invitrogen™

pcDNA™3.1/V5-His TOPO® TA Expression Kit

Five-minute cloning and expression of *Taq* polymerase-amplified PCR products in mammalian cells

Catalog nos. K4800-01, K4800-40

Rev. Date: 10 April 2009
Part no. 25-0203

MAN0000055

User Manual

Table of Contents

Kit Contents and Storage

Shipping and Storage

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

Types of Kits

Ordering information for the pcDNA™3.1/V5-His TOPO® TA Expression Kits is provided below.

Kit	Reactions	Catalog no.
pcDNA™3.1/V5-His TOPO® TA Expression Kit	20	K4800-01
	40	K4800-40

TOPO® TA Cloning® Reagents

pcDNA™3.1/V5-His TOPO® TA Cloning® reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pcDNA™3.1/V5-His-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton® X-100 100 µg/ml BSA 30 µM phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µl
50 mM dNTPs	12.5 mM dATP; 12.5 mM dCTP; 12.5 mM dGTP; 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl; 0.06 M $MgCl_2$	50 µl
T7 Sequencing Primer	0.1 µg/µl in TE Buffer	20 µl
BGH Reverse Sequencing Primer	0.1 µg/µl in TE Buffer	20 µl
Control PCR Template	0.05 µg/µl in TE Buffer	10 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer	10 µl
Expression Control Plasmid	0.5 µg/µl in TE Buffer	10 µl
Sterile Water	--	1 ml

continued on next page

Kit Contents and Storage, continued

One Shot® Reagents

The table below describes the items included in the One Shot® chemically competent cell kit. Store at –80°C.

Item	Composition	Amount
TOP10 cells	--	21 × 50 µl
SOC 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
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Sequencing Primers

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

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

Genotype of TOP10 Cells

TOP10: Use this strain for general cloning. Note that this strain cannot be used for single-strand rescue of DNA.

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

Additional Products

The table below lists additional products available from Invitrogen which you may use in conjunction with the pcDNA™3.1/V5-His TOPO® TA Expression Kit. For details on the product, visit www.invitrogen.com.

Item	Amount	Catalog no.
One Shot® Kit (TOP10 **Electrocompetent** Cells)	10 reactions	C4040-50
One Shot® TOP10 **Chemically Competent** Cells	10 reactions	C4040-10
	20 reactions	C4040-03
T7 Promoter Primer	2 µg (328 pmoles)	N560-02
BGH Reverse primer	2 µg (358 pmoles)	N575-02
Anti-V5 Antibody	25 westerns	R960-25
Anti-V5-HRP Antibody	25 westerns	R961-25
Anti-V5-AP Antibody	25 westerns	R962-25
Anti-His (C-term) Antibody	25 westerns	R930-25
Anti-His (C-term)-HRP Antibody	25 westerns	R931-25
Anti-His(C-term)-AP Antibody	25 westerns	R932-25
ProBond™ Purification System	6 reactions	K850-01
ProBond™ Metal-Binding Resin	50 ml	R801-01
PureLink™ HiPure Plasmid Miniprep Kit	100 preps	K2100–03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100–04

Introduction

Overview

Introduction

The pcDNA™3.1/V5-His TOPO® TA Expression Kit provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. Once cloned, analyzed, and transfected, the PCR product will express directly in mammalian cell lines.

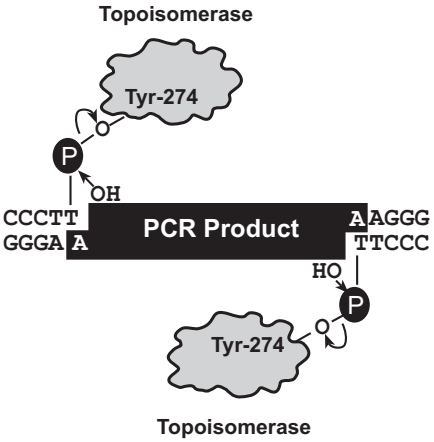
How It Works

The plasmid vector (pcDNA™3.1/V5-His-TOPO®) is supplied linearized with:

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

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

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



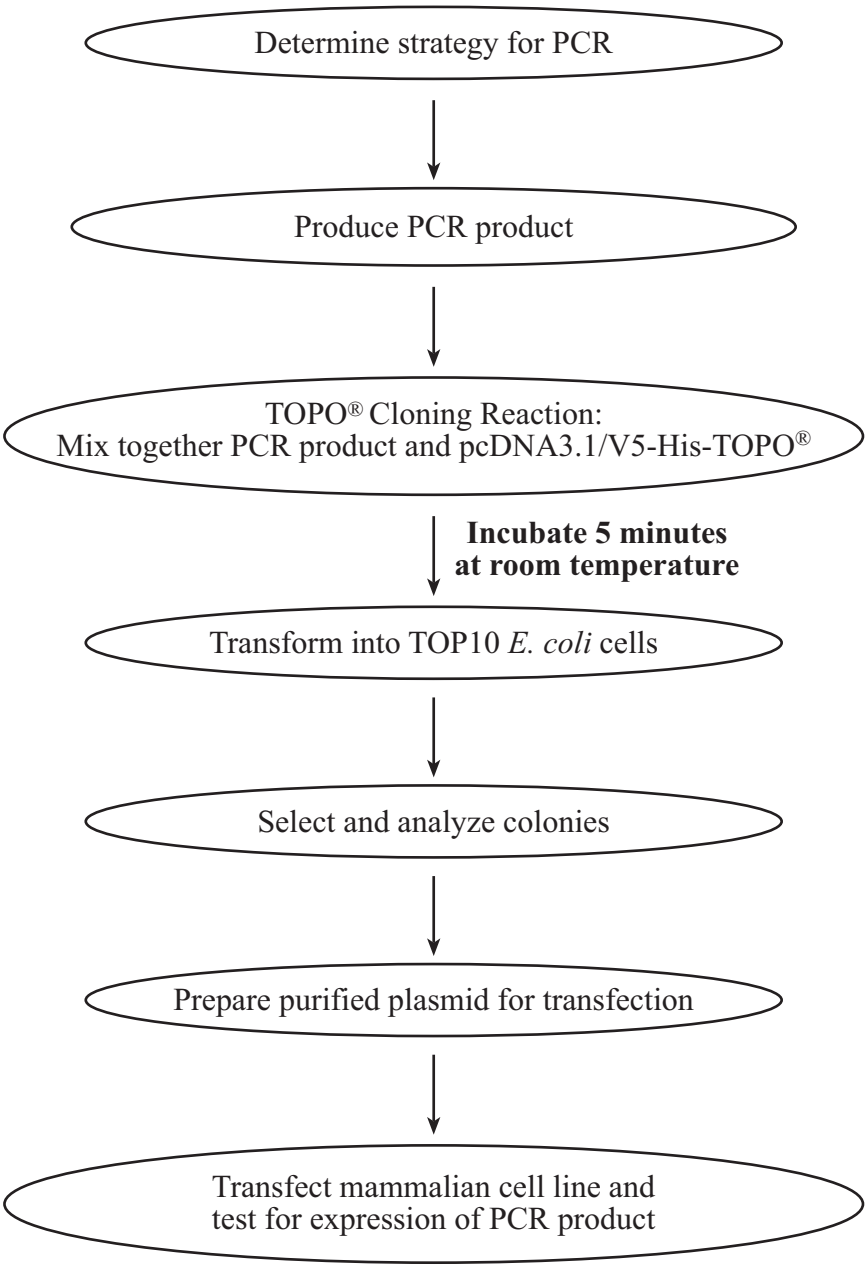
Once the PCR product is cloned into pcDNA™3.1/V5-His-TOPO® and transformants analyzed for the correct orientation of the PCR product, the plasmid is transfected into mammalian cells for expression. The PCR product may be expressed as a fusion to the V5 epitope and polyhistidine tag for detection and purification; or, by designing the 3´ PCR primer with a stop codon, the PCR product may be expressed as a native protein.

continued on next page

Overview, continued

Experimental Outline

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



Methods

PCR Primer Design

Designing Your PCR Primers

Design of the PCR primers to clone your DNA sequences of interest is critical for expression. This is a C-terminal fusion vector that does not contain an ATG initiation codon. If there is no initiating ATG codon or optimal sequences for translation initiation (Kozak sequences) in the DNA to be amplified, then these features need to be incorporated into your forward primer.

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

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

- Clone in frame with the V5 epitope and polyhistidine tag (C-terminal peptide) in order to detect and/or purify your PCR product.

OR

- Include the native stop codon to express the native protein.

Note: Cloning efficiencies may vary depending on the 5´ primer nucleotide sequence (page 15).

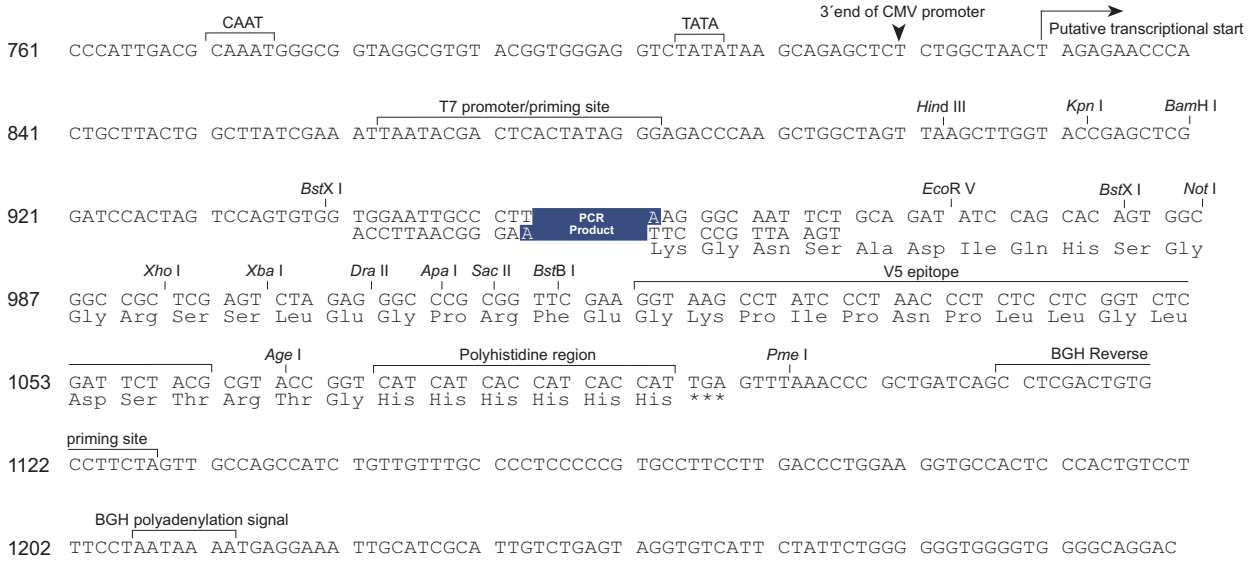
Use the diagram below to design your PCR primers. Once you have designed your PCR primers, proceed to the next page.

Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pcDNA™3.1/V5-His-TOPO®.

TOPO® TA Cloning® Site

Restriction sites are labeled to indicate the actual cleavage site. The vector is supplied linearized between base pair 953 and 954. This is the TOPO® Cloning site. **Note that the full sequence of pcDNA™3.1/V5-His-TOPO® may be downloaded from www.invitrogen.com or requested from Technical Support (see page 24).** A map of pcDNA™3.1/V5-His-TOPO® is provided on page 19.



Producing PCR Products

Introduction

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

Materials Supplied by the User

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product (i.e. Expand™ or eLONGase™).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 18.

Producing PCR Products

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

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

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

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the pcDNA™3.1/V5-His TOPO® TA Expression Kit (see page 16). Take special care to avoid sources of nuclease contamination and long exposure to UV light. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) from Invitrogen can help you optimize your PCR. Contact Technical Support for more information (page 24).

TOPO® Cloning Reaction and Transformation

Introduction

TOPO® Cloning technology allows you to produce your PCR products, ligate them into pcDNA™3.1/V5-His-TOPO®, and transform the recombinant vector into TOP10 *E. coli* in one day. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. If this is the first time you are using TOPO® Cloning, perform the control reactions on pages 13-15 in parallel with your samples.

Note

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

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

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **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 below).** For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results. Review the following information carefully.

Chemically Competent *E. coli*

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

Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see next page).

Materials Supplied by the User

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

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Note

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

Preparing for Transformation

For each transformation, you 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*.
- For electroporation, dilute a small portion of the Salt Solution 4-fold to prepare Dilute Salt Solution (e.g. add 5 µl of the Salt Solution to 15 µl sterile water)
- Warm the vial of SOC medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent One Shot® TOP10 *E. coli* (provided) or electrocompetent *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 9.

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

Reagent*	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 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

*Store all reagents at -20°C when finished. Store Salt solutions and water at room temperature or 4°C.

Performing the TOPO® Cloning Reaction

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 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

2. Place the reaction on ice and proceed to **One Shot® Chemical Transformation** (next page) or **Transformation by Electroporation** (next page). **Note**: You may store the TOPO® Cloning reaction at -20°C overnight.

continued on next page

TOPO® Cloning Reaction and Transformation, continued

One Shot® TOP10 Chemical Transformation

1. Add 2 µl of the TOPO® Cloning reaction from Step 2 previous page 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 (see page 9).
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 SOC medium.
6. Cap and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 25-200 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 will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

Transformation by Electroporation

1. Add 2 µl of the TOPO® Cloning reaction into a 0.1 cm cuvette containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see below.
3. Immediately add 250 µl of room temperature SOC medium.
4. Transfer the solution to a 15 ml snap-cap tube (i.e. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance gene.
5. Spread 10-50 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 SOC. 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 will produce hundreds of colonies. Pick ~10 colonies for analysis (see **Analysis of Positive Clones**, next page).

Note

Addition of the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. 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:

- 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

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Analysis of Positive Clones

1. Pick 10 colonies and culture them overnight in LB medium containing 50 µg/ml ampicillin (3-5 ml).
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page vi for ordering information).
3. Note that PCR products clone bidirectionally. Analyze the plasmids for insertion and orientation by restriction analysis or by sequencing. The T7 and BGH Reverse sequencing primers are included to help you sequence your insert. Refer to the diagram on page 3 for restriction sites and sequence surrounding the TOPO Cloning® site. For the complete sequence of the vector, see www.invitrogen.com or contact Technical Support (page 24).

   If you need help with setting up restriction enzyme digests or DNA sequencing, refer to general molecular biology texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. Use a combination of either the T7 or the BGH Reverse sequencing primer with a primer that binds within your insert as PCR primers. You will have to determine the amplification conditions. If this is the first time you have used this technique, we recommend that you perform restriction analysis in parallel to confirm that PCR gives you the correct result. Artifacts may be obtained because of mispriming or contaminating template.

The following protocol is provided for your convenience. Other protocols are suitable.

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µl reaction volume. Multiply by the number of colonies to be analyzed (e.g. 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. (Don't forget to make a patch plate to preserve the colonies for further analysis.)
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles using parameters previously determined (see text, above).
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 13–15. These reactions will help you troubleshoot your experiment.

continued on next page

TOPO® Cloning Reaction and Transformation, continued

Long-Term Storage

Once you have identified the correct clone, be sure to isolate a single colony and prepare 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 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. Grow until culture reaches stationary phase.
3. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
4. Store at -80°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high cloning efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Transfection

Introduction

Once you have the desired construct, you are ready to transfect the plasmid into the mammalian cells of choice. Note the following guidelines for transfection. Included in the kit is an expression control vector (pcDNA™3.1/V5-His-TOPO/*lac*Z) which you can use to check both transfection efficiencies and expression in your particular cell line.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants kill the cells and salt interferes with lipids decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HiPure Miniprep Kit or the PureLink™ HiPure Midiprep Kit (see page vi for ordering information), 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 cells, we recommend using Lipofectamine™ 2000 Reagent available from Invitrogen. For more information on Lipofectamine™ 2000 and other transfection reagents available, visit www.invitrogen.com or contact **Technical Support** (page 24).

Positive Control

pcDNA™3.1/V5-His-TOPO/*lac*Z is provided as a positive control vector for mammalian transfection and expression (see page 21). It may be used to optimize transfection conditions for your cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the CMV promoter. A successful transfection will result in β-galactosidase expression that can be easily assayed (see below).

Assay for β-galactosidase Activity

You may assay for β-galactosidase expression by activity assay using cell-free lysates (Miller, 1972) or by staining the cells for activity. Invitrogen offers the 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.

Expression and Purification

Introduction

Expression of your PCR product can be performed in transiently transfected cells or stable cell lines (see page 12 for guidelines to create stable cell lines). You may use a functional assay to detect the protein encoded by your PCR product or a western blot analysis if you have an antibody to the protein. If you have elected to express your PCR product as a fusion to the V5 epitope and the polyhistidine tag, you may use antibodies to the V5 epitope or the polyhistidine C-terminus to detect the fusion protein. If you wish, the fusion protein may be purified using metal ion chromatography (see below).

Detection of Fusion Proteins

To detect the fusion protein by western blot, you need to prepare a cell lysate from transfected cells. We recommend that you perform a time course to optimize expression of the fusion protein (e.g., 24, 48, 72 hours, after transfection).

Note

The C-terminal peptide containing the V5 epitope and the polyhistidine region will add approximately ~5 kDa to your protein.

Antibodies for Detection

A number of antibodies are available from Invitrogen to detect expression of your fusion protein from pcDNA™3.1/V5-His-TOPO®. The table below describes the antibodies available and ordering information. The amount supplied is sufficient for 25 westerns.

Antibody	Purpose	Catalog no.
Anti-V5	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991)	R960-25
Anti-V5-HRP	See above. Provided as an HRP conjugate for time-saving detection.	R961-25
Anti-His(C-term)	Detects the C-terminal polyhistidine tag (requires the free carboxyl group for detection) (Lindner *et al.*, 1997)	R930-25
Anti-His(C-term)-HRP	See above. Provided as an HRP conjugate for time-saving detection.	R931-25

continued on next page

Expression and Purification, continued

Preparing Cells for Purification

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

1. Seed cells in five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are 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 (if necessary) and transfer the cells to a sterile microcentrifuge tube.
5. Centrifuge the cells at 1,500 rpm for 5 minutes. You may lyse the cells immediately or freeze in liquid nitrogen and store at -80°C until needed.
   Note: For cell lysis procedures, refer to the ProBond™ Purification System manual if using ProBond™. If you are using a different resin, refer to the manufacturer's instructions.

Creation of Stable Cell Lines

Introduction

If you wish to create stable cell lines, select for foci using Geneticin® Selective Antibiotic. General information and guidelines are provided below.

Geneticin® Selective Antibiotic

Geneticin® Selective Antibiotic 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 phosphotransferase gene (APH), derived from Tn*5*, results in detoxification of Geneticin® (Southern and Berg, 1982).

Geneticin® Selection Guidelines

Geneticin® Selective Antibiotic is available from Invitrogen (page vi). Use as follows:

1. Prepare Geneticin® in a buffered solution (e.g. 100 mM HEPES, pH 7.3).
2. Use 100 to 1,000 µg/ml of Geneticin® in complete medium.
3. Calculate concentration based on the amount of active drug.
4. Test varying concentrations of Geneticin® on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin®.

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.

Appendix

pcDNA™3.1/V5-His TOPO® TA Cloning Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions using the reagents included in the kit involves producing a control PCR product containing the *lac* promoter and the LacZα protein. Successful TOPO® Cloning of the control PCR product will yield blue colonies on LB agar plates containing ampicillin and X-gal.

Before Starting

Be sure to prepare the following reagents before performing the control reaction:

- 40 mg/ml X-gal in dimethylformamide (see page 22 for recipe)
- LB plates containing 50-100 µg/ml ampicillin and X-gal (two per transformation)

  To add X-gal to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Producing the Control PCR Product

1. To produce the 500 bp control PCR product containing the *lac* promoter and LacZα, set up the following 50 µl PCR:

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

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

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

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

pcDNA™3.1/V5-His TOPO® TA Cloning Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pcDNA™3.1/V5-His-TOPO® vector set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pcDNA™3.1/V5-His-TOPO® vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® TOP10 cells (page 7).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50-100 µg/ml ampicillin and X-Gal (see page 22). Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of SOC to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 90% of these will be blue and contain the 500 bp insert.

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 7. Plate 10 µl of the transformation mixture plus 20 µl SOC on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ~1 × 10^9 cfu/µg DNA.

continued on next page

pcDNA™3.1/V5-His TOPO® TA Cloning Control Reactions, continued

Factors Affecting Cloning Efficiency

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

Variable	Solution
pH>9	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products need a longer extension time.
Cloning large inserts (>3 kb)	Gel-purify as described on page 16.
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning blunt-ended fragments	Add 3´ A-overhangs by incubating with *Taq* polymerase (page 18).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 16).
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	*Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).

Purifying PCR Products

Introduction

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

Note

Note that cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band (see **Producing PCR Products**, page 4).

Using the PureLink™ Quick Gel Extraction Kit

The PureLink™ Quick Gel Extraction Kit (page vi) allows you to rapidly purify PCR products from regular agarose gels. Follow the protocol in the manual supplied with the kit to gel-purify your PCR fragment.

Use 4 µl of the purified DNA for the TOPO® Cloning reaction and proceed as described on page 10.

Low-Melt Agarose Method

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

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

Addition of 3´ A-Overhangs Post-Amplification

Introduction

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

Materials Needed

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

Procedure

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

1. After amplification with *Vent*® or *Pfu* polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pcDNA™3.1/V5-His-TOPO©.

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

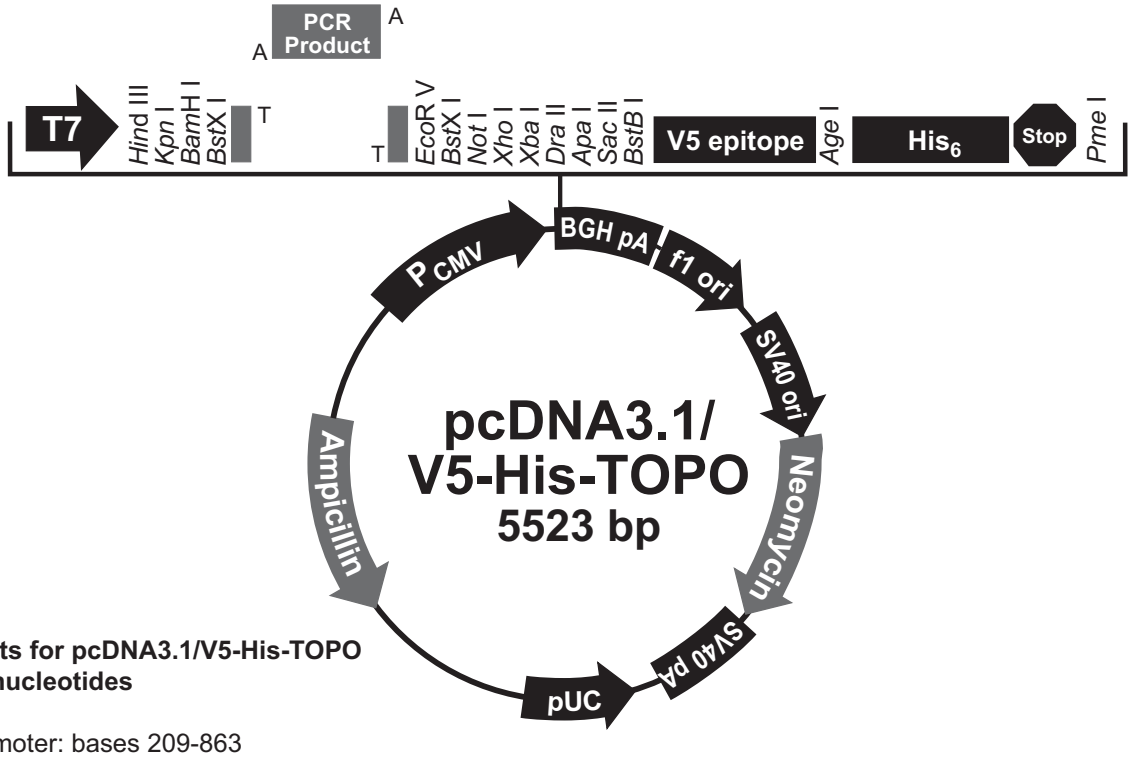
Note

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

pcDNA™3.1/V5-His-TOPO®

Map

The figure below summarizes the features of the pcDNA™3.1/V5-His-TOPO® vector. The vector is supplied linearized between base pairs 953 and 954. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from www.invitrogen.com or from Technical Support (page 24).**



Comments for pcDNA3.1/V5-His-TOPO 5523 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-1019
TOPO® Cloning site: 953-954
V5 epitope: bases 1020-1061
Polyhistidine tag: bases 1071-1088
BGH reverse priming site: bases 1111-1128
BGH polyadenylation signal: bases 1110-1324
f1 origin of replication: bases 1387-1800
SV40 promoter and origin: bases 1865-2190
Neomycin resistance gene: bases 2226-3020
SV40 polyadenylation signal: bases 3039-3277
pUC origin: bases 3709-4382
Ampicillin resistance gene: bases 4527-5387

continued on next page

pcDNA™3.1/V5-His-TOPO®, continued

Features of pcDNA™3.1/V5-His-TOPO©

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

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits 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	Allows insertion of your PCR product in frame with the V5 epitope and polyhistidine C-terminal tag
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 Antibody or Anti-V5-HRP Antibody (Southern *et al.*, 1991)
C-terminal polyhistidine tag	Permits purification of your recombinant protein on metal-chelating resin such as ProBond™ In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His (C-term) Antibody (Lindner *et al.*, 1997) and the Anti-His (C-term)-HRP Antibody
BGH reverse priming site	Permits sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
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 (G418) resistance gene	Selection of stable transfectants in mammalian cells (Southern and Berg, 1982)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC-derived origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

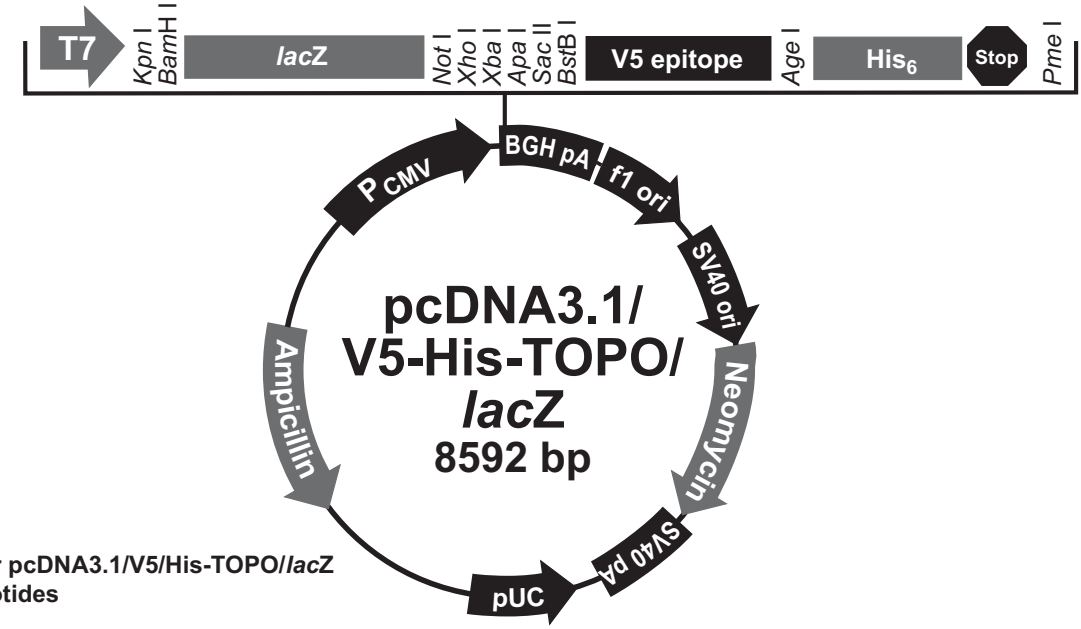
Map of pcDNA™3.1/V5-His-TOPO®/*lacZ*

Description

pcDNA™3.1/V5-His-TOPO®/*lacZ* is a 8,592 bp control vector containing the gene for β-galactosidase. The *lacZ* gene was amplified and TOPO® Cloned into pcDNA™3.1/V5-His-TOPO© such that it is in frame with the C-terminal peptide.

Map of Control Vector

The figure below summarizes the features of the pcDNA™3.1/V5-His-TOPO®/*lacZ* vector. **The complete nucleotide sequence for pcDNA™3.1/V5-His-TOPO®/*lacZ* is available for downloading from www.invitrogen.com or by contacting Technical Support (page 24).**



Comments for pcDNA3.1/V5/His-TOPO/*lacZ*
8592 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
LacZ portion of fusion: bases 963-4019
V5 epitope: bases 4089-4130
Polyhistidine tag: bases 4140-4157
BGH Reverse priming site: bases 4180-4197
BGH polyadenylation signal: bases 4179-4393
f1 origin of replication: bases 4456-4869
SV40 promoter and origin: bases 4934-5259
Neomycin resistance gene: bases 5295-6089
SV40 polyadenylation signal: bases 6107-6346
pUC origin: bases 6778-7451 (C)
Ampicillin resistance gene: bases 7596-8456 (C)

C = complementary strand

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

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

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

X-Gal Stock Solution

1. To make a 40 mg/ml stock solution, dissolve 400 mg X-Gal in 10 ml dimethylformamide.
2. Protect from light by storing in a brown bottle at -20°C.
3. To add to previously made agar plates, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml stock solution onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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

Corporate Headquarters:
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_support@invitrogen.com

Japanese Headquarters:
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:
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

MSDS

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

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited Warranty

Invitrogen (a part of Life Technologies Corporation) 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 Support Representatives. All Invitrogen products are warranted to 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 the Company's liability to only the price 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. The Company reserves the right to select the method(s) used to analyze a product unless the Company 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 the Company 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 Support Representatives.
Life Technologies Corporation shall have 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

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. For products that are subject to multiple limited use label licenses, the terms of the most restrictive limited use label license shall control. Life Technologies Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Life Technologies 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, Life Technologies is willing to accept return of the product with a full refund. For information about purchasing a license to use this product or the technology embedded in it for any use other than for research use contact Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008 or outlicensing@lifetech.com

Limited Use Label License No. 22: Vectors and Clones Encoding Histidine Hexamer

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

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.

Brownstein, M. J., Carpten, J. D., and Smith, J. R. (1996). Modulation of Non-Templated Nucleotide Addition by *Taq* DNA Polymerase: Primer Modifications that Facilitate Genotyping. BioTechniques *20*, 1004-1010.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Molec. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Felgner, P. L., Holm, M., and Chan, H. (1989). Cationic Liposome Mediated Transfection. Proc. West. Pharmacol. Soc. *32*, 115-121.

Felgner, P. L., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Goodwin, E. C., and Rottman, F. M. (1992). The 3´-Flanking Sequence of the Bovine Growth Hormone Gene Contains Novel Elements Required for Efficient and Accurate Polyadenylation. J. Biol. Chem. *267*, 16330-16334.

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. S. (1990) PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.

Lindner, P., Bauer, K., Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Pluckthun, A. (1997). Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions. BioTechniques *22*, 140-149.

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

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.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

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

continued on next page

References, continued

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.

Southern, P. J., and Berg, P. (1982). Transformation of Mammalian Cells to Antibiotic Resistance with a Bacterial Gene Under Control of the SV40 Early Region Promoter. J. Molec. Appl. Gen. *1*, 327-339.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

invitrogen™

Corporate Headquarters
Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech_support@invitrogen.com

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