invitrogen™

pLenti6/V5 Directional TOPO® Cloning Kit

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an expression vector for the ViraPower™ Lentiviral Expression System

Catalog nos. K4955-00, K4955-10

Version E
8 June 2010
25-0502

User Manual

Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Step	Action
Design PCR Primers	• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer. • Design the primers such that your gene of interest will be optimally expressed and fused in frame with the V5 epitope tag, if desired.
Amplify Your Gene of Interest	1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product. 2. Use agarose gel electrophoresis to check the integrity and determine the yield of your PCR product.
Perform the TOPO® Cloning Reaction	1. Set up the following TOPO® Cloning reaction. **For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.** Fresh PCR product — 0.5 to 4 µl Salt Solution — 1 µl Water — add to a final volume of 5 µl TOPO® vector — 1 µl Total volume — 6 µl 2. Mix gently and incubate for 5 minutes at room temperature. 3. Place on ice and proceed to transform One Shot® Stbl3™ chemically competent *E. coli*, below.
Transform One Shot® Stbl3™ Chemically Competent *E. coli*	1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® Stbl3™ chemically competent *E. coli* and mix gently. 2. Incubate on ice for 30 minutes. 3. Heat-shock the cells for 45 seconds at 42°C without shaking. Immediately transfer the tube to ice for 2 minutes. 4. Add 250 µl of pre-warmed LB or S.O.C. Medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 25-100 µl of bacterial culture on a prewarmed LB agar plate containing 100 µg/ml ampicillin, and incubate overnight at 37°C.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 25-26 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Catalog no.
ViraPower™ Lentiviral Directional TOPO® Expression Kit	K4950-00
pLenti6/V5 Directional TOPO® Cloning Kit	K4955-10

Kit Components

Catalog nos. K4950-00 and K4955-10 include the following components. For a detailed description of the reagents supplied with the pLenti6/V5-D-TOPO® and the One Shot® Stbl3™ Chemically Competent *E. coli* kits, see pages viii-ix. For a detailed description of the reagents supplied with the ViraPower™ Bsd Lentiviral Support Kit, or instructions to culture and maintain the 293FT Cell Line, see the ViraPower™ Lentiviral Expression System or 293FT Cell Line manuals, respectively. Both manuals are supplied with the ViraPower™ Lentiviral Directional TOPO® Expression Kit, but are also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 34).

Component	Catalog no.	
	K4950-00	K4955-10
pLenti6/V5-D-TOPO® Reagents	√	√
One Shot® Stbl3™ Chemically Competent *E. coli*	√	√
ViraPower™ Bsd Lentiviral Support Kit	√	
293FT Cell Line	√	

Shipping/Storage

The ViraPower™ Directional TOPO® Kits are shipped as described below. Upon receipt, store each item as detailed below.

Note: Catalog no. K4955-10 includes Box 1 and Box 2 only.

Box	Component	Shipping	Storage
1	pLenti6/V5-D-TOPO® Reagents	Dry ice	-20°C
2	One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	-80°C
3	ViraPower™ Bsd Lentiviral Support Kit	Blue ice	ViraPower™ Packaging Mix: -20°C Lipofectamine™ 2000: +4°C Blasticidin: -20°C
4	293FT Cell Line	Dry ice	Liquid nitrogen

continued on next page

Kit Contents and Storage, continued

pLenti6/V5-D-TOPO® Reagents

pLenti6/V5-D-TOPO® reagents (Box 1) are listed below. **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Reagent	Concentration	Amount
pLenti6/V5-D-TOPO®, TOPO®-adapted	15-20 ng/µl linearized 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 bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Water	---	1 ml
CMV Forward Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
V5(C-term) Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl
pLenti6/V5-GW/*lacZ* Expression Control Plasmid	Lyophilized in TE Buffer, pH 8	10 µg

continued on next page

Kit Contents and Storage, continued

Sequences of the Primers

The table below provides the sequences of CMV Forward and V5(C-term) Reverse sequencing primers. Two micrograms of each primer are supplied.

Primer	Sequence
CMV Forward	5´-CGCAAATGGGCGGTAGGCGTG-3´
V5(C-term) Reverse	5´-ACCGAGGAGAGGGTTAGGGAT-3´

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

The table below lists the items included in the One Shot® Stbl3™ Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^8 cfu/µg plasmid DNA. **Store Box 2 at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at +4°C or room temperature)	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+

ViraPower™ Bsd Lentiviral Support Kit and 293FT Cell Line

The ViraPower™ Lentiviral Directional TOPO® Expression Kit includes the ViraPower™ Bsd Lentiviral Support Kit and the 293FT Cell Line to facilitate production of replication-incompetent lentiviral stocks. For a detailed description of the reagents provided in the ViraPower™ Bsd Lentiviral Support Kit and their use, refer to the ViraPower™ Lentiviral Expression System manual. For a detailed description of the 293FT Cell Line and instructions to culture and maintain the cells, refer to the 293FT Cell Line manual.

Accessory Products

Introduction

The products listed in this section may be used with the pLenti6/V5 Directional TOPO® Cloning Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 34).

Additional Products

Some of the reagents supplied in the pLenti6/V5 Directional TOPO® Cloning Kit as well as other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® Stbl3™ Chemically Competent *E. coli*	20 x 50 µl	C7373-03
Ampicillin	200 mg	11593-019
Blasticidin	50 mg	R210-01
S.N.A.P.™ Midiprep Kit	20 reactions	K1910-01
PureLink™ HQ Plasmid Miniprep Kit	100 reactions	K2100-01
AccuPrime™ *Pfx* SuperMix	200 reactions	12344-040
E-Gel® 1.2% Starter Pak (6 gels + Powerbase™)	1 kit	G6000-01
E-Gel® 1.2% 18 Pak	18 gels	G5018-01
Lipofectamine™ 2000	0.75 ml	11668-027
	1.5 ml	11668-019
β-gal Antiserum, rabbit IgG fraction	500 µl*	A11132
β-Gal Assay Kit	1 kit	K1455-01
β-Gal Staining Kit	1 kit	K1465-01
S.N.A.P.™ Gel Purification Kit	1 kit	K1999-25

Accessory Products, continued

ViraPower™ Lentiviral Expression Products

The pLenti6/V5-D-TOPO® vector is designed for use with the ViraPower™ Lentiviral Expression System available from Invitrogen. Ordering information for other ViraPower™ lentiviral support products and expression vectors is provided below.

Item	Quantity	Catalog no.
ViraPower™ Lentiviral Gateway® Expression Kit	1 kit	K4960-00
ViraPower™ Zeo Lentiviral Gateway® Expression Kit	1 kit	K4980-00
ViraPower™ UbC Lentiviral Gateway® Expression Kit	1 kit	K4990-00
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970-00
ViraPower™ Zeo Lentiviral Support Kit	20 reactions	K4985-00
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 from pLenti6/V5-D-TOPO® can be detected using an antibody to the V5 epitope. The table below describes the antibodies available from Invitrogen. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemi-luminescent 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 immuno-staining reactions (FITC-conjugated antibody only).

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/V5 Directional TOPO® Cloning Kit combines the ViraPower™ Lentiviral Expression System with TOPO® Cloning technology to provide a highly efficient, rapid cloning strategy for insertion of blunt-end PCR products into a vector for expression in dividing and non-dividing mammalian cells. TOPO® Cloning requires no ligase, post-PCR procedures, or restriction enzymes.

Features of the pLenti6/V5-D-TOPO® Vector

pLenti6/V5-D-TOPO® is a ~7.0 kb expression vector designed to facilitate rapid, directional TOPO® Cloning and high-level expression of PCR products in mammalian cells using the ViraPower™ Lentiviral Expression System available from Invitrogen. Features of the vector include:

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

  Note: The U3 region of the 3′ LTR is deleted (ΔU3) and facilitates self-inactivation of the 5′ LTR 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 (CMV) immediate early 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)
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- C-terminal V5 epitope for detection of the recombinant protein of interest (Southern *et al.*, 1991)
- Blasticidin (*bsd*) 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 and maintenance of the plasmid in *E. coli*

The control plasmid, pLenti6/V5-GW/*lacZ*, is included for use as a positive expression control in the mammalian cell line of choice.

For more information about the ViraPower™ Lentiviral Expression System, see the next page.

continued on next page

Overview, continued

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. To express your gene of interest in mammalian cells using the ViraPower™ Lentiviral Expression System, you will:

1. TOPO® Clone a PCR fragment encoding your gene of interest into pLenti6/V5-D-TOPO® to create an expression construct.
2. Cotransfect your pLenti6/V5-D-TOPO® expression plasmid 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 or generate a stable cell line using Blasticidin selection.

For more information about the ViraPower™ Lentiviral Expression System, the ViraPower™ Packaging Mix, and the biosafety features of the System, refer to the ViraPower™ 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 our Web site (www.invitrogen.com) or by contacting Technical Service (see page 34).

How Directional TOPO® Cloning Works

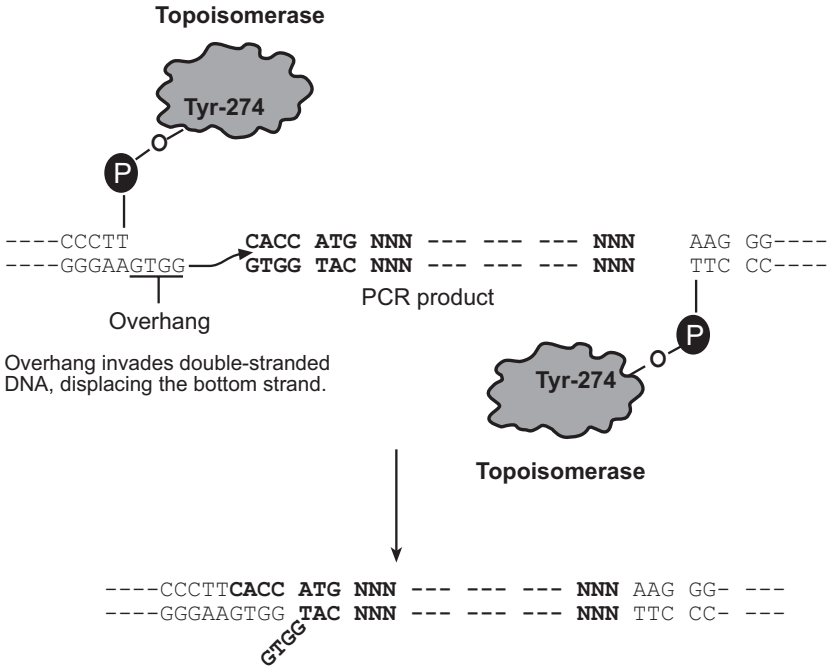
How Topoisomerase I Works

Topoisomerase I from vaccinia virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone 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.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

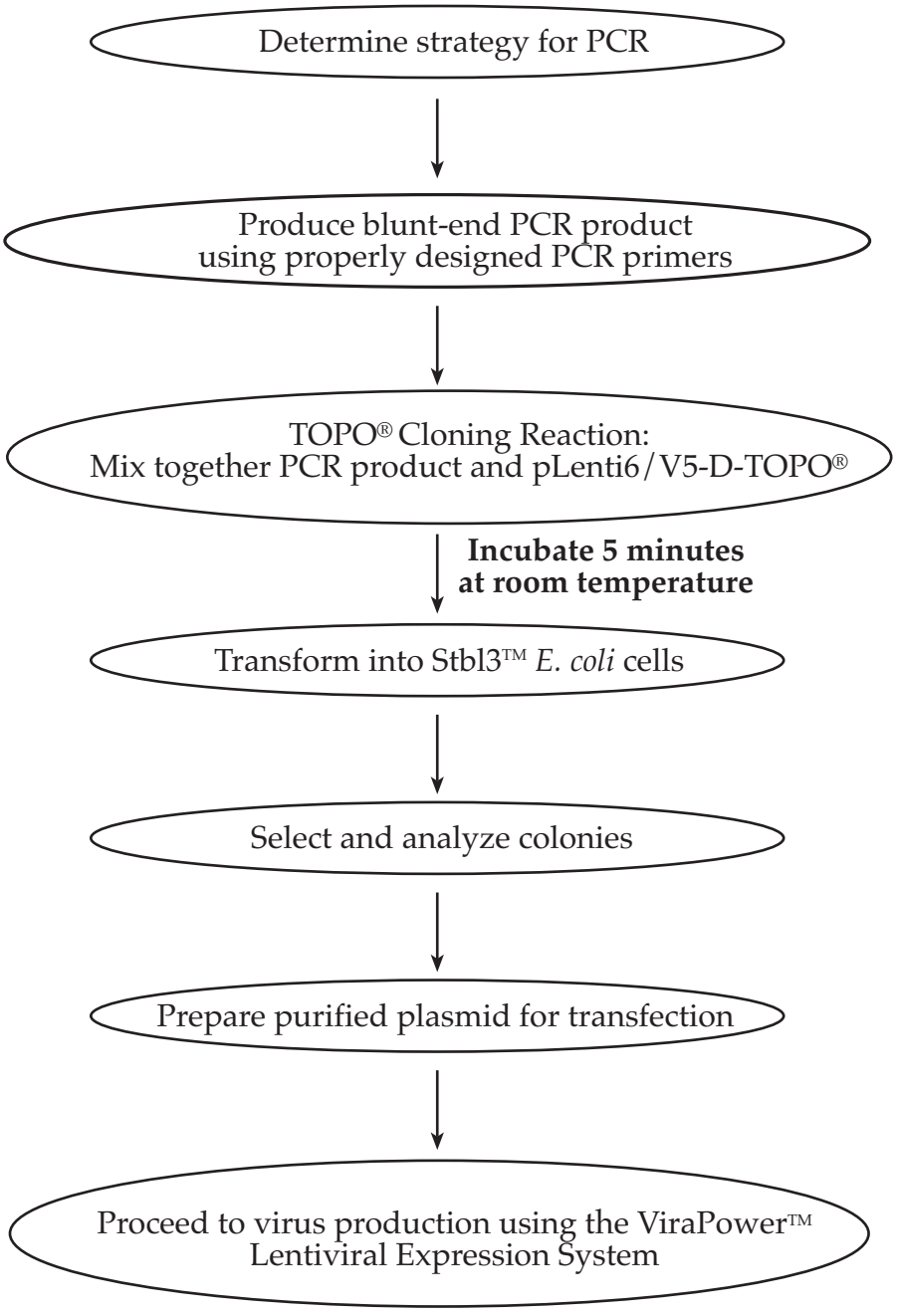
In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and clone your blunt-end PCR product.



Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with the C-terminal V5 epitope tag

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the points below. Refer to page 7 for diagrams of the TOPO® Cloning site for pLenti6/V5-D-TOPO®.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in the pLenti6/V5-D-TOPO® vector.
- Your sequence of interest should include a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)**NNATG**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then:

- The primer includes the 4 nucleotides, CACC, required for directional cloning.
- The ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

continued on next page

Designing PCR Primers, continued

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the points below. Refer to page 7 for diagrams of the TOPO® Cloning site for pLenti6/V5-D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 below).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with the C-terminal tag containing the V5 epitope, then design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you do NOT wish to fuse your PCR product in frame with the C-terminal tag, then include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

Important

If you plan to amplify DNA from a cDNA clone, make sure that your reverse PCR primer **does not** contain sequences encoding the polyA tail. The presence of polyA sequences between the viral LTR's prevents production of functional lentivirus.

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with the C-terminal tag in pLenti6/V5-D-TOPO®. The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

continued on next page

Designing PCR Primers, continued

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

…GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′

- To fuse the ORF in frame with the C-terminal tag in pLenti6/V5-D-TOPO®, remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with the C-terminal tag.

- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

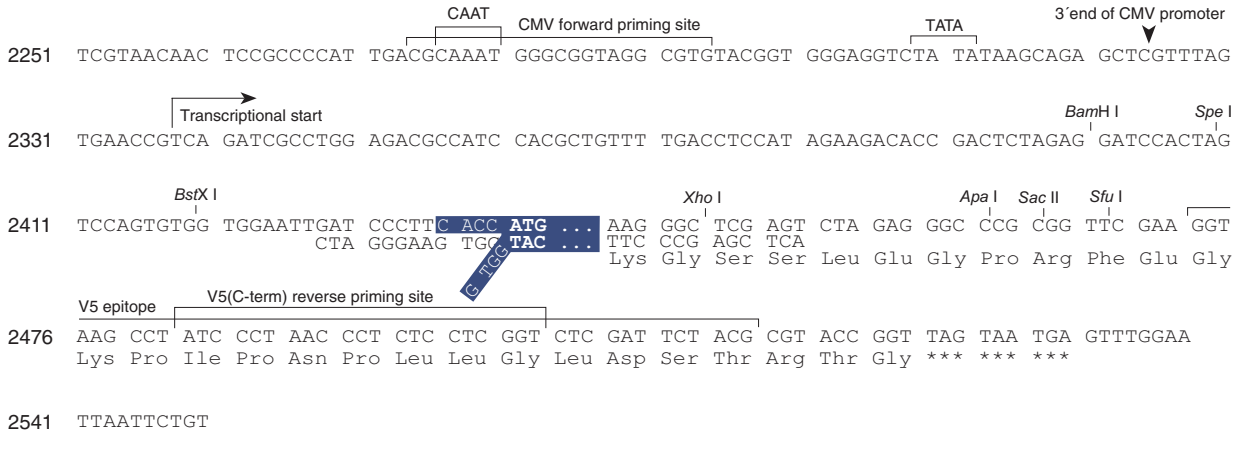
  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that pLenti6/V5-D-TOPO® accepts blunt-end PCR products.
- Do not add 5´ phosphates to your primers for PCR. This will prevent ligation into the pLenti6/V5-D-TOPO® vector.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

TOPO® Cloning Site for pLenti6/V5-D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pLenti6/V5-D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. Note that pLenti6/V5-D-TOPO® is supplied linearized with both ends adapted with topoisomerase I (see diagram on page 30). **The sequence of pLenti6/V5-D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 34).** For more information about the features of pLenti6/V5-D-TOPO®, see the **Appendix**, pages 30-31.



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

You will need the following reagents and equipment for PCR.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers to produce the PCR product

Producing Blunt-End PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below:

1. Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
2. Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
3. Use a 7- to 30-minute final extension to ensure that all PCR products are completely extended.
4. After cycling, place the tube on ice or store at –20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below:

- You have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice.
- We strongly recommend that you gel-purify the desired PCR product, even if you obtain a discrete band. This is especially important if your PCR template was an ampicillin-resistant plasmid, because amp-resistant plasmids can be carried through the cloning reaction and can grow favorably over the lentiviral plasmid construct after transformation. For a protocol to gel-purify your PCR product see pages 27-28.
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

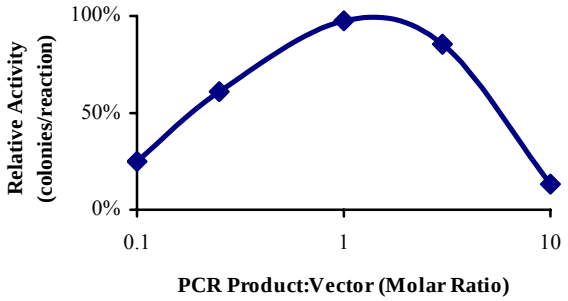
continued on next page

Producing Blunt-End PCR Products, continued

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For the pLenti6/V5-D-TOPO® vector, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



Performing the TOPO® Cloning Reaction

Introduction

Once you have produced the desired blunt-end PCR product, you are ready to TOPO® Clone it into the pLenti6/V5-D-TOPO® vector and transform the recombinant vector into competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent *E. coli*** (page 12) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 25-26 in parallel with your samples.

Recommended *E. coli* Host

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. One Shot® Stbl3™ Chemically Competent *E. coli* are included in the kit for transformation. For instructions, see **One Shot Stbl3™ Chemical Transformation Procedure**, page 13. Note that transformants containing unwanted recombinants are generally **not** obtained when Stbl3™ *E. coli* are used for transformation.

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). Use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction, continued

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Amount
Fresh PCR product	0.5 to 4 µl
Salt Solution	1 µl
Dilute Salt Solution (1:4)	--
Water	add to a final volume of 5 µl
TOPO® vector	1 µl
Total volume	6 µl

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

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°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 (1-3 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Stbl3™ Competent Cells**, next page.

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

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pLenti6/V5-D-TOPO® construct into competent *E. coli*. One Shot® Stbl3™ Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation and are recommended (see page 10).

Note: The transformation efficiency of One Shot® Sbl3™ Chemically Competent *E. coli* is ≥ 1 x 10^8 cfu/µg plasmid DNA.

Materials Needed

Have the following materials on hand before beginning:

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot™ Stbl3™ Chemically Competent *E. coli* (supplied with the kit, Box 2; one vial per transformation; thaw on ice immediately before use)
- pUC19 positive control (if desired to verify the transformation efficiency; supplied with the kit, Box 2)
- LB Medium pre-warmed to 37ºC

  Note: You may use S.O.C. Medium provided with the kit in place of LB Medium for cell recovery
- 15-ml sterile, capped tubes
- 42°C water bath
- LB plates containing 100 µg/ml ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmid with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

continued on next page

Transforming One Shot® Competent Cells, continued

One Shot® Stbl3™ Chemical 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 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, 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. Transfer cells gently to a sterile, 15-ml tube containing 1 ml of pre-warmed LB Medium
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 prewarmed selective 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. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 10-20 colonies for analysis (see **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.

Note: PCR screening of clones should never be used 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.

You will screen colonies by performing miniprep and restriction analysis to validate the clones. You may also perform PCR analysis or sequencing of your clones with the primers provided 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 or large scale DNA preparation.

Experimental Outline

Step	Action
1	For each transformation, pick 10-20 ampicillin-resistant colonies from plating the transformation mix. Culture cells overnight in LB medium containing 100 µg/ml ampicillin.
2	Isolate plasmid DNA for each colony using a miniprep kit (see **Important**, next page).
3	Analyze the plasmids by restriction analysis to confirm the presence and orientation of your insert as well as the integrity of the vector.
Optional	Sequence the plasmids to determine that your gene of interest is in frame with the C-terminal V5 epitope tag.
4	Re-transform One Shot® Stbl3™ Chemically Competent *E. coli* separately with the validated clones.
5	Inoculate LB-ampicillin with a fresh colony and grow for 6 -8 hours to generate a starter culture.
6	Inoculate the starter culture into at least 100 ml LB-ampicillin and grow for 18 hours.
7	Isolate plasmid DNA using a midiprep kit or large scale DNA preparation (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 S.N.A.P.™ MidiPrep Kits (page x).

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 the pLenti6/V5-D-TOPO® vector is available at www.invitrogen.com.

Materials Needed

You will need the following materials:

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

Screening Colonies by Miniprep

For each transformation, pick 10-20 colonies from plates obtained after plating the transformation mix. Culture them overnight in LB medium containing 100 µg/ml ampicillin.

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.

Perform restriction digests on plasmid DNA, and analyze the digested DNA on 1.2% agarose gels to confirm the correct pLenti6/V5-D-TOPO® clones.

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 TOPO® cloning into the lentiviral vector. Additional or unexpected bands indicate aberrant recombination of the lentiviral vector.

Continued on next page

Analyzing Transformants, continued

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a combination of the CMV Forward primer or the V5(C-term) Reverse primer and a primer that hybridizes within your insert. 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:

AccuPrime™ *Pfx* SuperMix (cat no. 12344-040)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µl of AccuPrime™ *Pfx* SuperMix into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5-10 colonies that have been analyzed by restriction digest (see page 15) and resuspend them individually in 50 µl of SuperMix 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 for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation and in frame with the V5 epitope. The CMV Forward and V5(C-term) Reverse primers are included in the kit to help you sequence your insert (see diagram on page 7 for the location of the priming sites in the pLenti6/V5-D-TOPO® vector).
The complete sequence of pLenti6/V5-D-TOPO®, is available on our Web site (www.invitrogen.com).

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 25-26 or refer to the **Troubleshooting** section, page 23 for tips to help you troubleshoot your experiment.

Analyzing Transformants, continued

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 at least 100 ml volume for large scale or midiprep isolation of DNA.
6. Isolate plasmid DNA using S.N.A.P.™ MidiPrep Kit or equivalent (see **Important**, page 15).

   Note: For best results using the S.N.A.P.™ MidiPrep Kit, split the 100 ml culture into two 50-ml tubes and process as if they were separate samples. Run both samples over the same DNA binding column B, (*i.e.* perform two spins) and treat as a single DNA prep in subsequent steps.
7. Perform restriction analysis (see 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 next page).

   Note: Typical DNA yield should be ~300-400 µg and the O.D. $_{260/280}$ ratio should be between 1.8 and 2.1.

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 S.N.A.P.™ MidiPrep Kit.

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

Maintaining the Expression Clone

Maintain and propagate the pLenti6/V5-D-TOPO® expression plasmid in LB medium containing 100 µg/ml ampicillin. Addition of other antibiotics is not required.

continued on next page

Analyzing Transformants, continued

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out for single colonies on LB plates 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 until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Verifying Expression of Recombinant Protein (Optional)

Before proceeding to generate a lentiviral stock of your pLenti6/V5-D-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:

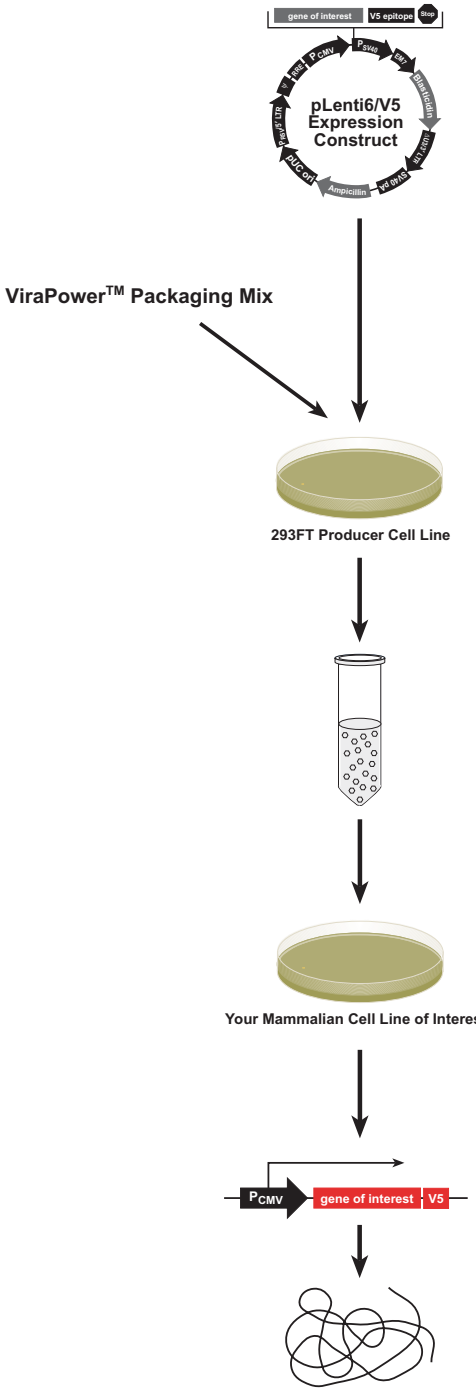
- Use an easy-to-transfect, dividing mammalian cell line (*e.g.* HEK 293 or COS-7).
- Use a transfection reagent that allows high-efficiency transfection; we recommend using Lipofectamine™ 2000 Reagent.

  Note: Lipofectamine™ 2000 is supplied with the ViraPower™ Lentiviral Directional TOPO® Expression Kit, but is also available separately from Invitrogen (see page x for ordering information).
- 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.

Expressing Your Recombinant Protein

Introduction

Once you have TOPO® Cloned your gene of interest into pLenti6/V5-D-TOPO®, you are ready to use Invitrogen's ViraPower™ Lentiviral Expression System to produce a viral stock, which may then be used to transduce your mammalian cell line of choice to express your recombinant protein (see outline below).



1. Generate the pLenti6-V5 expression construct containing your gene of interest.
2. Cotransfect the 293FT producer cell line with your pLenti6/V5 expression construct and the optimized packaging mix.
3. Harvest viral supernatant and determine the titer.
4. Add the viral supernatant to your mammalian cell line of interest. Select for stably transduced cells using blasticidin, if desired.
5. Assay for recombinant protein of interest.

continued on next page

Expressing Your Recombinant Protein, continued

Materials to Have on Hand

To express your gene of interest from pLenti6/V5-D-TOPO® using the ViraPower™ Lentiviral Expression System, you will need to have the following reagents:

- 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 pLenti6/V5-D-TOPO® plasmid 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 pLenti6/V5-D-TOPO® expression construct to 293FT cells. We recommend using Lipofectamine™ 2000 Reagent for optimal transfection efficiency.
- Blasticidin for selection of stably transduced cells (see the **Appendix**, page 29 for more information).

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™ Lentiviral Expression System manual. Both manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 34). The 293FT cell line and the ViraPower™ Bsd Lentiviral Support Kit (containing the ViraPower™ Packaging Mix, Lipofectamine™ 2000, and Blasticidin) are available separately from Invitrogen (see below for ordering information).

Item	Catalog No.
ViraPower™ Bsd Lentiviral Support Kit	K4970-00
293FT Cell Line	R700-07

continued on next page

DNA Isolation Guidelines

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency.

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

Note: We also recommend performing restriction analysis to verify the integrity of your expression construct after plasmid preparation. See page 15 for details.

Important

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

Positive Control

To optimize expression conditions in your mammalian cell line of interest, we recommend including the pLenti6/V5-GW/*lacZ* positive control plasmid supplied with the kit in your experiment. In pLenti6/V5-GW/*lacZ*, the gene encoding β-galactosidase is expressed in mammalian cells under the control of the CMV promoter. Once you have produced a lentiviral stock and stably transduced the lentivirus into your mammalian cell line of interest, you may easily assay for β-galactosidase expression (see the next page).

To propagate and maintain the pLenti6/V5-GW/*lacZ* plasmid:

1. Resuspend the vector in 10 µl sterile water to prepare a 1 µg/µl stock solution. Use the stock solution to transform Stbl3™ *E. coli.* Use 10 ng of plasmid DNA for transformation.
2. Select transformants on LB agar plates containing 100 µg/ml ampicillin. **Optional:** perform *Afl*II and *Xho*I double digest as described on page 15 to confirm plasmid.
3. Prepare a glycerol stock of a transformant containing plasmid for long-term storage (see page 18).

Producing Lentiviral Stocks

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

- Cotransfect your pLenti6/V5-D-TOPO® construct and the ViraPower™ Packaging Mix into the 293FT cell line to generate a lentiviral stock.
- Determine the titer of your viral stock.

continued on next page

Expressing Your Recombinant Protein, continued

Determining Blasticidin Sensitivity

Once you have produced a lentiviral stock with a suitable titer, you will 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 or use Blasticidin to select for stably transduced cells. 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. Generally, concentrations ranging from 2-10 µg/ml Blasticidin are sufficient to kill most untransduced mammalian cell lines. We recommend testing a range of concentrations to ensure that you determine the minimum concentration necessary for your cell line. For guidelines to generate a kill curve, refer to the ViraPower™ Lentiviral Expression System manual. For instructions to prepare and handle Blasticidin, see the **Appendix**, page 29.

Transducing Mammalian Cells

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

- Transduce your Lenti6/V5 construct into the mammalian cell line of interest at the appropriate multiplicity of infection (MOI).
- Generate stable cell lines using Blasticidin selection.

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 the Anti-V5-FITC antibody available from Invitrogen
- Functional analysis

For more information about the Anti-V5 antibodies, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 34). Ordering information is provided on page xi.

Note

The C-terminal peptide containing the V5 epitope will add approximately 2.9 kDa to the size of your protein.

Assay for β-galactosidase Activity

The β-galactosidase protein expressed from the Lenti6/V5-GW/*lacZ* control, lentiviral construct is approximately 121 kDa in size. You may assay for β-galactosidase expression by western blot analysis, activity assay using cell-free lysates (Miller, 1972), or by staining the cells for activity. Invitrogen offers an anti β-galactosidase antibody (A11132), β-Gal Assay Kit (Catalog no. K1455-01), and the β-Gal Staining Kit (Catalog no. K1465-01) for fast and easy detection of β-galactosidase expression.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 25-26) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	• Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector. • Increase the incubation time of the TOPO® reaction from 5-60 minutes (can also be increased to overnight). • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify your PCR product.
	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products need a longer extension time.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5-60 minutes (can also be increased to overnight) • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
Large number of incorrect inserts cloned	PCR cloning artifacts	Gel-purify your PCR product to remove primer-dimers and smaller PCR products. Optimize your PCR. Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	Make sure that the forward and reverse PCR primers are designed correctly.
Different sized colonies (*i.e.* large and small) appear when using TOP10 *E. coli* for transformation	Some transformants contain plasmids in which unwanted recombination has occurred between 5′ and 3′ LTRs	Do not use TOP10 *E. coli* for transformation. Use the One Shot® Stbl3™ Chemically Competent *E. coli* supplied with the kit for transformation. Stbl3™ *E. coli* are recommended for cloning unstable DNA including lentiviral DNA containing direct repeats and generally do not give rise to unwanted recombinants.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the 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 involves producing a control PCR product using the reagents included in the kit and using this product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 100 µg/ml ampicillin.

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the proofreading polymerase you are using.

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Component	Amount
Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile water	41.5 µl
Proofreading polymerase (1-2.5 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
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	55°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. Make sure that you see a single, discrete 750 bp band.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pLenti6/V5-D-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"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pLenti6/V5-D-TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 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™ cells using the protocol on page 13.
4. Spread 25-100 µl of each transformation mix onto LB plates containing 100 µg/ml ampicillin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformants, isolate plasmid DNA and digest with *Xho* I and *Eco*R I. *Xho* I cuts once in the vector and *Eco*R I cuts once in the insert. You should see the following digestion patterns:

- Correct orientation: 651, 7063 bp
- Reverse orientation: 109, 7605 bp
- Empty vector: 6964 bp

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation. Relatively few colonies should be produced in the vector-only reaction.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® Stbl3™ competent cells. Transform one vial of One Shot® Stbl3™ cells with 10 pg of pUC19 using the protocol on page 13. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^8 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (> 3 kb) may necessitate gel purification. If you wish 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.

Note

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 8).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 11.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 11) Be sure to make the gel slice as small as possible for best results.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that 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 11.
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 One Shot® Stbl3™ cells using the procedure on page 13.

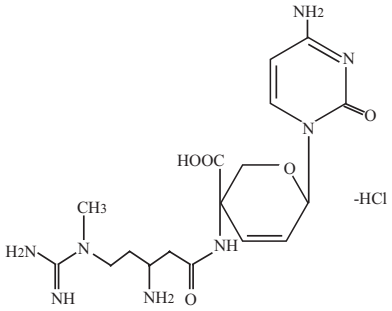
Blasticidin

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 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 (Catalog no. R210-01) 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**).

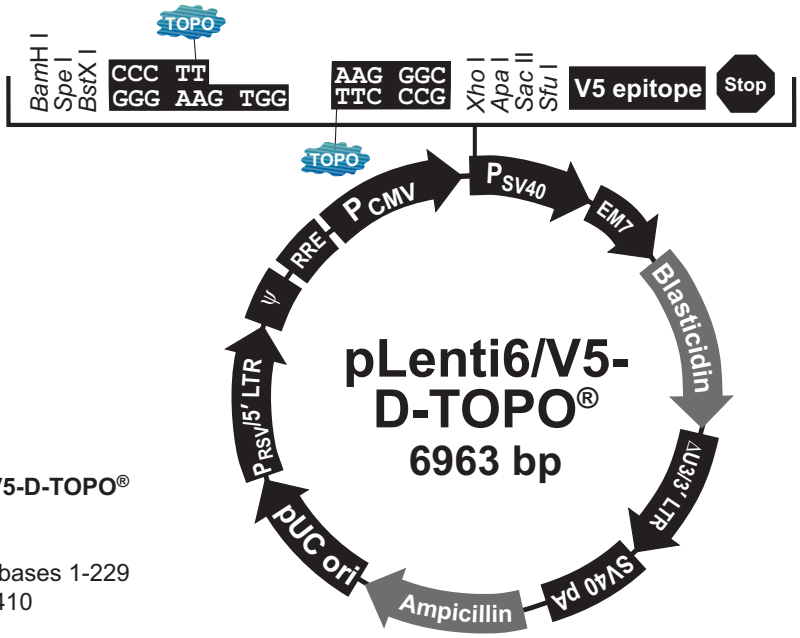
Upon thawing, use what you need and store the thawed stock solution at +4°C for up to 2 weeks.

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

Map and Features of pLenti6/V5-D-TOPO®

pLenti6/V5-D-TOPO® Map

The figure below shows the features of pLenti6/V5-D-TOPO® vector. **The complete sequence of pLenti6/V5-D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 34).**



Comments for pLenti6/V5-D-TOPO®
6963 nucleotides

RSV enhancer/promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging sequence: 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
CMV forward priming site: bases 2274-2294
Directional TOPO® site: bases 2431-2444
V5 epitope: bases 2473-2514
V5(C-term) reverse priming site: bases 2482-2502
SV40 early promoter and origin: bases 2569-2877
EM7 promoter: bases 2932-2998
Blasticidin resistance gene: bases 2999-3397
ΔU3/HIV-1 3′ LTR: bases 3484-3717
    ΔU3: bases 3484-3536
    Truncated HIV-1 3′ LTR: bases 3537-3717
SV40 polyadenylation signal: bases 3789-3920
bla promoter: bases 4779-4877
Ampicillin (*bla*) resistance gene: bases 4878-5738
pUC origin: bases 5883-6556

continued on next page

Map and Features of pLenti6/V5-D-TOPO®, continued

Features of pLenti6/V5-D-TOPO®

pLenti6/V5-D-TOPO® (6963 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the 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).
Human CMV promoter	Permits high-level expression of the gene of interest (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
CMV forward priming site	Permits sequencing of the insert.
TOPO® Cloning site (directional)	Permits rapid, directional cloning of your PCR product.
V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr)	Allows detection of your recombinant protein with the Anti-V5 antibodies (Southern *et al.*, 1991).
V5(C-term) reverse priming site	Allows sequencing of the insert.
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the Blasticidin resistance gene in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).
ΔU3/HIV-1 truncated 3′ LTR	Modified 3′ LTR that allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for efficient transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
bla promoter	Permits expression of the ampicillin (*bla*) resistance gene.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

Map of pLenti6/V5-GW/*lacZ*

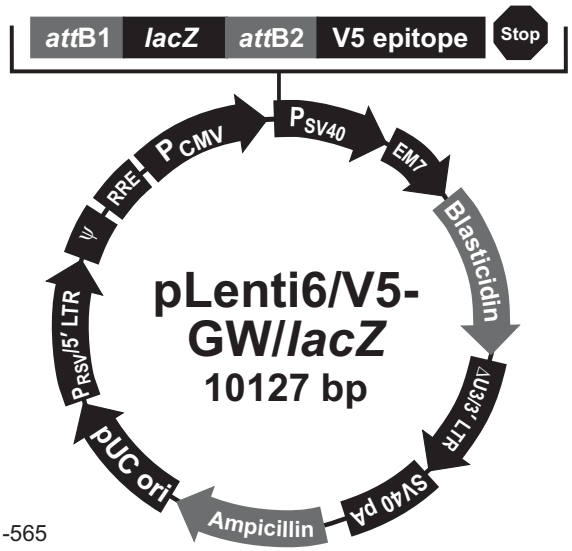
Description

pLenti6/V5-GW/*lacZ* is a 10127 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/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/V5-DEST, refer to the pLenti6/V5-DEST manual, which is available for downloading from our Web site or by contacting Technical Service.

pLenti6/V5-GW/*lacZ* Map

The figure below shows the features of the pLenti6/V5-GW/*lacZ* vector. **The complete sequence of pLenti6/V5-GW/*lacZ* is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 34).**



Comments for pLenti6/V5-GW/*lacZ*
10127 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
*att*B1 site: bases 2440-2464
lacZ ORF: bases 2484-5540
*att*B2 site: bases 5560-5584
V5 epitope: bases 5637-5678
SV40 early promoter and origin: bases 5733-6041
EM7 promoter: bases 6096-6162
Blasticidin resistance gene: bases 6163-6561
ΔU3/HIV-1 3′ LTR: bases 6647-6881
    ΔU3: bases 6647-6700
    Truncated HIV-1 3′ LTR: bases 6701-6881
SV40 polyadenylation signal: bases 6953-7084
bla promoter: bases 7943-8041
Ampicillin (*bla*) resistance gene: bases 8042-8902
pUC origin: bases 9047-9720

Recipes

LB (Luria-Bertani) Medium

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. Allow solution to cool to ~55°C and add antibiotic (100 µg/ml ampicillin), if desired.
4. Store at +4°C.

Technical Service

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

Contact Us

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

Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_service@invitrogen.com

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

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

Material Data Safety Sheets (MSDSs)

MSDSs (Material Safety Data Sheets) are available on our website at www.invitrogen.com/msds.

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

Use of the pLenti6/V5 Directional TOPO® Cloning Kit is covered under the licenses detailed below.

Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. Email: outlicensing@invitrogen.com

Limited Use Label License No. 27: RNA Transfection

Use of this product in conjunction with methods for the introduction of RNA molecules into cells may require licenses to one or more patents or patent applications. Users of these products should determine if any licenses are required.

Limited Use Label License No. 51: Blasticidin and the Blasticidin Selection Marker

Blasticidin and the blasticidin resistance gene (bsd) are the subject of U.S. Patent No. 5,527,701 sold under patent license for research purposes only. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500. email: outlicensing@invitrogen.com.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 108: Lentiviral Technology

The Lentiviral Technology (based upon the lentikat™ system) is exclusively licensed from Cell Genesys, Inc., under U.S. Patent Nos. 5,686,279; 5,834,256; 5,858,740; 5,994,136; 6,013,516; 6,051,427; 6,165,782 and 6,218,187 and corresponding patents and applications in other countries for internal research purposes only. Use of this technology for gene therapy applications or bioprocessing other than for non-human research use requires a license from Cell Genesys (Cell Genesys, Inc. 342 Lakeside Drive, Foster City, California 94404). The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer, including non-gene therapy research and target validation applications in laboratory animals (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Limited Use Label License No. 109: Retroviral Helper Lines

Retroviral helper cell lines are licensed from Wisconsin Alumni Research Foundation, under U.S. Patents and corresponding patents and applications in other countries for internal research purposes only. Use of these cell lines for Commercial Purposes requires a license from Life Technologies.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 304: Improved Transfection Reagent	This product is covered by U.S. Pat. Nos. 7,145,039, 7,166,745, 7,173,154, 7,323,594, and 7,479,573.
Limited Use Label License No. 317: LentiVector® Technology	This product is licensed under U.S. Pat. Nos. 5,817,491; 5,591,624; 5,716,832; 6,312,682; 6,669,936; 6,235,522; 6,924,123 and foreign equivalents from Oxford BioMedica (UK) Ltd., Oxford, UK, and is provided for use in academic and commercial in vitro and in vivo research for elucidating gene function, and for validating potential gene products and pathways for drug discovery and development, but excludes any use of LentiVector® technology for: creating transgenic birds for the purpose of producing useful or valuable proteins in the eggs of such transgenic birds, the delivery of gene therapies, and for commercial production of therapeutic, diagnostic or other commercial products not intended for research use where such products do not consist of or incorporate a lentiviral vector. Information about licenses for commercial uses excluded under this license is available from Oxford BioMedica (UK), Ltd., Medawar Centre, Oxford Science Park, Oxford OX4 4GA UK enquiries@oxfordbiomedica.co.uk or BioMedica Inc., 11622 EI Camino Real #100, San Diego CA 92130-2049 USA. LentiVector is a registered US and European Community trade mark of Oxford BioMedica plc.
Limited Use Label License No. 345: Gateway® Vectors	This product or one or more vectors made using this product is the subject of U.S. Patent No. 5,888,732 owned by Life Technologies Corporation.

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pLenti6/V5 Directional TOPO® Cloning Kit.

Vectors

The pLenti6/V5-DEST™ (parental vector of pLenti6/V5-D-TOPO®) and pLenti6/V5-GW/*lacZ* plasmids are qualified by restriction enzyme digestion. The pLenti6/V5-DEST™ vector is qualified by restriction digest prior to adaptation with topoisomerase I.

TOPO® Cloning Efficiency

After adaptation with topoisomerase I, the pLenti6/V5-D-TOPO® vector is lot-qualified using the control reagents included in the kit. Under conditions described on pages 25-26, a 750 bp control PCR product is amplified using a forward primer containing CACC at its 5′ end and a reverse primer. The PCR product is TOPO® Cloned into the pLenti6/V5-D-TOPO® vector and transformed into the One Shot® Stbl3™ chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency. Forty transformants are characterized using directional PCR. Of the transformants characterized, greater than 90% should contain an insert in the correct orientation.

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

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

Each lot of One Shot® Stbl3™ chemically competent *E. coli* is tested for transformation efficiency using the pUC19 control plasmid included in the kit and following the procedure on page 13. Test transformations are performed on 3 to 20 vials per lot, depending on batch size. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and incubated overnight. Transformation efficiency should be greater than 1×10^8 cfu/µg plasmid DNA. In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and the absence of phage contamination.

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.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

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.

Cheng, C., and Shuman, S. (2000). Recombinogenic Flap Ligation Pathway for Intrinsic Repair of Topoisomerase IB-Induced Double-Strand Breaks. Mol. Cell. Biol. *20*, 8059-8068.

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). Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233.

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

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

Kozak, M. (1987). An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148.

Kozak, M. (1991). An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903.

Kozak, M. (1990). Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305.

Luciw, P. A. (1996) Human Immunodeficiency Viruses and Their Replication. In *Fields Virology*, B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman and S. E. Straus, eds. (Philadelphia, PA: Lippincott-Raven Publishers), pp. 1881-1975.

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.

continued on next page

References, continued

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

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.

Shuman, S. (1994). Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. J. Biol. Chem. *269*, 32678-32684.

Shuman, S. (1991). Recombination Mediated by Vaccinia Virus DNA Topoisomerase I in *Escherichia coli* is Sequence Specific. Proc. Natl. Acad. Sci. USA *88*, 10104-10108.

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991). Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557.

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

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

Notes

Notes

invitrogen™

Corporate Headquarters
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech.service@invitrogen.com

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