USER GUIDE

invitrogen™
by life technologies™

pcDNA™3.4-TOPO® TA Cloning® Kit

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

Catalog Number A14697

Publication Number MAN0007209

Revision 2.0

For Research Use Only. Not for use in diagnostic procedures.

life technologies™

Information in this document is subject to change without notice.

DISCLAIMER

LIFE TECHNOLOGIES CORPORATION AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

NOTICE TO PURCHASER: LIMITED USE LABEL LICENSE: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008.

NOTICE TO PURCHASER: LIMITED USE LABEL LICENSE No. 308: WPRE Element

Notice to Purchaser: This product contains the Woodchuck Post-transcriptional Regulatory Element ("WPRE") which is the subject of intellectual property owned by The Salk Institute for Biological Studies, and licensed to Life Technologies Corporation. 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; and/or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. In addition, any use of WPRE outside of this product or the product's authorized use requires a separate license from the Salk Institute. Life Technologies will not assert a claim against the buyer of infringement of patents owned by Life Technologies and claiming 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 or for a Commercial Purpose. 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 on purchasing a license to this product for purposes other than research, contact Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008, Phone (760) 603-7200. Fax (760) 602-6500, or The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, Attn.: Office of Technology Management, Phone: (858) 453-4100 extension 1275, Fax: (858) 546-8093.

TRADEMARKS

The trademarks mentioned herein are the property of Life Technologies Corporation and/or its affiliate(s) or their respective owners.
Triton is a registered trademark of Union Carbide Corporation.

Table of Contents

Product Information 2

Kit Contents and Storage 2

Description of the System 4

Methods 6

Designing PCR Primers 6

Producing PCR Products 8

TOPO® Cloning Reaction 10

Transforming One Shot® Competent *E. coli* 12

Analyzing Positive Clones 14

Troubleshooting 15

Appendix A 17

Performing the Control Reactions 17

Addition of 3′ A-Overhangs Post-Amplification 19

Recipes 20

Map and Features of pcDNA™3.4-TOPO® Vector 21

Appendix B 23

Ordering Information 23

Documentation and Support 25

References 26

Product Information

Kit Contents and Storage

Shipping and Storage

The pcDNA™3.4-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™3.4-TOPO® TA Cloning® Reagents	–20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	–80°C

TOPO® TA Cloning Reagents

The pcDNA™3.4-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
pcDNA™3.4-TOPO® vector	TOPO® adapted, linearized plasmid DNA (5–10 ng/µL) in: 50% glycerol 50 mM Tris-HCl, pH 7.4 1 mM EDTA 1mM DTT 0.1% Triton® X-100 solution 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
pcDNA™3.4 reverse sequencing primer	2 µg/µL in TE buffer, pH 8.0	20 µL

***TE buffer, pH 8.0:** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Continued on next page

Kit Contents and Storage, continued

Primers

The pcDNA™3.4-TOPO® TA Vector Kit contains the following primers to sequence your insert.

Primer	Sequence
CMV forward	5′–CGC AAA TGG GCG GTA GGC GTG–3′
pcDNA™3.4 reverse	5′–CAA CAT AGT TAA GAA TAC CAG TC–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*	—	21 × 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

Product Use

For Research Use Only. Not for use in diagnostic procedures.

Description of the System

pcDNA™3.4-TOPO® Vector

The pcDNA™3.4-TOPO® vector allows the rapid TOPO® cloning of a PCR product containing a gene of interest downstream of the CMV promoter for high level expression of the native protein in adherent mammalian cell cultures, or for high level expression of secreted, native protein in suspension mammalian cell cultures following transient transfection.

Features of the Vector

The pcDNA™3.4-TOPO® vector contains the following elements:

- WPRE (Woodchuck posttranscriptional regulatory element) downstream of the cloning site to enhance transcript expression
- 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 and features of the pcDNA™3.4-TOPO® vector, see page 21.

CMV Promoter

The human cytomegalovirus immediate-early (HCMV IE1) gene promoter in the pcDNA™3.4-TOPO® vector is 680 bp and contains the native transcriptional start site (Hennighausen & Fleckenstein, 1986). This sequence results in high levels of transgene expression.

Continued on next page

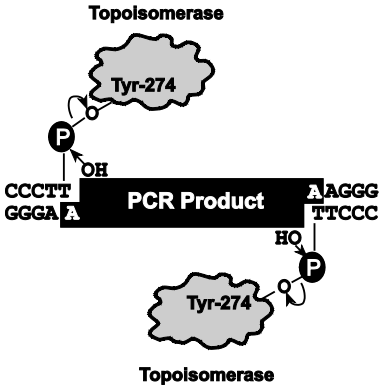
How TOPO® Works

The pcDNA™3.4-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™3.4-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.

Experiment Outline for Cloning

To TOPO® Clone your gene of interest into the pcDNA™3.4-TOPO® vector, 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™3.4-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.

Methods

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™3.4-TOPO® vector.

Points to Consider When Designing PCR Primers

To obtain the pcDNA™3.4-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

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

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.

IMPORTANT! 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™3.4-TOPO® vector. The complete vector sequence is available from **www.lifetechnologies.com** or by contacting Technical Support (page 25).

CMV Forward Primer Binding Site

```
571  TCCGCCCCAT TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA TATAAGCAGA GCTCGTTTAG TGAACCGTCA
     AGGCGGGGTA ACTGCGTTTA CCCGCCATCC GCACATGCCA CCCTCCAGAT ATATTCGTCT CTAGCAAATC ACTTGGCAGT

651  GATCGCCTGG AGACGCCATC CACGCTGTTT TGACCTCCAT AGAAGACACC GGGACCGATC CAGCCTCCGG ACTCTAGAGG
     CTAGCGGACC TCTGCGGTAG GTGCGACAAA ACTGGAGGTA TCTTCTGTGG CCCTGGCTAG GTCGGAGGCC TGAGATCTCC

731  ATCGAACCCT T                    AGGGTTCG ATCCCTACCG GTTAGTAATG AGTTTGATAT CTCGACAATC
     TAGCTTGGGA      PCR Product     TTCCCAAGC TAGGGATGGC CAATCATTAC TCAAACTATA GAGCTGTTAG

791  AACCTCTGGA TTACAAAATT TGTGAAAGAT TGACTGGTAT TCTTAACTAT GTTGCTCCTT TTACGCTATG TGGATACGCT
     TTGGAGACCT AATGTTTTAA ACACTTTCTA ACTGACCATA AGAATTGATA CAACGAGGAA AATGCGATAC ACCTATGCGA
                                     pcDNA™ Reverse Primer Binding Site
```

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™3.4-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 available from Life Technologies (see page 23 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 method 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°C 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.

Continued on next page

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

- The PCR Optimizer™ Kit from Life Technologies can help you optimize your PCR to eliminate multiple bands and smearing. See page 23 for ordering information.
- Gel-purify your fragment before performing the TOPO® Cloning reaction using the E-Gel® CloneWell™ system or PureLink® Gel Extraction Kit, available separately from Life Technologies. See page 24 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.

TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pcDNA™3.4-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 page 17 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 23 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 Life Technologies; see page 23), 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 page 11.

Continued on next page

TOPO® Cloning Reaction, continued

Materials Needed

- Your PCR product (freshly prepared)
- pcDNA™3.4-TOPO® vector
- Salt Solution or Dilute Salt Solution (see page 10)
- 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	0.5–4 µL	0.5–4 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°C–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

After 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 *E. coli* cells if desired (see page 23 for ordering information). Protocols for transforming chemically competent *E. coli* are provided below. For transforming electrocompetent *E. coli*, refer to the instruction provided with the electrocompetent cells.

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 13.
Clone large PCR products (>1000 bp)	
Obtain transformants as quickly as possible	Rapid chemical transformation protocol, page 13. **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, page 11)
- One Shot® TOP10 *E. coli*, either chemically competent (supplied with the kit, Box 2)
- 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)
- 2 selective LB plates containing 100 µg/mL ampicillin for each transformation. See page 20 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*
- 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, prewarm 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™3.4-TOPO® construct into *E. coli*, 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 Life Technologies' PureLink® HQ Mini Plasmid Purification Kit. See page 24 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 pcDNA™3.4 reverse primers included with the kit. Refer to page 3 for the sequences of the primers and the diagram on page 7 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

After obtaining the correct pcDNA™3.4-TOPO® plasmid construct, linearize and purify the plasmid prior to transfection into adherent or suspension cells. The pcDNA™3.4-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 24 for ordering information).

If you are using the Expi293™ Expression System Kit (Cat. no. A14635), refer to the user guide supplied with the system for specific instructions on transfecting high-density suspension Expi293F™ Cells using the ExpiFectamine™ 293 Reagent.

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 19.
	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 reaction 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. *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). • You may redesign your primers so that they contain a 5′ G instead of a 5′ T.

Continued on next page

Troubleshooting, continued

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 6), resynthesize your DNA, and reclone.
	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 A

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 20 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 PCR tube, set up the following 50-µL reaction.

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.

Continued on next page

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™3.4-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 12.
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. Be sure to 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 12. 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.

Addition 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.

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)

Protocol

This is just one method for adding 3´ A-overhangs. Other protocols may also 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 cloning into pcDNA™3.4-TOPO® vector.

   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.

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 of 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 of 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.

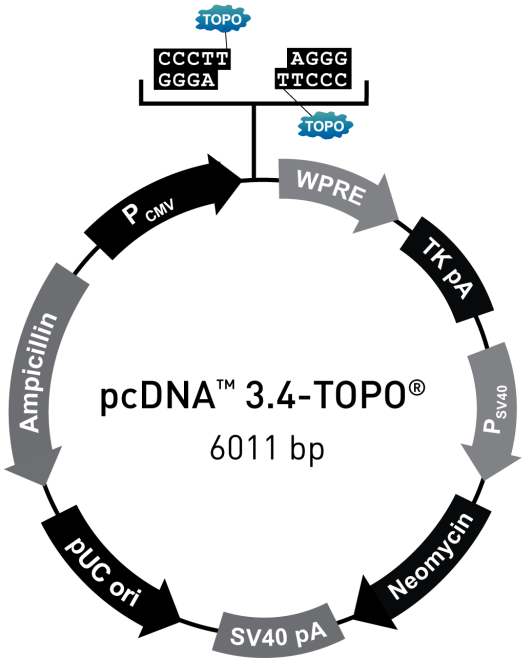
X-Gal Stock Solution

1. To make a 40 mg/mL stock solution, dissolve 400 mg of X-Gal in 10 mL of 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.

Map and Features of pcDNA™3.4-TOPO® Vector

Map of pcDNA™3.4-TOPO® vector

The map below shows the elements of the pcDNA™3.4-TOPO® vector. The vector sequence of pcDNA™3.4-TOPO® is available for downloading from **www.lifetechnologies.com** or by contacting Technical Support (page 25).



Features of pcDNA™ 3.4-TOPO®

6011 nucleotides

CMV promoter (P_{CMV}):	47–726
CMV for primer binding site:	584–604
TOPO® cloning site:	741
WPRE:	782–1379
pcDNA™ 3.4 rev primer binding site:	822–844 (c)*
TK polyadenylation signal (TK pA):	1384–1655
SV40 early promoter (P_{SV40}):	2124–2493
Neomycin resistance gene:	2529–3323
SV40 polyadenylation site (SV40 pA):	3499–3629
pUC origin:	4012–4685 (c)
Ampicillin resistance gene:	4830–5690 (c)
bla promoter:	5691–5789 (c)

*(c): complementary strand

Continued on next page

Map and Features of pcDNA™3.4-TOPO® Vector, continued

Features of pcDNA™3.4-TOPO® vector

The pcDNA™3.4-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)
WPRE (Woodchuck Posttranscriptional Regulatory Element)	Provides for increased transgene expression (Zufferey *et al.*, 1998)
CMV forward primer	Allows sequencing of the insert
TOPO® Cloning site	Allows insertion of your PCR product
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*

Appendix B

Ordering Information

Accessory Products

The following reagents are suitable for use with the pcDNA™3.4-TOPO® TA Cloning Kit and are available separately from Life Technologies. Ordering information is provided below. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (see page 25).

Item	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
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 Media	500 mL	10855-021
Ampicillin, sodium salt	200 mg	11593-027
Geneticin® Selective Antibiotic, liquid	20 mL	10131-035
E-Gel® 1.2% Starter Pak (6 gels + Powerbase™)	1 kit	G6000-01
E-Gel® 1.2% 18 Pak	18 gels	G5018-01
E-Gel® CloneWell™ 0.8% SYBR Safe™ Gel and iBase™ Starter Kit	1 kit	G6400ST
PureLink® Quick Gel Extraction System	1 kit	K2100-12
PCR Optimizer™ Kit	1 kit	K1220-01

Continued on next page

Plasmid Purification Products

The following plasmid purification products suitable for use with the kit are available separately from Life Technologies. Ordering information is provided below. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (see page 25).

Item	Amount	Catalog no.
Purelink® HiPure Plasmid Midiprep Kit	25 preps	K2100-14
PureLink® HiPure Plasmid Filter Midiprep Kit	25 preps	K2100-04
PureLink® HiPure Plasmid Maxiprep Kit	10 preps	K2100-06
PureLink® HiPure Plasmid Filter Maxiprep Kit	10 preps	K2100-16
PureLink® HiPure Plasmid Megaprep Kit	4 preps	K2100-08

Expi293™ Expression System and Related Products

The following reagents supplied in the Expi293™ Expression System and various other accessory products suitable for use with the kit are available separately from Life Technologies. Ordering information is provided below. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (see page 25).

Item	Amount	Catalog No.
Expi293F™ Cells (1×10^7 cells/vial)	1 vial	A14527
Expi293F™ Cells, 6 vial "Cell Bank" pack (1×10^7 cells/vial)	6 vials	A14528
Expi293™ Expression Medium	1000 mL 6 × 1000 mL	A14351-01 A14351-02
ExpiFectamine™ 293 Transfection Kit for 1 L of culture	1 kit	A14524
ExpiFectamine™ 293 Transfection Kit for 10 L of culture	1 kit	A14525
ExpiFectamine™ 293 Transfection Kit for 50 L of culture (5 × 10 L kit)	1 kit	A14526
Opti-MEM® I Reduced Serum Medium (1X), liquid	100 mL 500 mL	31985-062 31985-070
Opti-MEM® I Reduced Serum Medium (1X), liquid (for E.U. customers only)	500 mL	31985-047
Antibody Expressing Positive Control Vector (at 1 mg/mL)	150 µg	A14662

Documentation and Support

Obtaining Support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/sds**.

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support.**

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*, Greene Publishing Associates and Wiley-Interscience, New York

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

Cole, C. N., and Stacy, T. P. (1985) Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. Mol. Cell. Biol. *5*, 2104-2113

Coloma, M. J., Hastings, A., Wims, L. A., and Morrison, S. L. (1992) Novel Vectors for the Expression of Antibody Molecules Using Variable Regions Generated by Polymerase Chain Reaction. J. Imm. Methods *152*, 89-104

Hennighausen, L., and Fleckenstein, B. (1986) Nuclear factor 1 interacts with five DNA elements in the promoter region of the human cytomegalovirus major immediate early gen. Embo J *5*, 1367-1371

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

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

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

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987) Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129

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

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

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

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998) Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. J. Virol. 72. 9873-9880

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

lifetechnologies.com

17 July 2013