invitrogen™
by life technologies™

ViraPower™ Lentiviral Expression Systems

Lentiviral systems for high-level expression in dividing and non-dividing mammalian cells

Catalog nos. K4950-00, K4960-00, K4970-00, K4975-00, K4980-00, K4985-00, K4990-00, K367-20, K370-20, and K371-20

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User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the kits listed below. The ViraPower™ Lentiviral Support Kits includes the ViraPower™ Packaging Mix, Lipofectamine™ 2000, and a selection agent. The ViraPower™ and ViraPower™ II Lentiviral Expression Kits include the ViraPower™ Lentiviral Support Kit plus an expression vector and the 293FT producer cell line.

Product	Catalog no.
ViraPower™ Lentiviral Directional TOPO® Expression Kit	K4950-00
ViraPower™ Lentiviral Gateway® Expression Kit	K4960-00
ViraPower™ II Lentiviral Gateway® Expression Kit	K367-20
ViraPower™ II Lentiviral C-Lumio™ Gateway® Expression Kit	K370-20
ViraPower™ II Lentiviral N-Lumio™ Gateway® Expression Kit	K371-20
ViraPower™ Bsd Lentiviral Support Kit	K4970-00
ViraPower™ Lentiviral Packaging Mix	K4975-00
ViraPower™ Zeo Lentiviral Gateway® Expression Kit	K4980-00
ViraPower™ Zeo Lentiviral Support Kit	K4985-00
ViraPower™ UbC Lentiviral Gateway® Expression Kit	K4990-00

System Components

The following table shows the components associated with Lentiviral Expression Kit catalog numbers listed above.

Components	Catalog no.					
	K4950-00	K4960-00	K4980-00	K4990-00	K367-20	K370-20/K371-20
ViraPower™ Bsd Lentiviral Support Kit	✓	✓		✓	✓	✓
ViraPower™ Zeo Lentiviral Support Kit			✓			
pLenti6/V5-Directional TOPO® Cloning Kit	✓					
pLenti4/V5-DEST Gateway® Vector Kit			✓			
pLenti6/V5-DEST Gateway® Vector Kit		✓				
pLenti6.2/V5-DEST Gateway® Vector Kit					✓	
pLenti6.2/N- and C-Lumio™/ V5-DEST Vectors						✓
pLenti6/UbC/V5-DEST Gateway® Vector Kit				✓		
293FT Cell Line	✓	✓	✓	✓	✓	✓

Continued on next page

Shipping/Storage

The ViraPower™ Lentiviral products are shipped as described below. Upon receipt, store each component as detailed below.

Item	Shipping	Storage
ViraPower™ Bsd Lentiviral Support Kit: • ViraPower™ Packaging Mix • Lipofectamine™ 2000 • Blasticidin	Wet ice	 –20°C 4°C **(do not freeze)** –20°C
ViraPower™ Zeo Lentiviral Support Kit: • ViraPower™ Packaging Mix • Lipofectamine™ 2000 • Zeocin™	Wet ice	 –20°C 4°C **(do not freeze)** –20°C, protected from light
293FT Cell Line	Dry ice	Liquid nitrogen
pLenti6/V5 Directional TOPO® Cloning Kit: • pLenti6/V5-D-TOPO® Reagents • One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	 –20°C –80°C
pLenti4/V5-DEST Gateway® Vector Kit: • Vectors • One Shot® Stbl3™ Chemically Competent *E. coli*	 Wet ice Dry ice	 –20°C –80°C
pLenti6/V5-DEST Gateway® Vector Kit: • Vectors • One Shot® Stbl3™ Chemically Competent *E. coli*	 Wet ice Dry ice	 –20°C –80°C
pLenti6.2/V5-DEST Gateway® Vector Kit: • Vectors • One Shot® Stbl3™ Chemically Competent *E. coli*	 Wet ice Dry ice	 –20°C –80°C
pLenti6.2/C-Lumio™/V5-DEST Vector: • Vectors • One Shot® Stbl3™ Chemically Competent *E. coli*	 Wet ice Dry ice	 –20°C –80°C
pLenti6.2/N-Lumio™/V5-DEST Vector: • Vectors • One Shot® Stbl3™ Chemically Competent *E. coli*	 Wet ice Dry ice	 –20°C –80°C
pLenti6/UbC/V5-DEST Gateway® Vector Kit: • Vectors • One Shot® Stbl3™ Chemically Competent *E. coli*	 Wet ice Dry ice	 –20°C –80°C
ViraPower™ Packaging Mix	Wet ice	–20°C

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Expression Vectors

Each ViraPower™ or ViraPower™ II Lentiviral Expression Kit also includes a pLenti-based expression vector kit. The expression vector kit includes:

- A pLenti-based expression vector for cloning your gene of interest
- A corresponding expression control plasmid
- One Shot® Stbl3™ Chemically Competent *E. coli* for transformation

Expression vectors include pLenti6/V5-D-TOPO®, pLenti4/V5-DEST, pLenti6/V5-DEST, pLenti6.2/V5-DEST, pLenti6.2/C-Lumio™/V5-DEST, pLenti6.2/N-Lumio™/V5-DEST, and pLenti6/UbC/V5-DEST. Refer to the appropriate vector manual supplied with the kit for a detailed description of the reagents provided with each vector kit and instructions to generate an expression clone containing your gene of interest.

ViraPower™ Bsd Lentiviral Support Kit Contents

The ViraPower™ Bsd Lentiviral Support Kit includes the following vectors and reagents. Store as directed below.
Important: Store Lipofectamine™ 2000 at 4°C. **DO NOT FREEZE.**

Product	Composition	Quantity	Storage
ViraPower™ Packaging Mix	Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids, at 1 µg/µl in TE Buffer, pH 8.0	195 µg	–20°C
Lipofectamine™ 2000	Proprietary	0.75 ml	4°C
Blasticidin	Powder	50 mg	–20°C

ViraPower™ Zeo Lentiviral Support Kit Contents

The ViraPower™ Zeo Lentiviral Support Kit includes the following vectors and reagents. Store as directed below.

Important Tips

- Take care to store Lipofectamine™ 2000 at 4°C. **DO NOT FREEZE.**
- Zeocin™ is light sensitive. Store at –20°C in the dark.

Product	Composition	Quantity	Storage
ViraPower™ Packaging Mix	Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids, at 1 µg/µl in TE Buffer, pH 8.0	195 µg	–20°C
Lipofectamine™ 2000	Proprietary	0.75 ml	4°C
Zeocin™	100 mg/ml in sterile, deionized water	12.5 mg	–20°C, in dark

ViraPower™ Packaging Mix

Catalog no. K4975-00 contains 3 tubes with 195 µg DNA per tube of the ViraPower™ Packaging Mix; a mixture of pLP1, pLP2, and pLP/VSVG plasmids, supplied in solution at 1 µg/µl in TE Buffer, pH 8.0. Upon receipt, store at –20°C.

293FT Cell Line

Each ViraPower™ Lentiviral Expression Kit includes the 293FT producer cell line. The 293FT Cell Line is supplied as one vial containing 3×10^6 frozen cells in 1 ml of Freezing Medium. **Upon receipt, store in liquid nitrogen.**

For instructions to thaw, culture, and maintain the 293FT Cell Line, see the 293FT Cell Line manual, included with the ViraPower™ Lentiviral Expression Kit and available at www.invitrogen.com.

Accessory Products

Introduction

The products listed in this section may be used with the ViraPower™ Lentiviral Expression Kits. For more information, visit our web site at www.invitrogen.com or contact **Technical Support** (see page 36).

Additional Products

Many of the reagents supplied in the ViraPower™ Lentiviral Expression Kits as well as other products suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
pLenti6/V5 Directional TOPO® Cloning Kit	20 reactions	K4955–10
pLenti4/V5-DEST Gateway® Vector	6 µg (supplied as 40 µl of 150 ng/µl vector in 10 mM Tris-HCL, 1mM EDTA, pH 8.0)	V498–10
pLenti6/V5-DEST Gateway® Vector	6 µg (supplied as 40 µl of 150 ng/µl vector in 10 mM Tris-HCL, 1mM EDTA, pH 8.0)	V496–10
pLenti6.2/V5-DEST Gateway® Vector	6 µg (supplied as 40 µl of 150 ng/µl vector in 10 mM Tris-HCL, 1mM EDTA, pH 8.0)	V368–20
pLenti6/UbC/V5-DEST Gateway® Vector	6 µg (supplied as 40 µl of 150 ng/µl vector in 10 mM Tris-HCL, 1mM EDTA, pH 8.0)	V499–10
pLenti6.2-GW/EmGFP Expression Control Vector	20 µg (supplied as 40 µl of 5 µg/µl control vector in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.)	V369–20
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µl	C7373–03
S.N.A.P.™Midiprep DNA Isolation Kit	20 reactions	K1910–01
293FT Cell Line	3×10^6 cells, frozen	R700–07
Fetal Bovine Serum (FBS), Certified	500 ml	16000–044
Lipofectamine™ 2000	0.75 ml 1.5 ml	11668–027 11668–019
Opti-MEM® I Reduced Serum Medium	100 ml 500 ml	31985–062 31985–070
Blasticidin	50 mg	R210–01
Zeocin™	1 g 5 g	R250–01 R250–05
Geneticin®	20 ml 100 ml	10131–035 10131–027
Phosphate-Buffered Saline (PBS), pH 7.4	500 ml 1 L	10010–023 10010–031

Introduction

Overview

Introduction

The ViraPower™ Lentiviral Expression System allows creation of a replication-incompetent, HIV-1-based lentivirus that is used to deliver and express your gene of interest in either dividing or non-dividing mammalian cells. The major components of the system include:

- An expression plasmid containing the gene of interest under the control of a choice of promoters, and elements that allow packaging of the construct into virions
- An optimized mix of the three packaging plasmids (pLP1, pLP2, and pLP/VSVG) that supply the structural and replication proteins *in trans* that are required to produce the lentivirus
- The 293FT cell line, which allows production of lentivirus following cotransfection of the expression plasmid and the plasmids in the packaging mix
- Control expression plasmid to optimize virus production and cell transduction, containing either:
  - The *lacZ* gene which when packaged into virions and transduced into a mammalian cell line, expresses β-galactosidase (included with each expression vector), **or**
  - The Emerald Green Fluorescent Protein (EmGFP) gene which when packaged into virions and transduced into a mammalian cell line, expresses EmGFP (available separately; see page vii for ordering information)

For more information on expression vectors and the corresponding positive control vectors, refer to the manual for the specific expression or control vector you are using.

Advantages of the System

Use of the ViraPower™ Lentiviral Expression System to facilitate lentiviral-based expression of the gene of interest provides the following advantages:

- 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)
- 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)
- Produces a pseudotyped virus with a broadened host range (Yee *et al.*, 1994)
- Includes multiple features designed to enhance the biosafety of the system

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Purpose of this Manual

This manual provides an overview of the ViraPower™ Lentiviral Expression System and provides instructions and guidelines to:

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

For details and instructions to generate your expression vector, refer to the manual for the pLenti vector you are using. For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual. These manuals are supplied with the ViraPower™ Lentiviral Expression Kits, and are also available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (see page 36).

Components of the ViraPower™ Lentiviral Expression System

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

- A pLenti-based expression vector into which the gene of interest will be cloned. The vector also contains the elements required to allow packaging of the expression construct into virions (*e.g.*, 5′ and 3′ LTRs, Ψ packaging signal). For more information about the pLenti expression vectors, refer to the manual for the specific vector you are using.
- 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 30–34.
- 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.

You will cotransfect the ViraPower™ Packaging Mix and the pLenti vector containing your gene of interest into 293FT cells to produce a replication-incompetent lentivirus, which will be used to transduce a mammalian cell line of interest.

Continued on next page

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 your recombinant protein or use antibiotic selection to generate a stable cell line for long-term expression studies.

VSV Envelope Glycoprotein

Most retroviral vectors are limited in their usefulness as gene delivery vehicles by their restricted tropism and generally low titers. In the ViraPower™ 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).

Biosafety Features of the System

Introduction

The ViraPower™ Lentiviral Expression System is a third-generation system based on lentiviral vectors developed by Dull *et al.*, 1998. This third-generation lentiviral system includes a significant number of safety features designed to enhance its biosafety and to minimize its relation to the wild-type, human HIV-1 virus. These safety features are discussed below.

Biosafety Features of the ViraPower™ Lentiviral System

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

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

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Biosafety Features of the System, Continued

Biosafety Level 2

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

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories," 4th 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/bmbl4/bmbl4toc.htm

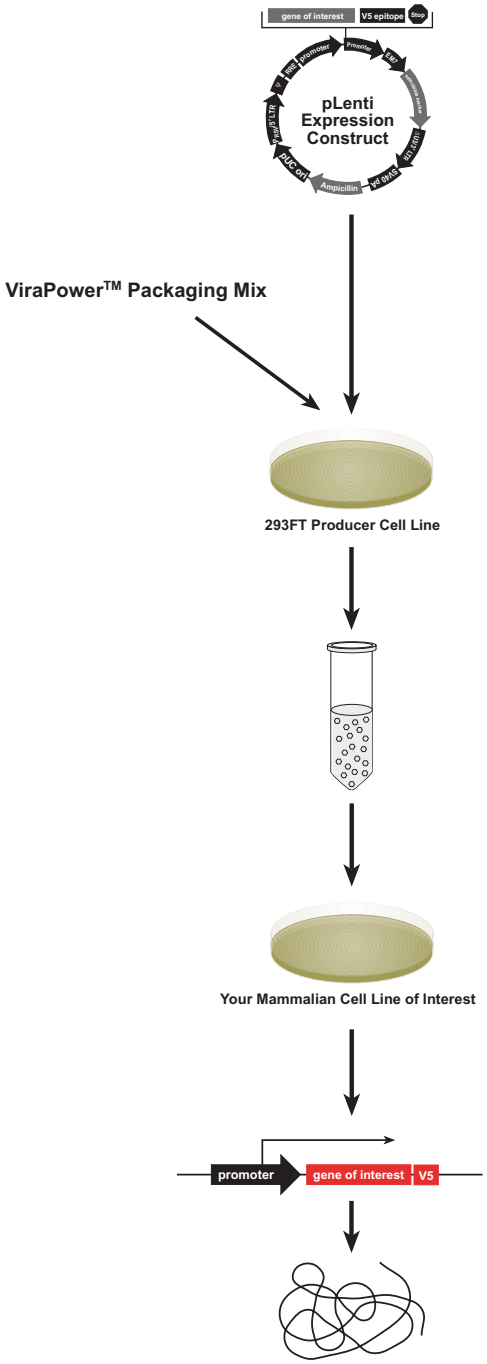
Important

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

Experimental Outline

Flow Chart

The diagram below describes the general steps required to express your gene of interest using the ViraPower™ Lentiviral Expression System. Refer to the appropriate manual for each pLenti expression vector for instructions to generate your pLenti expression construct.



1. Generate the pLenti expression construct containing your gene of interest.
2. Cotransfect the 293FT producer cell line with your pLenti expression construct and the optimized packaging mix.
3. Harvest viral supernatant and determine the titer.
4. Add the viral supernatant to your mammalian cell line of interest. Select for stably transduced cells, if desired.
5. Assay for recombinant protein of interest.

Methods

General Information

Introduction

The ViraPower™ 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 virus production and tissue culture techniques.

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

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

Generating Your pLenti Expression Construct

To generate a pLenti expression construct containing your gene of interest, refer to the manual for the vector you are using for instructions. Once you have created your expression construct, you will isolate plasmid DNA for transfection.

Important: You should verify that your lentiviral plasmid has not undergone aberrant recombination by performing an appropriate restriction enzyme digest. See the vector manual for details.

DNA Isolation Guidelines

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.

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

Important

Do not use mini-prep plasmid DNA for lentivirus production. We recommend preparing lentiviral plasmid DNA using the S.N.A.P.™ MidiPrep Kit, which contains 10 mM EDTA in the Resuspension Buffer (see page vii for ordering information).

ViraPower™ Packaging Mix

The pLP1, pLP2, pLP/VSVG plasmids are provided in an optimized mixture to facilitate viral packaging of your pLenti expression vector following cotransfection into 293FT producer cells. The amount of the packaging mix (195 µg supplied as 1 µg/µl in TE Buffer, pH 8.0) and Lipofectamine™ 2000 transfection reagent (0.75 ml) supplied in the ViraPower™ Lentiviral Expression kit is sufficient to perform 20 cotransfections in 10 cm plates.

Note: ViraPower™ Packaging Mix is available separately from Invitrogen or as part of the ViraPower™ Lentiviral Support Kits (page vii).

293FT Cell Line

The human 293FT Cell Line is supplied with the ViraPower™ Lentiviral Expression kits 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 ViraPower™ Lentiviral Expression kits, and is also available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (see page 36).

Note: The 293FT Cell Line is also available separately from Invitrogen (page vii).

The health of your 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 containing 0.1 mM MEM Non-Essential Amino Acids, 4 mM L-Glutamine, 1 mM sodium pyruvate, 500 µg/ml Geneticin® and 10% fetal bovine serum that is not heat-inactivated (page vii).
- Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 16 passages.

Positive Control

We recommend including a positive control vector in your cotransfection experiment to generate a control lentiviral stock that may be used to help you optimize expression conditions in your mammalian cell line of interest

- Each pLenti expression vector kit includes a positive control vector for use as an expression control (*e.g.* pLenti6/V5-GW/*lacZ*). For more information about the positive control vector supplied with each kit, refer to the appropriate expression vector manual.
- A control lentiviral expression vector (pLenti6.2-GW/EmGFP) containing Emerald Green Fluorescent Protein (EmGFP) for fluorescent detection is available separately from Invitrogen. For ordering information, see page vii.

Lipofectamine™ 2000

The Lipofectamine™ 2000 reagent supplied with the kit (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

Note: Lipofectamine™ 2000 is available separately from Invitrogen or as part of the ViraPower™ Lentiviral Support Kits (see page vii for ordering information).

Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine™ 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium available from Invitrogen (see page vii for ordering information).

Recommended Procedure

If you producing lentivirus for the first time using the ViraPower™ System and 293FT cells, you should perform the **Forward Transfection** procedure on page 12. This procedure requires plating the 293FT cells the day before transfection to obtain cells that are 90–95% confluent

Note: In previous ViraPower™ 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 **Reverse Transfection** procedure on page 13. In this procedure, 293FT cells are added to media containing the DNA-Lipofectamine™ 2000 complexes.

Producing Lentivirus in 293FT Cells

Introduction

Before you can create a stably transduced cell line expressing your gene of interest, you will first need to produce a lentiviral stock (containing the packaged pLenti expression construct) by cotransfecting the optimized packaging plasmid mix and your pLenti expression construct into the 293FT Cell Line. The following section provides protocols and instructions to generate a lentiviral stock.

Recommended Transfection Conditions

We produce lentiviral stocks in 293FT cells using the following **optimized** transfection conditions below. The amount of lentivirus produced using these recommended conditions (10 ml of virus at a titer of at least 1×10^5 transducing units (TU)/ml) is generally sufficient to transduce at least 1×10^6 cells at a multiplicity of infection (MOI) = 1. For example, 10 wells of cells plated at 1×10^5 cells/well in 6-well plates could each be transduced with 1 ml of a 1×10^5 TU/ml virus stock to achieve an MOI of 1.

Condition	Quantity
Tissue culture plate size	10 cm (one per lentiviral construct)
Number of 293FT cells to transfect	6×10^6 cells (see **Recommendation** on page 8 to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix	9 µg
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.

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Materials Needed

You will need the following items:

- ViraPower™ Packaging Mix (supplied in solution with the kit)
- pLenti expression vector containing your gene of interest (0.1–3.0 µg/µl)
- pLenti control vector containing lacZ (supplied with the kit) or EmGFP (available separately)
- 293FT cells cultured in the appropriate medium (*i.e.* D-MEM containing 10% FBS, 4 mM L-Glutamine, 1 mM MEM Sodium Pyruvate, 0.1 mM MEM Non-Essential Amino Acids, and 1% penicillin-streptomycin, and 500 µg/ml Geneticin®)

  Note: MEM Sodium Pyruvate provides an extra energy source for the cells and is available from Invitrogen as a 100 mM stock solution (Catalog no. 11360-070).
- Lipofectamine™ 2000 transfection reagent (supplied with the kit; store at 4°C and mix gently before use)
- Opti-MEM® I Reduced Serum Medium (pre-warmed to 37°C)
- Fetal bovine serum (FBS, Cat. no 16000-044)
- Complete growth medium **without antibiotics** (*i.e.* D-MEM containing 10% FBS, 4 mM L-Glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1 mM MEM Sodium Pyruvate), pre-warmed to 37°C
- 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
- **Optional:** Millex-HV 0.45 µm PVDF filters (Millipore, cat. no. SLHVR25LS) or equivalent
- Cryovials

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Forward Transfection Procedure

If you are a **first time user**, follow the procedure below to cotransfect 293FT cells. We recommend including a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help you evaluate your 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). **Do not include antibiotics in the medium.**
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 5 ml tube, dilute 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.
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 humidified 5% CO_2 incubator.
5. The next day (Day 3), remove the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with 10 ml complete culture medium **without antibiotics**. Incubate at 37°C in a humidified 5% 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 at either 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 page 4 for more information).
7. Centrifuge supernatants at 3000 rpm for 15 minutes at 4°C to pellet debris.
8. **Optional:** 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 Your Lentiviral Stock**, page 15.

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 you evaluate your 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, dilute 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 humidified 5% CO_2 incubator.
5. The next day (Day 2), remove the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with 10 ml complete culture medium **without antibiotics**. Incubate cells overnight at 37°C in a humidified 5% 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 page 4 for more information).
7. Centrifuge supernatants at 3000 rpm for 15 minutes at 4°C to pellet debris.
8. **Optional:** 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 Your Lentiviral Stock**, page 15.

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Note

If you plan to use your lentiviral construct for *in vivo* applications, we recommend filtering your viral supernatant through a sterile, 0.45 µm low protein binding filter after the low-speed centrifugation step (Step 8, previous page) to remove any remaining cellular debris. We recommend using Millex-HV 0.45 µm PVDF filters (Millipore, Catalog no. SLHVR25LS) for filtration.

If you wish to concentrate your viral stock to obtain a higher titer, perform the filtration step first before concentrating your viral stock.

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 to concentrate your virus supernatant by ultracentrifugation, refer to published reference sources (Yee, 1999).

Long-Term Storage

Store viral stocks at –80°C in cryovials 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, we recommend retitering your 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 Your Lentiviral Stock

Introduction

Before proceeding to transduction and expression experiments, we highly recommend determining the titer of your lentiviral stock. While this procedure is not required for some applications, it is necessary if:

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

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

Note: If you are using pLenti6.2-GW/EmGFP Expression Control Vector to produce a lentiviral stock, refer to the user manual for titer methods using fluorescent detection.

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 dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

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.

Continued on next page

Titering Your Lentiviral Stock, Continued

Factors Affecting Viral Titer

A number of factors can influence viral titers including:

- **The size of your gene of interest.** Titers will decrease as the size of the insert increases. We have determined that virus titer drops approximately 2-fold for each kb over 4 kb of insert size. If you wish to produce lentivirus with an insert of >4 kb, you will need to concentrate the virus to obtain a suitable titer (see page 14). The size of the wild-type HIV genome is approximately 10 kb. Since the size of the elements required for expression from pLenti vectors total approximately 4-4.4 kb, the size of your insert should not exceed 5.6 kb.
- **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, we recommend titering 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.

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Titering Your Lentiviral Stock, Continued

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). If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line. For more information on cells for titering, see **Factors Affecting Viral Titer**, previous page.

Antibiotic Selection

The pLenti expression constructs contain either the Blasticidin resistance gene (*bsd*) (Kimura *et al.*, 1994) or the Zeocin™ resistance gene (Calmels *et al.*, 1991; Drocourt *et al.*, 1990) to allow for Blasticidin selection (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) or Zeocin™ selection (Mulsant *et al.*, 1988), respectively of mammalian cells that have stably transduced the lentiviral construct.

If you have purchased a ViraPower™ Lentiviral Expression Kit, either Blasticidin or Zeocin™ is supplied. Blasticidin and Zeocin™ are also available separately from Invitrogen or as part of the appropriate ViraPower™ Lentiviral Support Kit (see page vii for ordering information).

Important

Cell density can affect the efficiency of Zeocin™ selection. For the most efficient Zeocin™ selection, cells should not be greater than 50% confluent.

Preparing Blasticidin or Zeocin™

For more information about how to prepare and handle Blasticidin and Zeocin™, refer to the **Appendix**, pages 25 and 29, respectively.

Determining Antibiotic Sensitivity

Since you will be selecting for stably transduced cells using Blasticidin or Zeocin™, you must first determine the minimum concentration of Blasticidin or Zeocin™ required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 2-10 µg/ml Blasticidin or 50–1000 µg/ml Zeocin™ are sufficient to kill most untransduced mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

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

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Titering Your Lentiviral Stock, Continued

Effect of Zeocin™ on Sensitive and Resistant Cells

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

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

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

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

Using Polybrene® During Transduction

Lentivirus transduction may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene®). For best results, we recommend performing transduction in the presence of Polybrene®. Note however, that some cells are sensitive to Polybrene® (*e.g.* primary neurons). Before performing any transduction experiments, you may want to test your cell line for sensitivity to Polybrene® at a range of 0-10 µg/ml. If your cells are sensitive to Polybrene® (*e.g.* exhibit toxicity or phenotypic changes), do not add Polybrene® during transduction. In this case, cells should still be successfully transduced with your lentivirus.

Preparing and Storing Polybrene®

Follow the instructions below to prepare Polybrene® (Sigma, Catalog 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 (up to 1 year). Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

Continued on next page

Titering Your Lentiviral Stock, Continued

Materials Needed

You will need the following items:

- Your pLenti lentiviral stock (store at –80°C until use)
- Adherent mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/ml Polybrene®, if desired
- 6-well tissue culture plates
- 10 cm tissue culture plates (for Zeocin™ selection only)
- Blasticidin (10 mg/ml stock) or Zeocin™ (100 mg/ml stock), as appropriate for selection
- Crystal violet (Sigma, Catalog no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
- Phosphate-Buffered Saline (PBS; Invitrogen, page vii)

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Titering Your Lentiviral Stock, Continued

Transduction and Titering Procedure

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. You will use **at least** one 6-well plate for every lentiviral stock to be titered (one mock well plus five dilutions).

Note: If you have generated a lentiviral stock of a *lacZ* expression control (*e.g.* pLenti6/V5-GW/*lacZ*), we recommend titering this stock as well. If you are using pLenti6.2-GW/EmGFP, refer to the user manual for the vector for the titering protocol.

1. The day before transduction (Day 1), trypsinize and count the cells, plating them in a 6-well plate such that they will be 30–50% confluent at the time of transduction. Incubate cells at 37°C overnight in a humidified 5% CO_2 incubator.

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

2. On the day of transduction (Day 2), thaw your lentiviral stock 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. Add Polybrene® (if desired) to each well to a final concentration of 6 µg/ml. Swirl the plate gently to mix. Incubate at 37°C overnight in a humidified 5% CO_2 incubator.
5. The following day (Day 3), remove the media containing virus and replace with 2 ml of complete culture medium. Incubate at 37°C overnight in a humidified 5% CO_2 incubator.
6. The following day (Day 4), treat cells as follows:
   - For Blasticidin selection, remove the medium and replace with complete culture medium containing the appropriate amount of Blasticidin to select for stably transduced cells.
   - For Zeocin™ selection, remove the medium and wash the cells once with PBS. **For each well of cells**, trypsinize the cells and replate the entire amount into one 10 cm plate containing complete culture medium with the appropriate amount of Zeocin™ to select for stably transduced cells.
7. Replace medium with fresh medium containing antibiotic 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 your lentiviral stock.

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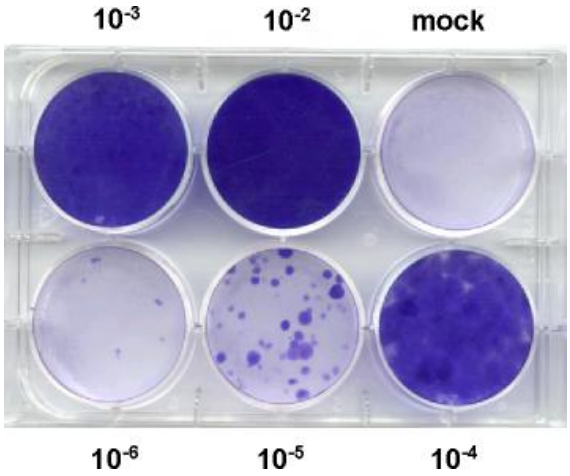
Titering Your Lentiviral Stock, Continued

What You Should See

When titering pLenti lentiviral stocks using HT1080 cells, we generally obtain titers ranging from 1 - 5 × 10^5 (for unconcentrated virus) up to 2 × 10^7 (for concentrated virus) transducing units (TU)/ml.

Example of Expected Results

In this experiment, a Lenti6/V5-GW/*lacZ* lentiviral stock was generated using the protocol on page 12 and was concentrated by ultracentrifugation. HT1080 cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^2 to 10^6 dilutions) or untransduced (mock) following the protocol on page 20. At 48 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

It is important to note that 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 **Transduction of Cells With Lentivirus**. If the titer of your concentrated lentiviral stock is less than 1 × 10^5 TU/ml, we recommend producing a new lentiviral stock. See the **Troubleshooting** section, page 25 for more tips and guidelines to optimize your viral yield.

Transduction and Analysis

Introduction

Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral construct into the mammalian cell line of choice and assay for expression of your recombinant protein. Guidelines are provided below.

Important

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

Transient vs. Stable Expression

After transducing your lentiviral construct into the mammalian cell line of choice, you may assay for expression of your gene of interest in the following ways:

- Pool a heterogeneous population of cells and test for expression 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.
- Select for stably transduced cells using Blasticidin or Zeocin™, as appropriate. This requires a minimum of 10-12 days after transduction, but allows generation of clonal cell lines that stably express the gene of interest.

  Note: We have observed stable expression of a target gene for at least 6 weeks following transduction and selection.

Determining Antibiotic Sensitivity for Your Cell Line

If you wish to select for stably transduced cells, you must first determine the minimum concentration of Blasticidin or Zeocin™, as appropriate, 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 17. If you titered your lentiviral construct in the same mammalian cell line that you are using to perform your stable expression experiment, then you may use the same concentration of Blasticidin or Zeocin™ for selection that you used for titering.

Multiplicity of Infection (MOI)

To obtain optimal expression of your gene of interest, you will need to transduce the lentiviral construct into your 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 of your gene of interest. Typically, expression levels increase linearly as the MOI increases.

Determining the Optimal MOI

A number of factors can influence optimal MOI including the nature of your mammalian cell line (*e.g.* non-dividing vs. dividing cell type; see **Recommendation** on the 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, we recommend using a range of MOI (*e.g.* 0, 0.5, 1, 2, 5, 10) to determine the MOI required to obtain the optimal expression of your protein for your application.

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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 transduce 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 (*e.g.* MOI = 10) to achieve optimal expression levels for your recombinant protein.

Positive Control

Control lentiviral vectors expressing lacZ or EmGFP are available for optimization (see your vector manual and page vii for information). If you have generated a lentiviral stock of a *lacZ* expression control (*e.g.* pLenti6/V5-GW/*lacZ*), we recommend using the stock to help you determine the optimal MOI for your particular cell line and application. Once you have transduced the control lentivirus into your mammalian cell line of choice, the gene encoding β-galactosidase or EmGFP will be constitutively expressed and can be easily assayed (refer to the expression vector or expression control vector manual for assay methods).

Note

Viral supernatants 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

You will need the following items:

- Your titered lentiviral stock (store at –80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/ml Polybrene®, if desired
- Appropriately sized tissue culture plates for your application
- Blasticidin or Zeocin™, as appropriate (if selecting for stably transduced cells)

Continued on next page

Transduction Procedure

Follow the procedure below to transduce the mammalian cell line of choice with your lentiviral construct.

Reminder: If you are performing Zeocin™ selection, remember that cells should not be confluent at the time of selection (see Step 6 below). Plate your cells accordingly.

1. Plate cells in complete media as appropriate for your application.
2. On the day of transduction (Day 1), thaw your lentiviral stock, and if necessary, dilute the appropriate amount of virus into fresh complete medium to obtain a suitable MOI. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration up to 10 µg/ml. Swirl the plate gently to mix. Incubate at 37°C in a humidified 5% 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 humidified 5% CO_2 incubator overnight.
6. The following day (Day 3), perform one of the following:
   - Harvest the cells and assay for expression of your recombinant protein if you are performing transient expression experiments.
   - Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin or Zeocin™, as appropriate to select for stably transduced cells. Proceed to Step 7.
7. Replace medium with fresh medium containing antibiotic every 3-4 days until antibiotic-resistant colonies can be identified (generally 10–12 days after selection).
8. Pick at least 5 antibiotic-resistant colonies (see **Note** below) and expand each clone to assay for expression of the recombinant 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. We recommend testing at least 5 antibiotic-resistant clones and selecting the clone that provides the optimal expression of your recombinant protein for further studies.

Detecting Recombinant Protein

You may use any method of choice to detect your recombinant protein of interest including functional analysis, immunofluorescence, or western blot. If you have cloned your gene of interest in frame with an epitope tag, you may easily detect your recombinant protein in a western blot using an antibody to the epitope tag (see your lentiviral vector manual for details).

Troubleshooting

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	Cause	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 S.N.A.P.™ Midiprep DNA Isolation Kit or CsCl gradient centrifugation to prepare plasmid DNA.
	• Unhealthy 293FT cells; cells exhibit low viability	• Use healthy 293FT cells under passage 16; 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.
	• Insufficient co-transfection	• Use more DNA/ Lipofectamine™ 2000 (keeping the ratios the same). For example, use 5 µg of lentiviral vector, 15 µg of packaging mix, and 60 µl of Lipofectamine™ 2000 for transfection.
	• 293FT cells plated too sparsely	• Plate cells such that they are 90–95% confluent at the time of transfection **OR** use the Reverse Transfection protocol (*i.e.* add cells to media containing DNA-lipid complexes; see page 13).
	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 your virus (Yee, 1999).
	Viral supernatant frozen and thawed multiple times	**Do not** freeze/thaw viral supernatant more than 3 times.
	Poor choice of titering cell line	Use HT1080 cells or another adherent cell line with the characteristics discussed on page 17.

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Generating the Lentiviral Stock, Continued

Problem	Cause	Solution
Low viral titer, continued	Gene of interest is toxic to cells	Do not generate constructs containing activated oncogenes or harmful genes.
	Gene of interest is large	Viral titers generally decrease as the size of the insert increases. Concentrate the virus if titer is low (see page 14) Inserts larger than 5.6 kb are not recommended.
	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. **Do not vortex.**
No colonies obtained upon titering	Too much antibiotic used for selection	Determine the antibiotic sensitivity of your cell line by performing a kill curve experiment, and use the minimum 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.
	Zeocin™ selection performed on confluent cells	Before adding selective medium, trypsinize transduced cells and replate in a larger tissue culture plate.
	Viral supernatant not diluted sufficiently	Titer lentivirus using a wider range of 10-fold serial dilutions (*e.g.* 10^{-2} to 10^{-8}).

Transducing Mammalian Cells

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

Problem	Cause	Solution
No expression of the gene of interest	Promoter silencing	• Lentiviral constructs may integrate into a chromosomal region that silences the CMV promoter. Screen multiple antibiotic-resistant clones and select the one with the highest expression levels. • Use pLenti6/UbC/V5-DEST to generate your lentiviral construct, containing a cellular promoter that is not subject to silencing.
	Viral stocks stored incorrectly	Aliquot and store stocks at –80°C. Do not freeze/thaw more than 3 times.

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Transducing Mammalian Cells, Continued

Problem	Cause	Solution
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.

Appendix

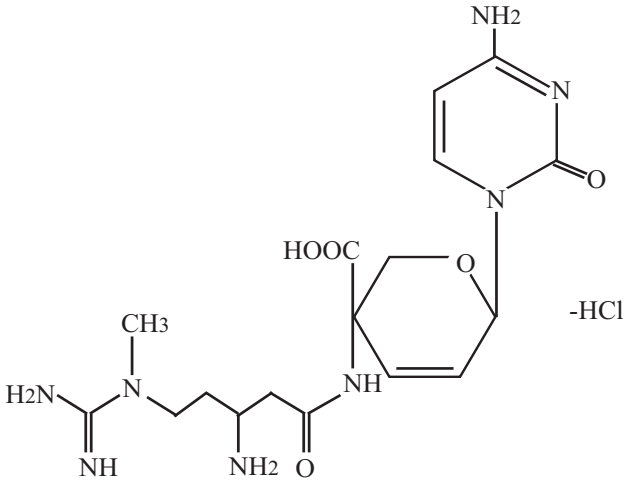
Blasticidin

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: 1350
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 5–10 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.

Zeocin™

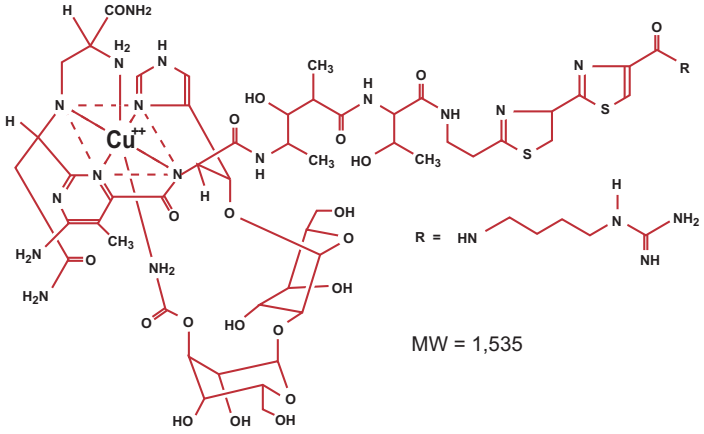
Zeocin™

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

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

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Zeocin™ Selection

Zeocin™ concentrations ranging from 50-1000 µg/ml are typically used for efficient selection in mammalian cells (Mulsant *et al.*, 1988).

Handling Zeocin™

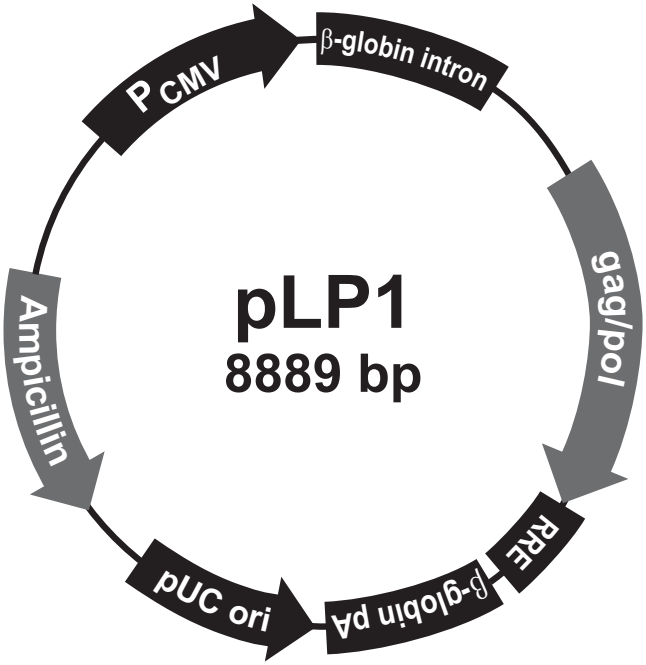
Zeocin™ is supplied in autoclaved, deionized water in 1.25 ml aliquots at a concentration of 100 mg/ml. Handle Zeocin™ using the following guidelines:

- Store Zeocin™ at –20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug, and medium containing drug, in the dark at 4°C. Culture medium containing Zeocin™ may be stored at 4°C protected from exposure to light for up to 1 month.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling Zeocin™-containing solutions.
- Zeocin™ is toxic. Do not ingest or inhale solutions containing the drug.

Map and Features of pLP1

Map of pLP1 Vector

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 then self-cleaved by the viral protease into individual Gag and Pol polyproteins. The complete sequence of pLP1 is available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (see page 36).



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 Vector

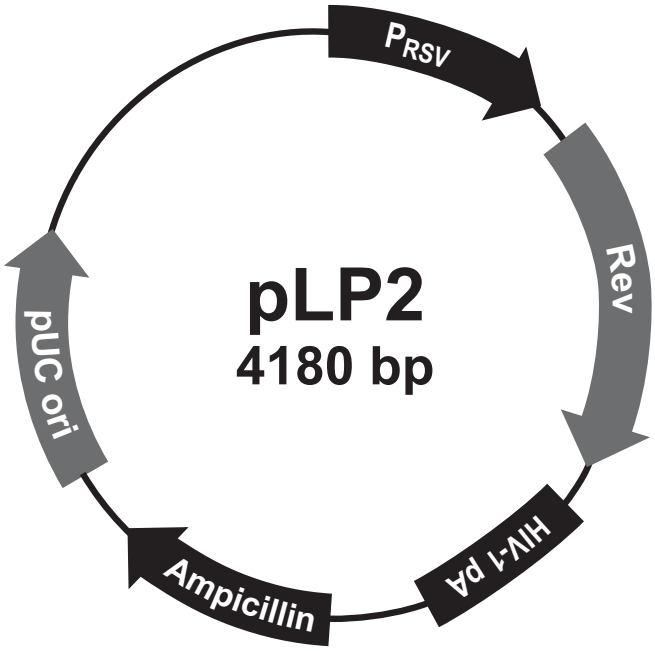
pLP1 (8889 bp) contains the following elements. Features have been functionally tested.

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

Map of pLP2 Vector

The figure below shows the features of the pLP2 vector. The complete sequence of pLP2 is available for downloading downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (see page 36).



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 Vector

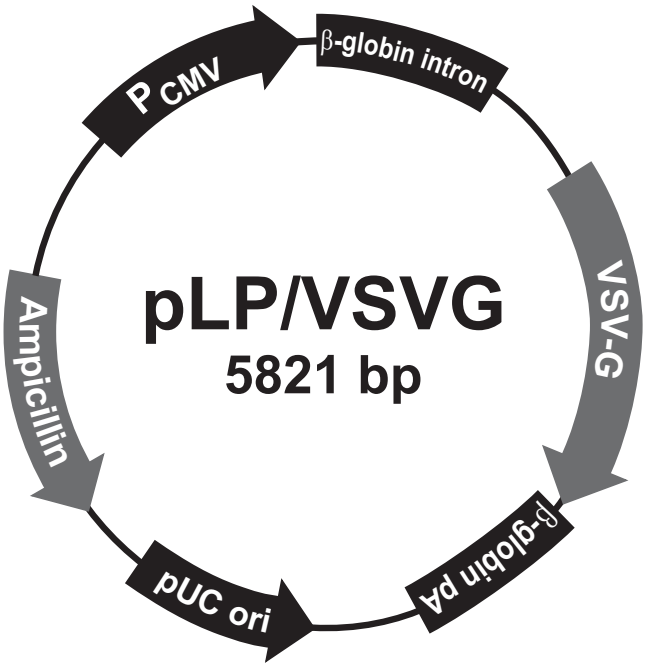
pLP2 (4180 bp) contains the following elements. Features have been functionally tested.

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 to induce Gag and Pol expression, and on the pLenti6/V5 expression vector to promote 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

Map of pLP/VSVG Vector

The figure below shows the features of the pLP/VSVG vector. The complete sequence of pLP/VSVG is available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support** (see page 36).



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 Vector

pLP/VSVG (5821 bp) contains the following elements. 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*.

Technical Support

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Visit the Invitrogen web site at www.invitrogen.com for:

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

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

European Headquarters:
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
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Purchaser Notification, Continued

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For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 40.

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

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.

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Gateway® Expression Clones

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Additional Terms and Conditions

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

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