USER GUIDE

invitrogen™
by life technologies™

pcDNA™3.3-TOPO® TA Cloning Kit

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

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For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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Table of Contents

Kit Contents and Storage

Shipping and storage

The pcDNA™3.3-TOPO® TA Cloning Kit is shipped on dry ice. Each kit contains two boxes. Upon receipt, store boxes as detailed below.

Box	Item	Storage
1	pcDNA™3.3-TOPO® TA Cloning Reagents	–20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	–80°C

TOPO® TA cloning reagents

The pcDNA™3.3-TOPO® TA cloning reagents (Box 1) are listed below. **Note that the user must supply *Taq* polymerase**. **Store the contents of Box 1 at –20°C.**

Item	Concentration	Amount
5–10 ng/µL pcDNA™3.3-TOPO® vector, TOPO® adapted	linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 1 mM EDTA 1mM 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
dNTP Mix	12.5 mM each dATP, dCTP, dGTP, and dTTP; neutralized at pH 8.0 in water	10 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL
Sterile Water	---	1 mL
Control PCR template	50 ng/µL in TE buffer, pH 8.0	10 µL
Control PCR primers	100 ng/µL each in TE buffer, pH 8.0	10 µL
CMV forward sequencing primer	100 ng/µL in TE buffer, pH 8.0	20 µL
TK polyA reverse sequencing primer	100 ng/µL in TE buffer, pH 8.0	20 µL
pcDNA™3.3-TOPO®/lacZ expression control plasmid	500 ng/µL in TE buffer, pH 8.0	10 µL

Continued on next page

Kit Contents and Storage, continued

Primers

The pcDNA™3.3-TOPO® TA Vector Kit contains the following primers to sequence your insert.

Primer	Sequence
CMV forward	5'-CGCAAATGGGCGGTAGGCGTG-3'
TK polyA reverse	5´-CTTCCGTGTTTCAGTTAGC-3´

One Shot® TOP10 reagents

The following reagents are included in the One Shot® TOP10 Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1×10^9 cfu/µg plasmid DNA. **Store the contents of Box 2 at -80°C**.

Item	Concentration	Amount
TOP10 *E. coli*	–	11 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL
S.O.C. Medium	2% Tryptone 0.5% Yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 mL

Genotype of TOP10 strain

F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*χ74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Overview

Description

The pcDNA™3.3-TOPO® vector is a TOPO®-adapted plasmid that allows rapid cloning of a PCR product containing a gene of interest downstream of the CMV promoter. The pcDNA™3.3-TOPO® vector can be used for high level expression of a native protein in adherent mammalian tissue culture cells following transient transfection, or high level expression of secreted, native protein using the FreeStyle™ MAX CHO and FreeStyle™ MAX 293 systems.

The pcDNA™3.3-TOPO® vector is also included with the Freedom™ DG44 Kit for cloning the for cloning the one or two ORFs of your protein(s) of choice.

Features of the vector

The pcDNA™3.3-TOPO® vector contains the following elements:

- Full-length human cytomegalovirus (CMV) immediate-early promoter/enhancer for high-level gene expression in a wide range of mammalian cells
- TOPO® Cloning site for rapid and efficient cloning of *Taq*-amplified PCR products
- Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
- Neomycin resistance gene for selection of stable cell lines with Geneticin®
- pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- Ampicillin (*bla*) resistance gene for selection in *E. coli*

For a map of the pcDNA™3.3-TOPO® vector, see page 24.

CMV promoter

The human cytomegalovirus immediate-early (HCMV IE1) gene promoter in the pcDNA™3.3-TOPO® vector is 680 bp and contains the native transcriptional start site (Hennighausen & Fleckenstein, 1986). This sequence results in high levels of transgene expression.

Overview, continued

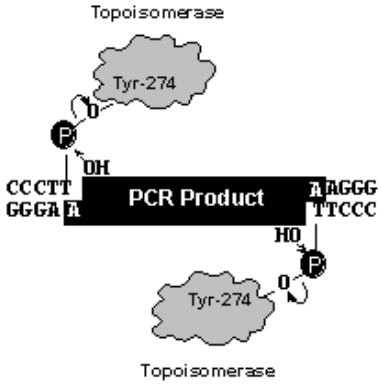
How TOPO® works

The pcDNA™3.3-TOPO® vector 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.



Once the PCR product is cloned into the pcDNA™3.3-TOPO® vector and the transformants are analyzed for correct orientation and reading frame, the expression plasmid may be transfected into your cell line of choice.

Experimental outline for general cloning

To TOPO® Clone your gene of interest into pcDNA™3.3-TOPO®, you will perform the following steps:

1. Generate a PCR product containing your gene of interest with *Taq* polymerase.
2. TOPO® Clone your PCR product into the pcDNA™3.3-TOPO® vector and use the reaction to transform One Shot® TOP10 Chemically Competent *E. coli*.
3. Pick colonies, isolate plasmid DNA, and screen for insert directionality by sequencing expression clones with the primers provided in the kit.

The following sections of this manual provide instructions and guidelines for these steps.

FreeStyle™ MAX system

The pcDNA™3.3-TOPO® vector is ideal for use with the FreeStyle™ MAX System for rapid, high-yield protein production from suspension-adapted CHO and 293 cells. The FreeStyle™ MAX System combines GIBCO® FreeStyle™ Media, FreeStyle™ MAX Reagent, and either FreeStyle™ CHO-S cells or FreeStyle™ 293 cell for rapid and high-yield mammalian protein production. See page 28 for ordering information.

Designing PCR Primers

Introduction

TOPO® Cloning provides a highly efficient, 5-minute, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required. The section below will help you design primers to produce your PCR product for cloning into the pcDNA™3.3-TOPO® vector.

Points to consider when designing PCR primers

To obtain the pcDNA™3.3-TOPO® expression construct containing your gene of interest, your PCR primer design must include:

- A Kozak consensus sequence
- A mammalian secretion signal upstream of your gene of interest (if you wish to produce secreted protein)
- A stop codon at the end of your gene of interest

See below for more information.

Kozak consensus sequence

Your gene of interest must contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. The ATG initiation codon is shown underlined.

(G/A)NNATGN

Other sequences are possible, but the G or A at position -3 (shown in bold) is critical for a functional Kozak sequence. At position +4 any of the four nucleotides can be present to form part of the Kozak sequence.

Secretion signal

If you wish to produce a secreted protein, you must include a mammalian secretion signal so the protein will be secreted from mammalian DG44 or CHO cells. To direct secretion of your protein of interest, you can include the endogenous secretion signal, or add one such as the murine Ig κ-chain leader sequence (Coloma *et al.*, 1992) using PCR.

Note

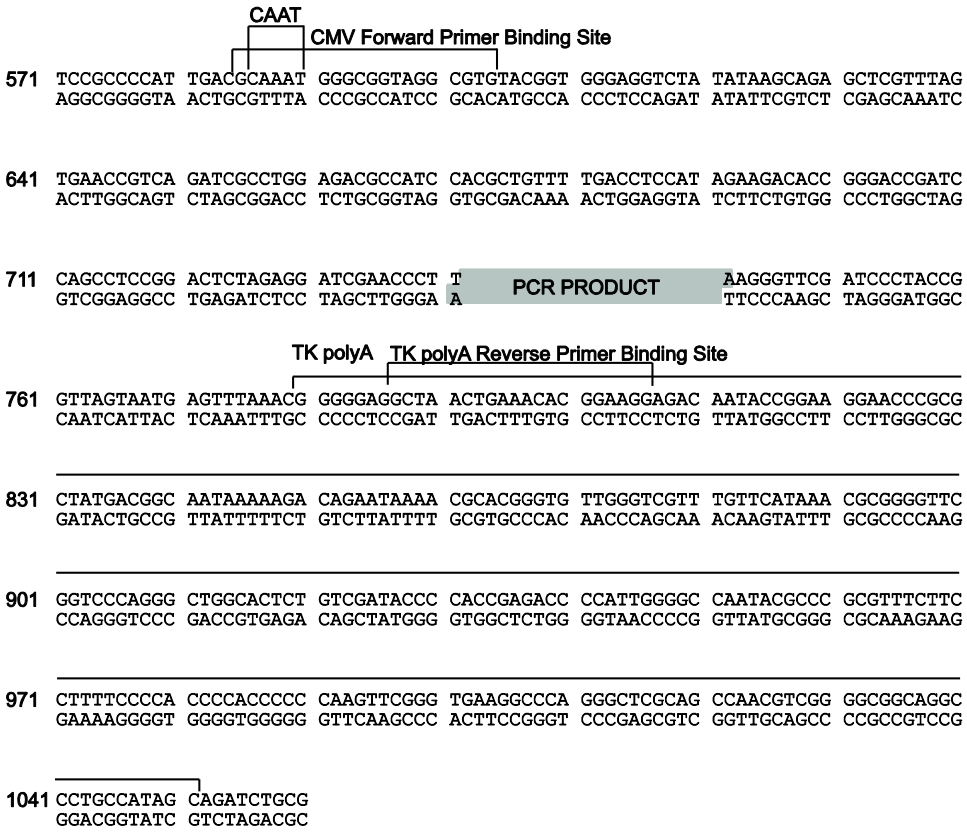
Do not add 5’ phosphates to your primers for PCR, because the synthesized PCR product will not ligate into the vector. Cloning efficiencies may vary depending on the primer nucleotide sequences.

Continued on next page

Designing PCR Primers, continued

TOPO® cloning site

Use the diagram below to help you design your PCR product for TOPO® Cloning into pcDNA™3.3-TOPO®. The complete vector sequence is available from **www.invitrogen.com** or by contacting Technical Support (page 28).



Producing PCR Products

Introduction

After you have designed primers to amplify your gene of interest, you are ready to produce your PCR product for TOPO® Cloning into the pcDNA™3.3-TOPO® vector.

Materials supplied by user

You will need the following reagents and equipment:

- *Taq* polymerase, such as Platinum® *Taq*
- Thermocycler
- DNA template
- Primers for PCR product

Polymerase mixtures

You may use an enzyme mixture containing *Taq* polymerase and a proofreading polymerase; however *Taq* must be used in excess of 10:1 to ensure the presence of 3' A-overhangs on the PCR product. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity (see page 28 for ordering information).

If you use polymerase mixtures that do not have enough *Taq* polymerase or use a proofreading polymerase only, you can add 3' A-overhangs after amplification using the procedure on page 23.

Producing PCR products

1. Set up the following reaction in a 50 µl volume. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template.

Reagent	Amount
DNA template	10–100 ng
10X PCR Buffer	5 µL
50 mM dNTPs	0.5 µL
PCR Primers	100–200 ng each
Sterile Water	to final volume of 49 µL
Taq polymerase (1 unit/µL)	1 µL
Total Volume	**50 µL**

2. Perform amplification using the cycling parameters suitable for your primers and template. Be sure to include a 7–30 minute extension at 72° after the last cycle to ensure that all PCR products are full-length and 3' adenylated.
3. Use agarose gel electrophoresis to verify the quality of your PCR product. You should see a single, discrete band of the correct size. If you do not see a single band, refer to the **Note** on the next page.

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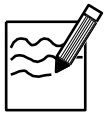

Note

If you do not obtain a single, discrete band from your PCR reaction, try the following:

- The PCR Optimizer™ Kit can help you optimize your PCR to eliminate multiple bands and smearing (see page 28 for ordering information).
- Gel-purify your fragment before performing the TOPO® Cloning reaction using the E-Gel® CloneWell™ system or the PureLink® Gel Extraction Kit (available separately, see page 28 for ordering information).

Alternatively, refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel *et al.*, 1994) for other common protocols for isolating DNA fragments.

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pcDNA™3.3-TOPO® vector and use this plasmid for transformation of competent *E. coli*. It is important to have everything you need to set up the reaction so that you can obtain the best results. We suggest that you read this entire section and the next section about transformation before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions detailed on pages 21–22 in parallel with your samples.

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt can also increase the number of transformants. This is in contrast to experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Including salt in the TOPO® Cloning reaction 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.

Using salt 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 28 for ordering information).

- If you are transforming chemically competent *E. coli* (included with the kit), use the stock Salt Solution as supplied, and set up the TOPO® Cloning reaction as directed on the next page.
- If you are transforming electrocompetent *E. coli* (available separately from Invitrogen; see page 28), 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 on the next page.

Continued on next page

Setting Up the TOPO® Cloning Reaction, continued

Materials needed

You should have the following materials on hand before beginning:

- Your PCR product (freshly prepared)
- pcDNA™3.3-TOPO® vector
- Salt Solution or Dilute Salt Solution (see previous page)
- Sterile Water

Performing the TOPO® cloning reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µL) to use for transformation of either chemically competent or electrocompetent *E. coli*.

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

Reagent	Chemically Competent *E. coli*	Electrocompetent *E. coli*
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 total volume of 5 µL	Add to total 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. 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 larger PCR products (>1 kb), increasing the reaction time may yield more colonies.

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

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

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you are ready to use your construct to transform competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit (Box 2) to facilitate transformation. You may also transform One Shot® Electrocompetent cells if desired (see page 28 for ordering information). Protocols for transforming chemically competent and electrocompetent *E. coli* are provided in this section.

Selecting a One Shot® chemical transformation protocol

Two protocols are provided to transform One Shot® TOP10 chemically competent *E. coli*. Consider the following factors and choose the protocol that best suits your needs.

If you wish to…	Then use the…
Maximize the number of transformants	Regular chemical transformation protocol, page 15.
Clone large PCR products (>1000 bp)	
Obtain transformants as quickly as possible	Rapid chemical transformation protocol, page 15. **Note:** This procedure is less efficient; the total number of transformants obtained may be lower than that obtained with the regular chemical transformation protocol.

Materials needed

In addition to general microbiological supplies (*i.e.* plates, spreaders), you will need the following:

- TOPO® Cloning reaction (From Step 2, previous page)
- One Shot® TOP10 *E. coli*, either chemically competent (supplied with the kit, Box 2) or electrocompetent (purchased separately, see page 28)
- S.O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (supplied with the kit, Box 2)
- 42°C water bath (chemically competent cells only)
- Electroporator with cuvettes (electrocompetent cells only)
- 15 mL sterile, snap cap plastic culture tubes (electrocompetent cells only)
- 2 selective LB plates containing 100 µg/mL ampicillin for each transformation. See page 27 for a recipe to prepare selective LB plates.
- 37°C shaking and non-shaking incubators

Continued on next page

Transforming One Shot® Competent *E. coli*, continued

Preparing for transformation

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

- Equilibrate a water bath to 42°C if using chemically competent *E. coli*, or set up your electroporator if using electrocompetent *E. coli*
- Warm the vial of S.O.C. Medium to room temperature
- Warm selective LB plates at 37°C for 30 minutes
- Thaw one vial of One Shot® cells **on ice** for each transformation

One Shot® chemical transformation protocol

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µL of the TOPO® Cloning reaction into a vial of One Shot® Chemically Competent *E. coli* with a sterile pipette tip and mix gently. Do not mix by pipetting up and down.

   Note: If you are using the pUC19 control plasmid for transformation, use 1 µL (10 pg).
2. Incubate cells/plasmid mix on ice for 5–30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency.
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 at 200 rpm in a 37°C shaking incubator for 1 hour.
7. Spread 10–50 µL from each transformation on a pre-warmed selective LB plate. To ensure even spreading of small volumes, you may add 20 µL of S.O.C. Medium to the transformation mixture. We recommend that you plate two different volumes to ensure that at least one plate contains well-spaced colonies. Incubate plates overnight at 37°C.

Rapid One Shot® chemical transformation protocol

Use the alternative protocol below to rapidly transform One Shot® TOP10 chemically competent *E. coli*. Before beginning, pre-warm LB plates containing 100 µg/mL ampicillin at 37°C for 30 minutes.

1. Add 4 µL of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently. Do not mix by pipetting up and down.
2. Incubate reaction on ice 5 minutes.
3. Spread 50 µL of cells on a prewarmed selective LB plate and incubate overnight at 37°C.

Analyzing Positive Clones

Introduction

After transformation of your pcDNA™3.3 construct into *E. coli*, you will select and analyze several colonies by sequencing using the specific primers included in the kit to determine the orientation of the insert.

Analyzing positive clones

1. Pick 10 colonies and culture them overnight in LB medium containing 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 the PureLink® HQ Mini Plasmid Purification Kit. See page 28 for ordering information.
3. Analyze plasmid DNA by sequencing (see below).

Sequencing

To confirm that your gene of interest is in the correct orientation, you may sequence your expression construct using the CMV forward and TK polyA reverse primers included with the kit. Refer to page 5 for the sequences of the primers and the diagram on page 9 for the location of the primer binding sites.

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.

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

We also recommend that you store a stock of plasmid DNA at –20°C.

Next Steps

Introduction

After obtaining the correct pcDNA™3.3-TOPO® plasmid construct, you will linearize and purify the plasmid prior to transfection into adherent or suspension cells. General guidelines for purifying the plasmid, performing a positive expression control, transfecting cells and generating stable cell lines are given below.

Note: If you are using the Freedom™ DG44™ Kit, refer to the product manual supplied with the system for specific instructions on preparing the plasmid.

Plasmid preparation

The pcDNA™3.3-TOPO® plasmid construct must be clean, sterile and free from contamination with phenol and sodium chloride for transfection into cells. Contaminants may kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HiPure DNA Midiprep Kit (see page 28 for ordering information).

Linearizing the plasmid

Prior to transfecting the pcDNA™3.3-TOPO® plasmid construct, you may linearize the plasmid. While linearizing your vector may not improve transfection efficiency, it increases the chances that the vector integrates into the host cell genome without disrupting the gene of interest or other elements required for expression in mammalian cells.

We suggest using *Pvu* I, which cuts once in the ampicillin resistance gene. Other unique restriction sites are possible. A complete restriction map of pcDNA™3.3-TOPO® is available at **www.invitrogen.com**. **Be sure that your insert does not contain the restriction enzyme site you wish to use to linearize your vector.**

After digestion, precipitate the DNA and resuspend pellet in sterile water and re-quantify using your method of choice.

Transfection reagents

A wide range of transfection reagents for plasmid DNA that provide high efficiency gene expression with minimal cytotoxicity across a broad range of adherent and suspension cell lines are available from Invitrogen. Go to **www.invitrogen.com\transfection** to learn more about transfection reagents for your particular application.

Positive control

pcDNA™3.3-TOPO®/*lacZ* is provided as a positive control vector for mammalian transfection and expression (see page 26). 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.

You may assay for β-galactosidase expression by activity assay using the FluoReporter® *lacZ*/Galactosidase Quantitation Kit (available separately, see page 28 for ordering information).

Continued on next page

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® selective antibiotic (Southern and Berg, 1982).

Geneticin® selection guidelines

If you wish to create stable cell lines, select using Geneticin® selective antibiotic (Cat. no. 10131-035). Use Geneticin® selective antibiotic as follows:

1. Use 100 to 1000 µg/mL of Geneticin® selective antibiotic in complete medium.
2. Calculate concentration based on the amount of active drug.
3. Test varying concentrations of Geneticin® selective antibiotic on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to Geneticin® selective antibiotic.

Cells will divide once or twice in the presence of lethal doses of Geneticin® selective antibiotic, 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

Introduction

The table below lists some potential problems solutions that may help you troubleshoot your TOPO® Cloning and expression of your gene of interest.

Problem	Possible Cause	Solution
Few or no colonies obtained from sample reaction, but transformation control yielded colonies	Incomplete extension during PCR	Include a final extension step of 7–30 minutes during PCR. Longer PCR products will need a longer extension time.
	Excess or dilute PCR product used in the TOPO® Cloning reaction	Reduce or concentrate the amount of PCR product.
	PCR primers contain 5’ phosphates	Do not add 5’ phosphates to your PCR primers.
	Used a proofreading polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use *Taq* polymerase to add 3’A-overhangs to your PCR product by following the method on page 23.
	Large PCR product	• Increase the amount of PCR product used in the TOPO® Cloning reaction. • Increase the incubation time of TOPO® Cloning reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers or other artifacts.
	PCR contains artifacts (*i.e.* not a single band on an agarose gel)	• Optimize your PCR conditions. • Gel-purify your PCR product.
	PCR product does not contain sufficient 3’A-overhangs even though you used *Taq* polymerase	• Increase the final extension time to ensure that all 3’ ends are adenylated. • You may redesign your primers so that they contain a 5’ G instead of a 5’ T. **Note:** *Taq* polymerase is most efficient at adding a non-template 3’A next to a C, and less efficient at adding a nontemplate 3’ A next to another A (Brownstein *et al.*, 1996).

Continued on next page

Problem	Possible Cause	Solution
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and other artifacts. • Optimize your PCR conditions. • Include a final extension step of 7–30 minutes during PCR.
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.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates with the wrong antibiotic	Use LB ampicillin plates for selection.
No protein expression in mammalian cells after transfection	PCR primer does not contain Kozak translation initiation sequence	Add a Kozak consensus site to the forward PCR primer (see page 8), resynthesize your DNA and re-clone.
	Premature stop codons	Remove stop codons by your method of choice.
	Poor secretion leader (for secreted proteins)	Include the endogenous secretion leader, if possible.
	Sequence not optimized	Optimize the codon sequence of the gene of interest.

Appendix

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product expressing the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product in either direction will yield > 85% blue colonies on LB plates containing ampicillin and X-gal.

Before starting

The following reagents should be prepared before performing the control reaction:

- Prepare stock X-gal solution (See page 27 for recipe)
- For each transformation, you will need two LB plates containing 100 µg/mL ampicillin and X-gal

Producing the control PCR product

1. In a 0.5 mL microcentrifuge tube, set up the following reaction in a 50 µL volume.

Reagent	Amount
Control DNA Template	1 µL
10X PCR Buffer	5 µL
50 mM dNTPs	0.5 µL
Control PCR Primers (0.1 µg/µL each)	1 µL
Sterile Water	41.5 µL
Taq polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

2. Amplify the control PCR product using the following cycling parameters:

Step	Time	Temp.	Cycles
Initial Denaturation	2 min.	94°C	1X
Denaturation	1 min.	94°C	25X
Annealing	1 min.	60°C	
Extension	1 min.	72°C	
Final Extension	7 min.	72°C	1X

3. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 500 bp band should be visible.

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Control TOPO® cloning reactions

Using the control PCR product generated in the steps above and the control vector, set up two 6 µL TOPO® Cloning reactions as described below:

Reagent	"Vector Only"	"Vector + PCR Insert"
Control PCR Product	–	1 µL
Sterile Water	4 µL	3 µL
Salt Solution or Dilute Salt Solution	1 µL	1 µL
pcDNA™3.3-TOPO® vector	1 µL	1 µL

1. Incubate at room temperature for 5 minutes and place on ice.
2. Use 2 µL of the reaction to transform two separate vials of One Shot® competent cells using the procedure on page 14.
3. Spread 10–50 µL of each transformation mix onto LB plates containing 100 µg/mL ampicillin and X-gal. When plating small volumes, add 20 µL of S.O.C. Medium to ensure even spreading. Plate two different volumes to ensure that at least one plate has well-spaced colonies.
4. Incubate plates overnight at 37°C.

What you should see

The "vector + PCR insert" reaction should produce hundreds of colonies. Greater than 85% of these will be blue.

The "vector only" reaction should yield very few colonies (<15% of the vector + PCR insert plate) and these should be white.

Transformation control

The pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 14. Plate 10 µL of the transformation reaction plus 20 µL of S.O.C. on LB plates containing 100 µg/mL ampicillin. The transformation efficiency should be 1×10^9 cfu/µg DNA.

Adding of 3’ A-Overhangs Post-Amplification

Introduction

TOPO® Cloning DNA amplified by proofreading polymerases into TOPO® Cloning vectors often results in very low cloning efficiencies. Proofreading polymerases remove the 3´ A-overhangs necessary for TOPO® Cloning.
A method for adding 3’As post-amplification is provided below.

Before starting

You will need the following items:

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

Procedure

This is just one method for adding 3´ A-overhangs. Other protocols may be suitable.

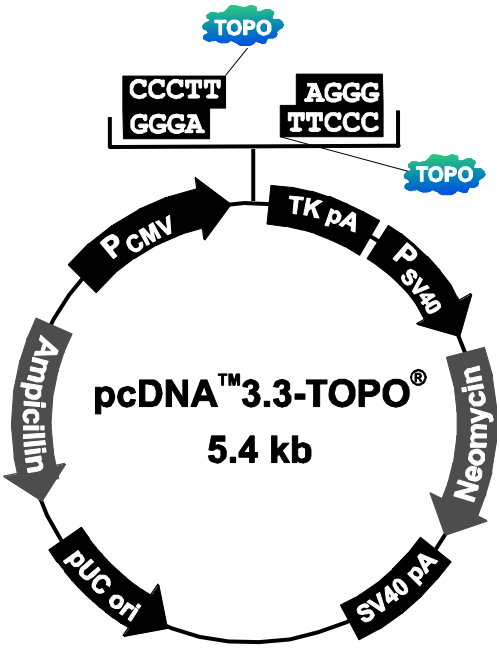
1. After amplification with a proofreading polymerase, place vials on ice and add 0.7–1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer.
2. Incubate at 72°C for 8–10 minutes (do not cycle).
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pcDNA™3.3-TOPO®.

   Note: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

Map and Features of pcDNA™3.3-TOPO®

Map

The map below shows the elements of the pcDNA™3.3-TOPO® vector. **The complete sequence is available for downloading from www.invitrogen.com or by contacting Technical Support (page 28).**



Comments for pcDNA™3.3-TOPO® 5407 nucleotides

CMV promoter:	47-726
CMV forward primer binding site:	584-604
TOPO® cloning site:	741
TK pA reverse primer binding site:	787-805
TK polyadenylation signal:	780-1051
f1 replication origin:	1087-1515
SV40 early promoter:	1520-1889
Neomycin Resistance gene:	1925-2719
SV40 polyadenylation signal:	2895-3025
pUC origin (c):	3408-4081
Ampicillin (*bla*) resistance gene (c):	4226-5086
bla promoter (c):	5087-5185

(c) = complementary strand

Continued on next page

Map and Features of pcDNA™3.3-TOPO®, continued

Features

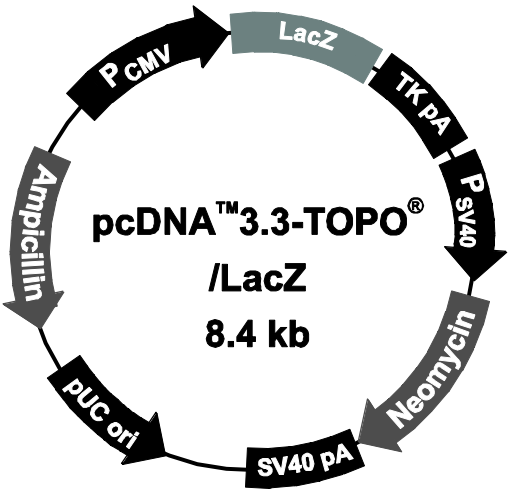
The pcDNA™3.3-TOPO® vector contains the following elements. Features have been functionally tested, and the vectors have been fully sequenced.

Feature	Benefit
Full length human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Hennighausen & Fleckenstein, 1986; Nelson *et al.*, 1987)
CMV forward primer	Allows sequencing of the insert
TOPO® Cloning site	Allows insertion of your PCR product
TK polyA reverse primer	Allows sequencing of the insert
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA (Cole & Stacy, 1985)
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 & 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*
Ampicillin (*bla*) resistance gene (β-lactamase)	Allows selection of transformants in *E. coli*

Map and Features of pcDNA™3.3-TOPO®/LacZ

Map

The map below shows the elements of the pcDNA™3.3-TOPO®/LacZ vector. **The complete sequence is available for downloading from www.invitrogen.com or by contacting Technical Support (page 28).**



Comments for pcDNA™3.3-TOPO®/LacZ 8464 nucleotides

CMV promoter:	47-726
CMV forward primer binding site:	584-604
LacZ ORF:	743-3799
TK pA reverse primer binding site:	3844-3862
TK polyadenylation signal:	3837-4108
f1 replication origin:	4144-4572
SV40 early promoter:	4577-4946
Neomycin Resistance gene:	4982-5776
SV40 polyadenylation signal:	5952-6082
pUC origin (c):	6465-7138
Ampicillin (*bla*) resistance gene (c):	7283-8143
bla promoter (c):	8144-8242

(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 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 if needed, and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C.

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.
4. Protect plates from light.

Accessory Products

Additional products

The products listed in this section are available from Invitrogen and may be used with the pcDNA™3.3-TOPO® TA Cloning Kit.

Product	Amount	Catalog no.
Platinum® *Taq* DNA Polymerase	100 reactions 250 reactions 500 reactions	10966-018 10966-026 10966-034
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
PCR Optimizer™ Kit	1 kit	K1220-01
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions 20 reactions	C4040-10 C4040-03
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions 20 reactions	C4040-50 C4040-52
LB Broth (1X), liquid	500 mL	10855-021
Ampicillin, sodium salt	200 mg	11593-027
Geneticin® Selective Antibiotic, liquid	20 mL	10131-035
PureLink® HQ Plasmid Miniprep Kit	100 preps	K2100-01
PureLink® HiPure DNA Midiprep Kit	25 preps	K2100-04
PureLink® Quick Gel Extraction System	1 kit	K2100-12
E-Gel® 1.2% Starter Pak (6 gels + Powerbase™)	1 kit	G6000-01
E-Gel® 1.2% 18 Pak	18 gels	G5018-01
FreeStyle™ MAX Transfection Reagent	1 mL	16447-100
FreeStyle™ CHO cells	1×10^7 cells	R800-07
FreeStyle™ 293-F cells	1×10^7 cells	R790-07
FreeStyle™ MAX CHO Expression System	1 kit	K9000-20
FreeStyle™ CHO Expression Medium	1 L 6 × 1 L	12651-014 12651-022
FreeStyle™ MAX 293 Expression System	1 kit	K9000-10
FreeStyle™ MAX 293 Expression Medium	1 L 6 × 1 L	12338-018 12338-026
OptiPRO™ SFM (1X)	100 mL	12309-050
OptiMEM® I Reduced-Serum Medium	100 mL	31985-062
FluoReporter® *lacZ*/Galactosidase Quantitation Kit	1000 assays	F-2905
Freedom™ DG44™ Kit	1 kit	A13737-01

Technical Support

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- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
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- Download software updates and patches

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Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit www.lifetechnologies.com/support or email techsupport@lifetech.com

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