



Vivid Colors[™] pLenti6.2- GW/EmGFP

**Lentiviral expression plasmid containing EmGFP
for optimization of lentivirus production, titer,
and transduction using the ViraPower[™] Lentiviral
Expression System**

Catalog no. V369-20

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By use of this product, you accept the terms and conditions of the Limited
Label License.**

User Manual

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Kit Contents and Storage

Shipping and Storage

The pLenti6.2-GW/EmGFP Expression Control Vector is shipped on wet ice. Upon receipt, store at -20°C .

Contents

20 μg of control vector is supplied in solution as 40 μl of 0.5 $\mu\text{g}/\mu\text{l}$ pLenti6.2-GW/EmGFP Expression Control Vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Accessory Products

Additional Products

Additional products available from Invitrogen are listed below. For more information, visit our website at www.invitrogen.com or contact **Technical Support** (page 27).

Product	Amount	Catalog no.
ViraPower™ Lentiviral Gateway® Expression Kit	1 kit	K4960-00
ViraPower™ UbC Lentiviral Gateway® Expression Kit	1 kit	K4990-00
ViraPower™ II Lentiviral Gateway® Expression Kit	1 kit	K367-20
ViraPower™ II Lentiviral C-Lumio™ Gateway® Expression Kit	1 kit	K370-20
ViraPower™ II Lentiviral N-Lumio™ Gateway® Expression Kit	1 kit	K371-20
pLenti6/V5 Directional TOPO® Cloning Kit	1 kit	K4950-00
pLenti6.3/V5-GW/EmGFP Expression Control Vector	20 µg (40 µl of 0.5 µg/µl in TE Buffer, pH 8.0.)	V370-06
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	31985-062
	500 ml	31985-070
One Shot® Stbl3™ Chemically Competent <i>E. coli</i>	20 x 50 µl	C7373-03
S.N.A.P.™ Midiprep DNA Isolation Kit	20 reactions	K1910-01
PureLink™ HiPure Plasmid Midiprep Kit	25 reactions	K2100-04
	50 reactions	K2100-05
Lipofectamine™ 2000 Transfection Reagent	0.75 ml	11668-027
	1.5 ml	11668-019
Blasticidin	50 mg	R210-01
Ampicillin	5 g	Q100-16

Introduction

Overview

Description

The Vivid Colors™ pLenti6.2-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. pLenti6.2-GW/EmGFP is designed for use with the ViraPower™ or ViraPower™ II Lentiviral Expression System 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
-

Features of the Vector

The pLenti6.2-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 (Δ U3) and facilitates self-inactivation of the 5' LTR after transduction to enhance the biosafety of the vector (Dull *et al.*, 1998)
- HIV-1 psi (Ψ) packaging sequence for viral packaging (Luciw, 1996)
- HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989)
- Human 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
- Murine PGK promoter for high-level expression of the Blasticidin resistance gene
- 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 pLenti6.2-GW/EmGFP Expression Control Vector, see page 25.

Continued on next page

Overview, Continued



Important

To produce lentivirus using the pLenti6.2-GW/EmGFP Expression Control Vector, you must supply the components of the ViraPower™ or ViraPower™ II Lentiviral Expression System, 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 vi.

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 such as pLenti6.2-GW/EmGFP that contains the elements required for packaging into virions (*e.g.* 5' and 3' LTRs, Ψ packaging signal).
 - The ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus.
 - The 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.
-

pLenti6.2-GW/EmGFP

The pLenti6.2-GW/EmGFP Expression Control Vector was generated by performing an LR recombination reaction between an entry vector containing the EmGFP gene and the pLenti6.2/V5-DEST vector. The *attB* sites flanking EmGFP are a result of the LR recombination reaction.

Note: There is a V5 epitope from the pDEST 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.

Continued on next page

Overview, Continued

Purpose of the Manual

This manual provides instructions and guidelines to:

1. Transfect pLenti6.2-GW/EmGFP Expression Control Vector into the 293FT Cell Line to determine transfection efficiency.
 2. Co-transfect the pLenti6.2-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™ or ViraPower™ II System, refer to the ViraPower™ 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™ Lentiviral Expression Kits, and are also available for downloading from www.invitrogen.com or by contacting **Technical Support** (page 27).

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.

Continued on next page

Overview, Continued

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 pLenti6.2-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).

Methods

Producing EmGFP Lentivirus in 293FT Cells

Introduction

You can use the Vivid Colors™ pLenti6.2-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™ 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™ Lentiviral Expression System. More information about the specific biosafety features of the ViraPower™ Lentiviral Expression System can be found in the System manual.

Using the Vector

The pLenti6.2-GW/EmGFP Expression Control Vector is supplied in solution as 40 µl of 5 µg/µl control vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. You can use this solution 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 pLenti6.2-GW/EmGFP Expression Control Vector, we recommend using 10 ng of the vector to transform One Shot® Stbl3™ Chemically Competent *E. coli* (page vi) This strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats.

Once you have transformed the Stbl3™ *E. coli*, select transformants on LB agar plates containing 100 µg/ml ampicillin (see page 23 for recipe).

For long-term storage, we recommend that you make a glycerol stock for long-term storage (see page 23).

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 vi).

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Producing EmGFP Lentivirus in 293FT Cells, Continued

Determining Transfection Efficiency

You may determine transfection efficiency of the pLenti6.2-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 293FT 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 cytometer, which may present biohazard concerns.

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

Materials Needed

You will need the following items:

- pLenti6.2-GW/EmGFP Expression Control Vector (0.5 µg/µl)

Materials available separately: (see page vi)

- ViraPower™ Packaging Mix (1 µg/µl)
- 293FT cells (6 × 10⁶ 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 Invitrogen (page vi)

- Lipofectamine™ 2000 transfection reagent (mix gently before use)
 - *Optional:* Irrelevant 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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Producing EmGFP Lentivirus in 293FT Cells, Continued

293FT Cell Line

The human 293FT Cell Line is optimized for 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 pCMVSPORT6TA_g.neo and must be maintained in medium containing Geneticin®.

The 293FT Cell Line is supplied with the ViraPower™ Lentiviral Expression kits and is also available separately from Invitrogen (page vi).

For more information about 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 www.invitrogen.com or by contacting **Technical Support** (page 27).



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 can 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 as recommended in the 293FT Cell Line manual. Do not allow cells to overgrow before passaging.
 - Use cells that have been subcultured for less than 20 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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Producing EmGFP Lentivirus in 293FT Cells, Continued

ViraPower™ Packaging Mix

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.

The ViraPower™ packaging Mix is supplied with the ViraPower™ Lentivirus Support Kits or is available separately from Invitrogen (page vi).

To prepare the stock solution of the ViraPower™ Packaging Mix, resuspend the contents of the tube (195 µg) in 195 µl of sterile water to obtain a 1 µg/µl stock.

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:

1. First prepare DNA-Lipofectamine™ 2000 complexes and add them to plates containing growth media, then
2. Add the 293FT cells to the media containing DNA-Lipofectamine™ 2000 complexes and allow the cells to attach and transfect overnight (see detailed procedure on the 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 Invitrogen (page vi).

Opti-MEM® I

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

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Producing EmGFP Lentivirus in 293FT Cells, Continued

293FT Transfection Protocol

Follow the protocol below to transfect 293FT cells with the pLenti6.2-GW/EmGFP Expression Control Vector. Note that this protocol differs from the transfection protocol in the ViraPower™ System manual, so 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 pLenti6.2-GW/EmGFP 1.5 ml of Opti-MEM® I Medium without serum	9 µg of an irrelevant plasmid DNA (see page 6) 3 µg of pLenti6.2-GW/EmGFP 1.5 ml of Opti-MEM® I Medium without serum

2. In a separate sterile 15 ml tube, mix Lipofectamine™ 2000 gently before use, then dilute 36 µl of Lipofectamine™ 2000 in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
3. After the 5 minute incubation, combine the diluted DNA from Step 1 with the diluted Lipofectamine™ 2000 from Step 2. Mix gently.
4. 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.
5. 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 FBS at the same concentration as the growth medium for that cell line. **Do not include antibiotics in the medium.**
6. 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.**
7. 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 CO₂ incubator.

Protocol continues on next page

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Producing EmGFP Lentivirus in 293FT Cells, Continued

293FT Transfection Protocol, Continued

Protocol continued from previous page

8. The next day, remove the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with complete culture medium containing sodium pyruvate. Return the cells to 37°C in a CO₂ incubator and continue to step 9 (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 6). Greater than 90% of the cells should be EmGFP positive.

9. Harvest virus-containing supernatants 48–72 hours post-transfection 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.
 10. Centrifuge the viral supernatant at 3000 rpm for 15 minutes at 4°C to pellet cell debris. You can perform a filtration step, if desired (see below).
 11. Pipet viral supernatants into cryovials in 1 ml aliquots. Store viral stocks at –80°C. See the next page for further details about long term virus stock storage.
-

Alternate Transfection Protocol

An alternative transfection procedure 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.* 6×10^6 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 dropwise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO₂ incubator.

Follow Steps 8-11 as instructed in the 293FT Transfection Protocol, above.

Filtering Virus

If you plan to use your EmGFP lentivirus 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 (see Step 10, above) 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 (see page 19).

Continued on next page

Producing EmGFP Lentivirus in 293FT Cells, Continued

Long-Term Storage

Aliquot lentiviral stocks in cryovials at -80°C for long-term storage. Repeated freezing and thawing is not recommended as it may result in loss of viral titer. When stored properly, viral stocks of an appropriate titer should be suitable for use for up to one year. After long-term storage, we recommend re-titering 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 stock, you will:

1. Prepare 10-fold serial dilutions of your EmGFP lentiviral stock.
 2. Transduce the different dilutions of lentivirus into cells in the presence of Polybrene®.
 3. Determine the lentiviral titer by fluorescence detection at 4 days post transduction.
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Note

The pLenti6.2-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™ 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.
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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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Titering EmGFP Lentivirus, Continued

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. Polybrene[®] is a registered trademark of Abbott Laboratories.

Materials Needed

You will need the following items:

- Your EmGFP lentiviral stock (store at -80°C until use)
 - Adherent mammalian cell line of choice (see page 12)
 - Complete culture medium for your cell line
 - **Optional:** 6 mg/ml Polybrene[®] (see above)
 - 6-well tissue culture plates
 - 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
 - **Optional:** Trypsin or cell dissociation solution of choice (if performing flow cytometry)
 - **Optional:** Flow cytometry buffer of choice, such as calcium/magnesium-free Phosphate-Buffered Saline containing 1% FBS or BSA (if performing flow cytometry)
-



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 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.
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Titering EmGFP Lentivirus, Continued

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 (Day 1), trypsinize and count the cells, plating them in a 6-well plate such that they will be 25% confluent at the time of transduction. For example when using HT1080 cells, plate 1×10^5 cells per well of a 6-well plate. Incubate cells at 37°C in a CO₂ incubator overnight.

Alternate plating method for HT1080 cells: The morning of transduction (Day 2), plate 2×10^5 cells/well in a 6-well plate. In the afternoon, after cells have adhered to the plate (approximately 4–5 hours) transduce as described below.

2. On the day of transduction (Day 2), thaw your lentiviral stock. In a biosafety cabinet, prepare five 10-fold serial dilutions ranging from 10^{-1} to 10^{-5} . You should also prepare a mock dilution containing no virus.

For each dilution, dilute the lentiviral stock into complete culture medium containing 6–8 µg/ml Polybrene® (optional, see page 13) to a final volume of 1 ml.

Important: Do NOT dilute virus in culture medium containing Blasticidin.

Note: You may prepare a wider range of serial dilutions (e.g. 10^{-1} to 10^{-8}) over several 6-well plates of cells, or perform multiple replicates if desired.

3. Remove the culture medium from the cells. Mix each virus dilution gently by inversion (**DO NOT** vortex) and add to each well of cells.
4. Swirl the plate gently to mix. Incubate at 37°C in a CO₂ incubator overnight.
5. The following day (Day 3), remove the virus-containing media from the plates and discard (See **Caution**, previous page). Replace with 2 ml of complete culture medium and return cells to the 37°C CO₂ incubator overnight.

Important: Do NOT add Blasticidin to the culture medium.

6. At 4 days post transduction (Day 6), determine the EmGFP lentivirus titer. You may confirm EmGFP expression by visualizing the cells using a fluorescence microscope. See page 7 for recommended filter sets. If determining titer by flow cytometry, see **Preparing Cells for Flow Cytometry**, next page.

Note: If you do not have access to a flow cytometry facility, you may estimate EmGFP lentiviral titer by an alternate method using fluorescence microscopy, on page 17. You may also determine titers using the Blasticidin selection protocol, which is described in the ViraPower™ System Manual.

Continued on next page

Titering EmGFP Lentivirus, Continued



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 4 for specific excitation/emission properties of EmGFP. The steps below provide simple guidelines, and other methods may be suitable.

1. At day 4 post transduction, dissociate the cells from the plate by using trypsin or cell dissociation buffer.
 2. 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).
 3. 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.
-

Continued on next page

Titering EmGFP Lentivirus, Continued

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 × 10⁵ (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 × 10⁷ TU/ml.

What You Can Expect

We typically obtain unconcentrated EmGFP lentivirus titers in the range of 5 × 10⁵–2 × 10⁶ TU/ml. To obtain higher lentivirus titer, you can concentrate your virus (see page 19). The titer of concentrated lentivirus stocks may be up to 1 × 10⁸ TU/ml.

Continued on next page

Titering EmGFP Lentivirus, Continued

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).
-

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 pLenti6.2-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 pLenti6.2-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. 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 pLenti6.2-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

Transduction and Analysis, Continued

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™ System Manual for more information about determining Blasticidin sensitivity. See page 24 for information on handling and storing Blasticidin.

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 and Analysis, Continued

Transduction Protocol

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 18) 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 a final concentration of 6 µg/ml. Swirl the plate gently to mix. Incubate at 37°C in a CO₂ 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 transient expression of EmGFP by flow cytometry (see page 15) or by fluorescence microscopy. If you wish to select for stably transduced cells, continue with Step 8, below.
 7. Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin, as appropriate to select for stably transduced cells (see page 19).
 8. Replace medium with fresh medium containing antibiotic every 3-4 days until antibiotic-resistant colonies can be identified (generally 10-12 days after selection).
 9. Pick at least 5 antibiotic-resistant colonies and expand each clone to analyze the expression of EmGFP by flow cytometry (see page 15) or by fluorescence microscopy.
-

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	Cause	Solution
Low viral titer	Low transfection efficiency: <ul style="list-style-type: none"> Used poor quality plasmid DNA (<i>i.e.</i> plasmid DNA from a mini-prep) Unhealthy 293FT cells; cells exhibit low viability Cells transfected in media containing antibiotics (<i>i.e.</i> Geneticin®) Plasmid DNA:transfection reagent ratio incorrect 293FT cells plated too sparsely 	<ul style="list-style-type: none"> Do not use mini-prep plasmid DNA for transfection. Use the PureLink™ Midiprep Isolation Kit (page vi) or CsCl gradient centrifugation to prepare plasmid DNA. Use healthy 293FT cells under passage 20; do not overgrow. 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. Use a DNA (in µg): Lipofectamine™ 2000 (in µl) ratio ranging from 1:2 to 1:3. 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

Troubleshooting, Continued

Problem	Cause	Solution
Low viral titer, continued	Polybrene [®] not included during transduction	Transduce pLenti6.2-GW/EmGFP into cells in the presence of Polybrene [®] .
	Lipofectamine [™] 2000 handled incorrectly	<ul style="list-style-type: none"> 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 pLenti6.2-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: <ul style="list-style-type: none"> Polybrene[®] not included during transduction Non-dividing cell type used 	<ul style="list-style-type: none"> Transduce pLenti6.2-GW/EmGFP into cells in the presence of Polybrene[®]. Transduce pLenti6.2-GW/EmGFP into cells using a higher MOI.
	MOI too low	Transduce pLenti6.2-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	pLenti6.2-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.
-

Making Glycerol Stocks for Long- Term Storage

1. Streak the original colony out to obtain single colonies on an LB agar plate containing 100 µg/ml ampicillin.
 2. Isolate a single colony and inoculate into 1–2 ml of LB containing 100 µg/ml ampicillin.
 3. Grow at 37°C with shaking until the culture reaches stationary phase.
 4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol.
 5. Transfer to a cryovial and store at -80°C.
-

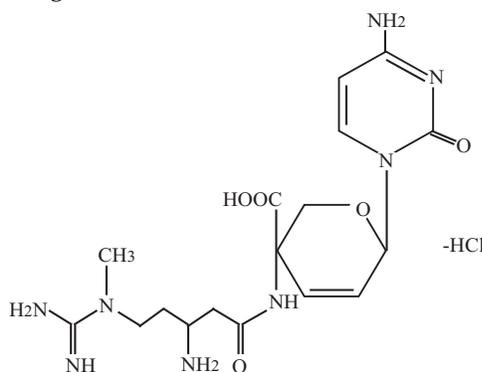
Blasticidin

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 Invitrogen (see page vi for ordering information). For information on preparing and handling Blasticidin see the Appendix, page 24.

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5 \cdot HCl$, 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 Invitrogen (page vi) in 50 mg aliquots. Blasticidin is soluble in water. Use sterile water to prepare stock solutions of 5 to 10 mg/ml.

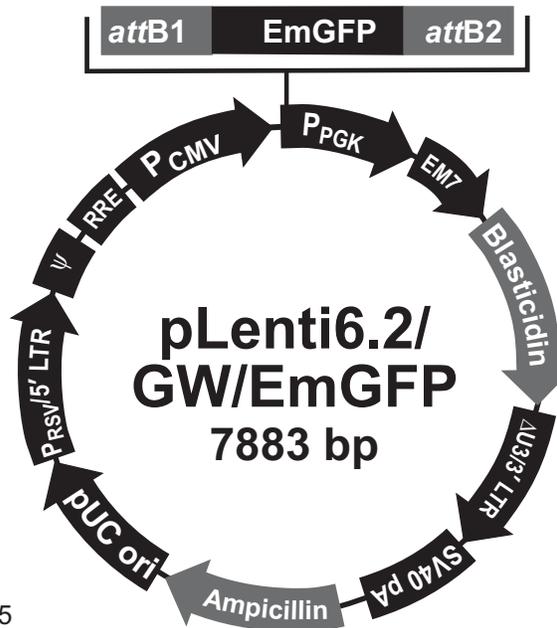
- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot solution in small volumes suitable for one time use (see next to last point below) and freeze at $-20^{\circ}C$ for long-term storage or store at $4^{\circ}C$ for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at $4^{\circ}C$ and 6–8 weeks at $-20^{\circ}C$.
- 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 $4^{\circ}C$ for up to 2 weeks.

Medium containing Blasticidin may be stored at $4^{\circ}C$ for up to 2 weeks.

Map of pLenti6.2-GW/EmGFP Expression Control Vector

Map of pLenti6.2-GW/EmGFP

The map below shows the elements of the pLenti6.2-GW/EmGFP Expression Control Vector (7883 bp). The complete sequence of this vector is available for downloading from our web site at www.invitrogen.com or by contacting **Technical Support**, page 27.



Comments for pLenti6.2/GW/EmGFP 7883 nucleotides

RSV enhancer/promoter: bases 1-229
HIV-1 5' LTR: bases 230-410
5' splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3' splice acceptor: base 1656
3' splice acceptor: base 1684
CMV promoter: bases 1809-2392
attB1 site: bases 2440-2464
EmGFP: bases 2470-3189
attB2 site: bases 3190-3214
PGK promoter: bases 3336-3841
EM7 promoter: bases 3852-3918
Blasticidin resistance gene: bases 3919-4317
 Δ U3/HIV-1 3' LTR: bases 4403-4637
 Δ U3: bases 4403-4456
 Truncated HIV-1 3' LTR: bases 4457-4637
SV40 polyadenylation signal: bases 4709-4840
bla promoter: bases 5699-5797
Ampicillin (*bla*) resistance gene: bases 5798-6658
pUC origin: bases 6803-7476

Continued on next page

Features of pLenti6.2-GW/EmGFP Expression Control Vector

Features of pLenti6.2-GW/EmGFP

The pLenti6.2-GW/EmGFP Expression Control Vector contains the following elements. All features have been functionally tested and the vector has been fully sequenced.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull <i>et al.</i> , 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 8 packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull <i>et al.</i> , 1998).
HIV-1 psi (ψ 8) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems <i>et al.</i> , 1991; Malim <i>et al.</i> , 1989).
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson <i>et al.</i> , 1989; Boshart <i>et al.</i> , 1985; Nelson <i>et al.</i> , 1987)
<i>attB1</i> and <i>attB2</i> 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
PGK Promoter	Allows high-level expression of Blasticidin in mammalian cell lines
EM7 promoter	Allows expression of Blasticidin in <i>E. coli</i>
Blasticidin (<i>bsd</i>) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura <i>et al.</i> , 1994)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
Ampicillin (<i>bla</i>) resistance gene (β -lactamase)	Allows selection of transformants in <i>E. coli</i>
pUC origin	Allows high-copy number replication and growth in <i>E. coli</i>

Technical Support

Web Resources



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

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
 - Complete technical support contact information
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-

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Introduction

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continued on next page

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Continued on next page

Purchaser Notification, Continued

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *attL1* and *attL2* sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *attB1* and *attB2* sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee (\$10 per clone) payable to Invitrogen.

Additional Terms and Conditions

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

References

- Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989) Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. *J. Biol. Chem.* 264, 8222-8229
- Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. *Cell* 41, 521-530
- 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
- Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991) Blastocidin 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) Blastocidin 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
- 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) Blastocidin 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
- 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 immunodeficiency virus elements. *J. Virology* 73, 2832-2840
- Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965) Inhibition of Protein Synthesis by Blastocidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. *J. Biochem (Tokyo)* 57, 667-677
- 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

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