to identify interacting proteins for proteome analysis or examine protein complexes that are part of specific cellular pathways, differentiation stages, or cell types.

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System Summary, Continued

NativePure™ Lentiviral Expression System, Continued

The NativePure™ Lentiviral Expression System is derived from the ViraPower™ Lentiviral Expression System, which 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. The System includes the following major components:

- pLenti6/capTEV™-DEST vectors adapted for use with the Gateway® Technology for the creation of expression plasmids containing the gene of interest fused to an N- or C-terminal capTEV™ tag. The vectors also contain modified lentiviral elements that allow packaging of the construct into virions (*e.g.*, 5′ and 3′ LTRs, Ψ packaging signal).
- The ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see the **Appendix**, pages 56–61.
- An optimized 293FT producer cell line that stably expresses the SV40 large T-antigen under the control of the human CMV promoter and facilitates optimal production of virus. For more information about the 293FT Cell Line, refer to the 293FT Cell Line manual, see page 62 for ordering information.

NativePure™ Affinity Purification System

The NativePure™ Affinity Purification Kit contains the following components to allow purification of biotinylated proteins and associated protein complexes expressed from vectors containing the capTEV™ Tag:

- Streptavidin Agarose
- NativePure™ Columns
- Pre-made, ready-to-dilute lysis, binding, and cleavage buffers
- AcTEV™ Protease
- NativePure™ Concentrators

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System Summary, Continued

How the System Works

To express your biotinylated protein of interest in mammalian cells, construct both N- and C-terminally tagged expression clones by performing LR recombination reactions between Gateway® entry vectors containing the gene of interest and the appropriate pLenti6/capTEV™-DEST vectors. Since individual protein expression and biotinylation may vary with an N- or C- terminal fusion tag in your cell line, it is necessary to construct both versions and determine which is best for your application. The resulting expression plasmids are each co-transfected with the ViraPower™ Packaging Mix in 293FT cells to produce two lentiviral stocks, which are titered to determine the number of infectious particles.

Transduce your mammalian cells with the lentivirus to allow expression of biotinylated proteins and to allow complex formation. Lyse the cells and use western analysis with a streptavidin conjugate to verify biotinylation of the protein of interest. Also, analyze the lysates using native gel electrophoresis to verify complex formation with the protein of interest.

After optimizing the expression and biotinylation of the bait protein of interest, purify the biotinylated protein and associated protein complexes under native conditions using the NativePure™ Affinity Purification Kit (supplied with Cat. nos. BN3005 and BN007, also available separately, see page 64). Analyze the associated complexes by western detection or mass spectrometry.

Advantages of the NativePure™ Lentiviral Expression System

Use of the NativePure™ Lentiviral Expression System to facilitate lentiviral-based expression of your protein of interest provides the following advantages:

- Gateway®-adapted vectors enable rapid and highly efficient transfer of DNA sequences for protein expression and functional analysis while maintaining orientation and reading frame
- Generates an HIV-1-based lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential applications beyond those of traditional Moloney Leukemia Virus (MoMLV)-based retroviral systems (Naldini, 1998)
- The number of lentivirus particles can be determined prior to delivery into mammalian cells by titering, which allows you to control expression of the *in vivo* biotinylated bait protein of interest thus allowing complex formation under physiological conditions
- Produces a pseudotyped virus with a broadened host range (Yee *et al.*, 1994)
- Efficiently delivers the gene of interest to mammalian cells in culture or *in vivo* (Dull *et al.*, 1998)
- Provides stable, long-term expression of a target gene beyond that offered by traditional adenoviral-based systems (Dull *et al.*, 1998; Naldini *et al.*, 1996)
- Includes multiple features designed to enhance the biosafety of the system

pLenti6/capTEV™-DEST Vectors

Features of the Vectors

The pLenti6/capTEV™-DEST vectors contain the following elements:

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

  Note: The U3 region of the 3′ LTR is deleted (ΔU3) and facilitates self-inactivation of the 5′ LTR after transduction to enhance the biosafety of the vector (Dull *et al.*, 1998)
- HIV-1 psi (Ψ) packaging sequence for viral packaging (Luciw, 1996)
- HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989)
- Human CMV promoter for constitutive expression of the gene of interest
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV 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 counterscreening
- The *ccd*B gene located between the *att*R sites for negative selection
- N or C-terminal capTEV™ tag, for *in vivo* biotinylation and affinity purification of recombinant proteins and associated complexes, consisting of:
  - BioEase™ Tag for *in vivo* biotinylation and purification using streptavidin agarose
  - 2 Tobacco Etch Virus (TEV) protease recognition sites to remove bound protein complexes after affinity purification with streptavidin agarose
  - 6XHis tag for identification of bait protein
- Blasticidin (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) resistance gene for selection in *E. coli* and mammalian cells
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

For plasmid map and features of the vectors, see pages 51–55.

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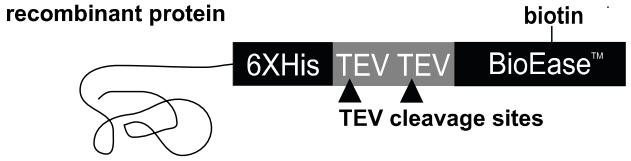
pLenti6/capTEV™-DEST Vectors, Continued

capTEV™ Tag

The NativePure™ Lentiviral Vectors allow N- or C- terminal fusion of your recombinant protein of interest to the capTEV™ Tag. The capTEV™ Tag consists of a BioEase™ *in vivo* biotinylation peptide, two Tobacco Etch Virus (TEV) protease recognition sites, and a 6XHis tag.



The capTEV™ Tag facilitates *in vivo* biotinylation of the recombinant "bait" protein of interest. The biotin-tagged protein of interest forms complexes in your cell line of choice, which is purified using streptavidin agarose affinity purification. The TEV sites allow removal of the bound biotinylated proteins/complexes of interest while endogenous biotinylated proteins remain bound to the streptavidin agarose column. After TEV cleavage, a 6XHis tag is present for identification of bait protein. These features are described in detail in the following sections.

BioEase™ Tag

The BioEase™ Tag is a 72 amino acid peptide derived from the C-terminus (amino acids 524–595) of *Klebsiella pneumoniae* oxalacetate decarboxylase α-subunit that contains a single covalent biotinylation site at lysine 561 of the protein (Schwarz *et al.*, 1988). When fused to a heterologous protein, the 72 amino acid BioEase™ domain is both necessary and sufficient to facilitate recognition and *in vivo* biotinylation of the recombinant protein of interest by cellular biotinylation enzymes. The high-affinity and selectivity of the streptavidin-biotin interaction is utilized to efficiently purify the biotinylated protein and associated complexes by streptavidin agarose affinity chromatography using the NativePure™ Affinity Purification Kit (supplied with Cat. nos. BN3005 and BN3007, also available separately, see page 64 for ordering information). For more information about cellular biotinylation processes, refer to published reviews (Chapman-Smith & J.E. Cronan, 1999).

TEV Protease Recognition Site

Since the streptavidin/biotin interaction is extremely strong, removal of the bound protein complexes from the streptavidin agarose is achieved by cleavage with a protease. The TEV (Tobacco Etch Virus) Protease is a site-specific protease that allows efficient release of bound materials under native conditions (Rigaut *et al.*, 1999).

The NativePure™ Lentiviral Vectors are designed with two tandem TEV cleavage sites that promote >90% cleavage of the biotinylated recombinant protein and associated protein complexes from the streptavidin agarose during purification under native conditions.

AcTEV™ Protease, an enhanced form of TEV protease that is highly active and specific (Nayak *et al.*, 2003) is supplied with Cat. nos. BN3005 and BN3007, and is available separately (page 64).

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The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989). Gateway® Technology enables rapid and highly efficient transfer of DNA sequences into multiple vector systems for protein expression and functional analysis while maintaining orientation and reading frame. You will:

1. Clone your gene of interest with and without a stop codon into Gateway® entry vectors to create **two** entry clones.
2. Generate **two** expression clones by performing LR recombination reactions between the appropriate entry clones and pLenti6/capTEV™-DEST vectors.

For more information on the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual, see www.invitrogen.com or contact Technical Support (page 65).

pLenti6/capTEV™-CT-GW/ARPC2 Control Plasmid

A control plasmid containing the *ARPC2* (actin protein complex component p34, Robinson *et al.*, 2001) gene fused to the capTEV™ Tag at the C-terminal end is included for use as a positive control for lentivirus production and as an expression control in the mammalian cell line of choice. For a map, see page 55.

Producing Lentivirus

How Lentivirus Works

Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). After the lentiviral construct has integrated into the genome, you may assay for transient expression of the 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 NativePure™ Lentiviral 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 lentiviral vector with a significantly broadened host cell range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).

ViraPower™ Packaging Mix

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

293FT Cell Line

The human 293FT Cell Line is supplied with NativePure™ Lentiviral Expression kits (Cat. nos. BN3004 and BN3007 only) to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo and must be maintained in medium containing Geneticin®. For more information about this cell line, refer to the 293FT Cell Line manual, which is available from www.invitrogen.com or by contacting Technical Support (see page 65). For ordering information about 293FT and Geneticin®, see pages 62–63.

Lentiviral Expression Techniques

The NativePure™ Lentiviral Expression System is designed to help you create a lentivirus to deliver and express a gene of interest 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. We highly recommend that users possess a working knowledge of viral and tissue culture techniques.

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

- Retrovirus biology and the retroviral replication cycle (Buchschacher & Wong-Staal, 2000; Luciw, 1996).
- Retroviral and lentiviral vectors (Naldini, 1998; Naldini, 1999; Pandya *et al.*, 2001; Yee, 1999).

Biosafety Features

Introduction

The NativePure™ Lentiviral Expression System is a system based on the ViraPower™ Lentiviral Expression System, which includes a 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 NativePure™ Lentiviral System

The NativePure™ Lentiviral Expression System includes the following key safety features:

- The pLenti6/capTEV™-DEST vectors contain a deletion in the 3′ LTR (ΔU3) that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yee *et al.*, 1987; Yu *et al.*, 1986; Zufferey *et al.*, 1998). Once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome.
- The number of genes from HIV-1 that are used in the system has been reduced to three (*i.e. gag*, *pol*, and *rev*).
- The VSV-G gene from Vesicular Stomatitis Virus is used in place of the HIV-1 envelope (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
- Genes encoding structural and other components required for packaging of 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 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 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).

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Biosafety Features, Continued

Biosafety Level 2

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

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

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

Important

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

Experimental Outline

Experimental Outline

Steps to create lentivirus stocks to express and analyze your recombinant biotinylated proteins of interest in the mammalian cell lines of choice are outlined below.

Step	Action
1	Clone the gene of interest with and without a stop codon into a Gateway® entry vector to create **two** entry clones.
2	Generate **two** expression clones (N- and C-terminally tagged) by performing LR recombination reactions between the appropriate entry clones and the pLenti6/capTEV™-NT-DEST1 and pLenti6/capTEV™-CT-DEST vectors.
Optional	Transiently transfect your expression clones into a mammalian cell line of choice and analyze cell lysate to confirm *in vivo* biotinylation of recombinant protein by SDS-PAGE, and detection on a western blot using a streptavidin conjugate.
3	Co-transfect each expression clone with the optimized packaging plasmid mix separately into the 293FT Cell Line to produce two lentivirus stocks.
4	Titer the two lentiviral stocks.
5	Use the two lentiviral stocks to separately transduce a mammalian cell line of interest.
6	Analyze cell lysate to confirm *in vivo* biotinylation of recombinant protein by SDS-PAGE and detection on a western blot using a streptavidin conjugate.
7	Confirm complex formation with biotinylated recombinant protein by native gel electrophoresis and detection on a western blot using a streptavidin conjugate.
Optional	Purify the biotinylated protein and associated protein complexes using NativePure™ Affinity Purification Kit.
8	Analyze protein complexes using native electrophoresis, SDS-PAGE, immunodetection, or mass spectrometry.

Methods

Generating Entry Clones

Introduction

Individual expression and *in vivo* biotinylation of the protein of interest in your mammalian cell line may vary depending on whether your protein of interest is fused to an N-terminal or C-terminal tag. Recombine your gene of interest into both pLenti6/capTEV™-DEST vectors to create both N- and C- terminally tagged expression clones. To recombine your gene of interest into both pLenti6/capTEV™-NT-DEST1 and pLenti6/capTEV™-CT-DEST vectors, you will generate two entry clones containing your gene of interest with and without a stop codon. This section provides guidelines for generating entry clones.

Creating Entry Clones

Entry vectors are available to facilitate generation of entry clones. We recommend pENTR™/D-TOPO® or pCR®8/GW/TOPO® for rapid cloning of your gene of interest using TOPO® technology (see page 62 for ordering information).

You may also perform a BP recombination reaction using a PCR product containing *att*B sites and an *att*P-containing pDONR™ vector to create your entry clone. Refer to page 62 for pDONR™201, pDONR™221, and pDONR™/Zeo ordering information.

For more information, go to www.invitrogen.com or contact Technical Support (page 65). Refer to the manual for the specific vector you are using for detailed instructions to construct entry clones.

Note

Ultimate™ ORF (open reading frame) clones are fully-sequenced human and mouse clones supplied in a Gateway®-compatible entry vector. If you are using an Ultimate™ ORF clone as the source of your gene of interest, you may do the following:

For N-terminal tagged protein:

Use Ultimate™ ORF clones directly as an entry clone for LR recombination with pLenti6/capTEV™-NT-DEST1 to generate your entry clone.

For C-terminal tagged protein:

- Do **NOT** use Ultimate™ ORF clones directly as an entry clone for LR recombination with pLenti6/capTEV™-CT-DEST to generate your entry clone due to the presence of a TAG **stop codon**.
- Use Ultimate™ ORF clone as a template to amplify the gene of interest using primers that modify the stop codon, and clone the template **without** the stop codon into the entry vector of choice.

For more information about the Ultimate™ ORF collection, go to www.invitrogen.com or contact Technical Support (page 65).

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N- and C-Terminal Expression Clones

Recombine your gene of interest into both pLenti6/capTEV™-DEST vectors to create both N- and C- terminally tagged expression clones. You will need to create two entry vectors containing your gene of interest with either a stop codon (N-terminal tagged, recombine with pLenti6/capTEV™-NT-DEST1) or a Kozak translation initiation sequence and no stop codon (C-terminal tagged, recombine with pLenti6/capTEV™-CT-DEST). These required elements are summarized below.

To make an entry clone to recombine with…	Then your gene of interest must contain…
pLenti6/capTEV™-NT-DEST1	• **Stop codon**
pLenti6/capTEV™-CT-DEST	• **Kozak consensus sequence** (see below) • **No stop codon**

Make sure that your gene of interest is in frame with the N- or C-terminal capTEV™ tag and other vector elements after performing the LR recombination reaction with the pLenti6/capTEV™-DEST vectors. Refer to pages 14–15 to see the recombination regions of the vectors.

Kozak Consensus Sequence

To recombine your entry clone with a destination vector for mammalian expression, your insert should contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (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**

Creating N- and C-Terminal Tagged Expression Clones

Introduction

After generating an entry clone, you will perform LR recombination reactions to transfer the gene of interest into the pLenti6/capTEV™-DEST vectors to create your expression clones. See the next pages for illustrations of the recombination regions of expression clones in pLenti6/capTEV™-CT-DEST and pLenti6/capTEV™-NT-DEST1 vectors. To ensure that you obtain the best possible results, read this section and the sections entitled **Performing the LR Recombination Reaction** (pages 16–17) and **Transforming One Shot® Stbl3™ Competent *E. coli*** (pages 18–19) before beginning.

Experimental Outline

To generate expression clones:

1. Perform LR recombination reactions using the *att*L-containing entry clones and the *att*R-containing pLenti6/capTEV™-DEST vectors

   Note: Both entry clones and destination vectors should be supercoiled.

2. Transform the reaction mixtures separately into One Shot® Stbl3™ Chemically Competent *E. coli*.
3. Select for expression clones.

Important

Do not propagate the pLenti6/capTEV™-DEST vectors due to the possibility of unwanted recombination. The amount of each plasmid supplied is sufficient for 40 reactions. For ordering information to purchase these plasmids separately, see page 62, or go to www.invitrogen.com.

Continued on next page

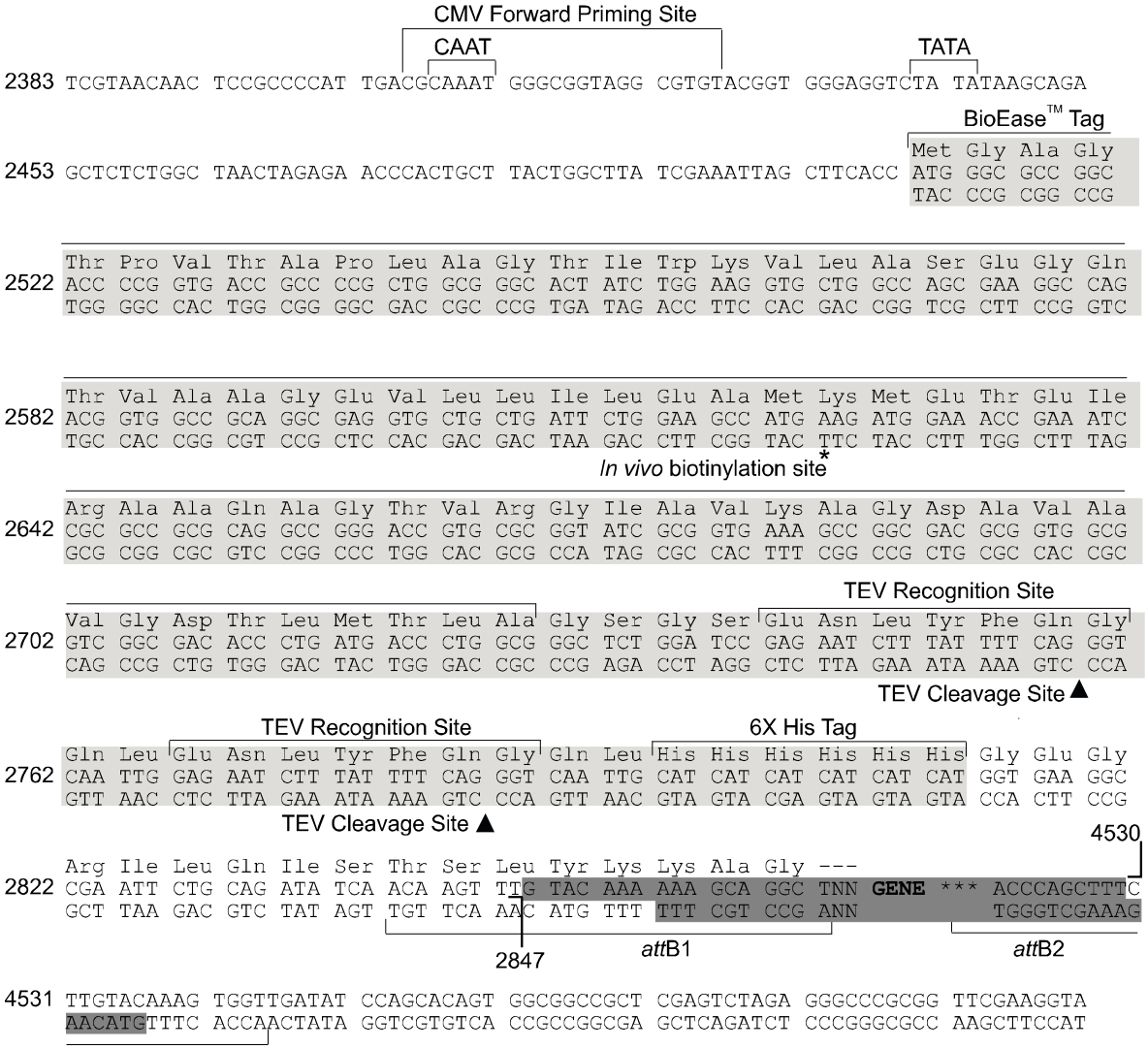
Creating N- and C-Terminal Tagged Expression Clones,
Continued

Recombination Region of pLenti6/capTEV™-NT-DEST1

The recombination region of the expression clone resulting from pLenti6/capTEV™-NT-DEST1 × entry clone is shown below.

Features of the Recombination Region:

- Dark shaded regions correspond to those DNA sequences transferred from the entry clone into the pLenti6/capTEV™-NT-DEST1 vector by recombination.
- Light shaded regions correspond to the capTEV™ Tag.
- Non-shaded and light shaded regions are derived from pLenti6/capTEV™-NT-DEST1.
- Bases 2,847 and 4,530 of the pLenti6/capTEV™-NT-DEST1 sequence are marked.



Continued on next page

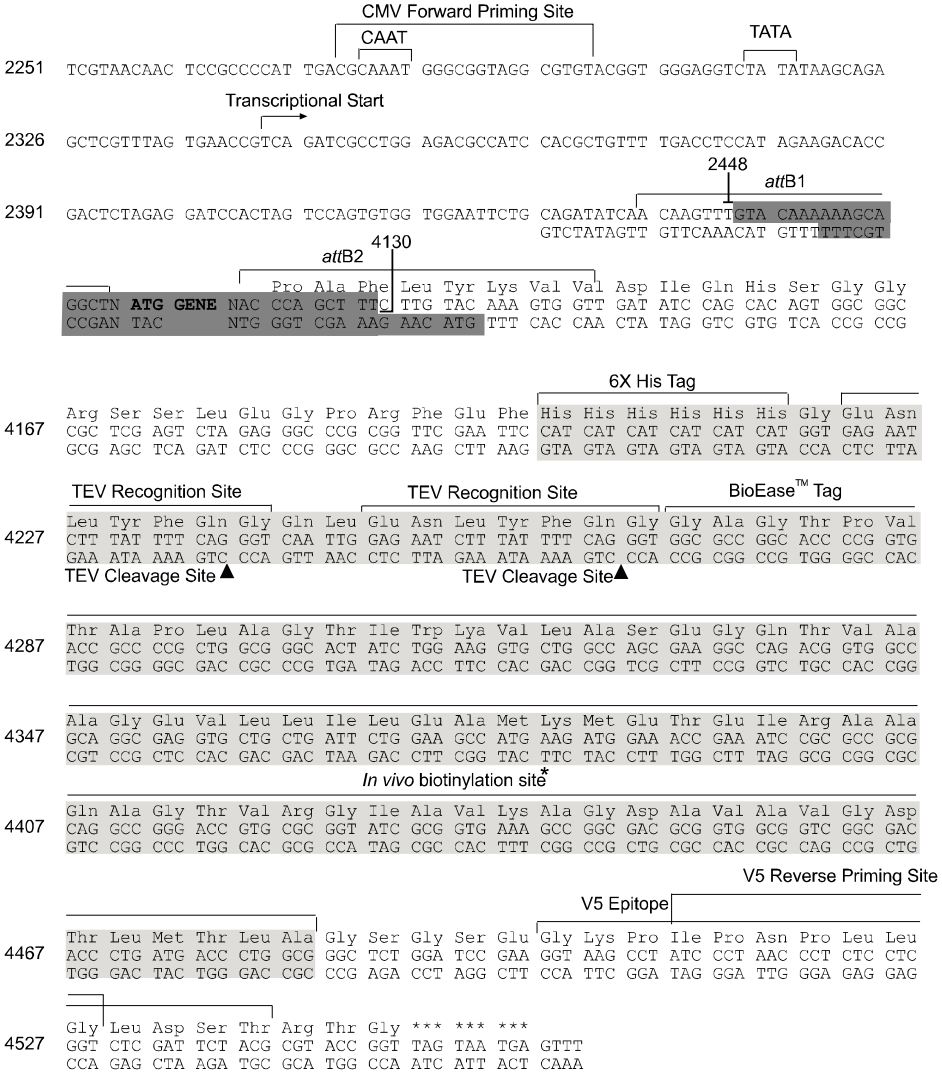
Creating N- and C-Terminal Tagged Expression Clones,
Continued

Recombination Region of pLenti6/capTEV™-CT-DEST

The recombination region of the expression clone resulting from pLenti6/capTEV™-CT-DEST × entry clone is shown below.

Features of the Recombination Region:

- Dark shaded regions correspond to those DNA sequences transferred from the entry clone into the pLenti6/capTEV™-CT-DEST vector by recombination.
- Light shaded regions correspond to the capTEV™ Tag.
- Non-shaded and light shaded regions are derived from the pLenti6/capTEV™-CT-DEST vector.
- Bases 2,448 and 4,130 of the pLenti6/capTEV™-CT-DEST sequence are marked.



Performing LR Recombination Reactions

Introduction

After obtaining the entry clones containing your gene of interest, perform LR recombination reactions between the entry clones and pLenti6/capTEV™-DEST vectors, and transform the reaction mixture into One Shot® Stbl3™ Competent *E. coli* to select for expression clones. Include a negative control (no Gateway® LR Clonase® II) in the experiment to help evaluate results.

E. coli Host

Transform the LR reactions into One Shot® Stbl3™ Competent *E. coli* (included with Cat. nos. BN3004 and BN3007, also available separately, page 62) to reduce the likelihood of unwanted recombination. **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.,* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Gateway® LR Clonase® II Enzyme Mix

Gateway® LR Clonase® II enzyme mix catalyzes the LR recombination reactions (included with Cat. nos. BN3004 and BN3007, also available separately, see page 62). Use the protocol provided on the next page to perform the LR recombination reactions using the Gateway® LR Clonase® II enzyme mix.

Materials Needed

- Purified plasmid DNA of your entry clone with stop codon (50–150 ng/µL in TE, pH 8.0)
- Purified plasmid DNA of your entry clone without stop codon (50–150 ng/µL in TE, pH 8.0)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), see page 62 for ordering information

Components supplied with the kits

- pLenti6/capTEV™-NT-DEST1 (150 ng/µL in TE, pH 8.0)
- pLenti6/capTEV™-CT-DEST (150 ng/µL in TE, pH 8.0)

Components supplied with the NativePure™ Lentiviral Expression Kit and the NativePure™ Lentiviral Expression and Affinity Purification System

- Gateway® LR Clonase® II enzyme mix (keep at −20°C until immediately before use)
- 2 µg/µL Proteinase K solution (thaw and keep on ice until use)
- pENTR™-gus (use as a control for the LR reaction; 50 ng/µL)

Continued on next page

LR Reaction

Follow this procedure to perform both LR reactions between each of your entry clones and pLenti6/capTEV™-DEST vectors. To include a negative control, set up a second sample reaction, but omit the Gateway® LR Clonase® II enzyme mix.

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

Component	NT-tag Entry Clone	CT-tag Entry Clone	Positive Control
Entry clone (without stop, 50–150 ng/rxn)	–	1–7 µL	–
Entry clone (with stop, 50–150 ng/rxn)	1–7 µL	–	–
pLenti6/capTEV™-NT-DEST1 (150 ng/µL)	1 µL	–	1 µL
pLenti6/capTEV™-CT-DEST (150 ng/µL)	–	1 µL	–
pENTR™-gus (50 ng/µL)	–	–	2 µL
TE Buffer, pH 8.0	to 8 µL	to 8 µL	5 µL

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

   Reminder: Return Gateway® LR Clonase® II enzyme mix to −20°C immediately after use.
5. Incubate reactions 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 the next section to transform the LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli*.

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

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

Introduction

Follow the instructions in this section to transform the LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli* (supplied with Cat. nos. BN3004 and BN3007 only; see page 62 to order separately). 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 6, previous page)
- LB Medium (if performing the pUC19 control transformation)
- 42°C water bath
- LB plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- 37°C shaking and non-shaking incubators

Components supplied with the NativePure™ Lentiviral Expression Kit and the NativePure™ Lentiviral Expression and Affinity Purification System

- One Shot® Stbl3™ Chemically Competent *E. coli* (one vial per transformation; thaw on ice immediately before use); may also be purchased separately (see page 62)
- S.O.C. Medium (warm to room temperature)
- pUC19 positive control (if desired to verify the transformation efficiency)

One Shot® Stbl3™ Transformation Procedure

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

1. Thaw one vial of One Shot® Stbl3™ Chemically Competent *E. coli* on ice for each transformation.
2. Add 2–3 µL of the LR recombination reaction (from Step 6, previous page) into a vial of One Shot® Stbl3™ cells and mix gently. **Do not mix by pipetting up and down.** For the pUC19 control, add 10 pg (1 µL) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C water bath and place 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. Plate two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium (*e.g.*, add 100 µL of the transformation mix to 900 µL of LB Medium) and plate 25–100 µL.
9. Store the remaining transformation mix at 4°C. Plate out additional cells the next day, if desired.
10. Proceed to **Analyzing Transformants**, page 20.

Continued on next page

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

Expected Results

If you use *E. coli* cells with a transformation efficiency of 1×10^8 cfu/µg, the LR reaction should result in greater than 5,000 colonies if the entire LR reaction is transformed and plated.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be chloramphenicol-sensitive **and** ampicillin- and Blasticidin-resistant. Transformants containing a plasmid with a mutated *ccdB* gene will be chloramphenicol-, ampicillin-, and Blasticidin- resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/mL chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

Analyzing Transformants

Introduction

Analyze the transformants using restriction digestion as described below, even if you have observed a successful LR recombination. 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.

Screen colonies by performing miniprep and restriction analysis to validate the correct N- and C-terminal expression clones. After verifying the correct clones, use the miniprep DNA to re-transform Stbl3™ *E. coli*. Next, 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 or large scale DNA preparation.

Experimental Outline

Step	Action
1	For each transformation, pick 5 resistant colonies from plating the transformation mix (Step 8, page 18). Culture cells overnight in LB medium containing 100 µg/mL ampicillin.
2	Isolate plasmid DNA for each colony using a miniprep kit (see **Important**, next page).
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	Re-transform One Shot® Stbl3™ Chemically Competent *E. coli* separately with the validated clones.
5	Inoculate LB-ampicillin with a fresh colony and grow for 6–8 hours to generate a starter culture.
6	Inoculate the starter culture 1:1,000 into at least 100 mL LB-ampicillin and grow for 18 hours.
7	Isolate plasmid DNA for the N- and C-terminally tagged pLenti6/capTEV™ clones using a midiprep kit or large scale DNA preparation (see **Important**, next page).
Optional	Sequence the plasmids to determine that your gene of interest is in frame with the N- and C-terminal tags.
Optional	Transiently transfect a mammalian cell line of your choice with your pLenti6/capTEV™ clones to check for protein expression and biotinylation.

Continued on next page

Analyzing Transformants, Continued

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™ HiPure Plasmid DNA Purification MidiPrep Kits (page 62).

Restriction Digest

To confirm that no rearrangement in the LTR regions of the plasmids has taken place, perform restriction digests using *Afl* II and *Xho* I. *Afl* II sites are present in both LTRs. The *Xho* I site is present after the 3′ end of the *att*R recombination sites. 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 the pLenti6/capTEV™-DEST vectors are available at www.invitrogen.com.

Materials Needed

- LB medium containing 100 µg/mL ampicillin
- PureLink™ HQ Mini Plasmid Purification Kit (page 62) or equivalent
- Appropriate restriction enzymes (see above)
- E-Gels® 1.2% agarose gels (page 62) or equivalent
- PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit (page 62) or equivalent

Screening Colonies by Miniprep

1. For each transformation, pick 5 colonies from plates obtained after plating the transformation mix (Step 8, page 18). Culture them overnight in LB medium containing 100 µg/mL ampicillin.
2. Isolate plasmid DNA using PureLink™ HQ Mini Plasmid Purification Kit or equivalent (see **Important**, above).
3. Perform restriction digests on plasmid DNA.
4. Analyze the digested DNA on 1.2% agarose gels to confirm N- and C-terminally tagged pLenti6/capTEV™ expression clones.

Continued on next page

Restriction Enzyme Digest Results

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

Isolating Lentiviral Plasmid DNA

This protocol provides general steps to retransform Stbl3™ *E. coli* and perform isolation of plasmid DNA for lentivirus production. pLenti plasmid DNA midipreps often have lower yields; therefore, a 100 mL volume of culture must be used for one DNA midiprep.

1. Dilute 1 µL of miniprep plasmid DNA from the positive clones 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 18.
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 mL LB medium containing 100 µg/mL ampicillin for 6–8 hours at 37°C to obtain a starter culture.
5. Inoculate 1:1,000 of the starter culture into LB medium containing 100 µg/mL ampicillin (*e.g.*, inoculate 100 µL of starter culture in 100 mL LB-ampicillin) and culture at 37°C overnight.

   Note: Use at least 100 mL volume for large scale or midiprep isolation of DNA.
6. Isolate plasmid DNA using PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit or equivalent (see **Important**, page 21).
7. Perform restriction analysis to confirm the presence of the insert.
8. Use the purified plasmid DNA from the positive clone for producing the lentivirus (see below and page 24).

DNA Isolation Guidelines

Once you have generated and validated the pLenti6/capTEV™ expression clones, isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating lentiviral plasmid DNA using the PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit (see page 62 for ordering information).

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

Maintaining the Expression Clone

Once you have generated your expression clone, maintain and propagate the plasmid in LB medium containing 100 µg/mL ampicillin.

Continued on next page

Sequencing

Confirm that your gene of interest is in frame with the N- or C-terminal tag by sequencing your expression constructs. We recommend using the following primers. Refer to the diagrams on pages 14–15 for the location of the primer binding sites in each pLenti6/capTEV™ vector.

Vector	Primer	Sequence
pLenti6/capTEV™-NT-DEST1	CMV forward	5′-CGCAAATGGGCGGTAGGCGTG-3′
pLenti6/capTEV™-CT-DEST	V5 reverse	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Note: For custom primer synthesis information go to www.invitrogen.com or contact Technical Support (see page 65).

Transient Transfection

Prior to lentivirus production, you may transfect your expression constructs separately into a mammalian cell line of choice to determine protein expression, biotinylation and complex formation. Perform transient transfection using any method of choice, and analyze cells as described in **Detecting Protein Biotinylation and Complex Formation,** page 38.

Producing Lentivirus in 293FT Cells

Introduction

To produce lentiviral stocks (containing the packaged pLenti expression constructs), cotransfect the optimized packaging plasmid mix and your pLenti expression constructs into the 293FT Cell Line. The following section provides protocols and instructions to generate lentiviral stocks.

293FT Cell Line

The human 293FT Cell Line is supplied with NativePure™ Lentiviral Expression Kits (Cat. nos. BN3004 and BN3007, also available separately) to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo and must be maintained in medium containing Geneticin®.

For more information about pCMVSPORT6TAg.neo and how to culture and maintain 293FT cells, refer to the 293FT Cell Line manual. This manual is supplied with the NativePure™ Lentiviral Expression kits, and is also available from www.invitrogen.com or by contacting Technical Support (see page 65).

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

- Make sure that cells are healthy and greater than 90% viable.
- Subculture and maintain cells in complete medium supplemented with 0.1 mM MEM Non-Essential Amino Acids, 2 mM Glutamine, 1 mM sodium pyruvate, 500 µg/mL Geneticin®, and 10% fetal bovine serum that is not heat-inactivated (see page 62).
- Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 20 passages.

Positive Control

Include a positive control vector in the cotransfection experiment to generate a control lentiviral stock that may be used to optimize expression conditions in your mammalian cell line of interest. A positive control vector pLenti6/capTEV™-CT-GW/ARPC2 is included for use as an expression control. See page 55 for a plasmid map of the control vector.

Continued on next page

Lipofectamine® 2000

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 are not required, although complexes can be removed after 4–6 hours without loss of activity

Lipofectamine® 2000 is supplied with Cat. nos. BN3004 and BN3007, and is available separately or as part of the ViraPower™ Bsd Lentiviral Support Kits (see page 62 for ordering information).

Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine® 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium (see page 62).

Recommended Procedure

If you are a first time or inexperienced user with producing lentivirus using the ViraPower™ System and 293FT cells, perform the recommended procedure (**Forward Transfection**) on page 27. This procedure requires plating the cells the day before transfection to obtain cells that are 90–95% confluent.

Note: In previous ViraPower™ Lentivirus manuals, this protocol was called the Alternate Transfection Method.

If you are an experienced lentivirus user and are familiar with the growth characteristics of 293FT cells, you may choose to perform the rapid procedure (**Reverse Transfection**) on page 28. In this procedure, the 293FT cells are added to media containing the DNA-Lipofectamine® 2000 complexes and allowed to attach.

Continued on next page

Producing Lentivirus in 293FT Cells, Continued

Materials Needed

- pLenti6/capTEV™ N- and C-tagged expression clones (0.1–3.0 µg/µL in sterile water or TE, pH 8.0)
- Opti-MEM® I Reduced Serum Medium (pre-warmed to 37°C)
- Fetal bovine serum (FBS)
- Complete growth medium containing sodium pyruvate (*i.e.* D-MEM supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin-streptomycin, and 1 mM MEM Sodium Pyruvate)

  Note: D-MEM already contains 4 mM L-glutamine, which is enough to support cell growth of the 293FT Cell Line. However, since L-glutamine slowly decays over time, supplement the medium with 2 mM L-glutamine. 293FT cells grow well in 6 mM L-glutamine, but higher concentrations of L-glutamine may reduce growth.
- Sterile, 10 cm tissue culture plates (one each for the lentiviral construct, positive control, and negative control)
- Sterile tissue culture supplies
- 15 mL sterile, capped, conical tubes
- Millex®-HV 0.45 µm PVDF filters or equivalent
- Cryovials

Components supplied with the kits

- Control vector pLenti6/capTEV™-CT-GW/ARPC2

Components supplied with the NativePure™ Lentiviral Expression Kit and the NativePure™ Lentiviral Expression and Affinity Purification System only

- ViraPower™ Packaging Mix
- 293FT cells cultured in the appropriate medium
- Lipofectamine® 2000 transfection reagent (store at 4°C, mix gently before use)

Recommended Transfection Conditions

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

Condition	Amount
Tissue culture plate size	10 cm (one per lentiviral construct)
Number of 293FT cells to transfect	5–6 × 10^6 cells depending on method (see **Recommendation** on page 24 to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix	9 µg (9 µL of 1 µg / µL stock)
Amount of pLenti expression plasmid	3 µg
Amount of Lipofectamine® 2000	36 µL

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

Continued on next page

Forward Transfection Procedure

If you are a **first time or inexperienced user**, follow the procedure below to cotransfect 293FT cells. Include a negative control (no DNA, no Lipofectamine® 2000) in your experiment to help you evaluate results.

1. The day before transfection (Day 1), plate 293FT cells in a 10 cm tissue culture plate so that they will be 90–95% confluent on the day of transfection (*i.e.* 5×10^6 cells in 10 mL of growth medium containing serum).
2. On the day of transfection (Day 2), remove the culture medium from the 293FT cells and replace with 5 mL of growth medium (or Opti-MEM® I Medium) containing serum. **Do not include antibiotics in the medium.**
3. **For each transfection sample**, prepare DNA-Lipofectamine® 2000 complexes as follows:
   a. In a sterile 15 mL tube, combine 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti expression plasmid DNA (12 µg total) in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently.
   b. In a separate sterile 15 mL tube, mix Lipofectamine® 2000 gently before use, then dilute 36 µL in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine® 2000. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine® 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
4. Add the DNA-Lipofectamine® 2000 complexes dropwise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO_2 incubator.
5. The next day (Day 3), remove the medium containing the DNA-Lipofectamine® 2000 complexes and replace with complete culture medium. Incubate at 37°C in a CO_2 incubator.

   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia. This morphological change is normal and does not affect production of the lentivirus.
6. Harvest virus-containing supernatants 48–72 hours posttransfection (Day 4–5) by removing medium into to a 15 mL sterile, capped, conical tube. Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours posttransfection.

   Caution: Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see pages 8 and 32 for more information).
7. Centrifuge supernatants at 2,000 × *g* for 15 minutes at 4°C to pellet debris.
8. Filter the viral supernatants through a Millex®-HV 0.45 µm or equivalent PVDF filter.
9. Pipet viral supernatants into cryovials in 1 mL aliquots. Store viral stocks at −80°C. Proceed to **Titering Lentiviral Stocks**, page 30.

Continued on next page

Reverse Transfection Procedure

If you are an **experienced user**, you may use the rapid procedure below to cotransfect 293FT cells. We recommend including a negative control (no DNA, no Lipofectamine® 2000) in your experiment to help evaluate results. You will need 6×10^6 293FT cells for each sample.

1. On Day 1, prepare DNA-Lipofectamine® 2000 complexes **for each transfection sample** as follows:
   a. In a sterile 5 mL tube, combine 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti expression plasmid DNA (12 µg total) in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently.
   b. In a separate sterile 5 mL tube, mix Lipofectamine® 2000 gently before use, then dilute 36 µL in 1.5 mL of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine® 2000. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine® 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
2. While DNA-lipid complexes are forming, trypsinize and count the 293FT cells. Resuspend the cells at a density of 1.2×10^6 cells/mL in growth medium (or Opti-MEM® I Medium) containing serum. **Do not include antibiotics in the medium.**
3. Add the DNA-Lipofectamine® 2000 complexes to a 10 cm tissue culture plate containing 5 mL of growth medium (or Opti-MEM® I Medium) containing serum. **Do not include antibiotics in the medium.**
4. Add 5 mL of the 293FT cell suspension (6×10^6 total cells) to the plate containing media and DNA-Lipofectamine® 2000 complexes. Mix gently by rocking the plate back and forth. Incubate cells overnight at 37°C in a CO_2 incubator.
5. The next day (Day 2), remove the medium containing the DNA-Lipofectamine® 2000 complexes and replace with complete culture medium. Incubate cells overnight at 37°C in a CO_2 incubator.

   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of large, multinucleated cells known as syncytia. This morphological change is normal and does not affect production of the lentivirus.
6. Harvest virus-containing supernatants 48–72 hours posttransfection (Day 3–4) by removing medium into a 15 mL sterile, capped, conical tube. Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours posttransfection.

   Caution: Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see pages 8 and 32 for more information).
7. Centrifuge supernatants at 2,000 × *g* for 15 minutes at 4°C to pellet debris.
8. Filter the viral supernatants through a Millex®-HV 0.45 µm or equivalent PVDF filter.
9. Pipet viral supernatants into cryovials in 1 mL aliquots. Store viral stocks at −80°C. Proceed to **Titering Lentiviral Stocks**, page 30.

Continued on next page

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their ability to transduce cells. If your cell transduction experiment requires that you use a relatively high MOI, you may wish to concentrate your virus before titering and proceeding to transduction. For details and guidelines, refer to published reference sources (Yee, 1999).

Long-Term Storage

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

Scaling Up Virus Production

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

Titering Lentiviral Stocks

Introduction

Before proceeding to transduction and expression experiments, we highly recommend that you determine the titer of your pLenti6/capTEV™ lentiviral stocks. Determining the viral titer is necessary if:

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

Guidelines and protocols are provided in this section to titer your lentiviral stocks.

Experimental Outline

To determine the titer of lentiviral stocks, you will:

1. Prepare 10-fold serial dilutions of your lentiviral stocks.
2. Transduce the different dilutions of lentivirus in the presence of the polycation Polybrene® into a mammalian cell line (HT1080 is recommended).
3. Select for stably transduced cells using Blasticidin.
4. Stain and count the number of Blasticidin-resistant colonies in each dilution.

Factors Affecting Viral Titer

A number of factors can influence viral titers including:

- **The size of your gene of interest-** Titers will generally decrease as the size of the insert increases. The size of the wild-type HIV-1 genome is approximately 10 kb. Since the size of the elements required for expression from pLenti6/capTEV™ vectors total approximately 4 kb, the size of your gene of interest should theoretically not exceed 6 kb for efficient packaging.
- **The characteristics of the cell line used for titering-** We strongly recommend the human fibrosarcoma line HT1080 as the "gold standard" for reproducibly titering lentivirus. However, other cell lines may be used. In general, these cells should be an adherent, non-migratory cell line, and exhibit a doubling time in the range of 18–25 hours.
- **The age of your lentiviral stock-** Viral titers may decrease with long-term (>1 year) storage at –80°C. If your lentiviral stock has been stored for longer than 6 months, titer your lentiviral stock prior to use.
- **Number of freeze/thaw cycles-** Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- **Improper storage of your lentiviral stock-** Lentiviral stocks should be stored at –80°C in cryovials.

Selecting a Cell Line for Titering

We strongly recommend the human fibrosarcoma line HT1080 (ATCC, Cat. no. CCL-121) as the "gold standard" for reproducibly titering lentivirus. However, you may wish to use the same mammalian cell line to titer your lentiviral stocks as you will use to perform your expression studies (*e.g.,* if you are performing expression studies in a dividing cell line or a non-primary cell line). For more information on cells for titering, see **Factors Affecting Viral Titer**, above.

Continued on next page

Titering Lentiviral Stocks, Continued

Antibiotic Selection

The pLenti6/capTEV™ expression constructs contain the Blasticidin resistance gene (*bsd*) (Kimura *et al.*, 1994) to allow for Blasticidin selection (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) of mammalian cells that have stably transduced the lentiviral construct. Blasticidin is available separately or as part of the ViraPower™ Bsd Lentiviral Support Kit (see page 62 for ordering information).

Preparing Blasticidin

For more information about how to prepare and handle Blasticidin, refer to the **Appendix**, page 50.

Determining Antibiotic Sensitivity

Since you will be selecting for stably transduced cells using Blasticidin during the titer procedure, 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. Test a range of concentrations (see protocol below) to ensure that you determine the minimum Blasticidin concentration necessary for your cell line.

1. Plate cells at approximately 25% confluence. Prepare a set of 6–7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Blasticidin.
3. Replenish selective media every 3–4 days, and observe percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10–14 days after addition of antibiotic.

Using Polybrene® During Transduction

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

Continued on next page

Titering Lentiviral Stocks, Continued

Preparing and Storing Polybrene®

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

1. Prepare a 6 mg/mL stock solution in deionized, sterile water.
2. Filter-sterilize and dispense 1 mL aliquots into sterile microcentrifuge tubes.
3. The working stock may be stored at 4°C for up to 2 weeks. Store at −20°C for long-term storage. Stock solutions may be stored at −20°C for up to 1 year. Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

Materials Needed

- Your pLenti6/capTEV™ lentiviral stocks (store at −80°C until use)
- Adherent mammalian cell line (HT1080 human fibrosarcoma or other)
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired
- 6-well tissue culture plates
- Crystal violet (Sigma-Aldrich®, Cat. no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
- Phosphate-Buffered Saline (PBS; page 62)

Components supplied with the NativePure™ Lentiviral Expression Kit and the NativePure™ Lentiviral Expression and Affinity Purification System

- Blasticidin (10 mg/mL stock) for selection

Remember that you will be working with media containing infectious virus. Follow the recommended Federal and institutional guidelines for working with BL-2 organisms.

- Perform all manipulations within a certified biosafety cabinet.
- Treat media containing virus with bleach.
- Treat used pipettes, pipette tips, and other tissue culture supplies with bleach and use biohazardous waste disposal.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

Continued on next page

Titering Lentiviral Stocks, Continued

Transducing and Titering Lentivirus

Follow the procedure below to determine the titer of your pLenti6/capTEV™ stocks. You will use **at least** one 6-well plate of cells for every lentiviral stock to be titered (one mock well plus five dilutions).

1. The day before transduction (Day 1), trypsinize and count the cells. Plate them in a 6-well plate such that they will be 30–50% confluent at the time of transduction. Incubate cells at 37°C in a CO_2 incubator overnight.

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

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

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

3. Remove the culture medium from the cells. Mix each dilution gently by inversion and add to one well of cells (total volume = 1 mL).
4. If using Polybrene® (see page 31), add to each well for a final concentration of 6 µg/mL. Swirl the plate gently to mix. Incubate cells at 37°C in a CO_2 incubator overnight
5. The following day (Day 3), remove the media containing virus and replace with 2 mL of complete culture medium. Incubate cells at 37°C in a CO_2 incubator overnight
6. The following day (Day 4), remove the medium and replace with complete culture medium containing the appropriate amount of Blasticidin to select for stably transduced cells (see page 31). Incubate cells at 37°C in a CO_2 incubator.
7. Replace medium with fresh medium containing Blasticidin every 3–4 days.
8. After 10–12 days of selection (day 14–16), you should see no live cells in the mock well and discrete antibiotic-resistant colonies in one or more of the dilution wells. Remove the medium and wash the cells twice with PBS.
9. Add crystal violet solution (1 mL for 6-well dish; 5 mL for 10 cm plate) and incubate for 10 minutes at room temperature.
10. Remove the crystal violet stain and wash the cells with PBS. Repeat wash.
11. Count the blue-stained colonies and determine the titer of each lentiviral stock.

Expected Results

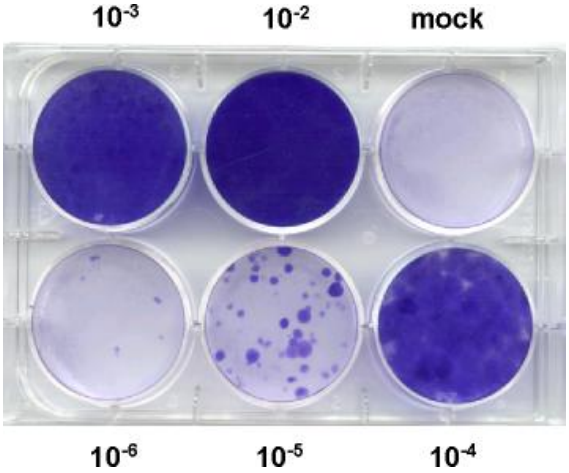
When titering lentiviral stocks using HT1080 cells, we generally obtain titers ranging from $1–5 \times 10^5$ (for unconcentrated virus) to 2×10^7 (for concentrated virus) transducing units (TU)/mL.

Continued on next page

Titering Lentiviral Stocks, Continued

Example of Expected Results

In this experiment, a control lentiviral stock was generated using the protocol on page 27. HT1080 cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^{-2} to 10^{-6} dilutions) or untransduced (mock). Forty-eight hours post-transduction, the cells were placed under Blasticidin selection (10 µg/mL). After 10 days of selection, the cells were stained with crystal violet (see plate below), and colonies were counted.



In the plate above, the colony counts were:

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

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

Next Steps

User experience, the nature of the gene, and vector backbone may affect virus titer. If the titer of your unconcentrated virus is suitable (*i.e.* 1×10^5 TU/mL or higher), proceed to **Transducing Cells with Lentivirus**, next page. If the titer of your concentrated lentiviral stock is less than 1×10^5 TU/mL, produce a new lentiviral stock. See **Troubleshooting**, page 46, for more tips and guidelines to optimize viral yield.

Transducing Cells with Lentivirus

Introduction

Once you have generated lentiviral stocks with suitable titers, transduce the lentiviral constructs into the mammalian cell line of choice and assay your recombinant proteins. Guidelines are provided below.

Important

Your lentiviral constructs contain a deletion in the 3′ LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. Once integrated into the cellular genome, the lentivirus can no longer produce packageable virus.

Transient vs. Stable Expression

After transducing lentiviral constructs into the mammalian cell line of choice, you can:

- Pool a heterogeneous population of cells and confirm biotinylation of your protein of interest and the ability of the tagged protein to form complexes directly after transduction (*i.e.* "transient" expression). Note that you must wait for a minimum of 48–72 hours after transduction before harvesting your cells to allow expressed protein to accumulate in transduced cells.
- After selecting the optimal *in vivo* biotinylated protein and the ability of the tagged protein to form complexes in your cell line, select for stably transduced cells using Blasticidin. This requires a minimum of 10–12 days following transduction, but allows generation of clonal cell lines that stably express the gene of interest (see page 45).

Determining Antibiotic Sensitivity for Your Cell Line

To select for stably transduced cells, first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). For guidelines to perform a kill curve experiment, see page 31. If you titered your lentiviral construct in the same mammalian cell line that you are using to perform the stable expression experiment, then you may use the same concentration of Blasticidin for selection that you used for titering.

Multiplicity of Infection (MOI)

To obtain optimal expression of your gene of interest, transduce the lentiviral construct into a mammalian cell line of choice using a suitable MOI. MOI is defined as the number of virus particles per cell and generally correlates with the number of integration events and as a result, expression. Typically, expression levels increase linearly as the MOI increases.

Determining the Optimal MOI

A number of factors influence the optimal MOI including the nature of your mammalian cell line (*e.g.*, non-dividing vs. dividing cell type; see **Note**, next page), its transduction efficiency, your application of interest, and the nature of your gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, use a range of MOIs (*e.g.*, 0, 0.05, 0.1, 0.5, 1, 2, 5) to determine the MOI required to obtain optimal expression of your recombinant protein for your particular application.

Continued on next page

Transducing Cells with Lentivirus, Continued

Note

In general, we have found that 80–90% of the cells in an actively dividing cell line (*e.g.*, HT1080) express a target gene when transduced at an MOI of ~1. Some non-dividing cell types are transduced with lentiviral constructs less efficiently. For example, only about 50% of the cells in a culture of primary human fibroblasts express a target gene when transduced at an MOI of ~1. If you are transducing your lentiviral construct into a non-dividing cell type, you may need to increase the MOI to achieve optimal expression levels for your recombinant protein.

Positive Control

If you have generated a lentiviral stock of an expression control (*e.g.*, pLenti6/capTEV™ CT-GW/ARPC2), use the stock to help you determine the optimal MOI for your particular cell line and application.

A control lentiviral vector containing EmGFP (pLenti6.2-GW/EmGFP) for optimizing MOI using fluorescence detection is available separately (page 62).

Important

Remember that lentiviral stocks are generated by harvesting spent media containing virus from the 293FT producer cells. Spent media lacks nutrients and may contain some toxic metabolic waste products. If you are using a large volume of viral supernatant to transduce your mammalian cell line (*e.g.*, 1 mL of viral supernatant per well in a 6-well plate), note that growth characteristics or morphology of the cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

Materials Needed

- Your titered lentiviral stocks (store at −80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired (see page 32)
- Appropriately sized tissue culture plates for your application

Components supplied with the NativePure™ Lentiviral Expression Kit and the NativePure™ Lentiviral Expression and Affinity Purification System

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

Continued on next page

Transducing Cells with Lentivirus, Continued

Transduction Procedure

1. Plate the mammalian cell line of choice in complete media as appropriate for your application.
2. On the day of transduction (Day 1), thaw your lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. If using Polybrene® (see page 31), add the optimized amount to each well for a final concentration of up to 10 µg/mL. Swirl the plate gently to mix. Incubate at 37°C in a CO_2 incubator overnight.

   Note: If you are transducing cells with undiluted viral stock and are concerned about possible toxicity or growth effects caused by overnight incubation, it is possible to incubate cells for as little as 6 hours prior to changing medium.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium. Incubate at 37°C in a CO_2 incubator overnight.
6. The following day (Day 3) harvest the cells and assay for recombinant protein biotinylation and ability to form complexes by western analysis and native gel electrophoresis (next section).

Detecting Protein Biotinylation and Complex Formation

Introduction

After transducing your cells with the pLenti6/capTEV™ N- and C-terminal lentivirus stocks, you must confirm biotinylation of your protein of interest and the ability of the tagged protein to form complexes prior to proceeding with purification or analysis experiments.

This section includes instructions to verify biotinylation using SDS-PAGE and to verify complex formation using native electrophoresis followed by western detection with a streptavidin conjugate.

Note: You may also analyze cells that have been transiently transfected with your pLenti6/capTEV™ expression clones (page 23) using these protocols, prior to producing lentivirus stocks.

Experimental Outline

To detect protein biotinylation and complex formation:

1. Prepare cell lysate using freeze-thaw cycles (no SDS buffers).
2. Analyze lysate by:
   - SDS-PAGE
   - Native gel electrophoresis
3. Perform two western blots:
   - SDS-PAGE gel to detect protein biotinylation
   - Native gel electrophoresis to detect protein complex formation
4. Develop the blots with streptavidin conjugate using the WesternBreeze® Kits (see page 64 for ordering information).

Important

The cell lysate is prepared using mild conditions for lysis to enable analysis of protein complexes. The cell lysis protocol included in this section allows you to use the same lysate for analysis using native (non-denaturing) electrophoresis and denaturing SDS-PAGE.

- Use freeze-thaw cycles for cell lysis to obtain intact protein complexes. Trypsin treatment or scraping the cells is not recommended as these methods cause cell damage and dissociation of protein complexes.
- If you have already performed trypsin treatment, inactivate trypsin using medium with 10% FBS. Wash cells three times with 1X PBS before lysing the cells.
- Perform cell lysis in the absence of NP40, as some protein complexes may be unstable in the presence of NP40.
- During lysate preparation, avoid vortexing the lysate as it can dissociate protein complexes.
- If your sample is in a SDS-PAGE sample buffer, prepare a fresh lysate **without** SDS using the protocol on page 41 for native electrophoresis. **Do not** use SDS-PAGE samples for native gel electrophoresis.

Continued on next page

Detecting Protein Biotinylation and Complex Formation, Continued

Streptavidin Conjugates

Use the strong interaction between biotin and streptavidin to easily detect your recombinant biotinylated protein with one of the following streptavidin conjugates:

Conjugate	Catalog Number
Streptavidin-HRP	SA100-01
Streptavidin-AP	43-4322

Use WesternBreeze® Chromogenic and Chemiluminescent Kits to facilitate detection of streptavidin conjugates (see page 64 for ordering information).

NativePAGE™ Gel Electrophoresis

The NativePAGE™ Novex® Bis-Tris Gel system is a near neutral pH, pre-cast polyacrylamide mini gel system used to perform **native** (non-denaturing) electrophoresis. The near neutral pH 7.5 environment during electrophoresis results in maximum stability of both proteins and gel matrix, providing better band resolution than other gel systems including the traditional Tris-glycine native electrophoresis (Laemmli) system. The NativePAGE™ Novex® Bis-Tris Gel system provides a sensitive and high-resolution method for analysis of native membrane protein complexes, native soluble proteins, molecular mass estimations, and purity assessments of native proteins.

A variety of NativePAGE™ gels and pre-made buffers for native electrophoresis are available (see page 64).

SDS-PAGE

Use NuPAGE® Novex Bis-Tris Gels, Novex® Tris-Glycine Gels, or any other SDS/PAGE gel of choice for performing SDS/PAGE. Select an appropriate acrylamide percentage that will best resolve your proteins of interest.

Note

The N-terminal fusion tag adds approximately 12.3 kDa to the size of your protein. The C-terminal fusion tag adds approximately 15.1 kDa to the size of your protein.

Continued on next page

Detecting Protein Biotinylation and Complex Formation, Continued

Materials Needed

- Transiently transduced cells (*i.e.* 48 hours after transduction)
- 1X phosphate-buffered saline (PBS, see page 62)
- Complete protease inhibitor (Roche Cat. no. 04693116001 or equivalent)
- Pepstatin (Roche Cat. no. 10253286001 or equivalent)
- Deionized water
- Protein quantification kit (such as Quant-iT™ Protein Assay Kit, page 62)
- Protein standards
- *Optional:* Benzonase nuclease
- NativePAGE™ gels (page 64) for native electrophoresis
- NuPAGE® Novex® Bis-Tris Gels or Tris-Glycine gels (page 63) for SDS-PAGE
- Appropriate units for electrophoresis and blotting
- Streptavidin conjugate (page 64)
- WesternBreeze® Detection Kits (page 64) or equivalent

Components supplied with the NativePure™ Lentiviral Affinity Purification Kit and the NativePure™ Lentiviral Expression and Affinity Purification System

- NativePure™ 5X Lysis/Binding Buffer (see below for buffer composition)

1X Lysis Buffer

For each experiment, you will have 4 transiently transduced cell samples: (N- and C-terminal, positive and negative controls). Make ~2–4 mL of 1X Lysis Buffer per sample, depending on the volume of your samples (*i.e.* 30 mL flask, 10 cm dish, T-175 flask, see next section).

Prepare 1X Lysis Buffer:

100 mM Tris-HCl, pH 8.0

100 mM KCl

200 µM EDTA

1.5 mM $MgCl_2$

1X (700 ng/mL) Pepstatin (Roche Cat. no. 10253286001 or equivalent)

Complete protease inhibitor (Roche Cat. no. 04693116001 or equivalent)

Store the buffer on ice until use. Aliquot the buffer and store the aliquots at −20ºC, if needed.

For Cat. nos. BN3005 and BN3007, refer to the manual supplied with the NativePure™ Affinity Purification Kit for details.

Continued on next page

Detecting Protein Biotinylation and Complex Formation, Continued

Preparing Cell Lysate Under Native Conditions

1. Harvest **suspension cells** by centrifugation. We generally use cells from a 30 mL flask. Wash the cells twice in phosphate buffered saline (PBS). Resuspend the cell pellet in 4 mL 1X Lysis Buffer (see previous page for a recipe). Proceed to Step 4.
2. Wash **adherent cells** with PBS. Remove the PBS and add 0.5–1 mL 1X Lysis Buffer/10 cm culture dish containing adherent cells. For a T-175 flask, use 2 mL 1X Lysis Buffer.
3. Harvest cells by pipetting up and down. Transfer the cells to a sterile tube.
4. Perform 3 freeze-thaw cycles to lyse the cells.
5. Centrifuge the lysate at 10,000 × g for 10 minutes at 4ºC.
6. Transfer the post-nuclear supernatant to a sterile tube. Aliquot the supernatant and perform protein estimation on an aliquot of the lysate using the Quant-iT™ Protein Kit (page 62) or Bradford protein assay. Store aliquots at –80ºC until use.

Native Gel Electrophoresis

For samples with high DNA content, pretreat the samples with benzonase (endonuclease) to reduce protein streaking as follows:

1. To the sample from Step 6 of the lysis treatment (above), add $MgCl_2$ to a final concentration of 2 mM and 1–2 units of benzonase per µL of sample. Mix well and incubate at room temperature for 30–60 minutes.
2. Centrifuge the lysate at 20,000 × g for 30 minutes at 4°C.
3. For NativePAGE™ electrophoresis, add NativePAGE™ Sample Buffer (4X) to obtain a final concentration of 1X in the sample. **Do not heat the samples.**
4. Load the samples onto the NativePAGE™ Gel and load NativeMark™ Unstained Protein Standard (page 64).
5. Perform electrophoresis using the conditions listed in the NativePAGE™ manual.

SDS-PAGE

1. To the sample from Step 6 of **Preparing Cell Lysate Under Native Conditions**, above, add NuPAGE® LDS Sample Buffer (4X) or Tris-Glycine SDS Sample Buffer (2X) to obtain a final concentration of 1X in the sample.
2. Add reducing agent (DTT) to a final concentration of 50 mM.
3. Heat the samples at 85°C for 2–5 minutes.
4. Load the samples onto the SDS gel and load an appropriate molecular weight standard.
5. Perform electrophoresis using the conditions listed in the manual supplied with the gel.

Continued on next page

Detecting Protein Biotinylation and Complex Formation, Continued

Western Analysis

Perform western blotting with nitrocellulose or PVDF membranes (see page 63). After blocking, probe the blot with a suitable dilution of streptavidin-AP or -HRP conjugate and develop the blot using the WesternBreeze® Chromogenic or Chemiluminescent Kits.

Expected Results

After SDS-PAGE and western blotting with a streptavidin conjugate, the protein of interest should exhibit biotinylation of the protein. Expect to see background bands due to endogenous biotinylated proteins. The expression level of the N- and C-terminally tagged proteins may vary or may be similar. In rare cases, conformation or subcellular compartmentalization variations may result in under-biotinylation of the single biotinylation site within the capTEV™ tag. If you do not observe any biotinylation on your protein of interest, see **Troubleshooting**, page 46.

Under native electrophoresis conditions, the protein of interest should migrate as a complex, indicating the ability to interact with endogenous binding partners. The ability for complex formation may vary between the N- and C-terminally tagged proteins or may be similar.

The next section shows results of a SDS-PAGE and native electrophoresis experiment and provides guidelines for interpreting your results.

The Next Steps

Based on the observed biotinylation and complex formation of your protein, choose the appropriate N-or C-terminal construct for stable transduction into your cell line (page 45) and further purification and analysis of your protein of interest. Select the construct that provides better biotinylation signal and demonstrates complex formation.

If you are performing purification of your protein of interest, refer to the NativePure™ Affinity Purification manual available from www.invitrogen.com and supplied with Cat. nos. BN3005 and BN3007.

Expected Results

Introduction

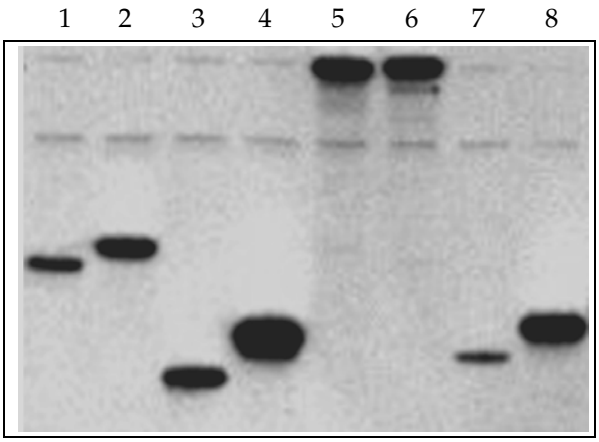
Examples of results obtained by SDS-PAGE and native gel electrophoresis followed by western blot detection to confirm biotinylation and native complex formation of a number of N- and C-terminally tagged proteins of interest are shown in this section.

Protein Biotinylation

N- and C- terminal (NT and CT) tagged expression clones for the following genes were constructed as described in this manual: actin related protein complex component p34 (ARPC2), Golgi associated protein (Bet-3), β-galactosidase (LacZ), and human proteosome subunit beta-2 (PSMB2). Freestyle™293 cells were transiently transfected with the plasmid DNA using 293fectin™.

At 24 hours post transfection, cells were harvested and lysed using the protocol on page 41. Ten micrograms of post-nuclear supernatant was electrophoresed per well on a 4–12% NuPAGE® Novex® Bis-Tris gel.

Proteins were transferred to a nitrocellulose membrane (0.45 µm) and subjected to western detection using streptavidin-alkaline phosphatase conjugate (1:4,000) and the WesternBreeze® Chemiluminescent Kit.



Lane 1: ARPC2 NT, 47.4 kDa
Lane 2: ARPC2 CT, 49.4 kDa
Lane 3: Bet-3 NT, 32.8 kDa
Lane 4: Bet-3 CT, 35.5 kDa
Lane 5: LacZ NT, 129 kDa
Lane 6: LacZ CT, 131.6 kDa
Lane 7: PSMB2 NT, 35.3 kDa
Lane 8: PSMB2 CT, 37.9 kDa

HiMark™ multicolored protein standard (not shown, see page 63) was used to determine the molecular weights of the N- and C-terminally tagged proteins. The faint band detected in all lanes is endogenous biotinylation from the lysate. The presence of multiple bands in lane 5 represents slight protein degradation.

Results

These results show that the proteins of interest are biotinylated. For some proteins, there is a difference in expression level of the N- and C-terminally tagged proteins. A difference exists in the amount of N- and C-terminally tagged versions of the same protein (*e.g.*, Lane 1 vs. Lane 2, Lane 3 vs. Lane 4, and Lane 7 vs. Lane 8). However, for LacZ (Lane 5 vs. Lane 6), both N- and C- terminally tagged constructs have similar levels of biotinylation.

Continued on next page

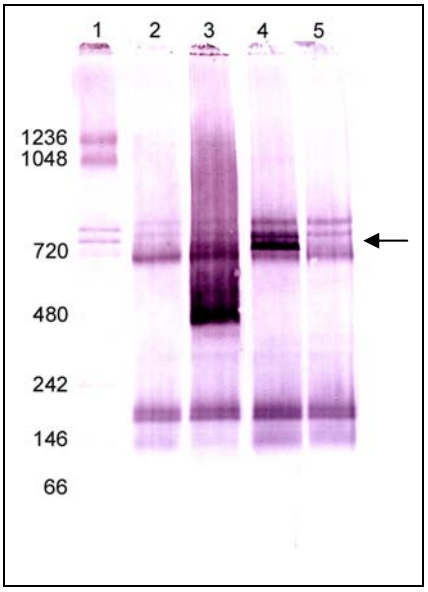
Expected Results, Continued

Native Complex Formation

N- and C- terminal tagged (NT and CT) human proteosome subunit beta-2 (PSMB2) lentivirus stocks were constructed and produced as described in this manual. GripTite™293 MSR cells were transiently transduced with the lentiviruses or with a control lentivirus (APRC2-CT).

At 48 hours post transduction, cells were harvested and lysed using the native protocol on page 41. Ten micrograms of post-nuclear supernatant was loaded per well on a 3–12% NativePAGE® Novex® Bis-Tris gel and electrophoresed.

Proteins were transferred to Invitrolon™ PVDF membrane and subjected to western detection using streptavidin-alkaline phosphatase conjugate (1:4,000) and the WesternBreeze® Chromogenic Kit. Apparent molecular weights (kDa) are listed on the left, which correspond to NativeMark™ Unstained Protein Standard.



Lane 1: 5 µL of 1:20 diluted NativeMark™ Unstained Protein Standard

Lane 2: Untransfected cell lysate (negative control)

Lane 3: APRC2 CT

Lane 4: PSMB CT tagged

Lane 5: PSMB NT tagged

Results

These results show that in the case of the human proteasome subunit beta-2 protein (PSMB), the C-terminally tagged protein forms a complex (20S proteosome complex, shown by the arrow) in lane 4, while the N-terminally tagged protein (lane 5) does not. These data also show the size of the protein complex formed.

Background bands detected in all lanes may be protein present in the cell lysate with endogenous phosphatase activity, endogenous biotinylation, or nonspecific binding.

Stable Transduction of Cells with Lentivirus

Introduction

Guidelines for transducing pLenti6/capTEV™ lentiviral constructs into your mammalian cell line and selecting for stable transformants using Blasticidin selection are provided below.

Materials Needed

- Your titered lentiviral stocks (store at −80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/mL Polybrene®, if desired (see page 31)
- Appropriately sized tissue culture plates for your application

Components supplied with the NativePure™ Lentiviral Expression Kit and the NativePure™ Lentiviral Expression and Affinity Purification System

- Blasticidin (see page 35)

Stable Transduction Procedure

1. Plate cells in complete media as appropriate for your application.
2. On the day of transduction (Day 1), thaw your lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. If using Polybrene® (see page 31), add the optimized amount to each well for a final concentration of up to 10 µg/mL. Swirl the plate gently to mix. Incubate at 37°C in a CO_2 incubator overnight.

   Note: If you are transducing cells with undiluted viral stock and are concerned about possible toxicity or growth effects caused by overnight incubation, it is possible to incubate cells for as little as 6 hours prior to changing medium.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium. Incubate at 37°C in a CO_2 incubator overnight.
6. The following day (Day 3), remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin (page 35) to select for stably transduced cells. Incubate at 37°C in a CO_2 incubator.
7. Replace medium with fresh medium containing Blasticidin every 3–4 days until Blasticidin-resistant colonies can be identified (generally 10–12 days after selection).
8. Pick at least 5 Blasticidin-resistant colonies (see **Note**, below) and expand each clone to assay for expression of the recombinant biotinylated protein.

Note

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of recombinant protein expression from different antibiotic-resistant clones. Test at least 5 antibiotic-resistant clones and select the clone that provides the optimal expression of your recombinant, biotinylated protein, and which exhibits proper complex formation for further studies.

Troubleshooting

Creating an Expression Clone

The table below lists some potential problems and possible solutions that may help you troubleshoot creating an expression clone.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Check the antibiotic resistance marker and use the correct antibiotic to select for entry clones or expression clones.
	Recombination reactions were not treated with Proteinase K	Treat reactions with Proteinase K before transformation.
	Too much entry clone was used in an LR reaction	Use equal fmol of destination vector and entry clone.
High background in the absence of the entry clone	LR reaction transformed into an *E. coli* strain containing the F′ episome and the *ccd*A gene	Use One Shot® Stbl3™ Chemically Competent *E. coli.*
Few or no colonies obtained from the transformation control	Competent cells stored incorrectly	Store competent cells at −80°C.
	Loss of transformation efficiency due to repeated freeze/thawing	Once you have thawed a tube of competent cells, discard any unused cells.

Generating the Lentiviral Stock

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

Problem	Reason	Solution
Low viral titer	Low transfection efficiency:	
	• Used poor quality expression construct plasmid DNA (*i.e.* plasmid DNA from a mini-prep)	• **Do not** use mini-prep plasmid DNA for transfection. Use the PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit or similar kit to prepare plasmid DNA.
	• Unhealthy 293FT cells; cells exhibit low viability	• Use healthy 293FT cells under passage 20; do not overgrow.
	• Cells transfected in media containing antibiotics (*i.e.* Geneticin®)	• Although Geneticin® is required for stable maintenance of 293FT cells, **do not** add Geneticin® to media during transfection as this reduces transfection efficiency and causes cell death.
	• Plasmid DNA:transfection reagent ratio incorrect	• Use a DNA (in µg):Lipofectamine® 2000 (in µL) ratio ranging from 1:2 to 1:3.
	• 293FT cells plated too sparsely	• Plate cells such that they are 90–95% confluent at the time of transfection.

Continued on next page

Generating the Lentiviral Stock, Continued

Problem	Reason	Solution
Low viral titer, continued	Transfected cells not cultured in media containing sodium pyruvate	One day after transfection, remove media containing DNA-lipid complexes and replace with media containing sodium pyruvate. Sodium pyruvate provides an extra energy source for the cells.
	Viral supernatant harvested too early	Viral supernatants can generally be collected 48–72 hours posttransfection. If many cells are still attached to the plate and look healthy at this point, wait an additional 24 hours before harvesting the viral supernatant.
	Viral supernatant too dilute	Concentrate virus using method of choice (Yee, 1999).
	Viral supernatant frozen and thawed multiple times	**Do not** freeze/thaw viral supernatant more than 3 times.
	Poor choice of titering cell line	Use HT1080 cells.
	Gene of interest is large	Viral titers generally decrease as the size of the insert increases; inserts larger than 6 kb are not recommended.
	Gene of interest is toxic to cells	Do not generate constructs containing activated oncogenes or potentially harmful genes.
	Polybrene® not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene®.
	Lipofectamine® 2000 handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion before use. **Do not vortex.**
No colonies obtained upon titering	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve experiment. Use the minimum antibiotic concentration required to kill your untransduced cell line.
	Viral stocks stored incorrectly	Aliquot and store stocks at −80°C. Do not freeze/thaw more than 3 times.
	Polybrene® not included during transduction	Transduce the lentiviral construct into cells in the presence of Polybrene®.
Titer indeterminable; cells confluent	Too little antibiotic used for selection	Increase amount of antibiotic used for selection.
	Viral supernatant not diluted sufficiently	Titer lentivirus using a wider range of 10-fold serial dilutions (*e.g.*, 10^{-2} to 10^{-8}).

Continued on next page

Transducing Mammalian Cells

The table below lists some potential problems and possible solutions that may help you troubleshoot transduction and expression experiments.

Problem	Reason	Solution
No expression of the gene of interest	Promoter silencing	Lentiviral constructs may integrate into a chromosomal region that silences the CMV promoter controlling expression of the gene of interest. Screen multiple antibiotic-resistant clones and select the clone that gives optimal biotinylated protein expression.
	Viral stocks stored incorrectly	Aliquot and store stocks at –80°C. Do not freeze/thaw more than 3 times.
Poor expression of the gene of interest	Low transduction efficiency: • Polybrene® not included during transduction • Non-dividing cell type used	• Transduce the lentiviral construct into cells in the presence of Polybrene®. • Transduce your lentiviral construct into cells using a higher MOI.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve. Use the minimum antibiotic concentration required to kill your untransduced cell line.
	Cells harvested too soon after transduction	Do not harvest cells until at least 48–72 hours after transduction to allow expressed protein to accumulate in transduced cells.
	Gene of interest is toxic to cells	Generating constructs containing activated oncogenes or potentially harmful genes is not recommended.
Cytotoxic effects observed after transduction	Large volume of viral supernatant used for transduction	• Remove the "spent" media containing virus and replace with fresh, complete media. • Concentrate the virus (Yee, 1999).
	Polybrene® used during transduction	Verify the sensitivity of your cells to Polybrene®. If cells are sensitive, omit the Polybrene® during transduction.
	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve. Use the minimum concentration of antibiotic required to kill your untransduced cell line.
	Gene of interest is toxic to cells	Try a different cell line.

Continued on next page

Protein Biotinylation and Complex Formation

The table below lists some potential problems and solutions for troubleshooting biotinylation and complex formation.

Problem	Possible Cause	Solution
No biotinylation of recombinant protein observed	Incorrect detection method	Use streptavidin conjugated to alkaline phosphatase or horseradish peroxidase followed by western detection as described (page 39).
	Gene of interest not in-frame with capTEV™ Tag	Make sure that the gene of interest is in frame with the capTEV™ Tag as shown on pages 14–15.
Recombinant protein recovered but not as a complex	N- or C-terminal tag interfering with complex formation	Test both N- and C-terminal tagged constructs to determine the construct that results in optimal complex formation (page 11).
	Complexes dissociated during lysate preparation	To avoid dissociation of protein complexes: • Perform cell lysis using freeze-thaw cycles. Avoid trypsinizing the cells or scraping the cells. • Perform cell lysis in the absence of NP40 as some protein complexes may be unstable in the presence of NP40. • Avoid vortexing the lysate during lysate preparation. • **Perform all purification steps at 4°C and use chilled buffers.**
	Complexes unable to form in mammalian cell line of choice	Optimize using another mammalian cell line.
Protein complexes not observed	Protein degraded	• **Perform all purification steps at 4°C**. • Check to make sure that the CapTEV™-tag is not cleaved during processing or purification. • Include protease inhibitors during cell lysis.

Appendix

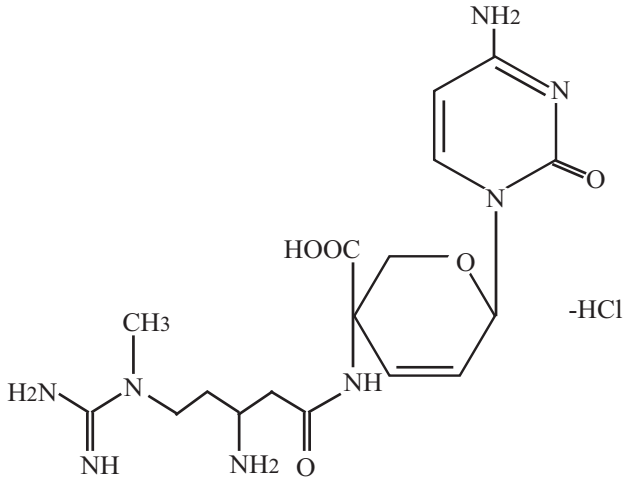
Blasticidin

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).

Molecular Weight, Formula, and Structure

Merck Index: 12: 1,350
MW: 458.9
Formula: $C_{17}H_{26}N_8O_5$-HCl



Handling Blasticidin

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

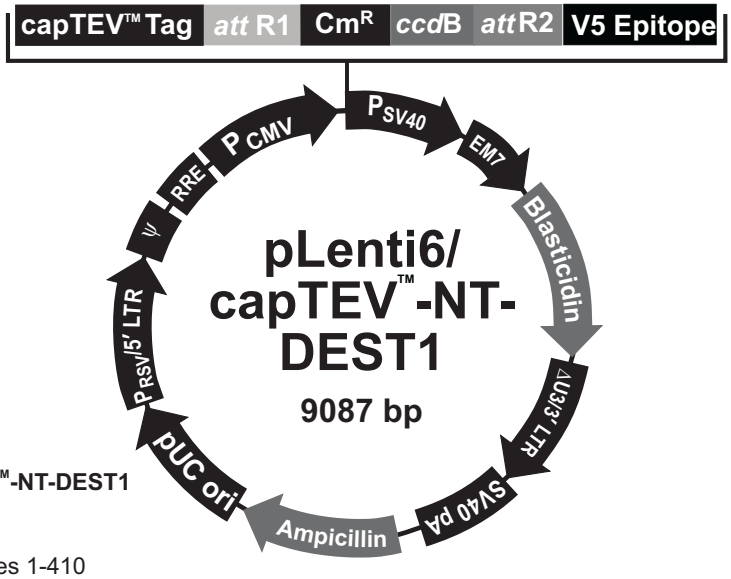
Preparing and Storing Stock Solutions

- 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.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Map and Features of pLenti6/capTEV™-NT-DEST1

Map of pLenti6/capTEV™-NT-DEST1

The figure below shows the features of the pLenti6/capTEV™-NT-DEST1 vector. DNA from the entry clone replaces the region between bases 2,847 and 4,530. The sequence is available at www.invitrogen.com or by contacting Technical Support (see page 65).



Comments for pLenti6/capTEV™-NT-DEST1
9087 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
  RSV promoter: bases 1-229
  HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV promoter: bases 1873-2460
capTEV™ tag: bases 2510-2815
  BioEase™ tag: bases 2510-2728
  TEV cleavage site: bases 2741-2761
  TEV cleavage site: bases 2768-2788
  6X His tag: bases 2795-2815
*att*R1 site: bases 2840-2964
Chloramphenicol resistance gene (Cm^R): bases 3073-3732
*ccd*B gene: bases 4074-4379
*att*R2 site: bases 4420-4544
V5 epitope: bases 4597-4638
SV40 promoter: bases 4693-5001
EM7 promoter: bases 5056-5122
Blasticidin resistance gene: bases 5123-5521
ΔU3/3′ LTR: bases 5607-5841
  ΔU3: bases 5607-5660
  3′ LTR: bases 5661-5841
SV40 polyadenylation signal: 5913-6043
bla promoter: bases 6903-7007
Ampicillin (*bla*) resistance gene: bases 7002-7862
pUC origin: bases 8007-8680

Continued on next page

Map and Features of pLenti6/capTEV™-NT-DEST1, Continued

Features of pLenti6/capTEV™-NT-DEST1

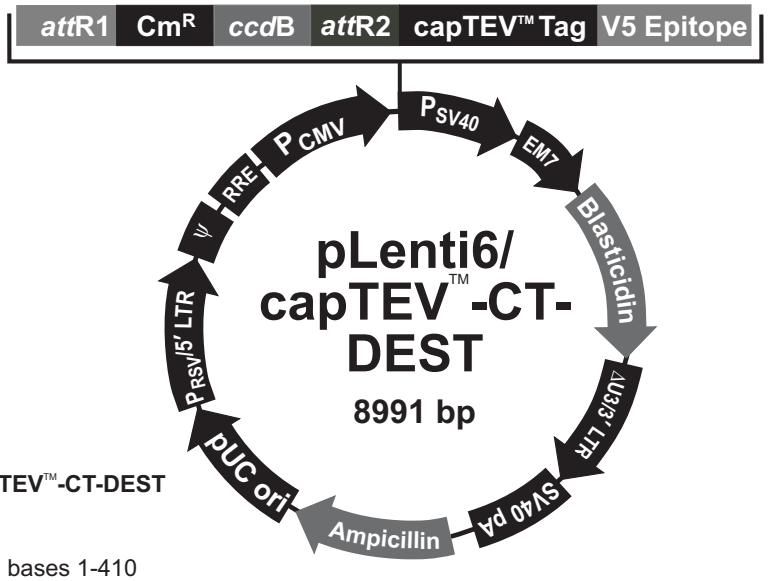
pLenti6/capTEV™-NT-DEST1 (9,087 bp) contains the following elements. Features have been functionally tested and the vector has been fully sequenced.

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).
CMV promoter	Permits high-level, constitutive expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
capTEV™ tag	Allows *in vivo* biotinylation and affinity purification of recombinant proteins and associated complexes.
*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 counterscreening of plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
V5 epitope	Allows detection of recombinant fusion protein by Anti-V5 Antibodies (Southern *et al.*, 1991). **Note:** V5 epitope will not be expressed in pLenti6/capTEV™-NT-DEST1 expression clones due to the stop codon at the end of the gene of interest.
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).

Map and Features of pLenti6/capTEV™-CT-DEST

Map of pLenti6/capTEV™-CT-DEST

The figure below shows the features of the pLenti6/capTEV™-CT-DEST vector. DNA from the entry clone replaces the region between bases 2,448 and 4,130. The sequence is available at www.invitrogen.com or by contacting Technical Support (see page 65).



Comments for pLenti6/capTEV™-CT-DEST
8991 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV promoter: bases 1808-2392
*att*R1 site: bases 2440-2564
Chloramphenicol resistance gene (Cm^R): bases 2673-3332
*ccd*B gene: bases 3674-3979
*att*R2 site: bases 4020-4144
capTEV™ tag: bases 4200-4484
    6X His tag: bases 4200-4220
    TEV cleavage site: bases 4221-4241
    TEV cleavage site: bases 4248-4268
    BioEase™ tag: bases 4269-4484
V5 epitope: bases 4500-4541
SV40 promoter: bases 4596-4904
EM7 promoter: bases 4959-5025
Blasticidin resistance gene: bases 5026-5424
ΔU3/3′ LTR: bases 5510-5744
    ΔU3: bases 5510-5563
    3′ LTR: bases 5564-5744
SV40 polyadenylation signal: 5816-6019
bla promoter: bases 6807-6911
Ampicillin (*bla*) resistance gene: bases 6906-7766
pUC origin: bases 7911-8584

Continued on next page

Map and Features of pLenti6/capTEV™-CT-DEST, Continued

Features pLenti6/capTEV™-CT-DEST

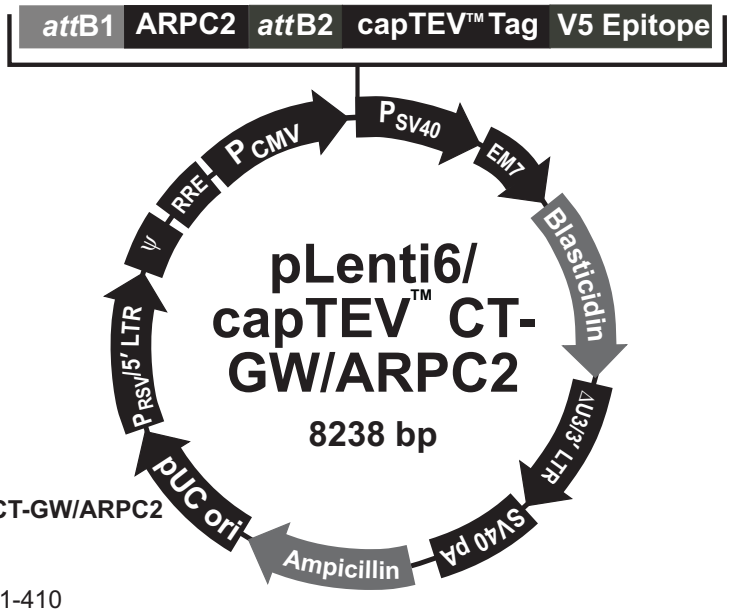
pLenti6/capTEV™-CT-DEST (8,991 bp) contains the following elements. Features have been functionally tested and the vector has been fully sequenced.

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).
CMV promoter	Permits high-level, constitutive expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
*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 counterscreening of plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
capTEV™ tag	Allows *in vivo* biotinylation and affinity purification of recombinant proteins and associated complexes.
V5 epitope	Allows detection of fusion protein by Anti-V5 Antibodies (Southern *et al.*, 1991). **Note:** V5 epitope will not be present in pLenti6/capTEV™-CT-DEST expression clones after TEV cleavage.
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).

Map of pLenti6/capTEV™-CT-GW/ARPC2

Map of pLenti6/capTEV™-CT-GW/ARPC2

The figure below shows the features of the pLenti6/capTEV™-CT-GW/ARPC2 control vector. The sequence is available at www.invitrogen.com or by contacting Technical Support (see page 65).



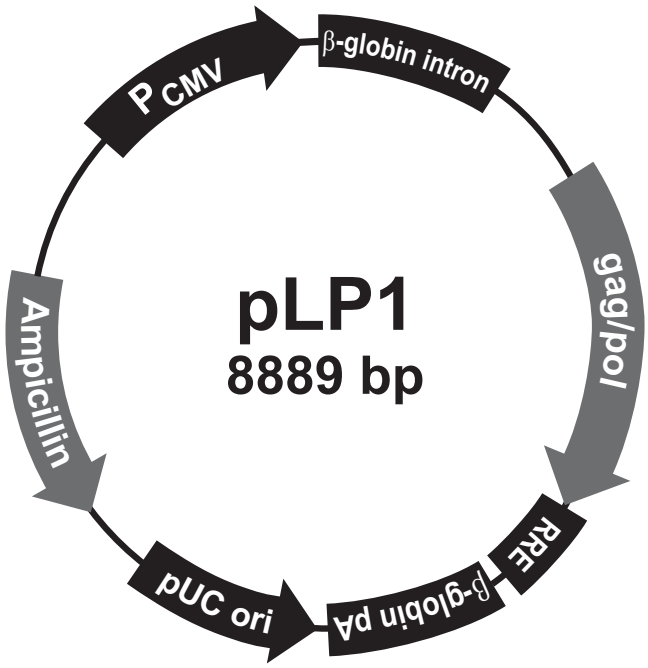
Comments for pLenti6/capTEV™-CT-GW/ARPC2
8238 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
CMV promoter: bases 1808-2392
*attB*1 site: bases 2440- 2464
ARPC2 gene: bases 2467-3366
*att*B2 site: bases 3368-3392
capTEV™ tag: bases 3448-3732
    6X His tag: bases 3448-3468
    TEV cleavage site: bases 3469-3489
    TEV cleavage site: bases 3496-3516
    BioEase™ tag: bases 3517-3732
V5 epitope: bases 3748-3789
SV40 promoter: bases 3844-4152
EM7 promoter: bases 4207-4273
Blasticidin resistance gene: bases 4274-4672
ΔU3/3′ LTR: bases 4758-4992
    ΔU3: bases 4758-4811
    3′ LTR: bases 4812-4992
SV40 polyadenylation signal: 5064-5194
bla promoter: bases 6054-6158
Ampicillin (*bla*) resistance gene: bases 6153-7013
pUC origin: bases 7158-7831

Map and Features of pLP1

pLP1 Map

The figure below shows the features of the pLP1 vector. Note that the *gag* and *pol* genes are initially expressed as a gag/pol fusion protein, which is self-cleaved by the viral protease into individual Gag and Pol polyproteins. The sequence of pLP1 is available at **www.invitrogen.com** or by contacting Technical Support (see page 65).



Comments for pLP1
8889 nucleotides

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

C=complementary strand

Continued on next page

Map and Features of pLP1, Continued

Features of pLP1

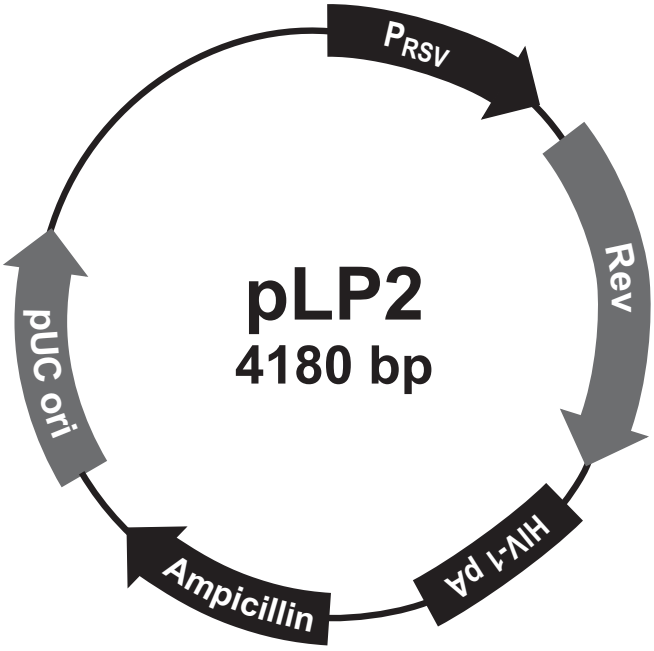
pLP1 (8,889 bp) contains the following elements. Features have been functionally tested.

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

Map and Features of pLP2

pLP2 Map

The figure below shows the features of the pLP2 vector. The sequence of pLP2 is available at **www.invitrogen.com** or by contacting Technical Support (see page 65).



Comments for pLP2
4180 nucleotides

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

Continued on next page

Map and Features of pLP2, Continued

Features of pLP2

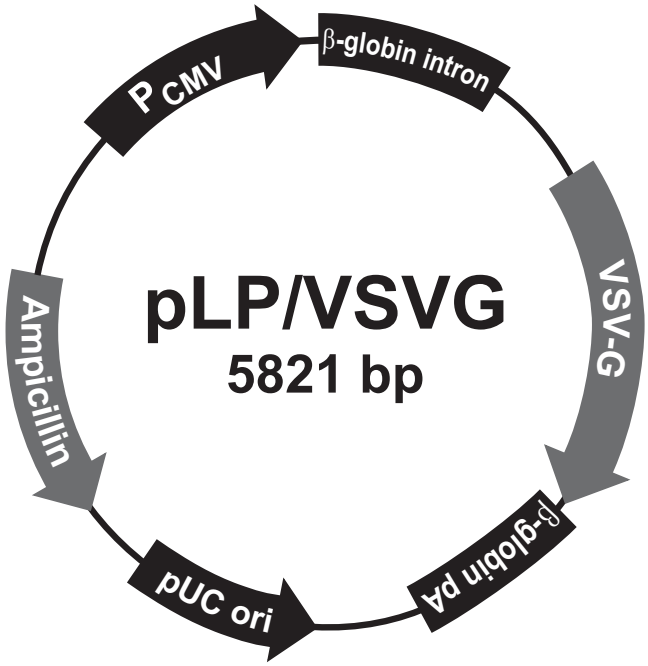
pLP2 (4,180 bp) contains the following elements. All features have been functionally tested.

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

Map and Features of pLP/VSVG

pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. The sequence of pLP/VSVG is available at www.invitrogen.com or by contacting Technical Support (see page 65).



Comments for pLP/VSVG 5821 nucleotides

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

C=complementary strand

Continued on next page

Map and Features of pLP/VSVG, Continued

Features of pLP/VSVG

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

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

Additional Products

Accessory Products

Some of the reagents supplied in the NativePure™ Lentiviral Expression System, as well as other products suitable for use with the kits are available separately. For more information, refer to **www.invitrogen.com** or contact Technical Support (see page 65).

Product	Amount	Cat. no.
pENTR™/D-TOPO® Cloning Kit	20 reactions	K2400-20
pCR®8/GW/TOPO® TA Cloning Kit	20 reactions	K2520-20
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µL	C7373-03
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 transformations	A10460
LB Media	500 mL	10855-021
Ampicillin	200 mg	11593-027
Carbenicillin	5 g	10177-012
PureLink™ HQ Plasmid Miniprep Kit	100 reactions	K2100-01
PureLink™ HiPure Plasmid DNA Purification MidiPrep Kit	25 reactions	K2100-04
E-Gel® 1.2% Starter Pak (6 gels + Powerbase™)	1 kit	G6000-01
E-Gel® 1.2% 18 Pak	18 gels	G5018-01
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970-00
293FT Cell Line	3 x 10^6 cells, frozen	R700-07
pDONR™201	6 µg	11798-014
pDONR™221	6 µg	12536-017
pDONR™/Zeo	6 µg	12535-035
TE, pH 8.0	500 mL	AM9849
	1 L	AM9858
Fetal Bovine Serum (FBS), Certified	500 mL	16000-044
Lipofectamine® 2000	0.75 mL	11668-027
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975-00
pLenti6.2-GW/EmGFP Control Vector	1 vector	V369-20
Opti-MEM® I Reduced Serum Medium	100 L	31985-062
Phosphate-Buffered Saline (PBS), pH 7.4	500 mL	10010-023
Quant-iT™ Protein Assay Kit	1 kit	Q33210

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Additional Products, Continued

Selection Agents

The table below lists ordering information for the selection agents required for use in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System. For more information, refer to www.invitrogen.com or contact Technical Support (see page 65).

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

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

Products for SDS-PAGE

A complete range of products for analysis using SDS-polyacrylamide gel electrophoresis is available. For more information, refer to www.invitrogen.com or contact Technical Support (see page 65).

Product	Amount	Cat. no.
NuPAGE® Novex® Bis-Tris Gels	varies	multiple
Novex® Tris-Glycine Gels	varies	multiple
NuPAGE® MOPS SDS Running Buffer (20X)	500 mL	NP0001
NuPAGE® MES SDS Running Buffer (20X)	500 L	NP0002
NuPAGE® LDS Sample Buffer (4X)	10 mL	NP0007
NuPAGE® Sample Reducing Agent (10X)	250 µL	NP0004
NuPAGE® Transfer Buffer (20X)	1 L	NP0006-1
HiMark™ Pre-Stained Protein Standard	250 µL	LC5699
Novex® Tris-Glycine SDS Running Buffer (10X)	500 mL	LC2675
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 mL	LC2676
Nitrocellulose (0.45 µm) Membrane/Filter Paper Sandwiches	20 sandwiches	LC2001
Invitrolon™ PVDF (0.45 µm) Membrane/Filter Paper Sandwiches	20 sandwiches	LC2005

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Additional Products, Continued

Products for Native Protein Analysis

A complete range of products for purification of native protein complexes and analysis using native gel electrophoresis is available. For more information, refer to www.invitrogen.com or contact Technical Support (see page 65).

Product	Amount	Cat. no.
NativePure™ Affinity Purification Kit	1 kit	BN3003
NativeMark™ Unstained Protein Standard	5 × 50 µL	LC0725
NativePAGE™ Novex® 3-12% Bis-Tris Gels, 10-well	10 gels	BN1001BOX
NativePAGE™ Novex® 3-12% Bis-Tris Gels, 15-well	10 gels	BN1003BOX
NativePAGE™ Novex® 4-16% Bis-Tris Gels, 10-well	10 gels	BN1002BOX
NativePAGE™ Novex® 4-16% Bis-Tris Gels, 15-well	10 gels	BN1004BOX
NativePAGE™ Running Buffer (20X)	1 L	BN2001
NativePAGE™ Cathode Buffer Additive (20X)	250 mL	BN2002
NativePAGE™ Sample Buffer (4X)	10 mL	BN2003
NativePAGE™ 5% G-250 Sample Buffer Additive	0.5 mL	BN2004
NativePAGE™ Running Buffer Kit	1 kit	BN2007
NativePAGE™ Sample Prep Kit	1 kit	BN2008
10% DDM (n-dodecyl β-D-maltoside)	1 mL	BN2005
5% Digitonin	1 mL	BN2006
Streptavidin Agarose (sedimented bead suspension)	5 mL	S-951
AcTEV™ Protease	1000 units	12575-015
	10,000 units	12575-023

Products for Protein Detection

Reagents for detecting protein are available separately. For more information, refer to www.invitrogen.com or contact Technical Support (see page 65).

Product	Amount	Cat. no.
Streptavidin Agarose	5 mL	SA100-04
Streptavidin-HRP Conjugate	2.5 mg	43-4323
Anti-HisG-AP Antibody	125 µL	R942-25
WesternBreeze® Chromogenic Kit, Anti-Rabbit	20 reactions	WB7105
WesternBreeze® Chemiluminescent Kit, Anti-Mouse	20 reactions	WB7104

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Technical Support

Web Resources

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

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

Contact Us

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

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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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