invitrogen™

pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO® TA Cloning® Kits

Five-minute cloning of *Taq* polymerase-amplified PCR products for high level expression in mammalian cells using the ViraPower™ HiPerform™ Lentiviral Expression Systems

Catalog nos. K5315-20, K5325-20, K5310-00, K5320-00

Version B
7 June 2010
A10291

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the kits listed below.

Product	Catalog no.
pLenti6.3/V5-TOPO® TA Cloning® Kit	K5315-20
pLenti7.3/V5-TOPO® TA Cloning® Kit	K5325-20
ViraPower™ HiPerform™ Lentiviral TOPO® Expression Kit	K5310-00
ViraPower™ HiPerform™ Lentiviral FastTiter™ TOPO® Expression Kit	K5320-00

Shipping and Storage

The pLenti-TOPO® TA Cloning® Kits are shipped in **two boxes**. Box 1 contains the pLenti-TOPO® TA Cloning® reagents and is shipped on dry ice. Box 2 contains the One Shot® Stbl3™ Chemically Competent *E. coli* kit. and is shipped on dry ice. Upon receipt, store each box as follows:

Component	Shipping	Store Box at:
Box 1 (pLenti-TOPO® TA Cloning® reagents)	Dry ice	-20°C
Box 2 (One Shot® Stbl3™ Chemically Competent Cells)	Dry ice	-80°C

System Components

The following table shows the components supplied with the ViraPower™ HiPerform™ Lentiviral TOPO® Expression Kits. For details on the system components, refer to the ViraPower™ HiPerform™ Lentiviral System manuals.

Components	Catalog no.			
	K5315-20	K5325-00	K5310-00	K5320-00
pLenti6.3/V5-TOPO® TA Cloning Kit	✓		✓	
pLenti7.3/V5-TOPO® TA Cloning Kit		✓		✓
One Shot® Stbl3™ Chemically Competent *E. coli*	✓	✓	✓	✓
ViraPower™ Lentiviral Support Kit			✓	✓
293FT Cell Line			✓	✓
Blasticidin			✓	

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TOPO® TA Cloning® Reagents

Box 1 of the pLenti-TOPO® kits contains the TOPO® TA Cloning® Reagents. The contents, concentration, and quantity of each reagent are detailed below. Store Box 1 at -20°C.

Note: *Taq* polymerase is available separately from Invitrogen (page vii) and must be supplied by the user.

Reagent	Concentration	Quantity
pLenti6.3/V5-TOPO® Vector **or** pLenti7.3/V5-TOPO® (supplied linearized in solution)	5-10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM MgCl2 0.01% gelatin	100 µl
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP; 12.5 mM dGTP; 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl; 0.06 M $MgCl_2$	50 µl
CMV Forward Primer	0.1 µg/µl in TE Buffer	20 µl
V5 (C-term) Reverse Primer	0.1 µg/µl in TE Buffer	20 µl
Control PCR Template	0.05 µg/µl in TE Buffer	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer	10 µl
pLenti6.3/V5-GW/*lacZ* **or** pLenti7.3/V5-GW/*lacZ* expression control vector	0.5 µg/µl in TE Buffer	20 µl
Sterile Water	--	1 ml

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

Box 2 of the pLenti-TOPO® kits contains the One Shot® Stbl3™ Chemically Competent *E. coli* kit. The contents, concentration, and quantity of each reagent are detailed below. Store Box 2 at -80°C.

Reagent	Composition	Quantity
S.O.C. Medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
Stbl3™ Cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of Stbl3™ Cells

F$^-$ *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ *leu mtl*-1
Note: This strain is *end*A1+

Sequencing Primers

The table below provides the sequence and pmoles of the CMV sequencing primer and the V5 (C-term) reverse sequencing primer.

Primer	Sequence	Quantity
CMV forward primer	5′-CGCAAATGGGCGGTAGGCGTG-3′	306 pmoles
V5(C-term) reverse primer	5′-ACCGAGGAGAGGGTTAGGGAT-3′	305 pmoles

Accessory Products

Additional Products

Products listed in this section may be used with the pLenti-TOPO® TA Cloning kits. Many of the reagents supplied in the pLenti-TOPO® TA Cloning kits are also available separately from Invitrogen and are listed below. For more information visit our web site at www.invitrogen.com or contact **Technical Support** (page 36).

Item	Quantity	Catalog no.
Taq DNA Polymerase, Native	100 units	18038-018
	500 units	18038-042
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
Platinum® *Taq* DNA Polymerase	100 units	10966-018
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
PureLink™ HiPure Plasmid Midiprep Kit	25 reactions	K2100-04
	50 reactions	K2100-05
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink™ Quick Gel Extraction Kit	50 reactions	K2100-12
ViraPower™ Promoterless Lentiviral Gateway Expression System with MultiSite™ Gateway® Technology	1 kit	K5910-00
ViraPower™ Promoterless Lentiviral Gateway Vector Kit with MultiSite™ Gateway® Technology	1 kit	K591-10
Vivid Colors™ pLenti6.3/V5-GW/EmGFP Expression Control Vector	20 µg	V370-06
PCR Optimizer Kit	100 reactions	K1220-01
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 µl	C7373-03
Ampicillin	20 ml	11593-027
Blasticidin	50 mg	R210-01
Geneticin®	20 ml	10131-035
	100 ml	10131-027
Lipofectamine™ 2000 Reagent	1.5 ml	11668-019
	0.75 ml	11668-027
Phosphate Buffered Saline, pH 7.4	500 ml	10010-023
anti-β-galactosidase	0.5 ml	A-11132
β-Gal Assay Kit	100 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
X-gal	100 mg	15520-034
	1 g	15520-018

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ViraPower™ HiPerform™ Lentiviral Expression Products

The pLenti-TOPO® TA Cloning® Kits are designed for use with the ViraPower™ HiPerform™ Lentiviral Expression Systems, available from Invitrogen. Ordering information for the ViraPower™ HiPerform™ Lentiviral support products and expression kits is provided below.

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
Vivid Colors™ pLenti6.3-GW/EmGFP Expression Control Vector	20 µg	V370-06
ViraPower™ Lentiviral Support Kit	20 reactions	K4970-00
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975-00
293FT Cell Line	3 x 10^6 cells	R700-07

Detection of Recombinant Protein

Expression of your recombinant fusion protein can be detected using an antibody to the V5 epitope (see table below). Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods. A fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments. The amount of antibody supplied is sufficient for 25 western blots or 25 immunostaining reactions, as appropriate.

Item	Quantity	Catalog no.
Anti-V5 Antibody	50 µl	R960-25
Anti-V5-HRP Antibody	50 µl	R961-25
Anti-V5-AP Antibody	125 µl	R962-25
Anti-V5-FITC Antibody	50 µl	R963-25

Introduction

Overview

Introduction

The pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO® vectors are lentiviral expression vectors that are adapted for use with TOPO® Cloning technology. The pLenti-TOPO® vectors are designed to allow high-level expression of recombinant fusion proteins in dividing and non-dividing mammalian cells using Invitrogen's ViraPower™ HiPerform™ Lentiviral Expression Systems (Catalog nos: K5310-00 and K5320-00). Using the TOPO® Cloning technology, the pLenti-TOPO® vectors provide a highly efficient, 5-minute, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Once cloned, analyzed, and transfected, the PCR product expresses directly in mammalian cell lines.

pLenti6.3/V5-TOPO® (7691 bp) and pLenti7.3/V5-TOPO® (7935 bp) expression vectors contain two new 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) and allows for a two-fold increase in viral titer. Both WPRE and cPPT together, produce at least a four-fold increase in protein expression in most cell types, compared to other vectors that do not contain these elements. The pLenti7.3/V5-TOPO® vector kit (Catalog no K5320-00) allows for an accurate determination of titer of functional lentivirus in just **two days** using Emerald Green Fluorescent Protein (EmGFP).

Additional Information

For more information about the ViraPower™ HiPerform™ Lentiviral Expression Systems (Catalog nos: K5310-00and K5320-00), review the ViraPower™ HiPerform™ Lentiviral System manual. This manual is included with the kits (page iv) and is also available for downloading from our web site at www.invitrogen.com. For more information on TOPO® Cloning Technology or the ViraPower™ HiPerform™ Systems, visit our web site, or contact **Technical Support** (page 36).

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Features of pLenti-TOPO® Vectors

The pLenti-TOPO® vectors contain the following features:

- 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 (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)
- Polypurine Tract from HIV (cPPT) for increased viral titer (Park et al., 2001)
- Human cytomegalovirus (CMV) immediate early enhancer/promoter for high-level constitutive expression of the gene of interest in a wide range of mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987) See below for more information.
- TOPO® Cloning site for rapid and efficient cloning of PCR products with A-overhangs
- C-terminal V5 epitope for detection of the recombinant protein of interest (Southern *et al.*, 1991)
- Woodchuck Posttranscriptional Regulatory Element (WPRE) for increase transgene expression (Zufferey et al., 1999)
- SV40 promoter to drive expression of Blasticidin (pLenti6.3/V5-TOPO® vector), **or** EmGFP (pLenti7.3/V5-TOPO® vector).
- Blasticidin (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) resistance gene for stable transduction and selection in *E. coli* and mammalian cells (pLenti6.3/V5-TOPO® vector, only) **or**
- Emerald Green Fluorescent Protein (EmGFP, derived from *Aequorea Victoria* GFP, pLenti7.3/V5-TOPO® vector only) which allows you to easily determine the lentiviral titer by flow cytometry
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. Coli*

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How TOPO® Cloning Works

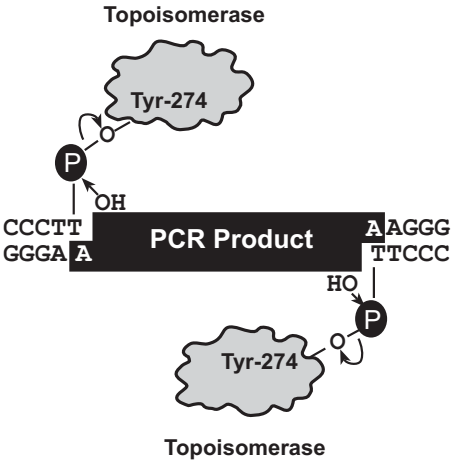
pLenti6.3/V5-TOPO® (7691 bp) and pLenti7.3/V5-TOPO® (7935 bp) are expression vectors designed to facilitate rapid cloning of TA PCR products for expression in mammalian cells.

The plasmid vector (pLenti6.3/V5-TOPO® or pLenti7.3/V5-TOPO®) is supplied linearized with:

- Single 3´ thymidine (T) overhangs for TA Cloning®
- Topoisomerase covalently bound to the vector (this is referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The supercoiled vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products (see below).



Once the PCR product is cloned into pLenti6.3/V5-TOPO® or pLenti7.3/V5-TOPO® and the transformants are analyzed for the correct orientation of the PCR product, the plasmid is transfected into mammalian cells for expression. The PCR product may be expressed as a fusion to the V5 epitope for detection and purification; or, by designing the 3´ PCR primer with a stop codon, the PCR product may be expressed as a native protein.

Continued on next page

How Topoisomerase Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.

The ViraPower™ HiPerform™ Lentiviral Expression System

The ViraPower™ HiPerform™ Lentiviral Expression System (Catalog nos: K5310-00 and K5320-00) facilitates 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. To express your gene of interest in mammalian cells using the ViraPower™ HiPerform™ Lentiviral Expression System, you will:

1. Create an expression clone in either the pLenti6.3/V5-TOPO® vector, or the pLenti7.3/V5-TOPO®.
2. Cotransfect your expression clone and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce lentivirus.
3. Use your lentiviral stock to transduce the mammalian cell line of choice.
4. Assay for "transient" expression of the recombinant protein (pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO® vectors) or generate a stable cell line using Blasticidin selection (pLenti6.3/V5-TOPO®, **only**).

For more information about the ViraPower™ HiPerform™ Lentiviral Expression System, the ViraPower™ Packaging Mix, and the biosafety features of the System, refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual. For more information about the 293FT Cell Line, refer to the 293FT Cell Line manual. Both manuals are available for downloading from www.invitrogen.com or by contacting **Technical Support** (page 36).

CMV Promoter

The pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO® vectors contain the human CMV immediate early promoter to allow high-level, constitutive expression of the gene of interest in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). Although highly active in most mammalian cell lines, activity of the viral promoter can be down-regulated in some cell lines due to methylation (Curradi *et al.*, 2002), histone deacetylation (Rietveld *et al.*, 2002), or both.

Note: If you experience silencing of your transgene expression, you may use the ViraPower™ Promoterless Lentiviral Gateway® Expression System with MultiSite™ Gateway® Technology (page vii) and use a gene-specific promoter

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Promoter Driving Blasticidin

The pLenti6.3/V5-TOPO® vector contains the SV40 promoter to drive mammalian expression of the Blasticidin selection marker. In some mammalian cell types, the activity of viral promoters such as SV40 may become significantly reduced over time due to promoter silencing from methylation (Curradi *et al.*, 2002) or histone deacetylation (Rietveld *et al.*, 2002).

Note: If you experience Blasticidin silencing, we recommend using any of the ViraPower™ II Lentiviral Gateway® Expression Systems. These kits contain lentiviral vectors in which expression of the Blasticidin gene is controlled by the PGK promoter. For more information, visit our web site at www.invitrogen.com, or contact **Technical Support** (page 36).

Positive Control Vector

A control plasmid containing the *lac*Z gene is included with each pLenti-TOPO® TA Cloning kit for use as a positive expression control in the mammalian cell line of choice. For more information on these vectors, refer to pages34-35.

A control lentiviral expression vector (Vivid Colors™ pLenti6.3/V5-GW/EmGFP) containing Emerald Green Fluorescent Protein (EmGFP) is available separately from Invitrogen (page vii).

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 can be detected using fluorescence microscopy or other methods, such as flow cytometry.

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 pLenti7.3/V5-TOPO® vector contains EmGFP in the vector backbone, to allow for rapid transient expression and determination of lentiviral titer within **two days (**see **Important**, page 22**)**. 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.

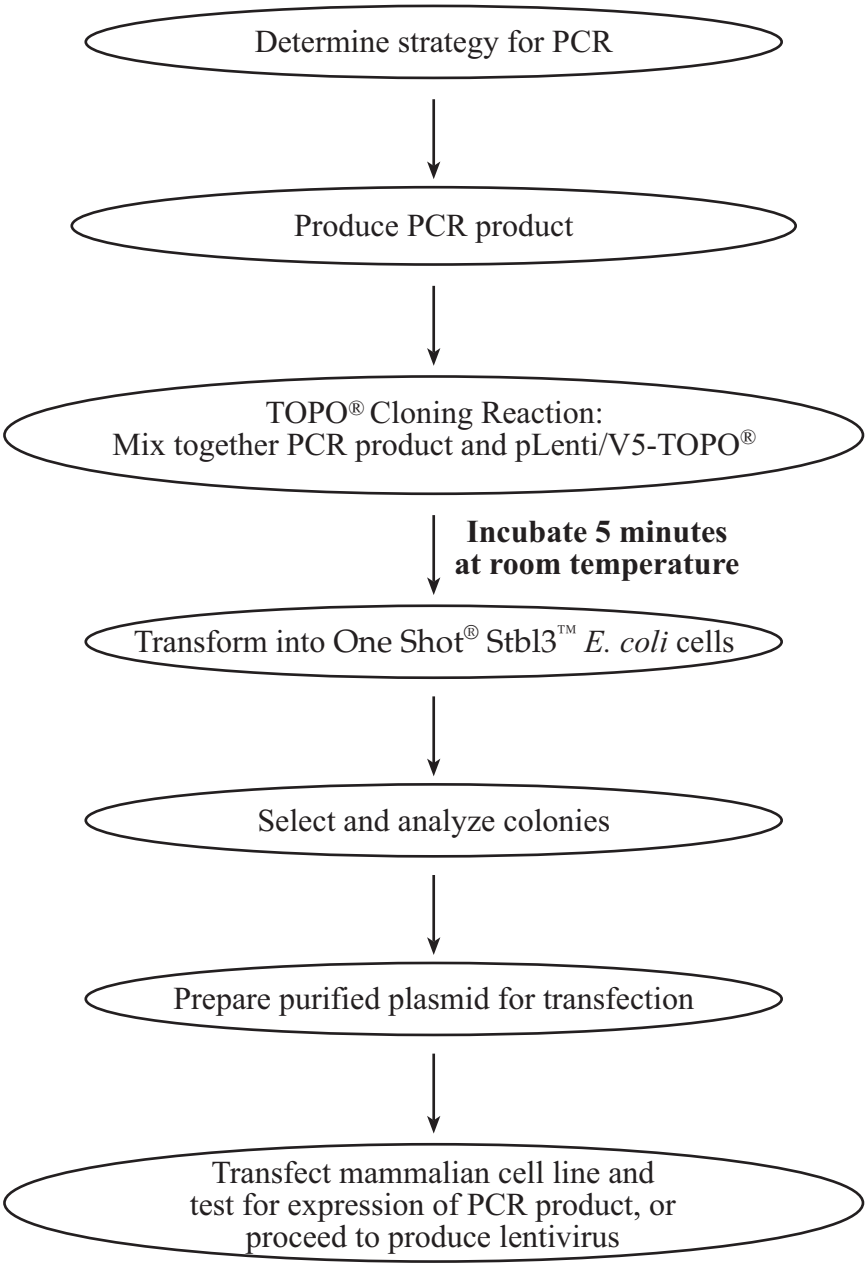
Fluorescent Protein	GFP Mutations*
EmGFP	S65T, S72A, N149K, M153T, I167T

*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.

Experimental Outline

Experimental Outline

The flow chart below outlines the experimental steps necessary to clone and express your blunt-end PCR product.



Methods

Designing PCR Primers

Designing Your PCR Primers

Design of the PCR primers to clone your DNA sequences of interest is critical for expression. pLenti6.3V5-TOPO® and pLenti7.3/V5-TOPO® are C-terminal fusion vectors that do not contain an ATG initiation codon. If there is no initiating ATG codon or optimal sequences for translation initiation (Kozak sequences) in the DNA to be amplified, then these features need to be incorporated into your forward primer.

Example: Kozak consensus sequence is (G/A)NNATGG

Depending on the nature of your PCR product you have two options to consider:

- Clone in frame with the V5 epitope (C-terminal peptide) to detect and/or purify your PCR product, **or**
- Include the native stop codon to express the native protein.

Note: Cloning efficiencies may vary depending on the 5´ primer nucleotide sequence (see page 17).

Use the diagram below to design your PCR primers. Once you have designed your PCR primers, proceed to **Producing PCR Products** (page 9)

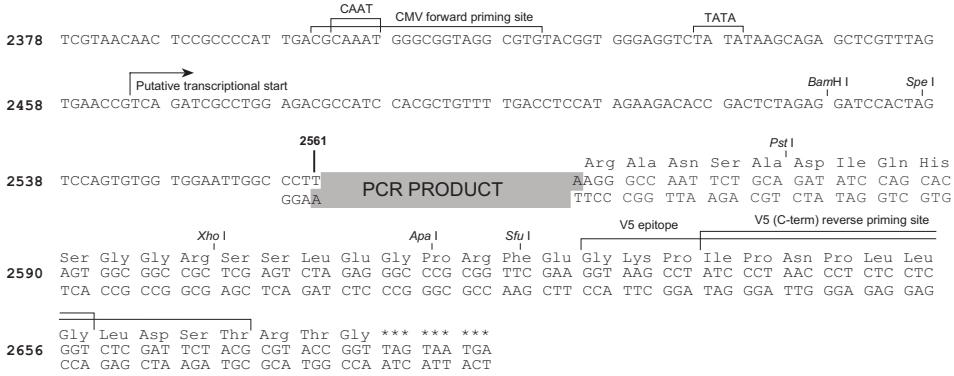
Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into the pLenti-TOPO® vector.

TOPO® TA Cloning® Site for pLenti6.3/V5-TOPO®

Restriction sites for pLenti6.3/V5-TOPO® are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 2561 and 2562. This is the TOPO® Cloning site.

Note: The full sequence of the pLenti6.3/V5-TOPO® vector (K5315-20) may be downloaded from our web site (www.invitrogen.com) or requested from **Technical Support** (see page 36). A map of pLenti6.3/V5-TOPO® vector is provided on page 31.



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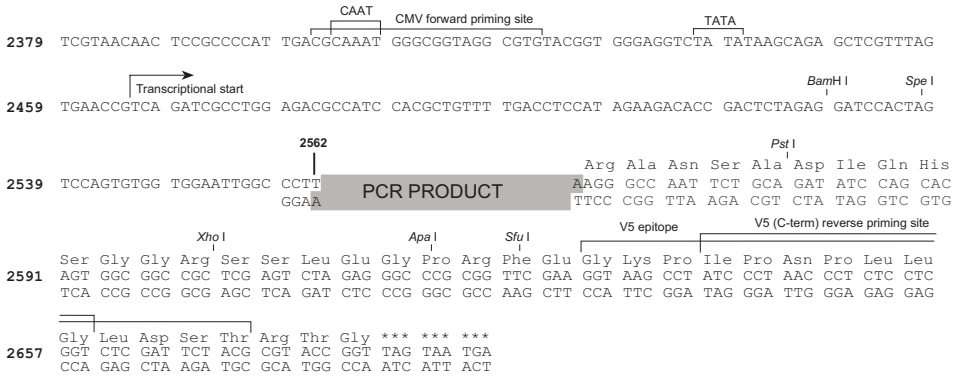
Designing PCR Primers, Continued

TOPO® TA Cloning® Site for pLenti7.3/V5-TOPO®

Restriction sites for pLenti7.3/V5-TOPO® are labeled to indicate the actual cleavage site. The vector is supplied supercoiled between base pair 2562 and 2563. This is the TOPO® Cloning site.

Note: The full sequence of the pLenti7.3/V5-TOPO® vector (K5325-20) may be downloaded from our web site: www.invitrogen.com or requested from **Technical Support** (see page 36). A map of pLenti7.3/V5-TOPO® vector is provided on page 32.



Producing PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers you are ready to produce your PCR product.

Materials Needed

You will need the items:

- Platinum® *Taq* DNA Polymerase High Fidelity (page vii), or equivalent
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, we strongly recommend using Platinum® *Taq* DNA Polymerase High Fidelity (page vii).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 30.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Primers	100-200 ng each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band (see **Note,** below).

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before performing TOPO® Cloning (see page 28). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (page vii) from Invitrogen can help you optimize your PCR. For additional information, contact **Technical Support** (page 36).

TOPO® Cloning Reaction

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into the pLenti-TOPO® vector, and transform the recombinant vector into One Shot® Stbl3™ Competent *E. coli*, in one day. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. If this is the first time you have TOPO® Cloned, perform control reactions parallel with your samples (see page 21 for information on positive controls).

Note

Experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. We have also observed that in the presence of salt, incubation times of greater than 5 minutes can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Inclusion of salt allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

Important

Because of the above results (see **Note**), we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose.

Transforming Chemically Competent *E. coli*

For TOPO® Cloning and transformation into One Shot® Stbl3™ Competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Materials Needed

You will need the following items:

- 42°C water bath
- LB plates containing 50-100 µg/ml ampicillin (two for each transformation)
- Reagents and equipment for agarose gel electrophoresis
- 37°C shaking and non-shaking incubator

Materials supplied with kit

- pLenti-TOPO® vector
- 10X PCR Buffer
- Salt Solution
- Sterile Water
- One Shot® Stbl3™ Competent E. coli

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TOPO® Cloning Reaction, Continued

Note

There is no blue-white screening for the presence of inserts. Individual recombinant plasmids need to be analyzed by restriction analysis or PCR sequencing for the presence and orientation of insert. Sequencing primers included in the kit can be used to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparation for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C.
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw **on ice**, 1 vial of One Shot® Stbl3™ Competent *E. coli* cells for each transformation.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for transformation into chemically competent One Shot® Stbl3™ Competent *E. coli* (supplied with kit) Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 9.

Note: The red or yellow color of the pLenti-TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	**Quantity**
Fresh PCR product	0.5 to 4 µl
Salt Solution	1 µl
Sterile Water	add to a final volume of 5 µl
pLenti-TOPO® vector	1 µl

*Store all reagents at -20°C when finished. Salt solution and water can be stored at room temperature or +4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for 5 minutes at room temperature (21-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Stbl3™ Competent *E.coli*** (next page).

   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

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

Introduction

Follow the instructions in this section to transform your TOPO® Cloning reaction (previous page) into One Shot® Stbl3™ Chemically Competent *E. coli* (supplied with kit). The transformation efficiency of One Shot® Stbl3™ Chemically Competent *E. coli* is ≥ 1 x 10^8 cfu/µg plasmid DNA.

Important

For optimal results, we recommend using Stbl3™ *E. coli* for transformation as this strain is particularly well suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats.

Note: Transformants containing unwanted recombinants are generally **not** obtained when Stbl3™ *E. coli* are used for transformation.

Materials Needed

You will need following items:

- TOPO® Cloning reaction (previous page)
- LB Amp plates containing 100 µg/ml Ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- LB Medium (if performing the pUC19 Control Transformation)
- 42°C water bath
- 37°C shaking and non-shaking incubator

Materials supplied with kit

- S.O.C Medium (pre-warmed to room temperature)
- One Shot® Stbl3™ Chemically Competent *E. coli* (one vial per transformation; thaw on ice immediately before use)
- pUC19 positive control (*Optional*: to verify the transformation efficiency)

Continued on next page

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

One Shot® Stbl3™ Transformation Procedure

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

1. Thaw, **on ice**, one vial of One Shot® Stbl3™ chemically competent cells for each transformation.
2. Add 2 to 3 µl of the TOPO® Cloning reaction (page 11) into a vial of One Shot® Stbl3™ cells and mix gently. **Do not mix by pipetting up and down**. For the pUC19 control, add 10 pg (1 µl) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C water bath and place them on ice for 2 minutes.
6. Add 225 µl S.O.C. media (pre-warmed to room temperature).
7. Cap the tube(s) tightly and shake horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 100 µl of the transformation mix on a pre-warmed LB-Ampicillin plate and incubate overnight at 37°C. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium (*e.g.* add 100 µl of the transformation mix to 900 µl of LB Medium) and plate 25-100 µl.
9. Store the remaining transformation mix at +4°C. Plate out additional cells the next day, if desired.
10. Proceed to **Analyzing Transformants** (next page).

Analyzing Transformants

Introduction

We recommend analyzing the transformants using both restriction digestion and sequencing or PCR analysis as described below. This allows you to confirm the presence of the insert as well as ensure the absence of any aberrant lentiviral vector recombination between the LTRs.

You will screen colonies by performing miniprep DNA isolation and restriction analysis to validate the clones. You may also perform PCR analysis and/or sequencing of your clones to determine that your insert is in the correct orientation and is in-frame with the V5 epitope tag.

After verifying the correct clones, you will use the miniprep DNA to re-transform Stbl3™ *E. coli*. You will then isolate plasmid DNA for transfection and lentivirus production. Plasmid DNA for transfection into 293FT cells must be very clean and free from contaminants and salts, and should be isolated by midiprep.

Note

Do **not** use PCR screening of clones in place of restriction analysis. For example, clones that contain both correct and aberrantly recombined DNA may look positive by PCR but may not be optimal for lentivirus production.

Experimental Outline

To analyze your transformants:

1. Pick 10-20 ampicillin-resistant colonies from plating the transformation mix. Culture cells overnight.
2. Isolate plasmid DNA for each colony using a miniprep kit (see **Important**, next page)
3. Analyze the plasmids by restriction analysis to confirm the presence and orientation of your insert as well as the integrity of the vector.
4. *Optional:* Sequence the plasmids or perform PCR to determine that your gene of interest is in frame with the C-terminal V5 epitope tag.
5. Re-transform One Shot® Stbl3™ Chemically Competent *E. coli* separately with the validated clones.
6. Inoculate LB-ampicillin with a fresh colony and grow to generate a starter culture.
7. Inoculate the starter culture into LB-ampicillin and grow.
8. Isolate plasmid DNA using a midiprep kit (see **Important**, next page) for lentivirus production.

Continued on next page

Analyzing Transformants, Continued

Important

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

We recommend using the PureLink™ HQ Mini Plasmid Purification Kit and preparing lentiviral plasmid DNA using PureLink™ MidiPrep Kits (page vii).

Materials Needed

You will need the following materials:

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

Screening Colonies by Miniprep

For each transformation:

1. Pick 10-20 colonies from plates obtained after plating the transformation mix (Step 9, page 13). Culture colonies overnight in LB medium containing 100 µg/ml ampicillin.
2. Isolate plasmid DNA using PureLink™ HQ Mini Plasmid Purification Kit or equivalent (see **Important**, above). The typical yield of pLenti DNA with PureLink™ HQ Mini Plasmid Purification Kit is ~ 5-7 µg, which is lower than the average DNA yield using this purification kit.
3. Perform restriction digests on plasmid DNA, then analyze the digested DNA on 0.8% or 1.2% agarose gels to confirm the correct clones.

Restriction Digest

To confirm that no rearrangement in the LTR regions of the plasmid has taken place, perform restriction digests using a combination of *Afl* II and *Xho* I. *Afl* II sites are present in both LTRs. The *Xho* I site is present in the plasmid backbone at the 3' end of the insert. Assuming there are no *Afl* II or *Xho* I sites in the insert, 3 DNA fragments are generated from the *Afl* II + *Xho* I digest. Any unexpected DNA fragments are a result of LTR recombination.

If *Afl* II and/or *Xho* I sites are present in the insert, you can use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert. The complete restriction enzyme maps of vectors are available at www.invitrogen.com.

What You Should See

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

Continued on next page

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to determine the presence and orientation of inserts, and analyze positive transformants using PCR. For PCR primers, use a primer such as the V5(C-term) Reverse primer that hybridizes in the vector downstream of your insert and a forward primer that hybridizes within your insert (see below for sequence). You will have to determine the amplification conditions. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template.

Materials Needed

You will need the items:

- Platinum® *Taq* DNA Polymerase High Fidelity (page vii), or equivalent
- DNA template and primers for PCR product
- Thermocycler

Procedure

1. For each reaction, add the following components to a DNase/RNase-free microcentrifuge tube. Total volume is 50 µl. For multiple reactions, prepare a master mix of common components to minimize reagent loss and enable accurate pipetting.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
Primers	100-200 ng each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Pick 10-20 colonies that have been analyzed by restriction digest with *Afl* II and *Xho* I (see previous page) and resuspend them individually in 50 µl of the master mix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify sample(s) in a thermocycler for 20 to 30 cycles.
   Note: Use the cycling parameters suitable for your primers and template.
5. Visualize PCR products by agarose gel electrophoresis.

Continued on next page

Analyzing Transformants, Continued

Sequencing

To confirm that your gene of interest is in frame with the C-terminal tag, you may sequence your expression construct to confirm that your gene is cloned in the correct orientation and in frame with the V5 epitope. We recommend using the following primers to help you sequence your expression construct. Refer to the diagrams on pages 31-32 for the locations of the primer binding sites in each vector.

Note: For your convenience, Invitrogen has a custom primer synthesis service. For more information, see our Web site (www.invitrogen.com) or contact **Technical Support** (page 36).

Vectors	Primer	Sequence
pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO®	CMV forward primer	5′-CGCAAATGGGCGGTAGGCGTG-3′
	V5(C-term) reverse primer	5′-ACCGAGGAGAGGGTTAGGGAT-3′

DNA Isolation Guidelines

Once you have generated and validated your clone you will isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating lentiviral plasmid DNA using the PureLink™ MidiPrep Kit.

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

Isolating Lentiviral Plasmid DNA

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

1. Dilute 1 µl of miniprep plasmid DNA from a positive clone 1:500 in TE.
2. Use 1 µl of this diluted DNA to retransform into One Shot® Stbl3™ Chemically Competent Cells as described on page 12.
3. Plate approximately one-tenth of the transformation on LB plates containing 100 µg/ml ampicillin and incubate at 37°C overnight.
4. Pick 1 colony and culture in 2-3 ml LB medium containing 100 µg/ml ampicillin for 6-8 hours at 37°C to obtain a starter culture.
5. Inoculate the entire volume of the starter culture into LB medium containing 100 µg/ml ampicillin and culture at 37°C overnight.

   Note: Use a 50-100 ml volume for large scale or midiprep isolation of DNA.
6. Isolate plasmid DNA using the PureLink™ MidiPrep Kit (page 15,**Important)**.
7. Perform restriction analysis (page 15) to confirm the presence of the insert.
8. Use the purified plasmid DNA from the positive clone for producing the lentivirus and to check protein expression (optional, see page 19).

   Note: Typical DNA yield should be ~150-300 µg and the O.D. $_{260/280}$ ratio should be between 1.8 and 2.1.

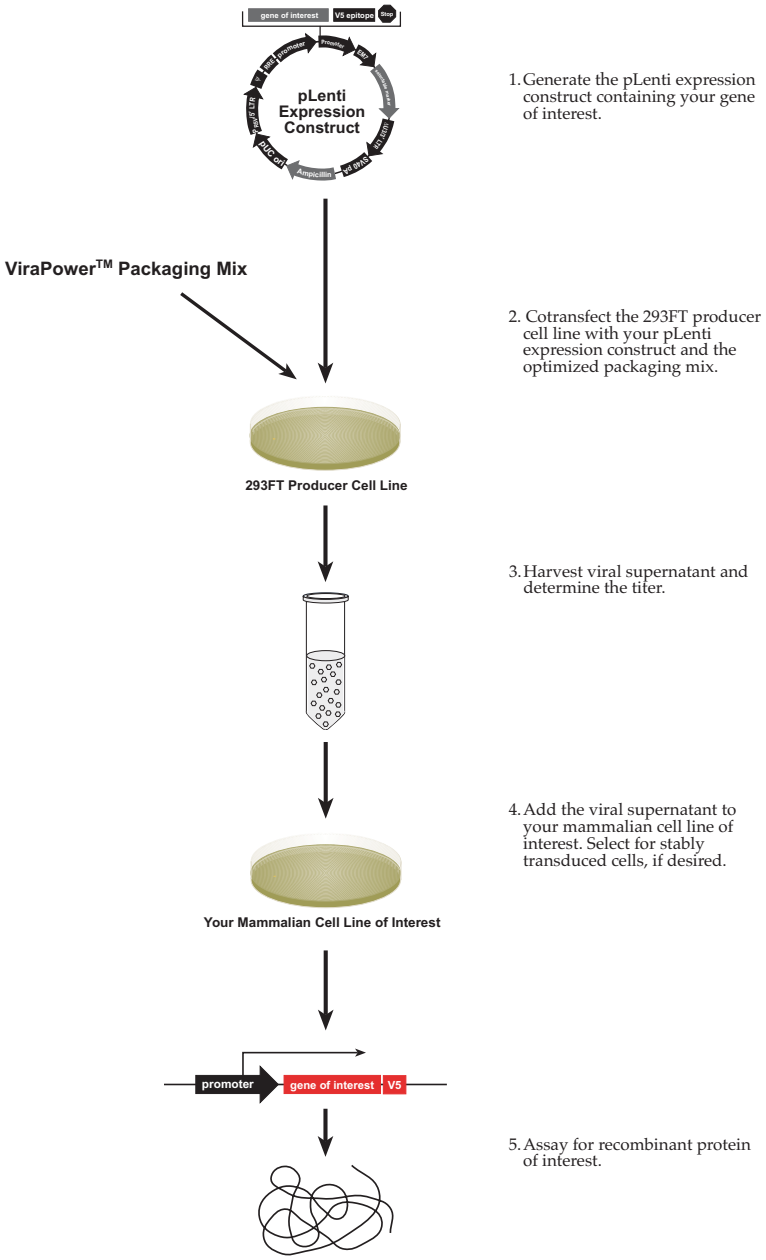
Maintaining the Expression Clone

Once you have generated your expression clone, maintain and propagate the plasmid in LB medium containing 100 µg/ml ampicillin. Addition of Blasticidin is not required.

Expression and Analysis

Introduction

Once you have obtained purified plasmid DNA of your pLenti-TOPO® expression construct, you are ready to use Invitrogen's ViraPower™ HiPerform™ Lentiviral Expression System to produce a lentiviral stock, which may then be used to transduce your mammalian cell line of choice to express your recombinant protein (see experimental outline below).



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Verifying Expression of Recombinant Protein (optional)

Optional: Before proceeding to generate a lentiviral stock of your pLenti-TOPO® expression construct, you may verify that the construct expresses the gene of interest by transfecting the plasmid directly into mammalian cells and assaying for your recombinant protein, if desired. Follow the guidelines below:

- Use an easy-to-transfect, dividing mammalian cell line (*e.g.* HEK 293 or COS-7).
- Use a transfection reagent that allows high-efficiency transfection; we recommend using Lipofectamine™ 2000 Reagent.

  Note: Lipofectamine™ 2000 is supplied with the ViraPower™ HiPerform™ Lentiviral TOPO® Expression Kits, but is also available separately from Invitrogen (page vii).
- Follow the manufacturer's instructions for the transfection reagent you are using to perform plasmid transfection. If you are using Lipofectamine™ 2000, follow the instructions included with the product.

Materials Needed

To express your gene of interest from the pLenti-TOPO® construct using Invitrogen's ViraPower™ HiPerform™ Lentiviral TOPO® Expression Kits (Catalog nos: K5310-00 and K5320-00), you will need the following reagents that are supplied with the Expression kits:

- 293FT Cell Line for producing maximized levels of virus (Naldini *et al.*, 1996). This cell line is derived from 293F cells and stably expresses the SV40 large T antigen for enhanced virus production.
- ViraPower™ Packaging Mix. When cotransfected with the pLenti-TOPO® expression construct into the 293FT producer cell line, this optimized mixture of plasmids supplies the viral proteins in *trans* that are required to create viral particles.
- Transfection reagent for efficient delivery of the ViraPower™ Packaging Mix and the pLenti-TOPO® expression construct to 293FT cells. We recommend using Lipofectamine™ 2000 Reagent for optimal transfection efficiency.
- Blasticidin for selection of stably transduced mammalian cells (pLenti6.3/V5-TOPO® vector **only**, see the **Appendix**, page 24 for more information).
- *Optional:* Control lentiviral expression vector (page 21).

For more information about the 293FT Cell Line, see the 293FT Cell Line manual. For more information about the ViraPower™ Packaging Mix, refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual. Both manuals are available for downloading from www.invitrogen.com or by contacting **Technical Support** (page 33).

Continued on next page

ViraPower™ Packaging Mix

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

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

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

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 vii).

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.invitrogen.com or by contacting **Technical Support** (page 36).

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

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

Additional Information

The 293FT Cell Line and the ViraPower™ Lentiviral Support Kits containing the ViraPower™ Packaging Mix, Lipofectamine™ 2000, and selection agent are included with the ViraPower™ HiPerform™ Lentiviral TOPO® Expression Kit and the ViraPower™ HiPerform™ Lentiviral FastTiter™ TOPO® Expression Kit (catalog nos: K5310-00 and K5320-00, respectively). These reagents are also available to order separately from Invitrogen (see page viii).

Continued on next page

Positive Controls

A positive control vector is included with each pLenti-TOPO® vector for use as an expression control in the ViraPower™ HiPerform™ Lentiviral Expression System (see table below). In each vector, β-galactosidase is expressed as a C-terminally tagged fusion protein that may be easily detected by western blot or functional assay.

Vector	Positive Control
pLenti6.3/V5-TOPO®	pLenti6.3/V5-GW/*lacZ*
pLenti7.3/V5-TOPO®	pLenti7.3/V5-GW/*lacZ*

A control lentiviral expression vector (pLenti6.3/V5-GW/EmGFP) containing Emerald Green Fluorescent Protein (EmGFP) to optimize transfection and virus production is available separately from Invitrogen. For ordering information, see page vii or visit www.invitrogen.com.

Note: The control vectors provided with the pLenti-TOPO® TA Cloning Kits and the ViraPower™ HiPerform™ Lentiviral Expression Systems are Gateway® Technology control vectors. These control vectors work well with the pLenti-TOPO® vectors. Visit our web site at www.invitrogen.com for more information on Gateway® Technology.

Propagating the Control Plasmids

The control plasmids are supplied in solution at a concentration of 0.5 µg/µl. To propagate and maintain the control plasmids:

1. Transform 10 ng of the stock solution into OneShot® Stbl3™ *E. coli* (see page 12).
2. Select transformants on selective plates containing 100 µg/ml ampicillin.
3. Propagate the plasmid in LB containing 100 µg/ml ampicillin.
4. Prepare a glycerol stock of a transformant containing plasmid for long-term storage.

Producing Viral Stocks

Refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual for detailed guidelines and protocols to:

- Cotransfect your pLenti-TOPO® expression construct and the ViraPower™ Packaging Mix into the 293FT Cell Line to generate a lentiviral stock.
- Determine the titer of your viral stock.

Determining Antibiotic Sensitivity

Note: pLenti7.3 vectors do not contain an antibiotic selection marker and therefore, do not generate antibiotic resistant clones

Once you have produced a lentiviral stock with a suitable titer, you use this stock to transduce your lentiviral construct into the mammalian cell line of choice. You may assay for transient expression of your recombinant protein (pLenti7.3/V5-TOPO®) or use Blasticidin to select for stably transduced cells (pLenti6.3/V5- TOPO®). Before generating your stably transduced cell line, we recommend that you generate a kill curve to determine the minimum concentration of Blasticidin required to kill your untransduced host cell line. For guidelines to generate a kill curve, refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual. For instructions to prepare and handle Blasticidin, see **Appendix**, page 24.

Transducing Mammalian Cells

Refer to the ViraPower™ HiPerform™ Lentiviral Expression System manual for instructions and guidelines to:

- Transduce your lentiviral construct into the mammalian cell line of interest at the appropriate multiplicity of infection (MOI).
- Generate stable cell lines using Blasticidin selection (pLenti6.3/V5- TOPO® vector, only).

Detecting Recombinant Fusion Proteins

To detect expression of your recombinant fusion protein, you may perform:

- Western blot analysis using the Anti-V5, Anti-V5-HRP, or Anti-V5-AP antibodies available from Invitrogen or an antibody to your protein
- Immunofluorescence using an Anti-V5-FITC antibody
- Functional analysis

For more information about the Anti-V5 antibodies, visit www.invitrogen.com or call **Technical Support** (page 36)..

Note

The C-terminal peptide containing the V5 epitope and the *att*B2 site will add approximately 4.5 kDa to the size of your protein.

Assay for β-galactosidase Activity

The β-galactosidase protein expressed from the pLenti6.3/V5-GW/*lacZ* and pLenti7.3/V5-GW/*lacZ* control lentiviral constructs is approximately 121 kDa in size. You may assay for β-galactosidase expression by western blot, using cell-free lysates (Miller, 1972), or by staining. Invitrogen offers an anti-β-galactosidase, β-Gal Assay Kit, and the β-Gal Staining Kit (see page vii for ordering details of the above products) for detection of β-galactosidase.

Important

For detecting EmGFP in pLenti7.3/V5-TOPO®, we recommend using flow cytometry. We do **not** recommend the use of fluorescence microscopy for detecting EmGFP in your cells from the pLenti7.3 vectors. The pLenti7.3 vectors are designed with EmGFP in their vector backbone which allows for quick-screens of transient expression in your cells and titering times of only 2-days. While the quantity of cells expressing your gene of interest is significantly greater than other pLenti vectors that do not contain the WPRE and cPPT elements, the signal intensity of EmGFP expressed in your cells is not optimal for viewing with fluorescence microscopy. For this reason, we recommend flow cytometry. For more details, refer to the ViraPower™ HiPerform™ Lentiviral System Manual.

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

Appendix

Recipes

LB (Luria-Bertani) Medium

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

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

LB Plates Containing Ampicillin

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

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add ampicillin to a final concentration of 100 g/ml and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

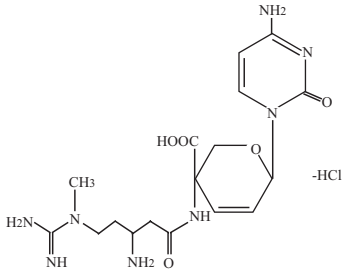
Blasticidin (pLenti6.3/V5-TOPO® Vector, Only)

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

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



Handling Blasticidin

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

Preparing and Storing Stock Solutions

Blasticidin may be obtained separately from Invitrogen (page vii) in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5 to 10 mg/ml.

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

pLenti-TOPO® TA Cloning Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions using the reagents included in the kit involves producing a 750 bp control PCR product.

Producing the Control PCR Product

1. To produce the 750 bp control PCR product containing the *lac* promoter and *lacZ* , set up the following 50 µl PCR:

Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Control PCR Primers (0.1 µg/µl each)	2 µl
Sterile Water	40.5 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	60°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

Continued on next page

pLenti6.3/V5-TOPO® TA Cloning Control Reactions, Continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pLenti/V5-TOPO© vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pLenti6.3/V5-TOPO© vector **or** pLenti7.3/V5-TOPO® Vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® Stbl3™ Chemically Competent *E. coli* (page 12).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 90% of these will be blue and contain the 750 bp insert.

Transformation Control

pUC19 plasmid is included in each vector kit to check the transformation efficiency of the One Shot® Stbl3™ Chemically Competent *E. col.*

1. Transform one vial of One Shot® Stbl3™ Chemically Competent *E. coli* with 10 pg of pUC19.
2. Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 100 µg/ml ampicillin.
3. Transformation efficiency: >85% will contain the 750 bp insert.

Continued on next page

Factors Affecting Cloning Efficiency

Please note that lower transformation and/or cloning efficiencies will result from the following variables. Most of these are easily corrected, but if you are cloning large inserts, you may not obtain the expected 90% (or more) cloning efficiency.

Variable	Solution
pH>9	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>3 kb)	Gel-purify as described on page 28.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 30).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 28).
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	*Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below for your convenience.

PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit **(**page vii**)** allows you to rapidly purify PCR products from regular agarose gels. To use the PureLink™ Quick Gel Extraction Kit:

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment.
3. Weigh the gel slice.
4. Add Gel Solubilization Buffer (GS1, supplied with kit) as follows:
   - For ≤2% agarose gels, place up to 400 mg gel into a sterile, 1.5 ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl GS1 for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5 ml polypropylene tubes and add 60 µl GS1 for every 10 mg of gel.
5. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate for an **additional** 5 minutes.
6. Preheat and aliquot of TE Buffer (TE) to 65-70°C
7. Place a Quick Gel extraction Column into a Wash Tube. Pipet the mixture from Step 5 above, onto the column. Use 1 column per 400 mg agarose.
8. Centrifuge at 12,000 x g for 1 minute. Discard the flow-through. Place the column back into the wash tube.
9. *Optional*: Add 500 µl GS1 to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 x g for 1 minute. Discard flow-through. Place the column back into the Wash Tube.
10. Add 700 µl Wash Buffer (W9) with ethanol (add 96-100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate at room temperature for 5 minutes. Centrifuge at >12,000 x g for 1 minute. Discard flow-through.
11. Centrifuge the column at >12,000 x g for 1 minute to remove any residual buffer. Place the column into a 1.5 ml Recovery Tube.
12. Add 50 µl **warm** (65-70°C) TE Buffer (TE) tp the cemter pf the cartridge. Incubate at room temperature for 1 minute.
13. Centrifuge at >12,000 x g for 2 minutes. *The Recovery Tube contains the purified DNA*. Store the purified DNA at -20°C. Discard the column.
14. Use 4 µl of the purified DNA for the TOPO® Cloning reaction.

Continued on next page

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Please note that the gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 10.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into chemically competent One Shot® TOP10 cells using the method on page 10.

Note

Please note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 9).

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by *Vent*® or *Pfu* polymerases into TOPO® TA Cloning® vectors is often difficult because of very low cloning efficiencies. These low efficiencies are caused by the lack of the terminal transferase activity associated with proofreading polymerases which adds the 3´ A-overhangs necessary for TA Cloning®. A simple method is provided below to clone these blunt-ended fragments.

Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pcDNA3.1/V5-His-TOPO©.

Note: If you plan to store your sample(s) overnight before proceeding with TOPO® Cloning, you may want to extract your sample(s) with phenol-chloroform to remove the polymerases. After phenol-chloroform extraction, precipitate the DNA with ethanol and resuspend the DNA in TE buffer to the starting volume of the amplification reaction.

Note

You may also gel-purify your PCR product after amplification with *Vent*® or *Pfu* (see previous page). After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase and incubate 10-15 minutes at 72°C. Use 4 µl in the TOPO® Cloning reaction.

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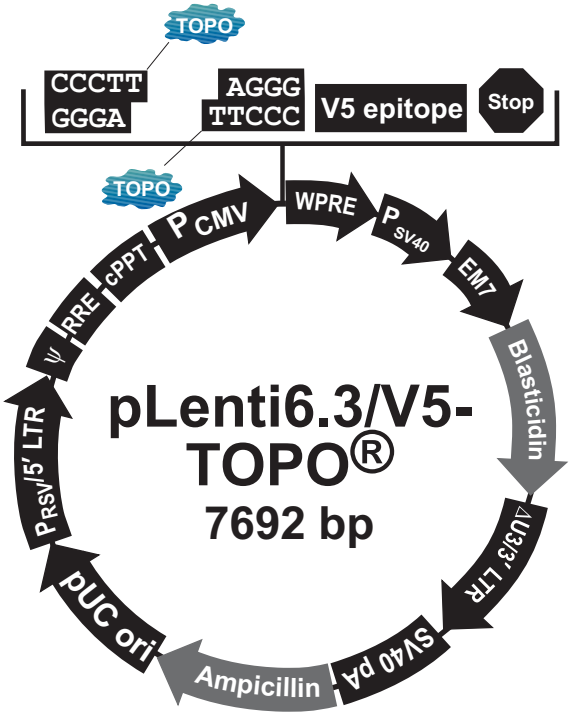
Map of pLenti6.3/V5-TOPO®

Map

The map below shows the elements of the pLenti6.3/V5-TOPO® vector. For more information, visit our web site at www.invitrogen.com or contact **Technical Support** (page 35).



Comments for pLenti6.3/V5-TOPO
7692 nucleotides

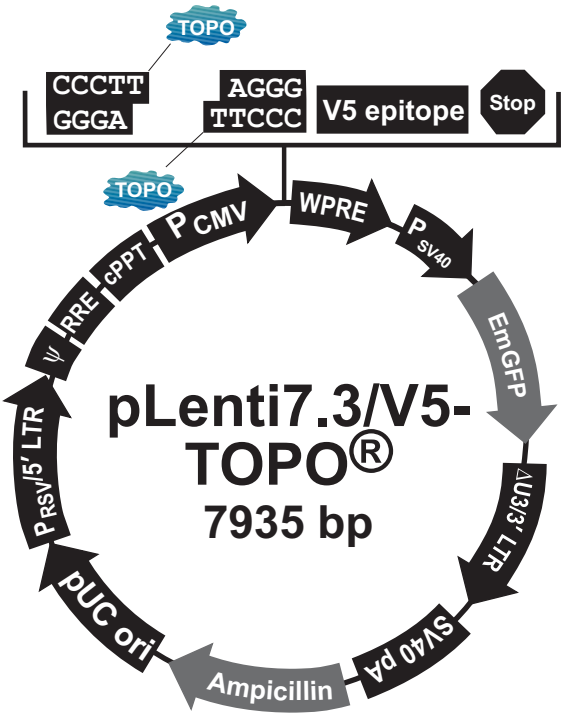
RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
cPPT: bases 1800-1922
CMV promoter: bases 1934-2518
TOPO® cloning site: bases 2557-2566
V5 epitope: bases 2629-2670
WPRE: bases 2689-3286
SV40 promoter: bases 3297-3605
EM7 promoter: bases 3660-3726
Blasticidin resistance gene: bases 3727-4125
ΔU3/3′ LTR: bases 4211-4445
    ΔU3: bases 4211-4264
    3′ LTR: bases 4265-4445
SV40 polyadenylation signal: bases 4517-4648
bla promoter: bases 5507-5605
Ampicillin (*bla*) resistance gene: bases 5606-6466
pUC origin: bases 6611-7284

continued on next page

Map of pLenti7.3/V5-TOPO®

Map

The figure below summarizes the features of the pLenti7.3/V5-TOPO® vector. For more information, visit our web site at www.invitrogen.com or contact **Technical Support** (page 36).



Comments for pLenti7.3/V5-TOPO
7935 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
TOPO® cloning site: bases 2558-2567
V5 epitope: bases 2630-2671
WPRE: bases 2690-3287
SV40 promoter: bases 3298-3606
EmGFP: bases 3665-4384
ΔU3/3′ LTR: bases 4455-4689
    ΔU3: bases 4455-4508
    3′ LTR: bases 4509-4689
SV40 polyadenylation signal: bases 4761-4892
bla promoter: bases 5751-5849
Ampicillin (*bla*) resistance gene: bases 5850-6710
pUC origin: bases 6855-7528

Features of pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO® Vectors

Features

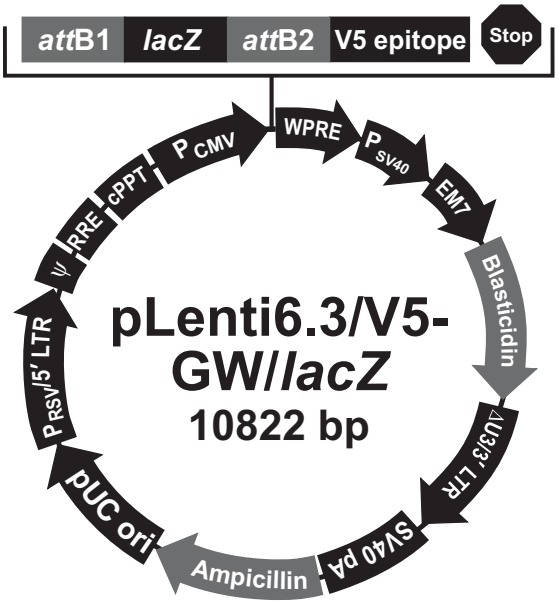
pLenti6.3/V5-TOPO® and pLenti7.3/V5-TOPO® vectors contain the following elements. All features have been functionally tested, and the vectors completely sequenced.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998)
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
Polypurine Tract from HIV (cPPT)	Provides for increased viral titer (Park, 2001)
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits high-level, constitutive expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
TOPO® Cloning site	Allows cloning of PCR products containing A-overhangs in frame with the V5 epitope.
V5 epitope	Allows detection of the recombinant fusion protein by Anti-V5 Antibodies (Southern *et al.*, 1991).
Woodchuck Posttranscriptional Regulatory Element (WPRE)	Provides for increased transgene expression (Zufferey *et al.*, 1998)
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene (pLenti6.3/V5-TOPO® only)	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).
Emerald Green Fluorescent Protein (EmGFP, pLenti7.3/V5-TOPO® only)	Allows for fluorescence detection by flow cytometry and quick-screen of transient expression in only 2 days post-transfection..
U3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli.*
pUC origin	Permits high-copy replication and maintenance in *E. coli.*

Map of pLenti6.3/V5-GW/*lacZ* Control Vector

Map

The map below shows the elements of the pLenti6.3/V5-GW/*lacZ* vector. pLenti6.3/V5-GW/*lacZ* is a 8675 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an Entry Clone containing the *lacZ* gene and pLenti6.3/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa. For more information about the Gateway® Cloning Technology and pLenti6.3/V5-DEST, refer to the pLenti6.3/V5-DEST manual, which is available for downloading from or web site (www.invitrogen.com) or contact **Technical Support** (page 36).



Comments for pLenti6.3/V5-GW/*lacZ*
10822 nucleotides

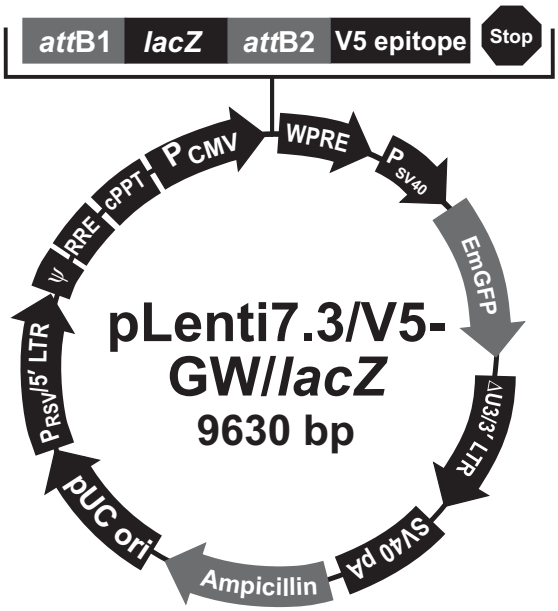
RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1655
3′ splice acceptor: base 1683
cPPT: bases 1800-1922
CMV promoter: bases 1934-2518
*att*B1 site: bases 2567-2591
lacZ ORF: bases 2606-5670
*att*B2 site: bases 5682-5706
V5 epitope: bases 5759-5800
WPRE: bases 5819-6416
SV40 promoter: bases 6427-6736
EM7 promoter: bases 6791-6857
Blasticidin resistance gene: bases 6858-7256
ΔU3/HIV-1 3′ LTR: bases 7342-7576
    ΔU3: bases 7342-7395
    Truncated HIV-1 3′ LTR: bases 7396-7576
SV40 polyadenylation signal: bases 7648-7779
bla promoter: bases 8638-8736
Ampicillin (*bla*) resistance gene: bases 8737-9587
pUC origin: bases 9742-10415

continued on next page

Map of pLenti7.3/V5-GW/*lacZ* Control Vector

Map

The map below shows the elements of the pLenti6.3/V5-GW/*lacZ* vector. pLenti6.3/V5-GW/*lacZ* is a 11066 bp control vector expressing β-galactosidase, and was generated using the Gateway® LR recombination reaction between an Entry Clone containing the *lacZ* gene and pLenti6.3/V5-DEST. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa. For more information about the Gateway® Cloning Technology and pLenti6.3/V5-DEST, refer to the pLenti6.3/V5-DEST manual, which is available for downloading from or web site (www.invitrogen.com) or contact **Technical Support** (page 36).



Comments for pLenti7.3/V5-GW/*lacZ*
9630 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
    RSV promoter: bases 1-229
    HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
cPPT: bases 1801-1923
CMV promoter: bases 1935-2519
*att*B1 site: bases 2568-2592
lacZ ORF: bases 2608-5672
*att*B2 site: bases 5684-5708
V5 epitope: bases 5761-5802
WPRE: bases 5821-6418
SV40 promoter: bases 6429-6738
EmGFP: bases 6796-7515
ΔU3/3′ LTR: bases 7586-7820
    ΔU3: bases 7586-7639
    3′ LTR: bases 7640-7820
SV40 polyadenylation signal: bases 7892-8023
bla promoter: bases 8882-8980
Ampicillin (*bla*) resistance gene: bases 8981-9841
pUC origin: bases 9986-10659

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
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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Continued on next page

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Continued on next page

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