invitrogen™

# TOPO TA Cloning® Kit for Sequencing

## Five-minute cloning of *Taq* polymerase-amplified PCR products for sequencing

**Catalog nos.** **K4530-20, K4575-J10, K4575-01, K4575-40, K4580-01, K4580-40, K4595-01, K4595-40, K4575-02**

**Version O**
10 April 2006
*25-0276*

User Manual

# Table of Contents

# Kit Contents and Storage

## Shipping and Storage

The TOPO TA Cloning® Kits for Sequencing are shipped on dry ice. Each kit contains a box with TOPO TA Cloning® reagents (Box 1) and a box with One Shot® Competent *E. coli* (Box 2).

TOPO TA Cloning® Kit for Sequencing supplied with the PureLink™ Quick Plasmid Miniprep (cat. no.K4575-02) is shipped with an additional box containing reagents for plasmid purification (Box 3).

**Store Box 1 at -20°C, Box 2 at -80°C, and Box 3 at room temperature.**

## Types of Kits

TOPO TA Cloning® Kits for Sequencing are available with either Mach1™-T1$^R$, TOP10, or DH5α-T1$^R$ One Shot® Chemically Competent cells or TO10 One Shot® Electrocomp™ cells (see page vi for the genotypes of the strains). Catalog no. K4575-02 also includes PureLink™ Quick Plasmid Miniprep Kit.

| Catalog no. | One Shot® Cells | Reactions |
|---|---|---|
| K4530-20 | Mach1™-T1$^R$ Chemically Competent | 20 |
| K4575-J10 | TOP10 Chemically Competent | 10 |
| K4575-01 | TOP10 Chemically Competent | 20 |
| K4575-40 | TOP10 Chemically Competent | 40 |
| K4595-01 | DH5α-T1$^R$ Chemically Competent | 20 |
| K4595-40 | DH5α-T1$^R$ Chemically Competent | 40 |
| K4580-01 | TOP10 Electrocomp™ | 20 |
| K4580-40 | TOP10 Electrocomp™ | 40 |
| K4575-02* | TOP10 Chemically Competent | 20 |

*Includes PureLink™ Quick Plasmid Miniprep Kit

## TOPO TA Cloning® Reagents

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 10 Reactions | Amount 20 Reactions | Amount 40 Reactions |
|---|---|---|---|---|
| pCR®4-TOPO® | 10 ng/µl plasmid DNA in:<br>50% glycerol<br>50 mM Tris-HCl, pH 7.4 (at 25°C)<br>1 mM EDTA<br>2 mM DTT<br>0.1% Triton X-100<br>100 µg/ml BSA<br>30 µM phenol red | 15 µl | 25 µl | 2 x 25 µl |

*continued on next page*

# Kit Contents and Storage, continued

### TOPO TA Cloning® Reagents, continued

| Item | Concentration | Amount 10 Reactions | Amount 20 Reactions | Amount 40 Reactions |
|---|---|---|---|---|
| 10X PCR Buffer | 100 mM Tris-HCl, pH 8.3 (at 42°C)<br>500 mM KCl<br>25 mM $MgCl_2$<br>0.01% gelatin | 100 µl | 100 µl | 2 x 100 µl |
| dNTP Mix | 12.5 mM dATP, 12.5 mM dCTP<br>12.5 mM dGTP, 12.5 mM dTTP<br>neutralized at pH 8.0 in water | 10 µl | 10 µl | 2 x 10 µl |
| Salt Solution | 1.2 M NaCl<br>0.06 M $MgCl_2$ | 50 µl | 50 µl | 2 x 50 µl |
| Water | -- | 1 ml | 1 ml | 2 x 1 ml |
| M13 Forward (-20) Primer | 0.1 µg/µl in TE Buffer, pH 8 | 20 µl<br>(407 pmoles) | 20 µl<br>(407 pmoles) | 2 x 20 µl<br>(814 pmoles) |
| M13 Reverse Primer | 0.1 µg/µl in TE Buffer, pH 8 | 20 µl<br>(385 pmoles) | 20 µl<br>(385 pmoles) | 2 x 20 µl<br>(770 pmoles) |
| T3 primer | 0.1 µg/µl in TE Buffer, pH 8 | 20 µl<br>(329 pmoles) | 20 µl<br>(329 pmoles) | 2 x 20 µl<br>(658 pmoles) |
| T7 primer | 0.1 µg/µl in TE Buffer, pH 8 | 20 µl<br>(328 pmoles) | 20 µl<br>(328 pmoles) | 2 x 20 µl<br>(656 pmoles) |
| Control PCR Template | 0.1 µg/µl in TE Buffer, pH 8 | 10 µl | 10 µl | 2 x 10 µl |
| Control PCR Primers | 0.1 µg/µl **each** in TE Buffer, pH 8 | 10 µl | 10 µl | 2 x 10 µl |

### Sequence of Primers

The table below lists the sequence of the sequencing primers included in the kit.

| Primer | Sequence |
|---|---|
| M13 Forward (-20) | 5´-GTAAAACGACGGCCAG-3´ |
| M13 Reverse | 5´-CAGGAAACAGCTATGAC-3´ |
| T3 | 5´-ATTAACCCTCACTAAAGGGA-3´ |
| T7 | 5´-TAATACGACTCACTATAGGG-3´ |

*continued on next page*

# Kit Contents and Storage, continued

## One Shot® Reagents

The tables below describe the items included in each One Shot® Kit.

**Store at -80°C.**

| Item | Composition | Amount 10 Reactions | Amount 20 Reactions | Amount 40 Reactions |
|---|---|---|---|---|
| S.O.C. Medium<br>(may be stored at +4°C or room temperature) | 2% Tryptone<br>0.5% Yeast Extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM $MgCl_2$<br>10 mM $MgSO_4$<br>20 mM glucose | 6 ml | 6 ml | 2 x 6 ml |
| Mach1™-T1$^R$, TOP10, or DH5α™-T1$^R$ cells<br>**OR**<br>TOP10 cells | Chemically Competent<br><br>Electrocomp™ | 11 x 50 µl | 21 x 50 µl | 41 x 50 µl |
| pUC19 Control DNA | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 | 50 µl | 50 µl | 2 x 50 µl |

## Genotypes

**TOP10**: Use this strain for general cloning and blue/white screening without IPTG.

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

**Mach1™-T1$^R$:** Use this strain for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage.

F$^-$ Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R($r_k^-$, $m_k^+$) Δ*recA*1398 *end*A1 *ton*A (confers resistance to phage T1)

**DH5α-T1$^R$:** Use this strain for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage.

F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17($r_k^-$, $m_k^+$) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A (confers resistance to phage T1)

## Information for Non-U.S. Customers Using Mach1™-T1$^R$ Cells

The parental strain of Mach1™-T1$^R$ *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S. A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

*continued on next page*

# Kit Contents and Storage, continued

### PureLink™ Quick Plasmid Miniprep Kit

For kit components of the PureLink™ Quick Plasmid Miniprep Kit (Box 3) supplied with cat. no. K4575-02, refer to the manual supplied with the miniprep kit.

# Accessory Products

## Additional Products

The table below lists additional products that may be used with TOPO® TA Cloning Kits for Sequencing. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 27).

| Item | Amount | Catalog no. |
|---|---|---|
| *Taq* DNA Polymerase, Native | 100 units | 18038-018 |
| | 500 units | 18038-042 |
| *Taq* DNA Polymerase, Recombinant | 100 units | 10342-053 |
| | 500 units | 10342-020 |
| Platinum® *Taq* DNA Polymerase High Fidelity | 100 units | 11304-011 |
| One Shot® TOP10 Chemically Competent *E. coli* | 10 reactions | C4040-10 |
| | 20 reactions | C4040-03 |
| | 40 reactions | C4040-06 |
| One Shot® TOP10 Electrocompetent *E. coli* | 10 reactions | C4040-50 |
| | 20 reactions | C4040-52 |
| One Shot® Mach1™-T1$^R$ Chemically Competent *E. coli* | 20 reactions | C8620-03 |
| One Shot® MAX Efficiency® DH5α-T1$^R$ Chemically Competent *E. coli* | 20 reactions | 12297-016 |
| PureLink™ Quick Plasmid Miniprep Kit | 50 reactions | K2100-10 |
| PureLink™ Quick Gel Extraction Kit | 50 reactions | K2100-12 |
| Ampicillin | 200 mg | 11593-019 |
| Kanamycin | 5 g | 11815-024 |
| | 25 g | 11815-032 |
| | 100 ml (10 mg/ml) | 18160-054 |
| S.O.C. Medium | 10 x 10 ml | 15544-034 |

# Methods

## Overview

### Introduction

The TOPO TA Cloning® Kits for Sequencing provide 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 for sequencing. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

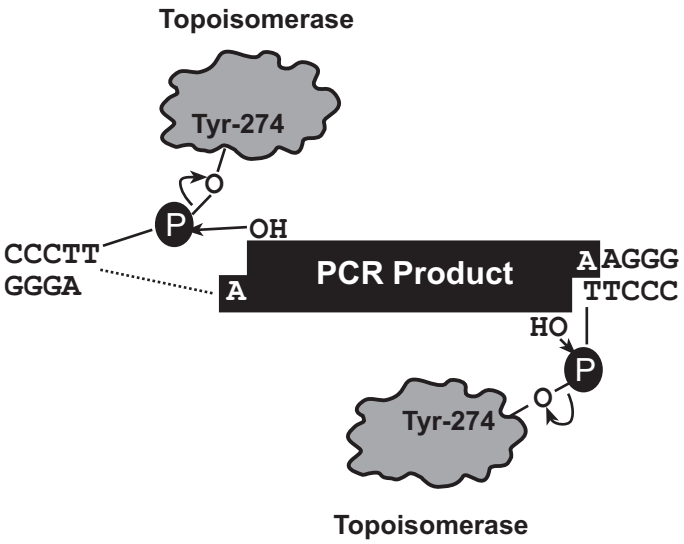
### How It Works

The plasmid vector (pCR®4-TOPO®) is supplied linearized with:

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

*Taq* polymerase has a nontemplate-dependent terminal transferase activity, which 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).




*continued on next page*

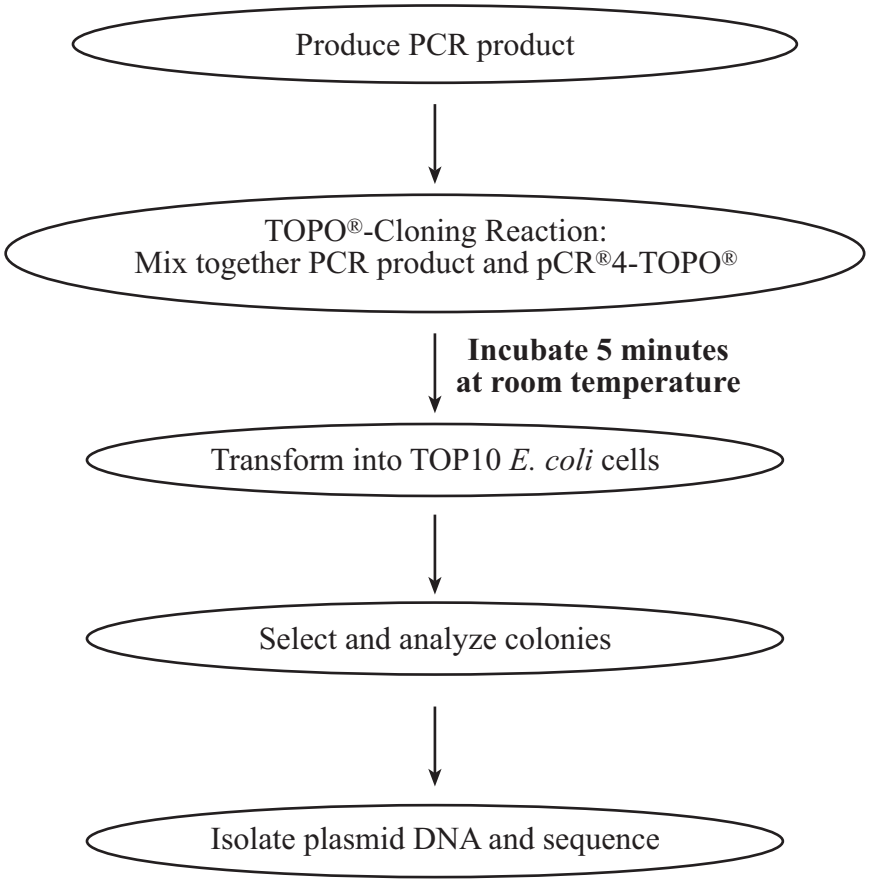
# Overview, continued

## Positive Selection

pCR®4-TOPO® allows direct selection of recombinants via disruption of the lethal *E. coli* gene, *ccd*B (Bernard and Couturier, 1992; Bernard *et al.*, 1994; Bernard *et al.*, 1993). The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment. Ligation of a PCR product disrupts expression of the *lac*Zα-*ccd*B gene fusion permitting growth of only positive recombinants upon transformation in TOP10 cells. Cells that contain non-recombinant vector are killed upon plating. Therefore, blue/white screening is not required.

## Experimental Outline

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



# Producing PCR Products

## Introduction

The TOPO TA Cloning® Kits for Sequencing are specifically designed to clone *Taq* polymerase-generated PCR products for sequencing. The first time you use the kit, we recommend performing the control TOPO® Cloning reaction on page 16 to evaluate your results.

**Note**

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®4-TOPO®.

## Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- *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.

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 21.

## Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use the cycling parameters suitable for your primers and template and be sure to end with a 7 to 30 minute extension at 72°C to ensure that all PCR products are full length and 3´ adenylated. Use less for plasmid template DNA and more DNA for genomic template DNA.

| | |
|---|---|
| DNA Template | 10-100 ng |
| 10X PCR Buffer | 5 µl |
| 50 mM dNTPs | 0.5 µl |
| Primers (~200 ng each) | 1 µM each |
| Water | add to a final volume of 49 µl |
| *Taq* Polymerase (1 unit/µl) | 1 unit |
| 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 TOPO® Cloning (see page 19). Take special care to avoid sources of nuclease contamination. Alternatively, you may elect to optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) incorporates many of the recommendations found in this reference.

# Performing the TOPO® Cloning Reaction

## Introduction

At this point you should have your PCR product ready for TOPO® Cloning and transformation into the One Shot® TOP10 cells. It is very important to proceed as soon as possible from the TOPO® Cloning reaction to transformation to ensure the highest cloning and transformation efficiencies.

**Note**

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction can increase 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 earlier 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 Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells or electrocompetent cells.**

- If you are transforming chemically competent *E. coli*, 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*, 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*

# Performing the TOPO® Cloning Reaction, continued

## Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction using the reagents in the order shown, and depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.*

**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 |
| Water | add to a final volume of 5 µl | add to a final volume of 5 µl |
| TOPO® vector | 1 µl | 1 µl |
| **Final Volume** | **6 µl** | **6 µl** |

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

1. Mix reaction gently and incubate for **5 minutes** at room temperature (22-23°C).

   **Note**: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 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 **General Guidelines for Transforming Competent Cells**, next page.

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

# General Guidelines for Transforming Competent Cells

## Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pCR®4-TOPO® construct into the competent *E. coli* provided with your kit. General guidelines for transformation are provided below. For transformation protocols, refer to the section entitled **Transforming One Shot® Mach1™-T1$^R$ Competent Cells** (pages 7-8) or **Transforming One Shot® TOP10 and DH5α™-T1$^R$ Competent Cells** (pages 9-11) depending on the competent *E. coli* you wish to transform.

## Selecting a One Shot® Chemical Transformation Protocol

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

| If you wish to… | Then use the… |
|---|---|
| maximize the number of transformants | regular chemical transformation protocol |
| clone large PCR products (>1000 bp) | |
| use kanamycin as the selective agent (see Important note below) | |
| obtain transformants as quickly as possible | rapid chemical transformation protocol |

**Important**

If you will be using kanamycin as the selective agent for chemical transformation, use the regular chemical transformation protocol. The rapid chemical transformation protocol is only suitable for transformations using ampicillin selection.


If you use a plasmid template for your PCR that carries either the ampicillin or kanamycin resistance marker, we recommend that you use the other selection agent to select for transformants. For example, if the plasmid template contains the ampicillin resistance marker, then use kanamycin to select for transformants. The template is carried over into the TOPO® Cloning and transformation reactions, resulting in transformants that are ampicillin resistant and white, but are not the desired construct.

# Transforming One Shot® Mach1™-T1R Competent Cells

## Introduction

Protocols to transform One Shot® Mach1™-T1R chemically competent *E. coli* are provided below. If are transforming cells other than Mach1™-T1R cells, refer to the section entitled **Transforming One Shot® TOP10 and DH5α™-T1R Competent Cells** (pages 9-11).

**Note**

The Mach1™-T1R strain allows you to visualize colonies 8 hours after plating on ampicillin selective plates. If you are using kanamycin selection, you will need to incubate plates overnight in order to visualize colonies.

With the Mach1™-T1R strain, you may also prepare plasmid DNA 4 hours after inoculating a single, overnight-grown colony. Note that you will get sufficient growth of transformed cells within 4 hours in either ampicillin or kanamycin selective media.

## Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2 (page 5)
- S.O.C. medium (included with the kit)
- LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin
- 42°C water bath
- 37°C shaking and non-shaking incubator

## Preparing for Transformation

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

- Equilibrate a water bath to 42°C.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes (see Important note below).
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

**Important**

If you are performing the rapid chemical transformation protocol or if you wish to visualize colonies within 8 hours of plating, it is essential that you prewarm your LB plates containing 50-100 µg/ml ampicillin prior to spreading.

*continued on next page*

# Transforming One Shot® Mach1™-T1R Competent Cells, continued

## One Shot® Chemical Transformation Protocol

For optimal growth of Mach1™-T1R *E. coli* cells, it is essential that selective plates are prewarmed to 37°C prior to spreading.

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® 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 do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a **prewarmed** selective plate. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Incubate plates at 37°C. If you are using ampicillin selection, visible colonies should appear within 8 hours. For kanamycin selection, incubate plates overnight.
9. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing Positive Clones**, page 12).

## Rapid One Shot® Chemical Transformation Protocol

An alternative protocol is provided below for rapid transformation of One Shot® Mach1™-T1R cells. This protocol is **only** recommended for transformations using **ampicillin** selection. For more information on selecting a transformation protocol, refer to page 6.

**Note:** It is essential that LB plates containing ampicillin are prewarmed to 37°C prior to spreading.

1. Add **4 µl** of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µl** of cells on a prewarmed LB plate containing 50-100 µg/ml ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing Positive Clones**, page 12).

# Transforming One Shot® TOP10 and DH5α™-T1$^R$ Competent Cells

## Introduction

Protocols to transform One Shot® TOP10 and DH5α™-T1$^R$ competent *E. coli* are provided below. Both chemical transformation and electroporation protocols are provided. If you are transforming Mach1™-T1$^R$ cells, refer to the section entitled **Transforming One Shot® Mach1™-T1$^R$ Competent Cells** (pages 7-8).

## Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2 (page 5)
- S.O.C. medium (included with the kit)
- LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin
- 15 ml snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath or an electroporator and 0.1 or 0.2 cm cuvettes
- 37°C shaking and non-shaking incubator

## Preparing for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes (see Important note below).
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

**Important**

If you are performing the rapid chemical transformation protocol, it is essential that you prewarm your LB plates containing 50-100 µg/ml ampicillin prior to spreading.

*continued on next page*

# Transforming One Shot® TOP10 and DH5α™-T1R Competent Cells, continued

## One Shot® Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® 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 do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 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 S.O.C. medium 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 should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing Positive Clones**, page 12).

## Rapid One Shot® Chemical Transformation Protocol

An alternative protocol is provided below for rapid transformation of One Shot® chemically competent *E. coli*. This protocol is **only** recommended for transformations using **ampicillin** selection. For more information on selecting a transformation protocol, refer to page 6.

**Note:** It is essential that LB plates containing ampicillin are prewarmed prior to spreading.

1. Add **4 µl** of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µl** of cells on a prewarmed LB plate containing 50-100 µg/ml ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing Positive Clones**, page 12).

*continued on next page*

# Transforming One Shot® TOP10 and DH5α™-T1$^R$ Competent Cells, continued

## One Shot® Electroporation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Carefully transfer solution to a 0.1 cm cuvette to avoid formation of bubbles.
3. Electroporate your samples using your own protocol and your electroporator.

   **Note**: If you have problems with arcing, see below.
4. Immediately add 250 µl of room temperature S.O.C. medium.
5. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance genes.
6. 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 S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
7. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing 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, try **one** of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

# Analyzing Transformants

## Analyzing Positive Clones

1. Take 2-6 colonies and culture them overnight in LB or SOB medium containing 50-100 µg/ml ampicillin or 50 µg/ml kanamycin.

   **Note:** If you transformed One Shot® Mach1™-T1$^R$ competent *E. coli*, you may inoculate overnight-grown colonies and culture them for 4 hours in **prewarmed** LB medium containing 50 µg/ml ampicillin or 50 µg/ml kanamycin before isolating plasmid. For optimal results, we recommend inoculating as much of a single colony as possible.

2. Isolate plasmid DNA using PureLink™ Quick Plasmid Miniprep Kit (supplied with cat. no. K4575-02 or available separately, page viii). The plasmid isolation protocol is included in the manual supplied with the PureLink™ Quick Plasmid Miniprep Kit and is also available for downloading from www.invitrogen.com. Other kits for plasmid DNA purification are also suitable for use.

3. Analyze the plasmids for inserts by restriction analysis (digest with *Eco*R I or refer to the vector map on page 15) or by PCR screening (see next page). You may also proceed directly to sequencing.

## Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation. Four primers (M13 Forward (-20), M13 Reverse, T3, and T7) are included to help you sequence your insert. Refer to the map on page 15 for the sequence surrounding the TOPO® Cloning site. For the full sequence of the vector, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 27).

If you discover that the primers included in the kit do not allow you to completely sequence your insert, you may try one or both of the following:

- Synthesize additional primers to sequence into the insert
- Prepare a set of nested deletions (refer to the protocol on page 22)

If you need help with sequencing, refer to general texts (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989) or the manufacturer of your sequencing enzyme.

*continued on next page*

# Analyzing Transformants, continued

## Analyzing Transformants by PCR

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use one of the four primers in the kit and a primer that hybridizes within your insert. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol is provided below for your convenience. Other protocols are suitable.

**Materials Needed**

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

Appropriate forward and reverse PCR primers (20 µM each)

**Procedure**

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 10 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above. 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.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

## 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. It is also a good idea to store a stock of purified DNA at -20°C.

1. Streak the original colony out for single colony on LB plates containing 100 µg/ml ampicillin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 100 µg/ml ampicillin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. 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 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

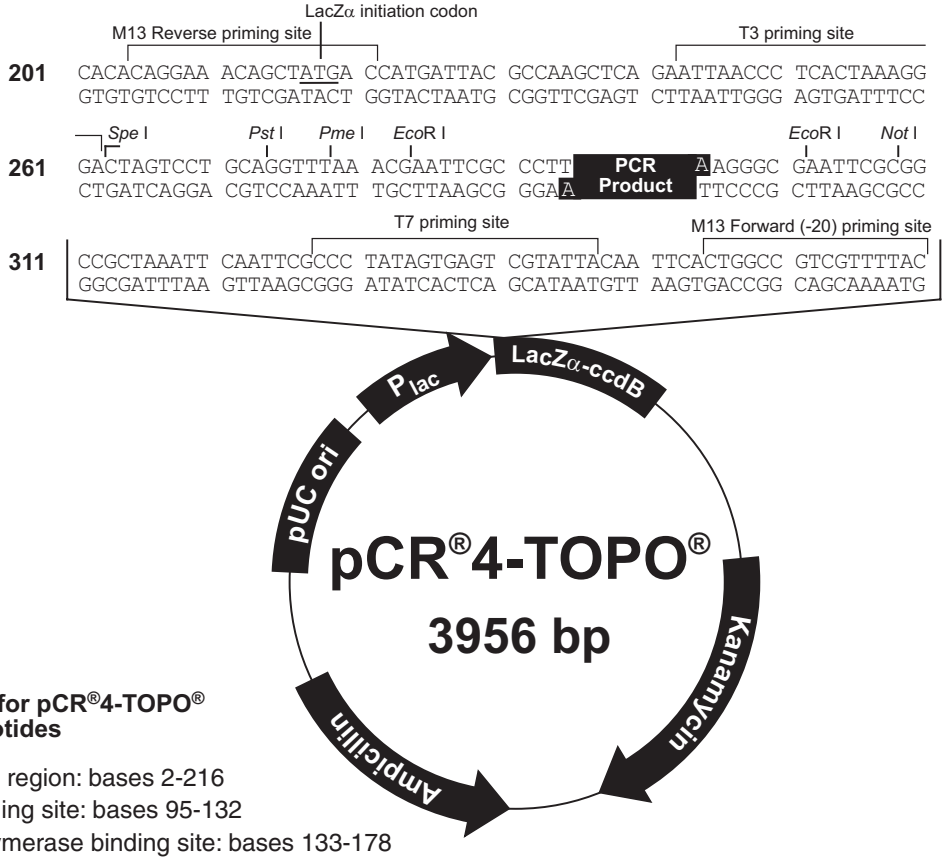
To clone dilute PCR products, you may:

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

# Map of pCR®4-TOPO®

## pCR®4-TOPO® Map

The map below shows the features of pCR®4-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. **The complete sequence of pCR®4-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 27).**




**Comments for pCR®4-TOPO®**
**3956 nucleotides**

*lac* promoter region: bases 2-216
  CAP binding site: bases 95-132
  RNA polymerase binding site: bases 133-178
  Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
  LacZα portion of fusion: bases 217-497
  *ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

# Performing the Control Reactions

## Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using it directly in a TOPO® Cloning reaction.

## Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

**Note**: Do not use plates containing ampicillin. The control template is a plasmid that encodes ampicillin resistance. This template is carried over into the TOPO® Cloning and transformation reactions. Transformants carrying this plasmid will also be ampicillin resistant, resulting in an apparent increase in TOPO® Cloning efficiency, but upon analysis, colonies do not contain the desired construct.

## Producing Control PCR Product

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

| | |
|---|---|
| Control DNA Template (100 ng) | 1 µl |
| 10X PCR Buffer | 5 µl |
| 50 mM dNTPs | 0.5 µl |
| Control PCR Primers (0.1 µg/µl **each**) | 1 µl |
| Water | 41.5 µl |
| *Taq* Polymerase (1 unit/µl) | 1 µl |
| Total Volume | 50 µl |

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

| Step | Time | Temperature | Cycles |
|---|---|---|---|
| Initial Denaturation | 2 minute | 94°C | 1X |
| Denaturation | 1 minute | 94°C | 25X |
| Annealing | 1 minute | 55°C | |
| Extension | 1 minute | 72°C | |
| Final Extension | 7 minutes | 72°C | 1X |

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

*continued on next page*

# Performing the Control Reactions, continued

## Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and pCR®4-TOPO®, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions. **If transforming by electroporation, use a 4-fold dilution of the Salt Solution to prevent arcing (see page 11).**

| Reagent | "Vector Only" | "Vector + PCR Insert" |
|---|---|---|
| Control PCR Product | -- | 1 µl |
| Water | 4 µl | 3 µl |
| Salt Solution | 1 µl | 1 µl |
| pCR®4-TOPO® | 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 (pages 7-11).
4. Spread 10-100 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin. Be sure to plate two different volumes to ensure well-spaced colonies. For plating small volumes (< 20 µl), add 20 µl of S.O.C. medium to allow even spreading.

   **Note: Do not use ampicillin to select for transformants.** The PCR product was generated from a template containing the ampicillin resistance gene. Carry-over from the PCR will produce transformants that are ampicillin-resistant but are not derived from pCR®4-TOPO®.
5. Incubate overnight at 37°C.

## Analyzing Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 90% (or more) of these will contain the 750 bp insert when analyzed by *Eco*R I digestion and agarose gel electrophoresis.

Relatively few colonies will be produced in the vector-only reaction. These colonies usually result from frameshift events (usually T-T mismatches) and results in disruption of the LacZα-*ccdB* reading frame.

## Transformation Control

The pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform with 10 pg per 50 µl of cells using the protocols on pages 7-11.

Use LB plates containing 100 µg/ml ampicillin. Just before plating the transformation mix for electrocompetent cells, dilute 10 µl of the mix with 90 µl of S.O.C. medium.

| Type of Cells | Volume to Plate | Transformation Efficiency |
|---|---|---|
| Chemically Competent | 10 µl + 20 µl S.O.C. | ~1 x $10^9$ cfu/µg DNA |
| Electrocompetent | 20 µl (1:10 dilution) | > 1 x $10^9$ cfu/µg DNA |

*continued on next page*

### Factors Affecting Cloning Efficiency

Note that lower cloning efficiencies will result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 95% (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 will need a longer extension time. |
| Cloning large inserts (>1 kb) | Increase amount of insert. Or gel purify as described on page 19. |
| 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 21) or use the Zero Blunt™ TOPO PCR Cloning Kit for Sequencing (Catalog no. K2875-20). |
| 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 19). |
| PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase | Increase the final extension time to ensure all 3´ ends are adenylated.<br>*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). |
| Cloning small PCR products (<100 bp) | Small PCR products may not completely disrupt the *lacZα-ccdB* gene fusion to allow growth of positive recombinants. Try TOP10F´ cells, which express the Lac repressor to repress expression of the fusion. Pick transformants and characterize. |

**Note**

Note that cloning efficiency may decrease with gel purification of the PCR product because of nuclease contamination or dilution of the DNA. You may wish to optimize your PCR to produce a single band.

# Appendix

## Purifying PCR Products

### Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 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. Two simple protocols are described below.

### Using the PureLink™ Quick Gel Extraction Kit

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

1. Equilibrate a water bath or heat block to 50°C.
2. Cut the area of the gel containing the desired DNA fragment using a clean, sharp blade. Minimize the amount of surrounding agarose excised with the fragment. Weigh the gel slice.
3. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
   - For ≤2% agarose gels, place up to 400 mg gel into a sterile, 1.5-ml polypropylene tube. Divide gel slices exceeding 400 mg among additional tubes. Add 30 µl Gel Solubilization Buffer (GS1) for every 10 mg of gel.
   - For >2% agarose gels, use sterile 5-ml polypropylene tubes and add 60 µl Gel Solubilization Buffer (GS1) for every 10 mg of gel.
4. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After gel slice appears dissolved, incubate for an **additional** 5 minutes.
5. Preheat an aliquot of TE Buffer (TE) to 65-70°C
6. Place a Quick Gel Extraction Column into a Wash Tube. Pipette the mixture from Step 4, above onto the column. Use 1 column per 400 mg agarose.
7. Centrifuge at >12,000 x g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
8. **Optional:** Add 500 µl Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at >12,000 x g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
9. Add 700 µl Wash Buffer (W9) with ethanol (add 96–100% ethanol to the Wash Buffer according to instructions on the label of the bottle) to the column and incubate at room temperature for 5 minutes. Centrifuge at >12,000 x g for 1 minute. Discard flow-through.
10. Centrifuge the column at >12,000 x g for 1 minute to remove any residual buffer. Place the column into a 1.5 ml Recovery Tube.
11. Add 50 µl **warm** (65-70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
12. Centrifuge at >12,000 x g for 2 minutes. *The Recovery Tube contains the purified DNA.* Store DNA at –20°C. Discard the column.
13. Use 4 µl of the purified DNA for the TOPO® Cloning reaction.

*continued on next page*

# Purifying PCR Products, continued

### Low-Melt Agarose Method

Note that gel purification will result in a dilution of your PCR product. Use only chemically competent cells for transformation.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Use 4 µl of the melted agarose containing your PCR product in the TOPO® Cloning reaction (page 5).
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 TOP10 One Shot® cells using the method on pages 7-11.

**Note**

Cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

# Addition of 3´ A-Overhangs Post-Amplification

## Introduction

Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is often difficult because of very low cloning efficiencies. This is because proofreading polymerases remove the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

## Materials Needed

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´ adenines. Other protocols may be suitable.

1. After amplification with the 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. A sufficient number of PCR products will retain the 3´ A-overhangs.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place on ice and use immediately in the TOPO® Cloning reaction.

**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.

**Note**

You may also gel-purify your PCR product after amplification with a proofreading polymerase. After purification, add *Taq* polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase. Incubate the reaction for 10-15 minutes at 72°C and use in the TOPO® Cloning reaction.

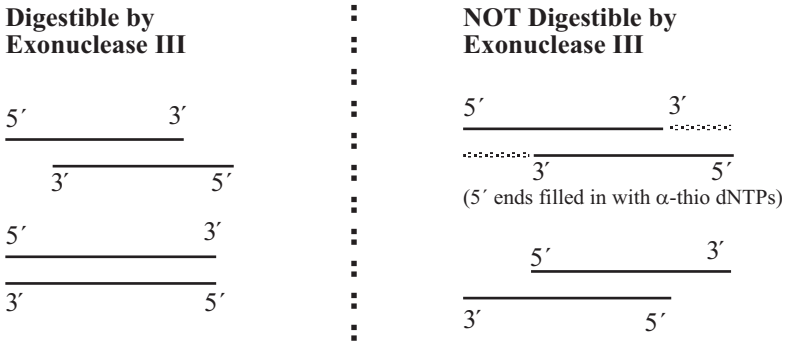
# Generating Nested Deletions

## Introduction

For large inserts, creating nested deletions is a method by which you can obtain additional sequence using the same sequencing primer. You may use your own method or the one provided below. The method below utilizes exonuclease III and mung bean nuclease to create nested deletions. Commercial kits are available to generate nested deletions.

## Background

Exonuclease III will progressively digest only double-stranded (ds) DNA containing a 5´ overhang or blunt ends to create single-stranded (ss) DNA. It will not digest the 3´ end of a ssDNA overhang or a 5´ overhang that is filled in with α-thio dNTPs. This activity can be exploited to create unidirectional, nested deletions in a DNA restriction fragment. After digestion of the DNA with exonuclease III, mung bean nuclease is used to remove all overhangs to produce blunt ends. The DNA fragment is then ligated back into a vector with blunt ends and transformed into *E. coli* competent cells.




## Strategy

Most nested deletion strategies involve digestion of the target DNA with two restriction enzymes. One enzyme should leave a 3´ overhang, which prevents digestion by exonuclease III. The other enzyme should leave a 5´ overhang or a blunt end for digestion of the DNA by exonuclease III.

Note that the multiple cloning site in this vector contains an *Sse*8387 I site, a rare site that leaves a 3´ overhang after digestion. In addition, there is also a *Pme* I site that leaves a blunt end when digested.

*continued on next page*

# Generating Nested Deletions, continued

## General Outline

The table below outlines the general steps necessary to prepare nested deletions.

| Step | Action |
|---|---|
| 1 | Prepare pure plasmid DNA. |
| 2 | Digest DNA with the first restriction enzyme. |
| 3 | Fill in 5´ overhangs with α-thio-dNTP mix and Klenow (optional). |
| 4 | Extract DNA with phenol-chloroform (1:1, v/v) and ethanol precipitate. |
| 5 | Check fill-in by digestion with exonuclease III and agarose gel electrophoresis (optional). |
| 6 | Digest DNA with the second restriction enzyme. |
| 7 | Extract DNA with phenol-chloroform (1:1, v/v) and ethanol precipitate. |
| 8 | Digest DNA with exonuclease III and collect time points. |
| 9 | Digest DNA with mung bean nuclease to remove ssDNA and create blunt ends. |
| 10 | Ligate the ends to recircularize vector. |
| 11 | Transform ligation into competent *E. coli* and select transformants |
| 12 | Analyze at least 5 transformants per time point to create an appropriate set of nested deletions. |

## Materials Needed

- Exonuclease III, deletion grade, 100 U/µl
- Klenow polymerase, 5 U/µl (optional)
- Mung bean nuclease, 100 U/µl
- T4 DNA ligase, 4 U/µl
- α-Thio phosphate dNTPs, 1 mM (optional)
- 2X Exonuclease III Buffer: 100 mM Tris-HCl, pH 8; 10 mM $MgCl_2$
- 10X Mung Bean Nuclease Buffer: 300 mM sodium acetate, pH 5; 500 mM NaCl; 10 mM $ZnCl_2$; 50% (v/v) glycerol
- 1X Mung Bean Dilution Buffer: 10 mM sodium acetate, pH 5; 0.1 mM zinc acetate; 0.1% Triton X-100; 50% (v/v) glycerol
- 10X Ligase Buffer: 500 mM Tris-HCl, pH 7.5; 70 mM $MgCl_2$, 10 mM dithiothreitol (DTT)
- β-mercaptoethanol
- 10 mM ATP (ribonucleotide form), pH 7 to 7.5
- Heat block with variable temperature settings
- Microcentrifuge tubes
- Dry Ice
- Agarose gel electrophoresis equipment and reagents

*continued on next page*

# Generating Nested Deletions, continued


We recommend that you first digest with *Sse*8387 I to linearize the vector and create ends that have 3´ overhangs. Then digest with *Pme* I to create a blunt end. Exonuclease III will digest from the *Pme* I site into the insert. After treating with mung bean nuclease to create blunt ends, simply ligate the vector back together. You can use either the M13 Reverse or the T3 primer to sequence into your insert.

**Important**

Be sure to check your insert for the presence of restriction sites of the enzymes you want to use.

## Other Considerations

The length of DNA to be sequenced will determine the number of time points taken during the exonuclease III digestion. The amount of enzyme, reaction temperature, and the time of incubation can control the rate of exonuclease III digestion. Use the table below as a guide to set up your digestion.

| Reaction Temperature | Exonuclease III Digestion (number of bases per minute) |
|---|---|
| 37°C | ~400 |
| 34°C | ~375 |
| 30°C | ~230 |
| 23°C | ~125 |

## Example

For a 3000 bp fragment, you might want to digest ~600 bases per time point. You will need to take 5 time points to progress through the fragment. Using the table above, you see that exonuclease III digests 400 bases/minute at 37°C; therefore your time points will be over 1 minute apart. Assume 5 µg DNA per time point.

**Note**: Exonuclease III digestion rates will vary. Use the information above as a guideline.

## Plasmid Preparation

You will need at least ~30 µg of DNA for restriction digestion and subsequent exonuclease III/mung bean nuclease digestion. Isolate DNA using the PureLink™ HQ Mini Plasmid Purification Kit(Catalog no. K2100-01) or CsCl gradient centrifugation.

## First Restriction Digest

Digest ~30 µg DNA in a 500 µl reaction volume with an enzyme that leaves a 3´ overhang (*e.g. Sse*8387 I). Use 5 U of enzyme per µg DNA and digest the DNA to completion. Remember to inactivate the restriction enzyme and check the digest (1 µl) on an agarose gel to ensure that the reaction went to completion. (If you used an enzyme that leaves a 5´ overhang, see the Important note on the next page.)

Extract with phenol:chloroform and ethanol precipitate. Resuspend the DNA in 200 µl of TE, pH 8.

*continued on next page*

# Generating Nested Deletions, continued

**Important**

If you find that you have to digest with an enzyme that leaves a 5´ overhang (*e.g. Spe* I), you will have to fill-in using α-thio-dNTPs and Klenow polymerase.

1. Add 2 µl of a 1 mM stock of α-thio-dNTPs and 5 U of Klenow polymerase to the restriction digest (previous page) and incubate the reaction at room temperature for 10 minutes.
2. Extract with phenol:chloroform and ethanol precipitate. Resuspend DNA in a volume of 200 µl.
3. Incubate 1 µg of the filled-in DNA with 20 U of exonuclease III for 15 minutes at 37° to check for protection against deletion. Analyze by agarose gel electrophoresis.

**Second Restriction Digest**

Digest the DNA as described above with an enzyme that leaves a 5´ overhang in your insert or a blunt end (*e.g. Pme* I). Use 5 U of enzyme per µg DNA and digest the DNA to completion. Remember to inactivate the restriction enzyme and check the digest (1 µl) on an agarose gel to ensure that the reaction went to completion. Extract with phenol:chloroform and ethanol precipitate. Resuspend the DNA at a concentration of ~1 µg/µl TE, pH 8.

**Exonuclease III/Mung Bean Nuclease Digestion**

For the exonuclease reaction, set up a single digestion reaction and remove 25 µl aliquots at various time points. Use 5 µg DNA/time point.

1. Set up 5 microcentrifuge tubes with the exonuclease III stop solution (155 µl water, 20 µl 10X mung bean nuclease buffer for each tube). Hold at room temperature.
2. Set up the following 125 µl exonuclease III digestion reaction:

| | |
|---|---|
| Double-digested DNA (~1 µg/µl) | 25 µl |
| 2X Exonuclease III buffer | 62.5 µl |
| 100 mM fresh β-mercaptoethanol | 12.5 µl |
| Water | 25 µl |
| Total Volume | 125 µl |

3. Add 5 µl of exonuclease III (100 U/µl) and incubate at the desired temperature (see page 24).
4. Remove 25 µl from the reaction for each time point (1-2 minutes per time point) and add to one of the tubes containing the stop solution. Place tubes on dry ice.
5. When all time points have been collected, heat the tubes at 68°C for 15 minutes to inactivate exonuclease III. Place the tubes on ice.
6. Dilute mung bean nuclease to 15 U/µl in 1X Mung Bean Nuclease buffer.
7. Add 1 µl of diluted mung bean nuclease to each time point tube. Incubate at 30°C for 30 minutes.
8. Extract each time point with phenol:chloroform and precipitate with ethanol. Resuspend each DNA pellet in 15 µl TE, pH 8.

*continued on next page*

# Generating Nested Deletions, continued

## What You Should See

Analyze 7 µl of each sample on an agarose gel. For each increasing time point you should see a single band that progressively decreases in size. There should be few other bands.

## Ligation and Transformation

Use the DNA from Step 8, previous page, to set up ligation reactions for each time point.

**Note:** Some ligase buffers already contain ATP. Be sure to check the composition of your ligase buffer before adding additional ATP.

1. Set up the following 20 µl ligation reaction:

| | |
|---|---|
| Digested DNA | 1 µl |
| 10X Ligase Buffer | 2 µl |
| 10 mM ATP, pH 7-7.5 (ribonucleotide form) | 1 µl |
| T4 DNA Ligase (1 U/µl) | 2 µl |
| Deionized Water | 14 µl |
| Total Volume | 20 µl |

2. Incubate at room temperature for 4 hours or at +4°C overnight.
3. Transform 1 µl of the ligation reaction into competent *E. coli* and select on LB plates containing 50-100 µg/ml ampicillin.
4. Choose ~5 colonies per time point and isolate DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. 2100-01) or similar kit.
5. Analyze for deleted inserts. Order the deletions by descending size and proceed to sequencing. Clones can be sequenced using the M13 Reverse or the T3 primer.

## For More Information

Refer to Current Protocols in Molecular Biology, pages 7.2.1 to 7.2.20 for more information on exonuclease III digestions (Ausubel *et al.*, 1994).

# Technical Service

## World Wide Web

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

## Contact Us

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

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

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

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

## MSDS

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

## Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives. Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order. Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

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

# Product Qualification

### Introduction

Invitrogen qualifies the TOPO TA Cloning® Kits for Sequencing as described below.

### TOPO® Vector

The parental supercoiled pCR®4 vector is qualified by restriction digest prior to adaptation with topoisomerase I. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

### TOPO® Cloning Efficiency

Once the pCR®4-TOPO® vector has been adapted with topoisomerase I, it is lot-qualified using the control reagents included in this kit. Under conditions described on pages 16-17, a 750 bp control PCR product was TOPO®-Cloned into the vector and subsequently transformed into the One Shot® competent *E. coli* included with the kit.

Each lot of vector should yield greater than 95% cloning efficiency.

### Primers

The primers included in this kit have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

### One Shot® Competent *E. coli*

All competent cells are qualified as follows:

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

# Purchaser Notification

**Information for European Customers**

The Mach1™-T1$^R$ *E. coli* strain is genetically modified to carry the *lac*ZΔM15 *hsd*R *lac*X74 *rec*A *end*A *ton*A genotype. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

**Limited Use Label License No. 5: Invitrogen Technology**

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

**Limited Use Label License No. 54: *ccd*B-Fusion Vectors**

This product is the subject of one or more of U.S. Patent Numbers 5,910,438 and 6,180,407 and corresponding foreign patents and is sold under license from the Université Libre de Bruxelles for research purposes only**.** 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). For licensing information, please contact: Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008. Tel: 760-603-7200; Fax: 760-602-6500.

# References


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).

Bernard, P., and Couturier, M. (1992). Cell Killing by the F Plasmid CcdB Protein Involves Poisoning of DNA-Topoisomerase II Complexes. J. Mol. Biol. *226*, 735-745.

Bernard, P., Gabant, P., Bahassi, E. M., and Couturier, M. (1994). Positive Selection Vectors Using the F Plasmid *ccd*B Killer Gene. Gene *148*, 71-74.

Bernard, P., Kezdy, K. E., Melderen, L. V., Steyaert, J., Wyns, L., Pato, M. L., Higgins, P. N., and Couturier, M. (1993). The F Plasmid CcdB Protein Induces Efficient ATP-dependent DNA Cleavage by Gyrase. J. Mol. Biol. *234*, 534-541.

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.

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.

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

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

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

invitrogen™

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

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