USER GUIDE

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Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector

Lentiviral expression plasmid containing EmGFP for optimization of lentivirus production, titer, and transduction using the ViraPower™ HiPerform™ Lentiviral Expression Systems

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Kit Contents and Storage

Shipping and Storage	The pLenti6.3/V5-GW/EmGFP Expression Control Vector is shipped on dry ice. Upon receipt, store the control vector at –20°C.
Contents	The pLenti6.3/V5-GW/EmGFP Expression Control Vector is supplied in a volume of 40 µl at a concentration of 0.5 µg/µl in TE Buffer, pH 8.0.
Intended Use	**For research use only.** Not intended for any human or animal diagnostic or therapeutic uses.

Accessory Products

Additional Products

The following additional products may be used with the pLenti6.3/V5-GW/EmGFP Expression Control Vector. For more information, visit our website at **www.lifetechnologies.com** or contact **Technical Support** (page 26)

Product	Quantity	Catalog no.
PureLink® HiPure Plasmid Midiprep Kit	25 reactions 50 reactions	K2100-04 K2100-05
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970-00
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975-00
293FT Cell Line	3 x 10^6 cells	R700-07
Opti-MEM® I Reduced Serum Medium	100 ml 500 ml	31985-062 31985-070
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 µl	C7373-03
Lipofectamine® 2000	0.75 ml 1.5 ml	11668-027 11668-019
Blasticidin	50 mg	R210-01
Ampicillin	5 g	Q100-16
TrypLE™ (1X) Liquid	500 ml	12563-029
Propodium Iodide	100 mg	P1304MP

ViraPower™ HiPerform™ Lentiviral Expression Products

The Vivid Colors™ pLenti6.3/V5-GW/EmGFP vector is designed for use with the following ViraPower™ HiPerform™ Lentiviral Expression Systems available from Life Technologies. For more information, visit our web site at **www.lifetechnologies.com**, or contact **Technical Support** (page 26).

Product	Quantity	Catalog no.
ViraPower™ HiPerform™ Lentiviral TOPO® Expression Kit	1 kit	K5310-00
ViraPower™ HiPerform™ Lentiviral FastTiter™ TOPO® Expression Kit	1 kit	K5320-00
ViraPower™ HiPerform™ Lentiviral Gateway® Expression Kit	1 kit	K5330-00
ViraPower™ HiPerform™ Lentiviral FastTiter™ Gateway® Expression Kit	1 kit	K5340-00
pLenti6.3/V5-TOPO® TA Cloning Kit	1 kit	K5315-20
pLenti7.3/V5-TOPO® TA Cloning Kit	1 kit	K5325-20
pLenti6.3/V5-DEST Gateway® Vector Kit	1 kit	V533-06
pLenti7.3/V5-DEST Gateway® Vector Kit	1 kit	V534-06

Introduction

Overview

Description

The Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector contains the Emerald Green Fluorescent Protein (EmGFP) under the control of a constitutive promoter, and viral elements that allow packaging of the control plasmid into virions. The pLenti6.3/V5-GW/EmGFP control vector is designed for use with the ViraPower™ HiPerform™ Lentiviral Expression Systems (see page v) for the following applications:

- As a positive transfection control for 293FT cells
- As a titer control to produce an EmGFP-expressing lentivirus stock
- As a transduction control to help you determine optimal lentiviral transduction conditions for your target mammalian cell line

The new pLenti6.3/V5-GW/EmGFP control vector contains two elements (WPRE and cPPT) to yield cell-specific, high performance results. The **WPRE** (Woodchuck Posttranscriptional Regulatory Element) from the woodchuck hepatitis virus, is placed directly downstream of the gene of interest, allowing for increased transgene expression(Zufferey *et al.*, 1998), with more cells expressing your gene of interest. **cPPT** (Polypurine Tract) from the HIV-1 integrase gene, increases the copy number of lentivirus integrating into the host genome (Park, 2001). Both WPRE and cPPT together, produce at least a four-fold increase in protein expression compared to vectors that do not contain these elements.

Features of the Vector

The pLenti6.3/V5-GW/EmGFP Expression Control Vector contains the following elements:

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

  Note: The U3 region of the 3′ LTR is deleted (ΔU3) and facilitates self-inactivation of the 5′ LTR after transduction to enhance the biosafety of the vector (Dull *et al.*, 1998)
- HIV-1 psi (Ψ) packaging sequence for viral packaging (Luciw, 1996)
- HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989)
- Polypurine Tract from HIV (cPPT) for increased viral titer (Park et al., 2001)
- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression of EmGFP in a wide range of mammalian cells
- Emerald Green Fluorescent Protein (EmGFP, derived from *Aequorea victoria* GFP) for fluorescence detection
- Woodchuck Posttranscriptional Regulatory Element (WPRE) for increased transgene expression (Zufferey et al., 1999)
- SV40 promoter driving expression of Blasticidin
- Blasticidin resistance gene for selection in *E. coli* and mammalian cells (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965)
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

For the map and features of the pLenti6.3/V5-GW/EmGFP Expression Control Vector, see pages 24-25.

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Important

To produce lentivirus using the pLenti6.3/V5-GW/EmGFP Expression Control Vector, you must supply the components of the ViraPower™ HiPerform™ Lentiviral Expression System (page v), including 293FT cells, Lipofectamine® 2000, Opti-MEM® I, and the ViraPower™ Packaging Mix. These components are described in the section below. Ordering information for these products can be found on page v.

Components of the ViraPower™ HiPerform™ Lentiviral Expression System

The ViraPower™ HiPerform™ Lentiviral Expression Systems (page v) facilitate highly efficient, *in vitro* delivery of a target gene to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. Based on the lentikat™ system developed by Cell Genesys (Dull *et al.*, 1998), the ViraPower™ HiPerform™ 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 (*e.g.* pLenti6.3/V5-DEST) into which the gene of interest will be cloned. The vector contains the WPRE and cPPT elements for higher levels of gene expression, with more cells expressing your gene of interest. The vector also contains the elements required to allow packaging of the expression construct into virions (*e.g.*, 5′ 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, refer to the ViraPower™ HiPerform™ Lentiviral System manual.
- VSV Envelope Glycoprotein: Most retroviral vectors are limited in their usefulness as gene delivery vehicles by their restricted tropism and generally low titers. In the ViraPower™ HiPerform™ 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).
- 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.

pLenti6.3/V5-GW/EmGFP

The pLenti6.3/V5-GW/EmGFP Expression Control Vector was generated by performing an LR recombination reaction between an entry vector containing the EmGFP gene and the pLenti6.3/V5-DEST vector. The *att*B sites flanking EmGFP are a result of the LR recombination reaction.

Note: There is a V5 epitope from the DEST vector backbone downstream of the EmGFP gene, but it will NOT be expressed due to a stop codon at the end of the EmGFP coding sequence.

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Green Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) is a naturally occurring bioluminescent protein derived from the jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962). GFP emits fluorescence upon excitation, and the gene encoding GFP contains all of the necessary information for posttranslational synthesis of the luminescent protein. GFP is often used as a molecular beacon because it requires no species-specific cofactors for function, and the fluorescence is easily detected using fluorescence microscopy and standard filter sets.

GFP and Spectral Variants

Modifications have been made to the wild-type GFP to enhance its expression in mammalian systems. These modifications include nucleic acid substitutions that correspond to the codon preference for mammalian use, and mutations that increase the brightness of the fluorescence signal, resulting in "enhanced" GFP (Zhang *et al.*, 1996). Mutations have also arisen or have been introduced into GFP that further enhance and shift the spectral properties of GFP such that these proteins will emit fluorescent color variations (reviewed in Tsien, 1998). The Emerald GFP (EmGFP) is a variant of enhanced GFP.

EmGFP

The EmGFP variant has been described in a published review (Tsien, 1998) and the amino acid changes are summarized in the table below. The mutations are represented by the single letter abbreviation for the amino acid in the consensus GFP sequence, followed by the codon number and the single letter amino acid abbreviation for the substituted amino acid.

Fluorescent Protein	GFP Mutations*
EmGFP	S65T, S72A, N149K, M153T, I167T

*Mutations listed are as described in the literature. When examining the actual sequence, the vector codon numbering starts at the first amino acid **after** the initiation methionine of the fluorescent protein, so that mutations appear to be increased by one position. For example, the S65T mutation actually occurs in codon 66 of EmGFP.

Spectral Properties of EmGFP Fluorescence

The EmGFP expressed from the pLenti6.3/V5-GW/EmGFP Expression Control Vector has the following excitation and emission wavelengths, as published in the literature (Tsien, 1998):

Fluorescent Protein	Excitation (nm)	Emission (nm)
EmGFP	487	509

Filter Set for Detecting EmGFP Fluorescence

The fluorescent signal from EmGFP can be detected with standard FITC filter sets for fluorescence microscopy systems. However, for optimal detection of the fluorescent signal, you may use a filter set which is optimized for detection within the excitation and emission ranges for each of the fluorescent proteins. This filter set and the manufacturer is listed below:

Fluorescent Protein	Filter Set for Fluorescence Microscopy	Manufacturer
EmGFP	Omega XF100	Omega (www.omegafilters.com)

For information on obtaining this filter set, contact Omega Optical, Inc. (www.omegafilters.com).

Purpose of the Manual

This manual provides instructions and guidelines to:

1. Transfect pLenti6.3/V5-GW/EmGFP Expression Control Vector into the 293FT Cell Line to determine transfection efficiency.
2. Co-transfect the pLenti6.3/V5-GW/EmGFP Expression Control Vector and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a control lentiviral stock.
3. Titer the control lentiviral stock using fluorescence detection methods
4. Use the control lentiviral stock to determine optimal transduction conditions for your mammalian cell line of choice.

Additional Information

For more information about the ViraPower™ HiPerform™ Lentiviral Expression Systems, refer to the ViraPower™ HiPerform™ Lentiviral System Manual. For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual. These manuals are supplied with the ViraPower™ HiPerform™ Lentiviral Expression Kits, and are also available for downloading from **www.lifetechnologies.com** or by contacting **Technical Support**, page 26.

Methods

Producing EmGFP Lentivirus in 293FT Cells

Introduction

You can use the Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector to transfect 293FT cells, estimate the transfection efficiency, and produce EmGFP lentiviral stocks. The following section provides guidelines and protocols for performing these steps.

Lentivirus produced with the ViraPower™ HiPerform™ System can pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat lentiviral stocks as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** For more information about 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

Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements may vary at individual institutions, we recommend consulting the health and safety guidelines and/or officers at your institution prior to use of the ViraPower™ HiPerform™ Lentiviral Expression System. More information about the specific biosafety features of the ViraPower™ HiPerform™ Lentiviral Expression System can be found in the System manual.

Using the Vector

The pLenti6.3/V5-GW/EmGFP Expression Control Vector is supplied in solution at 0.5 µg/µl plasmid DNA in TE Buffer (TE), pH 8.0. You can use this stock for production of lentivirus, or you can propagate and maintain the plasmid as described below.

Propagating the Vector

If you wish to propagate and maintain the pLenti6.3/V5-GW/EmGFP Expression Control Vector, we recommend using Stbl3™ Chemically Competent *E. coli* from Life Technologies (page v). This strain is particularly well suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats.

Use 1-10 ng of the plasmid to transform One Shot® Stbl3™ Chemically Competent *E. coli* and select transformants on LB agar plates containing 100 µg/ml ampicillin (see page 22 for recipe).

Plasmid DNA

Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride as contaminants may kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA for transfection using the PureLink® HiPure Plasmid Midiprep Kit (page v), or equivalent.

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Determining Transfection Efficiency

You may determine transfection efficiency of the pLenti6.3/V5-GW/EmGFP Expression Control Vector into 293FT cells in either of the following ways:

- **Qualitatively**; by examining transfected, EmGFP-expressing 293FT cells under a fluorescence microscope.
- **Quantitatively**; by analyzing transfected, EmGFP-expressing 239FT cells by the flow cytometry method of choice.

  Note: If you choose to perform flow cytometry, you can use an irrelevant plasmid DNA such as an empty DEST vector instead of the ViraPower™ Packaging mix to avoid using virus-producing cells in your flow cytometry, which may present biohazard concerns.

Depending on how you wish to determine transfection efficiency, follow the recommendations in the transfection protocol on page 9.

Materials Needed

You will need the following items:

- pLenti6.3/V5 GW/EmGFP Expression Control Vector (0.5 µg/µl)

Materials available separately: (see page v)

- ViraPower™ Packaging Mix (1 µg/µl)
- 293FT cells (6 x 10^6 cells for each transfection)
- Complete growth medium for 293FT cells (D-MEM containing 10% FBS, 2mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin-streptomycin, and 1 mM MEM Sodium Pyruvate)

  Note: MEM Sodium Pyruvate provides an extra energy source for the cells and is available from Life Technologies Catalog no. 11360-070)
- Lipofectamine® 2000 transfection reagent (mix gently before use)
- *Optional:* Irrevelant plasmid such as an empty DEST vector (1 µg/µl) if you do not intend to make virus-producing cells
- Opti-MEM® I Reduced Serum Medium (pre-warmed to 37°C)
- Fetal Bovine Serum
- Sterile 10 cm tissue culture plates
- Sterile tissue culture supplies
- 15 ml sterile, capped, conical tubes
- Cryovials
- *Optional:* Millex-HV 0.45 µm PVDF filters (Millipore, cat. no. SLHVR25LS) or equivalent, to filter viral supernatants
- Inverted fluorescence microscope with FITC filter or Omega XF100 filter (see next page) for detecting EmGFP-expressing cells in culture, or a flow cytometry system with a FITC filter to quantitatively detect EmGFP-expressing cells

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293FT Cell Line

The human 293FT Cell Line is supplied with the ViraPower™ HiPerform™ 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® (page v).

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™ HiPerform™ Lentiviral Expression kits, and is also available by downloading from **www.lifetechnologies.com** or by contacting **Technical Support** (page 26).

Note: The 293FT Cell Line is also available separately from Life Technologies (page v).

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:

- Ensure 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 v).
- Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 16 passages.

Detecting EmGFP by Fluorescence Microscopy

The fluorescent signal from EmGFP can be detected with standard FITC filter sets. However, for optimal detection of the fluorescent signal, you may use the Omega XF100 filter set for your cell culture (inverted) microscope that is optimized for detection of EmGFP:

Fluorescent Protein	Excitation/Emission (nm)	Filter Set for Fluorescence Microscopy
EmGFP	487/509	Omega XF100

For information on obtaining this filter set, contact Omega Optical, Inc. (www.omegafilters.com).

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ViraPower™ Packaging Mix

The ViraPower™ Packaging Mix is supplied with the ViraPower™ HiPerform™ Lentiviral Expression Kits or is available separately from Life Technologies (page v).

The ViraPower™ Packaging Mix contains a mixture of plasmids (pLP1, pLP2 and pLP/VSVG) which are cotransfected into 293FT cells to supply the structural and replication proteins *in trans* for producing lentivirus.

Lipofectamine® 2000

The Lipofectamine® 2000 reagent (Ciccarone *et al.*, 1999) is a proprietary, cationic lipid-based formulation for optimal transfection of nucleic acids into eukaryotic cells.

The recommended procedure to co-transfect 293FT cells differs from the traditional Lipofectamine® 2000 transfection procedure in that you will:

- First prepare DNA-Lipofectamine® 2000 complexes and add them to plates containing growth media, then
- Add the 293FT cells to the media containing DNA-Lipofectamine® 2000 complexes and allow the cells to attach and transfect overnight (see **293FT Transfection Protocol,** next page).

Using this procedure, we consistently obtain lentiviral stocks with titers that are **3 to 4-fold higher** than lentiviral stocks generated using the traditional Lipofectamine® 2000 transfection procedure.

Lipofectamine® 2000 is supplied with the ViraPower™ Lentiviral Support Kits or is available separately from Life Technologies (page v).

Opti-MEM® I

To facilitate the optimal formation of DNA-Lipofectamine® 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium available from Life Technologies, (page v).

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293FT Transfection Protocol

Follow the protocol below to transfect 293FT cells with the pLenti6.3/V5-GW/EmGFP Expression Control Vector.

Note: This protocol differs from the transfection protocol in the ViraPower™ HiPerform™ System manual. Use the protocol below **only** for producing EmGFP lentivirus.

1. Prepare DNA-Lipofectamine® 2000 complexes. In a sterile 15 ml tube, combine **one** of the following and mix gently.

To Generate Lentivirus from 293FT Cells, combine:	To Check 293FT Transfection Efficiency without Generating Lentivirus, combine:
9 µg of the ViraPower™ Packaging Mix 3 µg of pLenti6.3/V5-GW/EmGFP 1.5 ml of Opti-MEM® I Medium without serum	9 µg of an irrelevant plasmid DNA (**Materials Needed** page 6) 3 µg of pLenti6.3/V5-GW/EmGFP 1.5 ml of Opti-MEM® I Medium without serum

2. In a separate sterile 15 ml tube, dilute 36 µl Lipofectamine® 2000 (mix gently) into 1.5 ml of Opti-MEM® I Medium without serum. Mix gently.
3. Incubate diluted mixture for 5 minutes at room temperature.
4. Combine the diluted DNA (Step 1) with the diluted Lipofectamine® 2000 (Step 2). Mix gently.
5. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine® 2000 complexes to form.

   Note: The solution may appear cloudy, but this will not impede transfection.
6. While the 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 FBS at the same concentration as the growth medium for that cell line. **Do not include antibiotics in the medium.**
7. 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.**
8. Add 5 ml of the 293FT cell suspension (6×10^6 total cells, Step 6) to the plate containing media and DNA-Lipofectamine® 2000 complexes. Mix gently by rocking the plate back and forth.
9. Incubate cells overnight at 37°C in a CO_2 incubator.
10. The next day, remove the cells from the incubator and remove and discard the medium containing the DNA-Lipofectamine® 2000 complexes. Replace the medium with complete culture medium containing sodium pyruvate.
11. Return the cells to 37°C in a CO_2 incubator.
12. **Proceed to step 13 (next page) if you are producing lentivirus.**

Note: You may assay for transfection efficiency at 24-48 hours post-transfection by fluorescence microscopy (see page 7). Greater than 90% of the cells should be EmGFP positive.

Protocol continues on next page

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293FT Transfection Protocol, Continued

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13. 48-72 hours post-transfection, harvest virus-containing supernatants by removing medium to a 15 ml sterile, capped, conical tube.

    Caution: Remember that you are working with infectious virus at this stage. Follow your institution's guidelines for working with BL-2 organisms.

14. Centrifuge the viral supernatant at 2,000 x g for 15 minutes at +4°C to pellet cell debris.

    Note: You can perform a filtration step, if desired (see **Filtering Virus,** below).

15. Pipet viral supernatants into cryovials in 1 ml aliquots. Store viral stocks at -80°C. See **Long-Term Storage** (next page) for further details about long term virus stock storage.

Forward Transfection Protocol

An alternative transfection procedure (referred to as the Forward Transfection Protocol) is provided below to cotransfect 293FT cells. Note that use of this procedure generally results in production of lentiviral stocks with a slightly lower titer than those produced when using the 293FT Transfection Protocol, previous page.

1. The day before transfection, plate the 293FT cells in a 10 cm tissue culture plate such that they will be 90-95% confluent on the day of transfection (*i.e.* 6 x 10^6 cells in 10 ml of growth medium containing serum).
2. On the day of transfection, 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. Prepare DNA-Lipofectamine® 2000 complexes as instructed in the **293FT Transfection Protocol**, Step 1 (previous page).
4. Add the DNA-Lipofectamine® 2000 complexes drop wise 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. Follow Steps 8-11 as instructed in the **293FT Transfection Protocol**, (previous page).

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Filtering Virus

Note: It should be possible to use the new pLenti6.3/V5-GW/EmGFP vector for *in vivo* applications, however, we have not yet tested the new vector *in vivo*.

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 Multiplicity of Infection (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 EmGFP Lentivirus

Introduction

After you have produced your EmGFP lentiviral stock in 293FT cells, you are ready to determine the titer of your viral stock. Since cells that are transduced with EmGFP lentivirus produce EmGFP, the titer of the EmGFP lentivirus stock can be calculated at 4 days post transduction by fluorescence detection and without the need for lengthy antibiotic selection. Protocols and guidelines are provided in this section to titer your EmGFP lentiviral stock.

Experimental Outline

To determine the titer of your EmGFP lentiviral stocks, you will:

1. Prepare a 50-fold or 20-fold serial dilutions of your lentiviral stocks.
2. Transduce the different dilutions of lentivirus in the presence of the polycation Polybrene (page 13).
3. Determine the Lentiviral titer by fluorescence detection using flow cytometry or fluorescence microscopy at 2 days post-transduction.

Note

The pLenti6.3/V5-GW/EmGFP Expression Control Vector contains the Blasticidin resistance gene. You may use the standard titer method based on antibiotic selection described in the ViraPower™ HiPerform™ Lentiviral System manual.

Range of Dilutions

- If you are generating lentivirus for the first time and do not know what to expect, you may wish to generate a wider range (*i.e.* 10^{-1} – 10^{-8}) of dilutions in the event that your virus stock turns out to have a very high or very low titer.
- If calculating the most accurate titer is critical for your experiments, you may wish to set up triplicate transductions for each dilution and use the average percentage of GFP-positive cells for your calculations.

Selecting a Cell Line

You may titer your lentiviral stock using any mammalian cell line of choice. Generally, we recommend using the same mammalian cell line to titer your lentiviral stock as you will use to perform your expression studies. However, in some instances, you may wish to use a different cell line to titer your lentivirus (*e.g.* if you are performing expression studies in a non-dividing cell line or a primary cell line). In these cases, we recommend that you choose a cell line with the following characteristics to titer your lentivirus:

- Grows as an adherent cell line
- Easy to handle
- Exhibits a doubling time in the range of 18-25 hours
- Non-migratory

We generally use the HT1080 human fibrosarcoma cell line (ATCC, Catalog no. CCL-121) for titering purposes.

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Using Polybrene During Transduction

Transduction of lentivirus into mammalian cells may be enhanced if cells are transduced in the presence of hexadimethrine bromide (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. 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.

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 tubes.
3. 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.

   Note: The working stock of Polybrene may be stored at +4°C for up to 2 weeks.

Materials Needed

You will need the following items:

- Your EmGFP lentiviral stock (store at -80°C until use)
- Adherent mammalian cell line of choice (previous page)
- Complete culture medium for your cell line
- **Optional:** 6 mg/ml Polybrene (see above)
- 96-well tissue culture plates
- Optional: TrypLE™ (page v)
- Trypsin cell dissociation solution(or equivalent, see below) for flow cytometry
- *Optional:* Flow cytometry buffer of choice, such as calcium/magnesium-free Phosphate Buffered Saline containing 1% FBS or BSA
- *Optional:* Inverted fluorescence microscope with FITC filter or Omega XF100 filter (see page 7) for detecting EmGFP-expressing cells in culture, or a flow cytometry system with a FITC filter to quantitatively detect EmGFP-expressing cells

Trypsin Dissociation Solution

Before proceeding to analysis with flow cytometry, you need to dissociate your cells from the wells. To prepare the dissociation solution using TrypLE™:

1. Make a 1:3 mix of TrypLE™ and PBS, respectively (see page v to order).
2. Add 25 µl of a 1 mg/ml propidium iodide stock solution (page v).

Continued on next page

Remember that you will be working with media containing active 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 pipets, 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.

Transduction 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 each lentiviral stock to be titered (usually one mock well plus five dilutions).

1. **24 hours before transduction**, seed cells in a 96-well format at a density of 6,000 cells per well. Incubate in a 37°C CO_2 incubator overnight.
2. On the day of transduction (Day 1), thaw your lentiviral stock. In a biosafety cabinet, prepare a 50-fold or 20-fold serial dilution of the Lentiviral stock in DMEM growth medium supplemented with polybrene (page 13). Mix each virus dilution gently by inversion (**DO NOT** vortex).

   Important: Do **NOT** dilute virus in culture medium containing Blasticidin.
3. Remove the culture medium from each well of cells and replace with the diluted virus solution. We recommend allocating 3-6 replicate wells per sample.
4. Swirl the plate gently to mix. Incubate at 37°C in a CO_2 incubator overnight.
5. After 24 hours incubation (Day 2), remove the virus-containing media from each well and discard (See **Caution**, previous page). Replace with 100 µl of fresh growth medium in each well and incubate overnight in a 37°C CO_2 incubator.

   Important: Do **NOT** add Blasticidin to the growth medium.
6. After 24 hours incubation (Day 3), remove the growth media from each well and discard. Replace with dissociation solution (above) in each well.
7. Allow cells to dissociate for 5 minutes at 37°C then proceed **Preparing Cells for Flow Cytometry**, next page.

Continued on next page

Note

If you wish to fix your cells before flow cytometry, you can use 2% formaldehyde or paraformaldehyde in calcium/magnesium free PBS. However, these fixatives may increase autofluorescence of the cells, thus it is critical to include fixed, mock-transduced cells as a negative control for flow cytometry detection parameters.

Preparing Cells for Flow Cytometry

Prepare cells for flow cytometry using a FITC filter according to the established protocols in use at your flow cytometry facility. Refer to page 3 for specific excitation/emission properties of EmGFP. The steps below provide simple guidelines, and other methods may be suitable.

1. After cells have dissociated, (Steps 6-7, previous page), spin the cells at low speed to remove residual media components and resuspend the cell pellet in flow cytometry buffer such as calcium/magnesium free PBS with 1% FBS at the required density for analysis on your flow cytometer. Fixing the cells is not necessary but may be done (see **Note** above).
2. Use the mock-transduced cells and the lowest dilution of virus (*i.e.* 10^{-1}) as the negative and positive samples, respectively, to set up the parameters of your flow cytometer.

Alternate Protocol for Titering EmGFP Lentivirus

It is possible to estimate EmGFP lentiviral titer by counting EmGFP-positive cells using fluorescence microscopy (see page 7 for details). Note that this method is labor intensive and the results are much less accurate than using the flow cytometry method because of the necessity for a smaller sample size and reliance on visual discrimination of EmGFP-positive cells.

1. Using fluorescence microscopy, determine two dilutions that have a countable number of EmGFP positive cells (*i.e.* 100 or fewer).
2. Count the total number of cells for each dilution (the well may be divided into quarters to facilitate counting) and determine the number of EmGFP positive cells.
3. Calculate the titer by taking the average of the titers from the 2 wells. For example, if the 10^{-5} dilution has 46 green cells in the well, and the 10^{-6} dilution has 5 green cells in the well, the titer would be 4.8×10^6 TU/ml (average of 46×10^5 and 5×10^6).

Continued on next page

Calculating Lentiviral Titer

EmGFP lentivirus titers should be calculated from the dilutions at which the percentage of GFP-positive cells fall within the range of 1-30% (White *et al.*, 1999) (Sastry *et al.*, 2002). This is to avoid analyzing dilution samples containing multiple integrated lentiviral genomes, which may result in an underestimate of the viral titer, or dilution samples containing too few transduced cells, which will give inaccurate results. Titer is expressed as transducing units (TU)/ml.

In the following example, an EmGFP lentiviral stock was generated using the protocol on the previous page. The stock was concentrated and the following data were generated after performing flow cytometry:

Lentivirus Dilution	% EmGFP Positive Cells
10^{-2}	91.5%
10^{-3}	34.6%
10^{-4}	4.4%

The following formula (White *et al.*, 1999) (Sastry *et al.*, 2002) is used to calculate the titer: **$[F \times C/V] \times D$**

F = frequency of GFP-positive cells (percentage obtained divided by 100)

C = total number of cells in the well at the time of transduction

V = volume of inoculum in ml

D = lentivirus dilution

In the above example, the 10^{-4} dilution is used to calculate the titer since the percentage of EmGFP-positive cells falls into the desired range of 1-30%. The frequency of EmGFP-positive cells is 4.4/100 = 0.044, multiplied by 2×10^5 (the number of cells in the well) divided by 1 (the volume of inoculum). Thus the calculation is as follows:

$$[(0.044 \times 200{,}000)/1] \times 10^4$$

The titer for this example is 8.8×10^7 TU/ml.

What You Can Expect

We typically obtain unconcentrated EmGFP lentivirus titers in the range of 5×10^5 - 2×10^6 TU/ml. To obtain higher lentivirus titer, you can concentrate your virus (see page 18). The titer of concentrated lentivirus stocks may be up to 1×10^8 TU/ml.

Continued on next page

Transduction and Analysis

Introduction

Once you have generated an EmGFP lentiviral stock with a suitable titer, you are ready to optimize the transduction conditions for mammalian cell line of choice.

Reminder: Remember that the pLenti6.3/V5-GW/EmGFP Expression Control Vector 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.

Evaluating Transduction with EmGFP Expression

After transducing your mammalian cell line of choice with the pLenti6.3/V5-GW/EmGFP Expression Control Vector, you can assay for expression of EmGFP by either "transient" expression or stably transduced cells by performing one of the following:

- Pool a heterogeneous population of cells and test for EmGFP expression 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 optimal detection of EmGFP.
- Select for stably transduced cells using Blasticidin (pLenti6.3 vectors, only). This requires a minimum of 10-12 days after transduction, but allows generation of clonal cell lines that stably express EmGFP.

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

Multiplicity of Infection (MOI)

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

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell line (*e.g.* non-dividing vs. dividing cell type), its transduction efficiency, and your application of interest. If you are trying to optimize transducing your mammalian cell line of choice for the first time using pLenti6.3/V5-GW/EmGFP, we recommend using 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 EmGFP in your cell line.

Continued on next page

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their transducibility. If the titer of your lentiviral stock is relatively low (less than 5×10^5 TU/ml) and your experiment requires that you use a large volume of viral supernatant (*e.g.* a relatively high MOI), you may wish to concentrate your virus before proceeding to transduction. For details and guidelines to concentrate your virus, refer to published reference sources (Yee, 1999).

Determining Blasticidin Sensitivity for Your Cell Line

If you wish to select for stably transduced cells on your cell line for the first time, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). See the ViraPower™ HiPerform™ Lentiviral System Manual for more information about determining Blasticidin sensitivity. See page 23 for information on handling and storing Blasticidin (for pLenti6.3 vectors only).

Materials Needed

You will need the following items:

- Your titered lentiviral stock (stored at -80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/ml Polybrene (see page 13)
- Appropriately sized tissue culture plates for your application
- Inverted fluorescence microscope with FITC filter or Omega XF100 filter (see page 7) for detecting EmGFP-expressing cells in culture, or a flow cytometry system with a FITC filter to quantitatively detect EmGFP-expressing cells
- Blasticidin, if selecting for stably transduced cells.

Important

Remember that 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.

Continued on next page

Transduction Procedure

Follow the procedure below to transduce the mammalian cell line of choice.

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 (see **Determining Optimal MOI**, page 17) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting (**DO NOT** vortex) and add to the cells.
4. Add Polybrene (if desired) to the plate to a final concentration of 6 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 **without** Blasticidin.
6. The following day (Day 3), you may analyze the cells for expression of EmGFP by flow cytometry or fluorescence microscopy.
7. You may sort the cells expressing EmGFP with flow cytometry or fluorescence microscopy and use these cells for assaying protein expression.

Troubleshooting

Introduction

The table below lists some potential problems and solutions that may help you troubleshoot EmGFP lentivirus production, titering, and transduction of cells with EmGFP lentivirus.

Problem	Possible Cause	Solution
Low viral titer	Low transfection efficiency: • Used poor quality plasmid DNA (*i.e.* plasmid DNA from a mini-prep)	• **Do not** use mini-prep plasmid DNA for transfection. Use the PureLink® HiPure Plasmid Midiprep Kit or CsCl gradient centrifugation 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 as recommended in the transfection protocol (page 9), or try the alternate transfection protocol (page 10).
	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 post transfection. 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 any 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 or another adherent cell line with the characteristics discussed on page 12.

Continued on next page

Problem	Possible Cause	Solution
Low viral titer, continued	Polybrene not included during transduction	TransducepLenti6.3/V5-GW/EmGFP 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 EmGFP positive cells obtained after titering	Incorrect filter set on cell culture fluorescence microscope or detection parameters for flow cytometer	Make sure you are using a FITC or Omega XF100 filter set on your inverted fluorescence microscope (see page 7) or the FITC detection parameters on your flow cytometer.
	Viral stocks stored incorrectly	Aliquot and store stocks in cryovials at -80°C. Do not freeze/thaw more than 3 times.
	Polybrene not included during transduction	Transduce pLenti6.3/V5-GW/EmGFP into cells in the presence of Polybrene.
	Too soon to see EmGFP expression	For optimal EmGFP expression, wait 4 days post transduction.
Poor expression of EmGFP in transiently transduced mammalian cell lines	Low transduction efficiency: • Polybrene not included during transduction • Non-dividing cell type used	• Transduce pLenti6.3/V5-GW/EmGFP into cells in the presence of Polybrene. • Transduce pLenti6.3/V5-GW/EmGFP into cells using a higher MOI.
	MOI too low	Transduce pLenti6.3/V5-GW/EmGFP into cells using a higher MOI.
	Cells harvested too soon after transduction	Do not harvest cells until at least 48-72 hours after transduction to allow EmGFP to accumulate in transduced cells.
No expression of EmGFP after stable transduction into mammalian cell lines	Promoter silencing	pLenti6.3/V5 GW/EmGFP may integrate into a chromosomal region that silences the CMV promoter controlling expression of EmGFP. Screen multiple antibiotic-resistant clones and select the one with the highest expression levels.
	Incorrect filter used with cell culture fluorescence microscope or detection parameters for flow cytometer	Make sure you are using a FITC or Omega XF100 filter set on your inverted fluorescence microscope (see page 7) or the FITC detection parameters on your flow cytometer.
Poor expression of EmGFP after stable transduction into mammalian cell lines	Too much Blasticidin 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.

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

For LB agar plates:

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic if needed, and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

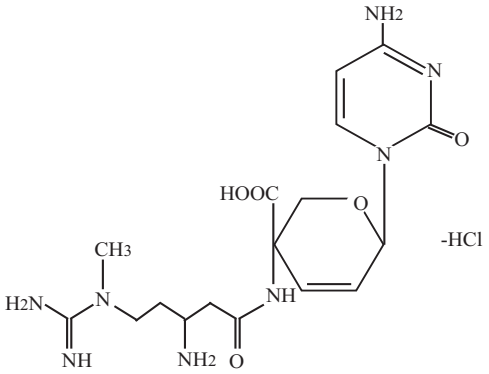
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd* from *Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a nontoxic deaminohydroxy derivative (Izumi *et al.*, 1991). Blasticidin is available separately from Life Technologies (see page v for ordering information). For information on preparing and handling Blasticidin, see the Appendix, page 23.

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.* a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

Blasticidin may be obtained separately from Life Technologies (Catalog no. R210-01) in 50 mg aliquots. Blasticidin is soluble in water. Use sterile water to prepare stock solutions of 5 to 10 mg/ml.

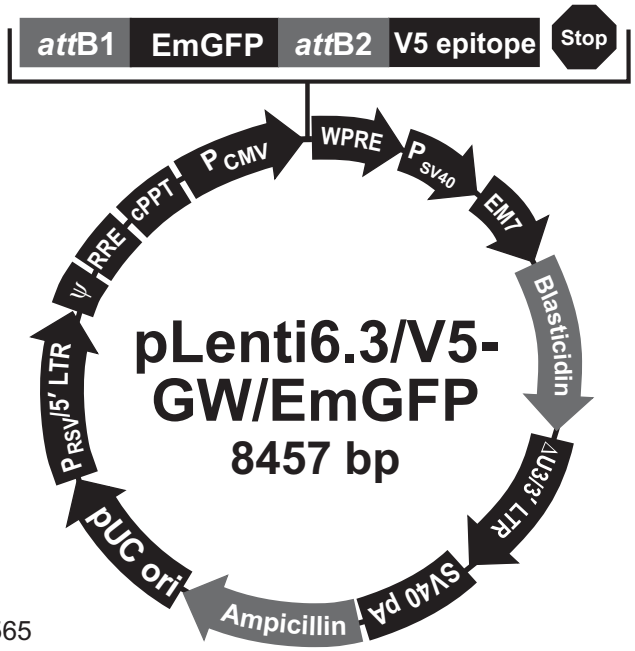
- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot solution in small volumes suitable for one time use (see next to last point below) and freeze at -20°C for long-term storage or store at +4°C for short-term storage.
- Aqueous stock solutions are stable for 1-2 weeks at +4°C and 6-8 weeks at -20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at +4°C for up to 2 weeks.

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

Map of pLenti6.3/V5-GW/EmGFP Expression Control Vector

Map

The map below shows the elements of the pLenti6.3/V5-GW/EmGFP Expression Control Vector (8675 bp). **The complete sequence of this vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (page 26).**



Comments for pLenti6.3/V5-GW/EmGFP
8457 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
- RSV promoter: bases 1-229
- HIV-1 5′ LTR: bases 230-410

HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
cPPT: bases 1801-1923
CMV promoter: bases 1935-2519
*att*B1 site: bases 2568-2592
EmGFP: bases 2598-3317
*att*B2 site: bases 3318-3342
V5 epitope: bases 3395-3436
WPRE: bases 3455-4052
SV40 promoter: bases 4063-4371
EM7 promoter: bases 4426-4492
Blasticidin resistance gene: bases 4493-4891
ΔU3/HIV-1 3′ LTR: bases 4977-5211
- ΔU3: bases 4977-5030
- Truncated HIV-1 3′ LTR: bases 5031-5211

SV40 polyadenylation signal: bases 5283-5414
bla promoter: bases 6273-6371
Ampicillin (*bla*) resistance gene: bases 6372-7232
pUC origin: bases 7377-8050

Continued on next page

Features of pLenti6.3/V5-GW/EmGFP Expression Control Vector

Features

The pLenti6.3/V5-GW/EmGFP Expression Control Vector contains the following elements. Features have been functionally tested.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	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 packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001).
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
*att*B1 and *att*B2 sites	Allow recombination-based transfer of EmGFP into any Gateway® expression vector via an LR and BP reaction.
EmGFP	Allows visual detection using fluorescence microscopy or flow cytometry.
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey *et al.*, 1998).
EM7 promoter	Allows expression of Blasticidin in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli.*
pUC origin	Allows high-copy number replication and growth in *E. coli.*

Technical Support

Obtaining Support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

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Purchaser Notification

Introduction

Use of the Vivid Colors™ pLenti6.3/V5-GW/EmGFP vector is covered under a number of different licenses including those detailed below.

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References

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

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

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

Ciccarone, V., Chu, Y., Schifferli, K., Pichet, J.-P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. (1999) Lipofectamine® 2000 Reagent for Rapid, Efficient Transfection of Eukaryotic Cells. Focus *21*, 54-55

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

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

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

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

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

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

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

Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient Transfer, Integration, and Sustained Long-Term Expression of the Transgene in Adult Rat Brains Injected with a Lentiviral Vector. Proc. Natl. Acad. Sci. USA *93*, 11382-11388

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

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

Sastry, L., Johnson, T., Hobson, M. J., Smucker, B., and Cornetta, K. (2002) Titering Lentiviral vectors:comparison of DNA, RNA and marker expression methods. Gene Ther. *9*, 1155-1162

Shimomura, O., Johnson, F. H., and Saiga, Y. (1962) Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous hHydromedusan, Aequorea. Journal of Cellular and Comparative Physiology *59*, 223-239

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

Tsien, R. Y. (1998) The Green Fluorescent Protein. Annu. Rev. Biochem. *67*, 509-544

References, Continued

White, S. M., Renda, M., Nam, N. Y., Klimatcheva, E., Y.Zhu, Fisk, J., Halterman, M., Rimel, B. J., Federoff, H., Pandya, S., Rosenblatt, J. D., and Planelles, V. (1999) Lentivirus vectors using human and simian imunodeficiency virus elements. J Virology *73*, 2832-2840

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

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

Yee, J. K. (1999) in *The Development of Human Gene Therapy* (Friedmann, T., ed), pp. 21-45, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Zhang, G., Gurtu, V., and Kain, S. (1996) An Enhanced Green Fluorescent Protein Allows Sensitive Detection of Gene Transfer in Mammalian Cells. Biochem. Biophys. Res. Comm. *227*, 707-711

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

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