invitrogen™

pcDNA™3.1 Directional TOPO® Expression Kit

Five-minute, directional TOPO® Cloning of blunt-end PCR products into a mammalian expression vector

Catalog nos. K4900-01, K4900-40

Version F
10 November 2010
25-0396

User Manual

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TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the directional 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 any epitope tags, 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 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**. **Note:** If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in the TOPO® Cloning reaction. Fresh PCR product 0.5 to 4 µl Salt Solution 1 µl Sterile 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® TOP10 chemically competent *E. coli*, below.
Transform TOP10 Chemically Competent *E. coli*	1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. 2. Incubate on ice for 5 to 30 minutes. 3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 4. Add 250 µl of room temperature S.O.C. medium. 5. Incubate at 37°C for 1 hour with shaking. 6. Spread 50-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kits to perform the control reaction. See the protocol on pages 22-23 for instructions.

Important Information

Shipping/Storage

The pcDNA™3.1 Directional TOPO® Expression Kit is shipped on dry ice. Each kit contains a box of pcDNA™3.1D/V5-His TOPO® reagents (Box 1) and a box of One Shot® TOP10 chemically competent *E. coli* (Box 2). **Store Box 1 at -20°C and Box 2 at -80°C.**

Types of Kits

This manual is supplied with the following kits.

Kit	Amount	Catalog no.
pcDNA™3.1 Directional TOPO® Expression Kit	20 reactions	K4900-01
	40 reactions	K4900-40

TOPO® Reagents

pcDNA™3.1D/V5-His 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.

Item	Concentration	Amount
pcDNA™3.1D/V5-His-TOPO®	15-20 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 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
Sterile Water	--	1 ml
T7 Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
BGH Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Expression Plasmid (pcDNA™3.1D/V5-His/*lac*Z)	0.5 µg/µl in TE Buffer, pH 8	10 µl

continued on next page

Sequencing Primers

The table below provides the sequence and pmoles of the T7 sequencing primer and the BGH Reverse sequencing primer.

Primer	Sequence	Amount
T7	5´-TAATACGACTCACTATAGGG-3´	328 pmoles
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	358 pmoles

One Shot® Reagents

The table below describes the items included in the One Shot® TOP10 chemically competent *E. coli* cell kit (Box 2). **Store at -80°C.**

Item	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
TOP10 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 TOP10 Cells

F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Accessory Products

Introduction

The products listed in this section may be used with the pcDNA™3.1 Directional TOPO® Expression Kit. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 30).

Additional Products

Many of the reagents supplied in the pcDNA™3.1 Directional TOPO® Expression Kit and other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Amount	Catalog no.
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink™ Quick Gel Extraction Kit	50 reactions	K2100-12
Ampicillin	20 ml	11593-027
Lipofectamine™ 2000 Reagent	1.5 ml	11668-019
	0.75 ml	11668-027
Geneticin® Selective Antibiotic	1 g	11811-023
	5 g	11811-031
	20 ml (50 mg/ml)	10131-035
	100 ml (50 mg/ml)	10131-027
Phosphate Buffered Saline, pH 7.4	500 ml	10010-023
β-Gal Antiserum	50 µl	R901-25
β-Gal Assay Kit	100 reactions	K1455-01
β-Gal Staining Kit	1 kit	K1465-01

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Accessory Products, continued

Detection of Recombinant Proteins

Expression of your recombinant fusion protein can be detected using Anti-V5 or Anti-His(C-term) Antibodies available from Invitrogen. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step detection using chemiluminescent or colorimetric detection methods. Fluorescein isothiocyanate (FITC)-conjugated antibodies allow one-step detection in immunofluorescence experiments.

The amount of antibody supplied is sufficient for 25 Western blots or 25 immunostaining reactions (FITC-conjugated antibodies only).

Product	Epitope	Catalog no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991) GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-V5-FITC Antibody		R963-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag (requires the free carboxyl group for detection (Lindner *et al.*, 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25
Anti-His(C-term)-FITC Antibody		R933-25

Purification of Recombinant Proteins

If your gene of interest in is frame with the C-terminal polyhistidine (6xHis) tag, you may use Invitrogen's ProBond™ or Ni-NTA Purification System to purify your recombinant fusion protein. See the table below for ordering information.

Product	Amount	Catalog no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
	150 ml	R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml	R901-01
	25 ml	R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

The pcDNA™3.1 Directional TOPO® Expression Kit provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a plasmid vector. Blunt-end PCR products clone directionally at greater than 90% efficiency, minimizing screening. No ligase, post-PCR procedures, or restriction enzymes are required. Once cloned, analyzed, and transfected, the gene of interest can be expressed directly in mammalian cell lines.

Features of pcDNA™3.1D/V5-His-TOPO®

pcDNA™3.1D/V5-His-TOPO® is a 5.5 kb expression vector designed to facilitate rapid directional cloning of blunt-end PCR products for expression in mammalian cells. The vector allows high-level expression, detection, and purification of heterologous proteins in most mammalian cells. The vector contains the following features:

- 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)
- TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see next page for more information)
- C-terminal peptide containing the V5 epitope and a polyhistidine (6xHis) tag for detection and purification of recombinant protein
- Neomycin resistance gene for selection of stable cell lines using Geneticin® (Southern and Berg, 1982)

The control plasmid, pcDNA™3.1D/V5-His/*lac*Z, is included for use as a positive control for transfection and expression in the mammalian cell line of choice.

Tag-On-Demand™ System

The pcDNA™3.1D/V5-His-TOPO® vector is compatible with the Tag-On-Demand™ System which allows expression of both native and C-terminally-tagged recombinant protein from the same expression construct.

The System is based on stop suppression technology originally developed by RajBhandary and colleagues (Capone *et al.*, 1985) and consists of a recombinant adenovirus expressing a $tRNA^{ser}$ suppressor. When an expression vector encoding a gene of interest with the TAG (amber stop) codon is transfected into mammalian cells and the $tRNA^{ser}$ suppressor supernatant is present, the stop codon will be translated as serine, allowing translation to continue and resulting in production of a C-terminally-tagged fusion protein.

For more information, refer to the Tag-On-Demand™ Suppressor Supernatant manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).

How Directional TOPO® Cloning Works

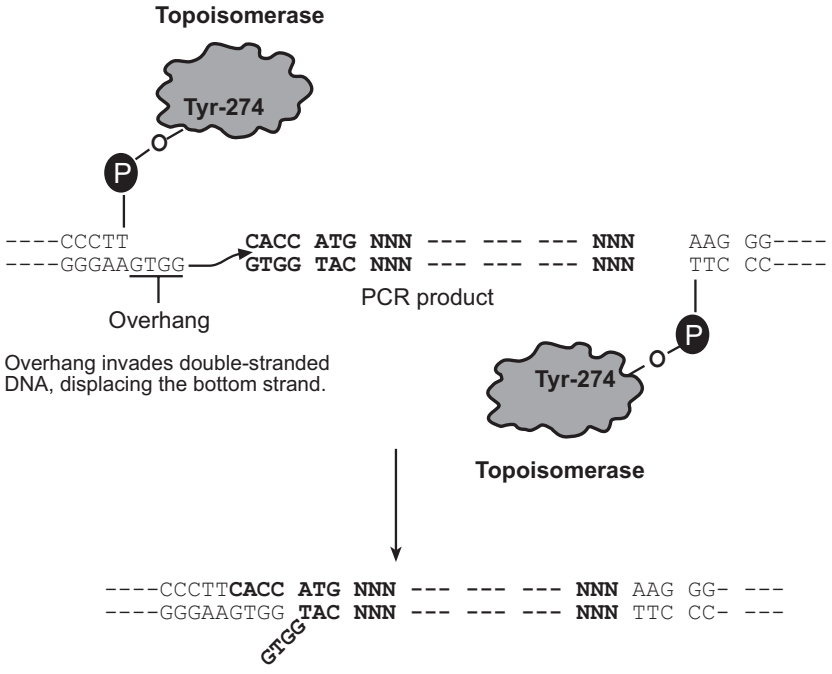
How Topoisomerase Works

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.

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.

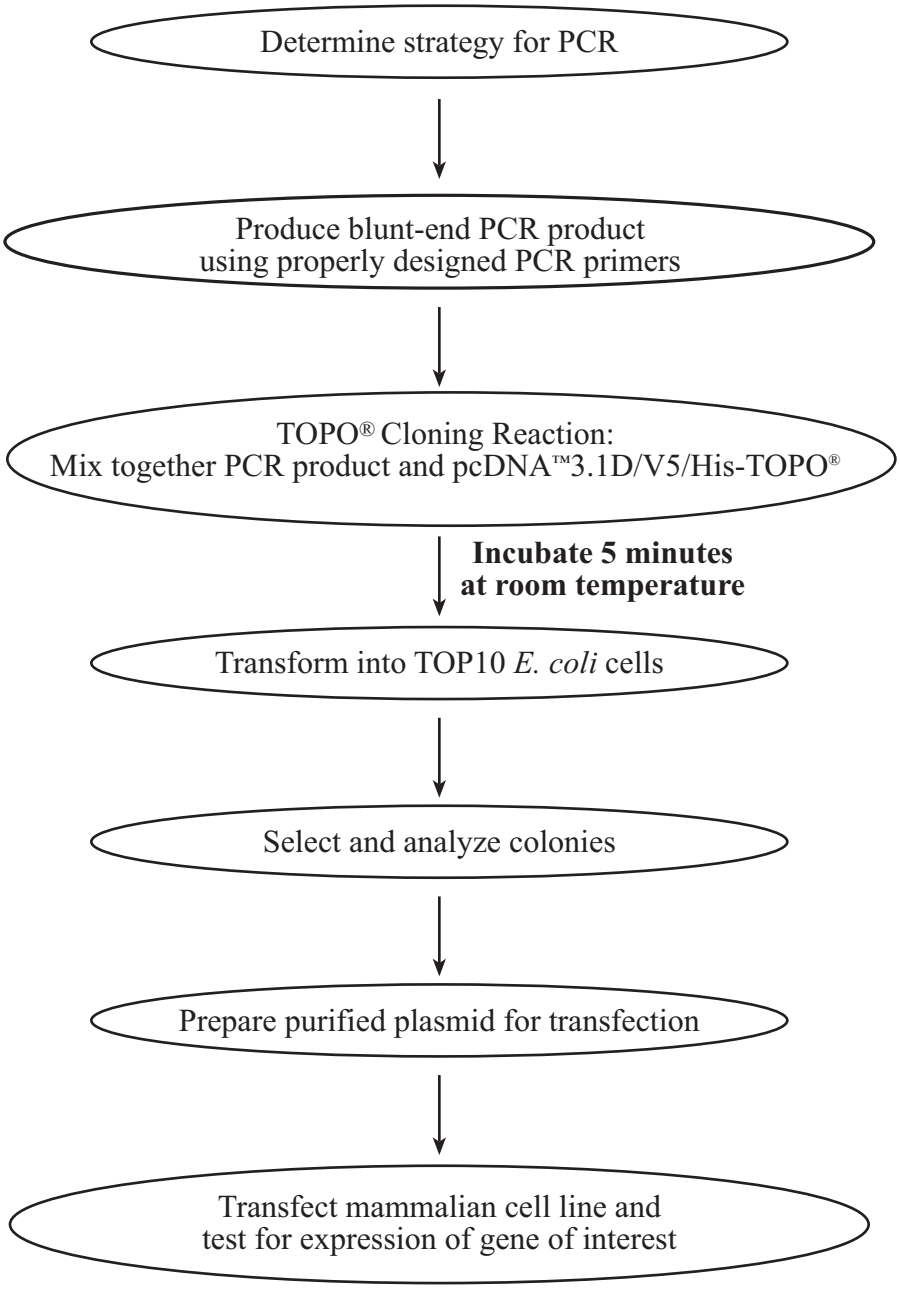
In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the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



Experimental Outline

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

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 and 6xHis tag

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to page 6 for a diagram of the TOPO® Cloning site.

- 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 pcDNA™3.1D/V5-His-TOPO®.
- Make sure your sequence of interest includes 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.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** below).

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 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.

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Designing PCR Primers, continued

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to page 6 for a diagram of the TOPO® Cloning site.

- **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 V5 epitope and 6xHis tag, 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 V5 epitope and 6xHis tag, 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).

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 a C-terminal tag. The stop codon is underlined.

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

One possibility is to design the reverse PCR primer to start with the codon just up-stream 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.

Another possibility is to design the reverse primer so that it hybridizes just down-stream 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 (see below).

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

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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 TGA-3′

- To fuse the ORF in frame with a C-terminal tag, 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 a 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′-TCA TGC AGT CGT CGA GTG CTC CGA CTT-3′

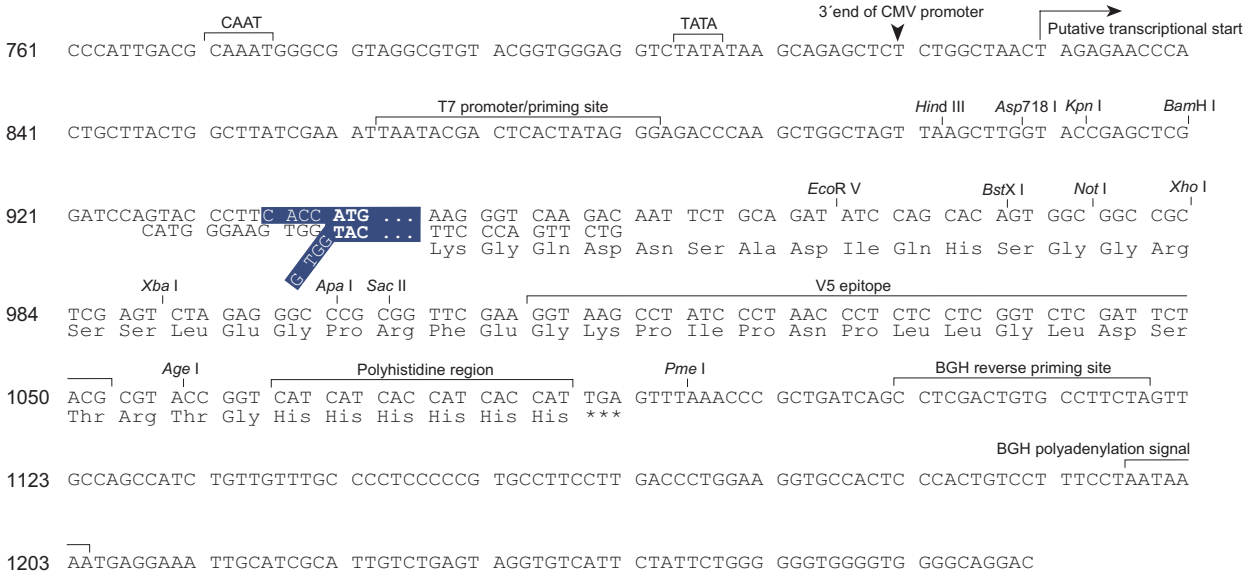
Important

- pcDNA™3.1D/V5-His-TOPO® vector accepts blunt-end PCR products.
- Do not add 5′ phosphates to your primers for PCR. This will prevent ligation into the pcDNA™3.1D/V5-His-TOPO® vector.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

TOPO® Cloning Site

Use the diagram below to help you design PCR primers to clone your PCR product into pcDNA™3.1D/V5-His-TOPO®. **The complete sequence of pcDNA™3.1D/V5-His-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).**

Note: If you are using the pcDNA™3.1D/V5-His-TOPO® vector in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page 1).



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 Needed

You should have the following materials on hand before beginning.

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.

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- 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.

- Be sure 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. Alternatively, you may gel-purify the desired product (see pages 24-25).
- 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).

Performing the TOPO® Cloning Reaction

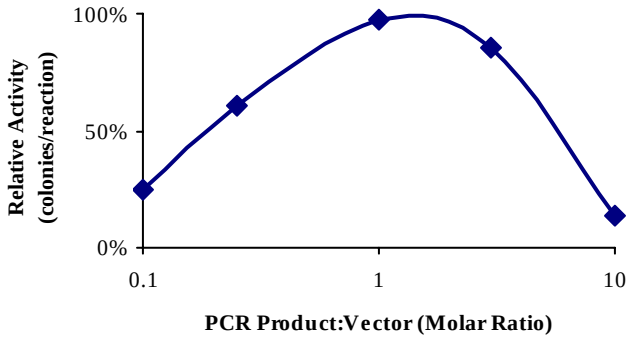
Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into pcDNA™3.1D/V5-His-TOPO® and transform the recombinant vector into TOP10 *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read the this section and the section entitled **Transforming One Shot® TOP10 Competent Cells** (pages 10-12) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 22-23 in parallel with your samples.

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 pcDNA™3.1D/V5-His-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.



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Performing the TOPO® Cloning Reaction, continued

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). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page ix for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **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*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	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 a sufficient number 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 may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

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

Transforming One Shot® TOP10 Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pcDNA™3.1D/V5-His-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page ix for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Needed

You should have the following materials on hand before beginning:

- 42°C water bath (or electroporator with cuvettes, optional)
- LB plates containing 50-100 µg/ml ampicillin (two for each transformation)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids 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.

Preparing for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm LB plates containing 50-100 µg/ml ampicillin at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® TOP10 cells from Box 2 for each transformation.

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Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 9 into a vial of One Shot® TOP10 Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 13). Refer to the **Troubleshooting** section on page 20 if you have problems obtaining transformants.

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 9 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
3. Immediately add 250 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the ampicillin resistance gene.
5. Spread 20-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick ~5 colonies for analysis (see **Analyzing Transformants**, page 13). Refer to the **Troubleshooting** section on page 20 if you have problems obtaining transformants.

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Transforming One Shot® TOP10 Competent Cells, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

- If you experience arcing during transformation, try one of the following suggestions:
- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation and in frame with the C-terminal V5 epitope and 6xHis tag. The T7 and BGH Reverse primers are included in the kit to help you sequence your insert (see the diagram on page6 for the location of the priming sites).

Important

If you download the sequence for pcDNA™3.1D/V5-His-TOPO® from our Web site, note that the overhang sequence (GTGG) will be shown already hybridized to CACC. No DNA sequence analysis program allows us to show the overhang without the complementary sequence.

Analyzing Transformants by PCR

You may analyze positive transformants using PCR. For PCR primers, use a combination of the T7 Promoter primer or the TK polyA 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. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above.
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.

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants, the correct insert, or inserts in the correct orientation, refer to the **Troubleshooting** section (see page 20). We also recommend that you perform the control reactions described on pages 22-23. These reactions will help you troubleshoot your experiment.

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 colony on LB plates containing 50-100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50-100 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.

Transfecting Cells

Introduction

Once you have the desired construct, you are ready to transfect the plasmid into the mammalian cells of choice. We recommend that you include the positive control vector pcDNA™3.1D/V5-His/*lac*Z, supplied with the kit, in your experiments to help you evaluate your results.

Plasmid Preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) or CsCl gradient centrifugation.

Methods of Transfection

For established cell lines (*e.g.* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner *et al.*, 1989; Felgner and Ringold, 1989) and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Catalog no. 11668-027) available from Invitrogen. For more information about Lipofectamine™ 2000 and the other transfection reagents available from Invitrogen, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 30).

Positive Control

pcDNA™3.1D/V5-His/*lac*Z is provided as a positive control vector for mammalian transfection and expression and may be used to optimize transfection conditions for your cell line. This vector allows expression of a β-galactosidase fusion protein that may be detected by Western blot or functional assay.

Detecting Recombinant Fusion Proteins

Introduction

You may express you gene of interest in either transiently transfected cells or stable cell lines (see page 19 for guidelines to create stable cell lines). You may use a functional assay or a Western blot analysis to detect your recombinant protein (see below).

Preparing Cell Lysates

To detect your fusion protein by Western blot, you will need to prepare a cell lysate from transfected cells. A sample protocol is provided below. Other protocols are suitable. To lyse cells:

1. Wash cell monolayers (~5 x 10^5 to 1 x 10^6 cells) once with phosphate-buffered saline (see page ix for ordering information).
2. Scrape cells into 1 ml PBS and pellet the cells at 1500 x g for 5 minutes.
3. Resuspend in 50 µl Cell Lysis Buffer (see the **Appendix**, page 26 for a recipe). Other cell lysis buffers are suitable. Vortex.
4. Incubate cell suspension at 37°C for 10 minutes to lyse the cells.

   Note: You may prefer to lyse the cells at room temperature or on ice if degradation of your protein is a potential problem.
5. Centrifuge the cell lysate at 10,000 x g for 10 minutes at +4°C to pellet nuclei and transfer the supernatant to a fresh tube. Assay the lysate for protein concentration.

   Note: Do not use protein assays utilizing Coomassie Blue or other dyes. NP-40 interferes with the binding of the dye with the protein.
6. Add SDS-PAGE sample buffer (see the **Appendix**, page 26 for a recipe) to a final concentration of 1X and boil the sample for 5 minutes.
7. Load 20 µg of lysate onto an SDS-PAGE gel and electrophorese. Use the appropriate percentage of acrylamide to resolve your fusion protein.

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 30).

continued on next page

Detecting Recombinant Fusion Proteins, continued

Detecting Fusion Proteins

To detect expression of your recombinant fusion protein by western blot analysis, you may use the Anti-V5 antibodies or the Anti-His(C-term) antibodies available from Invitrogen (see page x for ordering information) or an antibody to your protein of interest. In addition, the Positope™ Control Protein (Catalog no. R900-50) is available from Invitrogen for use as a positive control for detection of fusion proteins containing a V5 epitope or a polyhistidine (6xHis) tag. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 30).

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine region will add approximately 3.6 kDa to your protein.

Assay for β-galactosidase Activity

If you use the expression control plasmid, you may assay for β-galactosidase expression by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). Invitrogen offers the β-Gal Antiserum, the β-Gal Assay Kit, and the β-Gal Staining Kit (see page ix for ordering information) for fast and easy detection of β-galactosidase expression.

Purifying Recombinant Fusion Proteins

Introduction

You will need 5 x 10^6 to 1 x 10^7 transfected cells for purification of your protein on a 2 ml ProBond™ column (or other metal-chelating column). If you are using ProBond™ to purify your protein, refer to the protocol below to prepare cells for lysis. If you are using another metal-chelating resin, refer to the manufacturer's instructions to prepare the cells.

Preparing Cells for Lysis

Use the procedure below to prepare cells for lysis prior to purification of your protein on ProBond™. You will need 5 x 10^6 to 1 x 10^7 stably transfected cells for purification of your protein on a 2 ml ProBond™ column (see ProBond™ Purification System manual).

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are approximately 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with fresh medium and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1500 x g for 5 minutes. Resuspend the cell pellet in PBS.
6. Centrifuge the cells at 1500 x g for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -70°C until needed.

Lysing Cells

If you are using ProBond™ resin, refer to the ProBond™ Purification System manual for details about sample preparation for chromotography.

If you are using other metal-chelating resin, refer to the manufacturer's instructions for recommendations on sample preparation.

Creating Stable Cell Lines

Introduction

The pcDNA™3.1D/V5-His-TOPO® vector contains the neomycin resistance gene to allow selection of stable cell lines using Geneticin®. If you wish to create stable cell lines, transfect your pcDNA™3.1D/V5-His-TOPO® construct into the mammalian cell line of choice and select for foci using Geneticin®. General information and guidelines are provided below.

Geneticin®

Geneticin® blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin. Expression in mammalian cells of the bacterial aminoglycoside phospho-transferase gene (APH), derived from Tn*5*, results in detoxification of Geneticin® Selective Antibiotic (Southern and Berg, 1982).

Determining Geneticin® Sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Geneticin® required to kill your untransfected host cell line. Test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent. Let cells attach overnight before adding selective medium.
2. Prepare a set of 7 plates.
3. Prepare Geneticin® in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
4. Add the following concentrations of antibiotic to each plate: 0, 50, 125, 250, 500, 750, and 1000 µg/ml Geneticin®.
5. Replenish the selective media every 3-4 days, and observe the percentage of surviving cells.
6. Count the number of viable cells at regular intervals to determine the appropriate concentration of Geneticin® that prevents growth within 1-3 weeks.

Geneticin® Selection Guidelines

Once you have determined the appropriate Geneticin® concentration to use for selection, you can generate a stable cell line expressing your pcDNA™3.1D/V5-His-TOPO® construct.

1. Prepare Geneticin® in a buffered solution (*e.g.* 100 mM HEPES, pH 7.3).
2. Use the predetermined concentration of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Cells will divide once or twice in the presence of lethal doses of Geneticin®, so the effects of the drug take several days to become apparent. Complete selection can take from 2 to 4 weeks of growth in selective medium.

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 in parallel with your samples (see pages 22-23).

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 product too dilute	• Concentrate 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.
	Long PCR product	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • 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 choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a0.5:1 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 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 choice. • Gel-purify your PCR product to remove primer-dimers and smaller PCR products.
	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.
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.
	One Shot® transformation protocol not followed correctly	Follow the One Shot® transformation protocol provided on page 11.
	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 TOPO® Clone 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 50-100 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 polymerase you are using.

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

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
Thermostable polymerase (1-2.5 units/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. A discrete 750 bp band should be visible.
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 a pcDNA™3.1D/V5-His-TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

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
pcDNA™3.1D/V5-His-TOPO®	1 µl	1 µl
Final 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® TOP10 cells (see page 11).
4. Spread 50-200 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.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pcDNA™3.1D/V5-His-TOPO®	*Xba* I	Correct orientation: 4727, 5537 Reverse orientation: 167, 6097 Empty vector: 5514

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® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 11. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 x 10^9 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. Two simple protocols are provided below.

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit (page ix) allows you to rapidly purify PCR products from regular agarose gels.

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 in the 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 Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-ml polypropylene tubes and add 60 µl Gel Solubilization Buffer (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 an aliquot of TE Buffer (TE) to 65-70°C
7. Place a Quick Gel Extraction Column into a Wash Tube. Pipette 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 Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 x g for 1 minute. Discard the 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) to the center of 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 DNA at –20°C. Discard the column.
14. Use 4 µl of the purified DNA for the TOPO® Cloning reaction.

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. Use only chemically competent cells for transformation.

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 9.
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® TOP10 cells using the method on page 11.

Note

The cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 7).

Recipes

LB (Luria-Bertani) Medium and Plates

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (100 µg/ml ampicillin) if needed.
4. Store at room temperature or at +4°C.

LB agar plates

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

Cell Lysis Buffer

50 mM Tris, pH 7.8
150 mM NaCl
1% Nonidet P-40

1. This solution can be prepared from the following common stock solutions. For 100 ml, combine

1 M Tris base	5 ml
5 M NaCl	3 ml
Nonidet P-40	1 ml

2. Bring the volume up to 90 ml with deionized water and adjust the pH to 7.8 with HCl.
3. Bring the volume up to 100 ml. Store at room temperature.

To prevent proteolysis, you may add 1 mM PMSF, 1 µM leupeptin, or 0.1 µM aprotinin before use.

4X SDS-PAGE Sample Buffer

1. Combine the following reagents:

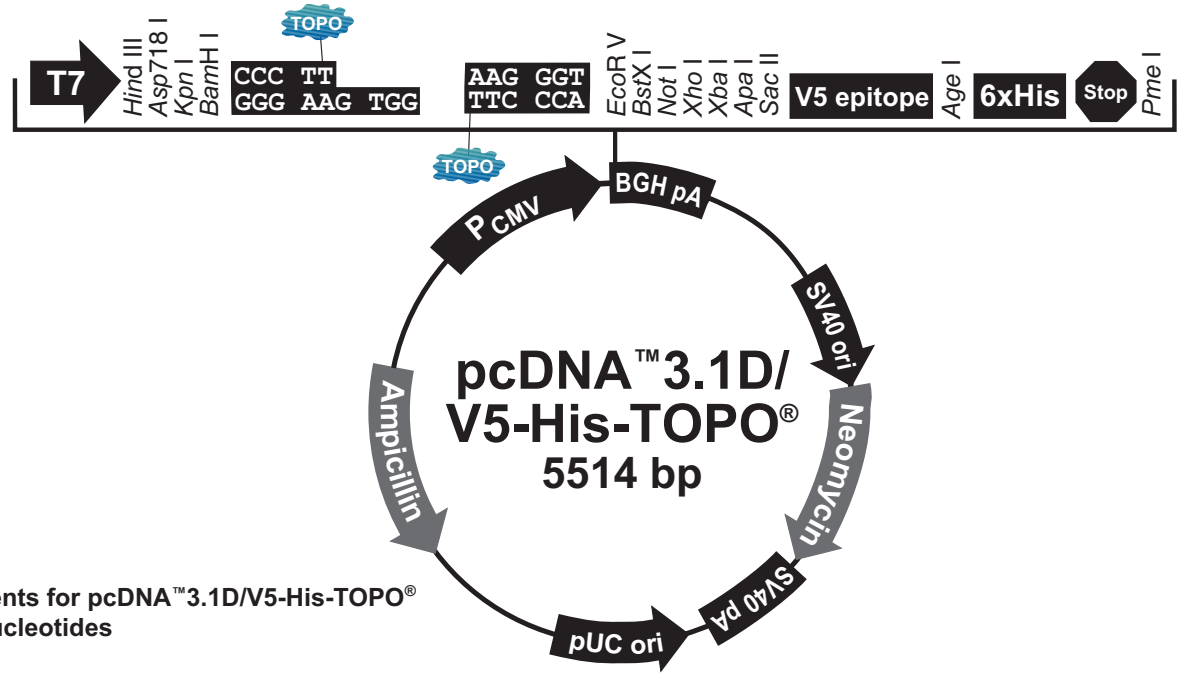
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol (100%)	4 ml
β-mercaptoethanol	0.8 ml
Bromophenol Blue	0.04 g
SDS	0.8 g

2. Bring the volume to 10 ml with sterile water.
3. Aliquot and freeze at -20°C until needed.

Map and Features of pcDNA™3.1D/V5-His-TOPO®

Map

The map below shows the elements of the pcDNA™3.1D/V5-His-TOPO® vector. **The complete nucleotide sequence is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).**



Comments for pcDNA™3.1D/V5-His-TOPO®
5514 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
TOPO® recognition site 1: bases 930-934
Overhang sequence (complementary strand): bases 935-938
TOPO® recognition site 2: bases 939-943
V5 epitope: bases 1011-1052
Polyhistidine (6xHis) tag: bases 1062-1079
BGH reverse priming site: bases 1102-1119
BGH polyadenylation signal: bases 1108-1332
SV40 early promoter and origin: bases 1833-2142
Neomycin resistance gene: bases 2217-3011
SV40 early polyadenylation signal: bases 3189-3319
pUC origin: bases 3700-4373 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4518-5378 (complementary strand)
bla promoter: bases 5379-5477 (complementary strand)

continued on next page

Map and Features of pcDNA™3.1D/V5-His-TOPO®, continued

Features

pcDNA™3.1D/V5-His-TOPO® contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
TOPO® Cloning site (directional)	Allows directional cloning of your PCR product in frame with the V5 epitope and polyhistidine C-terminal tag, if desired
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)
C-terminal polyhistidine tag	Allows purification of your recombinant protein on metal-chelating resin such as ProBond™ Allows detection of your recombinant protein with the Anti-His (C-term) antibodies (Lindner *et al.*, 1997)
BGH reverse priming site	Allows sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
SV40 early promoter and origin	Allows efficient, high-level expression of the neomycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Neomycin resistance gene	Allows selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
bla promoter	Allows expression of the ampicillin resistance gene
Ampicillin resistance gene (β-lactamase)	Allows selection of vector in *E. coli*

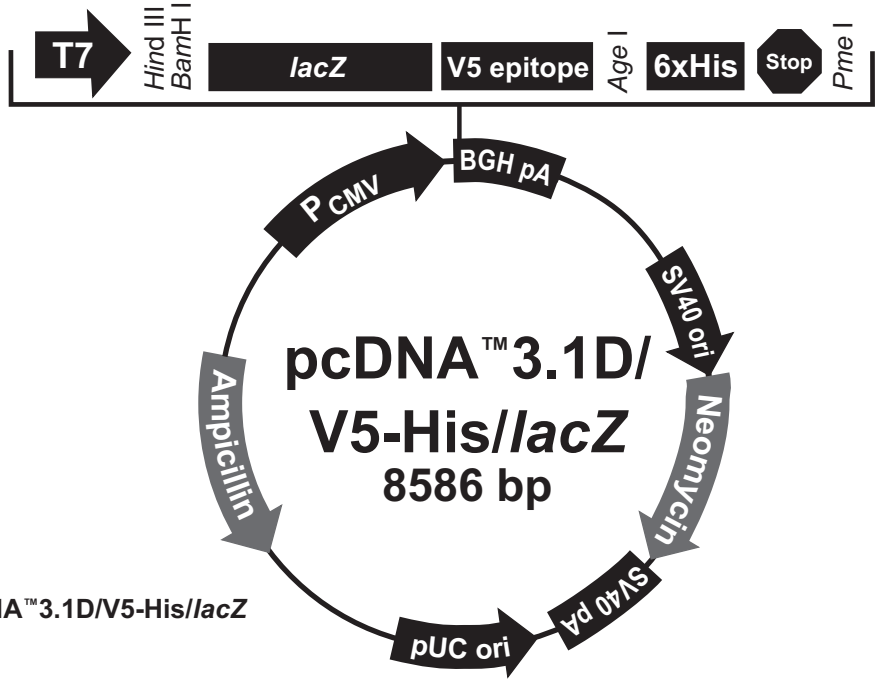
Map of pcDNA™3.1D/V5-His/*lac*Z

Description

pcDNA™3.1D/V5-His/*lac*Z is a 8586 bp control vector containing the gene for β–galactosidase. The *lac*Z gene was amplified and directionally TOPO® Cloned into pcDNA™3.1D/V5-His-TOPO® such that it is in frame with the C-terminal peptide. The size of the β-galactosidase fusion protein is approximately 120 kDa.

Map of Control Vector

The figure below summarizes the features of the pcDNA™3.1D/V5-His/*lac*Z vector. **The complete nucleotide sequence for pcDNA™3.1D/V5-His/*lac*Z is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 30).**



Comments for pcDNA™3.1D/V5-His/*lac*Z
8586 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
LacZ ORF: bases 939-3995
V5 epitope: bases 4083-4124
Polyhistidine (6xHis) tag: bases 4134-4151
BGH reverse priming site: bases 4174-4191
BGH polyadenylation signal: bases 4180-4404
SV40 early promoter and origin: bases 4905-5214
Neomycin resistance gene: bases 5289-6083
SV40 early polyadenylation signal: bases 6261-6391
pUC origin: bases 6772-7445 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 7590-8450 (complementary strand)
bla promoter: bases 8451-8549 (complementary strand)

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).

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Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pcDNA™3.1 Directional TOPO® Expression Kit.

Vectors

The pcDNA™3.1/V5-His (parental vector of pcDNA™3.1D/V5-His-TOPO®) and pcDNA™3.1D/V5-His/*lac*Z plasmids are qualified by restriction digest. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

TOPO® Cloning Efficiency

The pcDNA™3.1 Directional TOPO® vector is lot-qualified using the control reagents included in the kit. Under conditions described on pages 22-23, 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 pcDNA™3.1D/V5-His-TOPO® vector and transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 85% cloning efficiency. Forty transformants are characterized by restriction digest. Of the transformants characterized, greater than 90% should be in the correct orientation.

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® TOP10 Chemically Competent *E. coli*

1. One Shot® TOP10 chemically competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA.
2. To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
3. Untransformed cells are plated on LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Purchaser Notification

Introduction

Use of the pcDNA™3.1 Directional TOPO® Expression Kit is covered under a number of different licenses including those detailed below.

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