TOPO® Shotgun Subcloning Kit

Version H
7 April 2004
25-0336

TOPO® Shotgun Subcloning Kit

For preparation and rapid cloning of blunt-end DNA for sequencing

Catalog nos. K7000-01, K7010-01, K7050-01, and K7060-01

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Table of Contents

Kit Contents and Storage

Types of Kits

The following kits are available from Invitrogen and covered by this manual.

Kit	Contents	Catalog no.
TOPO® Shotgun Subcloning Kit with One Shot® TOP10 Chemically Competent *E. coli*	Vector Module Gel Purification Module Nebulizers One Shot® TOP10 Chemically Competent *E. coli*	K7000-01
TOPO® Shotgun Subcloning Kit with One Shot® TOP10 Chemically Competent *E. coli*—No Nebulizers	Vector Module Gel Purification Module One Shot® TOP10 Chemically Competent *E. coli*	K7010-01
TOPO® Shotgun Subcloning Kit with One Shot® TOP10 Electrocomp™ *E. coli*	Vector Module Gel Purification Module Nebulizers One Shot® TOP10 Electrocomp™ *E. coli*	K7050-01
TOPO® Shotgun Subcloning Kit with One Shot® TOP10 Electrocomp™ *E. coli*—No Nebulizers	Vector Module Gel Purification Module One Shot® TOP10 Electrocomp™ *E. coli*	K7060-01

Shipping and Storage

Each TOPO® Shotgun Subcloning Kit consists of three modules. Each module is listed below with the shipping and storage conditions. Please note that mussel glycogen, calf intestinal phosphatase (CIP), and 10X Dephosphorylation Buffer are stored at different temperatures.

Module	Shipping Temperature	Storage Temperature
Nebulizers/Gel Purification **Note**: Catalog nos. K7010-01 and K7060-01 do not contain Nebulizers	Room Temperature	Room Temperature
Vector	Dry Ice	-20°C, **EXCEPT** Mussel Glycogen: Room Temperature CIP: +4°C 10X Dephosphorylation Buffer: +4°C
One Shot® TOP10 Chemically Competent or Electrocomp™ *E. coli*	Dry Ice	-80°C

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Kit Contents

Vector Module

Component	Composition	Amount
pCR®4Blunt-TOPO® vector, linearized	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	30 µl
10 mM dNTPs	2.5 mM dATP 2.5 mM dCTP 2.5 mM dGTP 2.5 mM dTTP neutralized at pH 8.0 in water	40 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
T4 DNA Polymerase	4 U/µl in: 20 mM potassium phosphate, pH 6.5 5 mM dithiothreitol 50% glycerol	20 µl
Klenow Polymerase	4 U/µl in: 50 mM potassium phosphate, pH 7.0 0.25 mM dithiothreitol 50% glycerol	20 µl
10X Blunting Buffer	100 mM Tris-HCl 100 mM $MgCl_2$ 500 mM NaCl 10 mM dithiothreitol pH 7.9 (25°C)	40 µl
Bovine Serum Albumin	1 mg/ml in water	10 µl

Continued on next page

Vector Module, continued

Component	Composition	Amount
Calf Intestinal Phosphatase	1 U/µl in: 25 mM Tris-HCl 1 mM $MgCl_2$ 0.1 mM $ZnCl_2$ 50% glycerol pH 7.6 (+4°C)	40 µl
10X Dephosphorylation Buffer	0.5 M Tris-HCl 1 mM EDTA pH 8.5 (20°C)	60 µl
Mussel Glycogen	20 mg/ml in sterile, deionized water	40 µl
Sheared Herring Sperm DNA	0.2 µg/µl in TE, pH 8	4 µg
M13 (-20) Forward Sequencing Primer	0.1 µg/µl in TE, pH 8	20 µl
M13 Reverse Sequencing Primer	0.1 µg/µl in TE, pH 8	20 µl
T7 Sequencing Primer	0.1 µg/µl in TE, pH 8	20 µl
T3 Sequencing Primer	0.1 µg/µl in TE, pH 8	20 µl

Kit Contents

Nebulizers/Gel Purification Module

Component	Composition	Amount
Nebulizers	--	5
Nebulizer Tubing	Vinyl	5 x 1.5 in.
Purification Columns	--	5
Collection Vials	--	5
Sodium Iodide Solution	6.6 M Sodium iodide 16 mM Sodium sulfite	5 ml
Binding Buffer	7 M Guanidinium HCl	2 x 5 ml
4X Final Wash	400 mM NaCl	2.5 ml

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Kit Contents and Storage, Continued

One Shot® TOP10 *E. coli*

One Shot® TOP10 *E. coli* are provided as either chemically competent or electrocompetent. Each 10 reaction One Shot® TOP10 *E. coli* kit contains the following items. Three kits are included to provide a total of 30 reactions and 3 transformation controls. Transformation efficiency is at least 1 x 10^9 cfu/µg DNA for each kit. One Shot® kits can be ordered separately (see page viii).

Item	Composition	Amount
SOC Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 cells	--	33 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Genotype of TOP10

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

Primer Sequences

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

Primer	Sequence	pmoles Supplied
M13 Forward (-20)	5′-GTAAAACGACGGCCAG-3′	407
M13 Reverse	5′-CAGGAAACAGCTATGAC-3′	385
T3	5′-ATTAACCCTCACTAAAGGGA-3′	329
T7	5′-TAATACGACTCACTATAGGG-3′	327

Accessory Products

Products

The table below lists additional products available from Invitrogen separately which can be used with this kit.

Item	Quantity	Catalog no.
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	C4040-03
One Shot® TOP10 Electrocomp™ *E. coli*	10 reactions	C4040-50
One Shot® TOP10 Electrocomp™ *E. coli*	20 reactions	C4040-52
Zero Blunt® TOPO® PCR Cloning Kit for Sequencing (contains pCR®4Blunt-TOPO®)	20 reactions	K2875-20
Nebulizers	5	K7025-05
M13 Forward (-20) Primer	2 µg	N520-02
M13 Reverse Primer	2 µg	N530-02
T3 Primer	2 µg	N565-02
T7 Primer	2 µg	N560-02

Introduction

Overview

Description

The TOPO® Shotgun Subcloning Kit allows you to easily shear large DNA molecules, repair the ends of those molecules, and rapidly clone the resulting blunt-end molecules into pCR®4Blunt-TOPO® for sequencing.

Applications

The major application of the TOPO® Shotgun Subcloning Kit is to shear and subclone 2-3 kb DNA fragments from bacterial artificial chromosomes (BACs) for sequencing. Because of the low-copy number of these plasmids, it is difficult to prepare quality DNA for sequencing. The TOPO® Shotgun Subcloning Kit permits rapid blunt-end cloning and sequencing of sheared DNA fragments from small amounts of BAC DNA. In addition, the kit can be used to shear and subclone DNA from other artificial chromosomes (i.e. YACs, PACs), cosmid DNA, and genomic DNA.

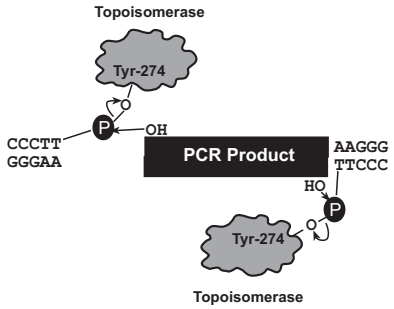
Nebulizers

A nebulizer is a small plastic device that uses compressed air to atomize liquids. They are easily adapted for shearing DNA and extremely effective and simple to use (Surzycki, 1990; Surzycki, 2000). Nebulization of BAC DNA routinely results in DNA that is fragmented into 1 to 6 kb fragments and can be easily cloned without gel purification, if desired.

How TOPO® Cloning Works

The plasmid vector (pCR®4Blunt-TOPO®) is supplied linearized with *Vaccinia* virus topoisomerase I covalently bound to the 3´ ends (referred to as "activated" vector).

The TOPO® Shotgun Subcloning Kit utilizes TOPO® technology, which exploits the ligation activity of topoisomerase I. 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 is subsequently attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase. These properties of topoisomerase are utilized to create the TOPO® Linker in the kit (Shuman, 1994).



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Overview, Continued

ccdB Gene

pCR®4Blunt-TOPO® allows direct selection of recombinants via disruption of the lethal *E. coli* gene, *ccd*B (Bernard *et al.*, 1994). The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment. Ligation of blunt-end, dephosphorylated DNA disrupts expression of the *lacZα-ccd*B gene fusion permitting growth of only positive recombinants upon transformation into TOP10 cells. Cells that contain non-recombinant vector are killed upon plating. Blue/white screening can be used to select colonies containing plasmids with inserts.

Experimental Outline

The table below outlines the steps necessary to fragment, repair, and subclone DNA using the TOPO® Shotgun Subcloning Kit.

Step	Action	Page
1	Shear BAC DNA with nebulizer.	3
2	Treat DNA ends with T4 DNA and Klenow polymerases to yield blunt-end DNA.	5
3	Dephosphorylate DNA with calf intestinal phosphatase.	6
4	Ligate DNA fragments into pCR®4Blunt-TOPO®.	7
5	Transform into One Shot® TOP10 Chemically Competent *E. coli* or Electrocomp™ *E. coli*.	9-11
6	Analyze colonies for inserts. Sequence clones using high throughput techniques or freeze clones for later analysis.	12

Methods

Shearing the DNA

Introduction

You will need purified BAC, cosmid, or genomic DNA in water or TE, pH 8 for shearing. Use your own protocol to purify the DNA or use the Easy-DNA™ Kit (Catalog no. K1800-01). BACs and cosmids can be purified using resin-based DNA purification kits for large constructs. If you purchased the TOPO® Shotgun Subcloning Kit without the nebulizers, use your own method to shear your DNA.

Before Starting

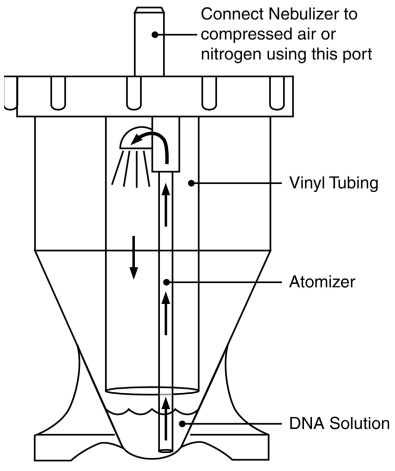
Be sure to have the following reagents on hand before starting. In addition you will need to modify the nebulizer before use (see below).

- Shearing Buffer (TE, pH 8 containing 20% glycerol)
- Sterile water
- 1% agarose gel in TAE (for checking the size of the DNA)
- 3 M sodium acetate, pH 5.2
- 100% isopropanol
- Cold 80% ethanol

Modifying and Connecting the Nebulizer

Please refer to the diagram below to modify and connect the nebulizer.

1. Unscrew the blue top of the nebulizer and slip the small piece of vinyl tubing over the atomizer (see diagram). The tubing acts to direct the atomized liquid back to the bottom of the nebulizer for easier collection.
2. Screw the cap back on (finger-tight) and **connect the nebulizer to a nitrogen or argon air tank, or laboratory compressed air line** using appropriate connectors and tubing. Use a regulator that allows you to easily vary the pressure from 10 to 30 psi (68 to 206 kPa or 0.63-0.7 bar). **Do not attach nebulizer to a vacuum line.**



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Shearing the DNA, Continued

Shearing Procedure

Once you have connected the nebulizer to the compressed gas or air source, you are ready to shear the DNA. You may have to experiment with the pressure to obtain the size of DNA you want. The size of the sheared DNA is inversely proportional to the amount of pressure (i.e. for smaller DNA, increase the pressure).

1. Add 3-25 µg DNA to 750 µl of Shearing Buffer and pipet into the bottom of the nebulizer. **Note**: Use more DNA for genomic DNA and less DNA for BAC DNA.
2. Screw on blue cap finger-tight. Do not over tighten.
3. Place nebulizer containing the DNA in an ice bucket to keep the DNA cold.
4. Shear the DNA for **30-90 seconds** at 9-10 psi (pounds per square inch) to obtain DNA that is 1-5 kb in size.

   Note: We recommend checking the DNA on a 1% agarose gel to ensure that you obtain the desired size range of DNA.
5. Transfer 700 µl of the sheared DNA to a sterile microcentrifuge tube.
6. Add 80 µl 3 M sodium acetate, pH 5.2; 4 µl 20 mg/ml glycogen as a carrier; and 700 µl 100% isopropanol.
7. Mix well and place on dry ice for 15 minutes. **Note**: DNA can be stored overnight at -20°C.
8. Centrifuge at 12,000g for 15 minutes at +4°C. Remove supernatant.
9. Wash DNA pellet with 800 µl of cold 80% ethanol.
10. Centrifuge for 5 minutes and decant the ethanol.
11. Centrifuge again for 1 minute and remove all traces of ethanol. **Be careful not to disturb pellet.** Alternatively, you may dry the pellet using a speed-vac.
12. Resuspend the DNA to a concentration of ~100-200 ng/µl in sterile water, assuming 80% recovery. Proceed to **Repairing and Dephosphorylating DNA**, next page. If you wish to gel-purify your DNA, see the **Note**, below.

Note

Whether to gel-purify the sheared DNA is a matter of personal preference. The nebulizer generates a very uniform range of fragment sizes that may not require purification. If you wish to purify the sheared DNA, a variety of methods exist for size fractionation and purification (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989). Reagents are supplied in the kit to gel-purify sheared DNA using the procedure on page 16.

Increasing the Volume

You may vary the volume of the DNA solution up to 2 ml. Because the time of nebulization depends on the volume, increasing the volume means increasing the time of nebulization. Volumes over 2 ml are not recommended (see below).

Troubleshooting

If you do not obtain complete nebulization, the gas pressure may be too low. Check the connections and tubing to ensure that you have no leaks. Volumes in excess of 2 ml may also contribute to poor nebulization.

Blunt-End Repairing and Dephosphorylating DNA

Introduction

Once you have sheared your DNA, you are ready to blunt-end repair and dephosphorylate the ends to create a suitable substrate for TOPO® Cloning.

A mixture of T4 DNA polymerase and Klenow DNA polymerase is used to fill in the ends of the DNA fragments by catalyzing the 5′ to 3′ incorporation of complementary nucleotides into double-stranded DNA. In addition, the 3′ to 5′ exonuclease activity of T4 DNA polymerase degrades 3′ overhangs.

To dephosphorylate the DNA for TOPO® Cloning, calf intestinal phosphatase (CIP) is added directly to the blunting reaction. After incubation, the DNA solution is extracted with phenol:chloroform and ethanol-precipitated.

Control Reaction

Sheared herring sperm DNA is provided as a positive control to monitor the blunt-end repair, dephosphorylation, and cloning efficiency. We recommend that you include the control DNA along with your samples the first time you perform the experiment.

Before Starting

Be sure to have the following reagents and equipment ready.

- Heat block
- Phenol:chloroform:isoamyl alcohol (25:24:1) (i.e. Sigma, Catalog no. P-3803)
- 3 M sodium acetate, pH 5.2
- Ethanol
- Dry ice
- Cold 80% ethanol

Blunt-End Repair

1. Set up the following 50 µl blunt-end repair reaction on ice. Be sure to add enzymes last.

Component	Your Reaction	Control Reaction
Sheared DNA (~1-3 µg)	X µl	--
Sheared Herring Sperm DNA (control)	--	10 µl (2 µg)
Deionized water	add to 35 µl total	25 µl
10X Blunting Buffer	5 µl	5 µl
BSA (1 mg/ml)	1 µl	1 µl
dNTP mix (final concentration is 250 µM)	5 µl	5 µl
T4 DNA polymerase	2 µl	2 µl
Klenow DNA polymerase	2 µl	2 µl

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Blunt-End Repairing and Dephosphorylating DNA, Continued

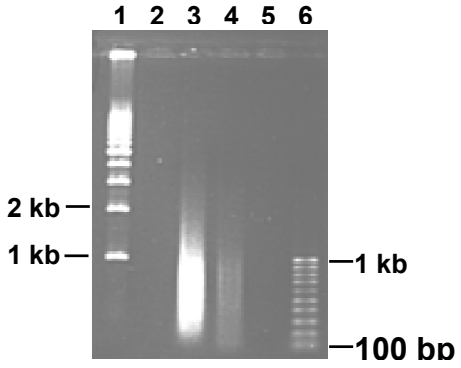
Blunt-End Repair, continued

2. Incubate at room temperature for 30 minutes.
3. Incubate at 75°C for 20 minutes to inactivate the enzymes. Proceed to the **Dephosphorylation Procedure**, below.

Dephosphorylation Procedure

1. To the 50 µl blunt-end repair reaction, above, add 35 µl sterile, deionized water, 10 µl 10X Dephosphorylation buffer, and 5 µl CIP (5 units). Total volume is 100 µl.
2. Incubate at 37°C for 60 minutes.
3. Extract the reaction with 100 µl phenol:chloroform, centrifuge for 3 minutes at maximum speed, and transfer the aqueous upper phase to a fresh tube.
4. Add 10 µl 3 M sodium acetate, pH 5.2, 1 µl 20 mg/ml glycogen, and 300 µl cold ethanol. Incubate the DNA on dry ice for 10 minutes to precipitate the DNA.
5. Centrifuge at 12,000g for 15 minutes at +4°C.
6. Wash the DNA pellet with 500 µl cold 80% ethanol. **Be careful not to dislodge pellet.**
7. Centrifuge for 5 minutes at 12,000g and decant the ethanol.
8. Centrifuge again for 1 minute and remove the last traces of ethanol. **Be careful not to dislodge pellet.**
9. Air-dry for 5 minutes and resuspend the pellet in 20 µl sterile water (~50-100 ng/µl).
10. Use the untreated control DNA to estimate the amount of DNA in both your sample and the treated control. You should have approximately 50-100 ng/µl. Proceed to **TOPO® Cloning Blunt-End DNA**, next page.

    Note: If you do not see any of your DNA, there may not be enough DNA on the gel or the pellet was lost in the preceding wash steps. Be sure to load at least 20 ng of DNA and be careful when washing the nucleic acid pellet.



Comparison of untreated sheared herring sperm DNA with treated sheared herring sperm DNA. **Important: Untreated sheared herring sperm DNA is not sheared using the nebulizer.** Our vendor supplies it sheared as shown in Lane 3. Lane 1: 1 kb ladder (0.8 µg); Lane 2: Blank; Lane 3: Untreated sheared herring sperm DNA (2 µg); Lane 4: Treated sheared herring sperm DNA, 5 µl of reaction (Step 9, above), ~50 ng/µl (estimated from Lane 3); Lane 5: Blank; Lane 6: 100 bp ladder (0.4 µg). **Note**: Brightness and contrast of the gel were adjusted uniformly to ensure the best reproduction.

TOPO® Cloning Blunt-End DNA

Introduction

At this point you are ready to clone your blunt-end, dephosphorylated DNA into pCR®4Blunt-TOPO®. Estimate the number of colonies you will need to cover the large DNA fragment you are interested in sequencing (see below). TOPO® Cloning technology allows you to ligate sheared DNA into pCR®4Blunt-TOPO®, and transform the recombinant vector into TOP10 *E. coli* in one day. To ensure that you obtain the best possible results, it is important to have everything you need set up and ready to use.

Creating a Sequencing Library

To create a sequencing library for your fragment, you will need to estimate the number of clones needed to cover the fragment. For example, if you are sequencing a 30-100 kb fragment, you might need 300 to 1000 colonies, assuming an average insert size of 1 kb, coverage of 10-fold, and that only 80% of the colonies contain plasmid with insert.

We recommend that you set up 3 TOPO® Cloning reactions to optimize the amount of DNA insert needed to obtain the most transformants. The TOPO® Cloning reactions can be transformed into either chemically competent TOP10 *E. coli* or electrocompetent TOP10 *E. coli*.

Generally speaking, you should obtain 800-1400 colonies per TOPO® Cloning reaction if you transform into chemically competent TOP10 *E. coli*. Approximately 10-fold more transformants are obtained with electrocompetent *E. coli*. For most applications, transforming into chemically competent cells will yield more than enough colonies for sequencing.

Materials Supplied by the User

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

- Sterile water
- Water bath or heat block at 37°C
- Sterile microcentrifuge tubes
- 42°C water bath or electroporator and 0.1 cm cuvettes
- Sterile, 15 ml snap-cap tubes
- Additional SOC for electroporation (see **Recipes**, page 14)
- LB plates containing 50-100 µg/ml ampicillin or 25-50 µg/ml kanamycin (estimate the number you will need)
- 40 mg/ml X-Gal in dimethylformamide (DMF, see page 15)
- 37°C shaking and non-shaking incubator

Continued on next page

TOPO® Cloning Blunt-End DNA, Continued

Preparation for Transformation

For each TOPO® Cloning reaction and transformation, you will need one vial of competent cells and two to three selective plates. For example, for 3 TOPO® Cloning reactions, you will need 3 vials of competent cells and 6-9 selective plates. If you are including the control reaction, you will need to double the reagents.

- Equilibrate a water bath to 42°C or set up your electroporator
- Warm the vial of SOC medium to room temperature.
- Spread 40 µl of 40 mg/ml X-Gal on each LB plate and incubate at 37°C until ready for use.
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

TOPO® Cloning Procedure

1. Serially dilute your blunt-end DNA 3-fold and 9-fold with water or TE, pH 8. Use the indicated amounts for the control DNA. Set up the following 6 µl TOPO® Cloning reactions for each sample (6 reactions total for one sample and one control).

Sample DNA

Component	Undiluted DNA	3-fold diluted DNA	9-fold diluted DNA
Blunt-end DNA	4 µl (~100 ng)	4 µl	4 µl
Salt Solution	1 µl	l µl	l µl
pCR®4Blunt-TOPO®	1 µl	1 µl	1 µl

Control DNA (optional)

Component	Reaction 1	Reaction 2	Reaction 3
Control DNA	60 ng	20 ng	5-6 ng
Salt Solution	1 µl	l µl	l µl
Water (control only)	to 4 µl	to 4 µl	to 4 µl
pCR®4Blunt-TOPO®	1 µl	1 µl	1 µl

2. Mix by tapping the bottom of each tube. Centrifuge briefly in a table top microcentrifuge.
3. Incubate the reactions for 5 minutes at room temperature. Place on ice and proceed to **Transforming Chemically Competent *E. coli***, next page. If you are planning to electroporate the TOPO® Cloning reaction, proceed to **Electroporation of *E. coli***, page 10.

Continued on next page

Transforming Chemically Competent *E. coli*

The procedure below is for transformation into chemically competent *E. coli*. **Do not use electrocompetent cells. The salt concentration of the TOPO® Cloning reaction will cause arcing.** If you wish to electroporate the TOPO® Cloning reaction, see page 10.

1. Add 2 µl of the TOPO® Cloning reaction to a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   Note: The remainder of the reaction (4 µl) can be stored at -20°C and transformed later if needed.
2. Incubate on ice for 15 minutes.
3. Heat-shock the cells for 45 seconds at 42°C without shaking.
4. Immediately transfer the tube to ice.
5. Add 500 µl of room temperature SOC medium.
6. Cap the tube tightly and shake the tube horizontally (225 rpm) at 37°C for 1 hour.
7. Plate 50-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. For your application, you may want to plate all of the transformation reaction to obtain the most colonies for analysis.
8. An efficient TOPO® Cloning reaction will produce 800-1400 colonies per transformation reaction. Pick ~10 white or light blue colonies for analysis (see page 12). **Do not pick dark blue colonies**.
9. Colonies may be picked into 96-well microtiter plates, cultured, and frozen for later analysis. Alternatively, the colonies may be sequenced using high-throughput techniques.

Low Transformation Efficiency

Low transformation efficiency can result from low TOPO® Cloning efficiency. Lower cloning efficiencies may result from the following variables. We recommend using the sheared herring sperm DNA as a control to ensure that the reagents are working correctly.

Variable	Solution
Incomplete blunting and/or dephosphorylation reaction.	Perform control reaction to ensure that the blunting and dephosphorylation reactions are working.
Excess or not enough DNA in TOPO® Cloning reaction	Vary amount of DNA to determine the optimal vector to insert ratio.

Electroporation of *E. coli*

Introduction

If you need more transformants for your particular application, we recommend transforming into electrocompetent *E. coli*. Use the TOPO® Shotgun Subcloning Kit with Electrocomp™ *E. coli* (Catalog no. K7050-01), which already includes electrocompetent *E. coli*, or use the One Shot® TOP10 Electrocomp™ Kit, which is available separately. Please see page viii for ordering information.

Precipitation of TOPO® Cloning Reaction

To electroporate your TOPO® Cloning reaction, **you will need to precipitate the reaction to prevent arcing during electroporation.**

1. Add 100 µl deionized water to the TOPO® Cloning reaction and incubate at 37°C for 10 minutes.
2. Add 10 µl 3 M sodium acetate, pH 5.2; 2 µl 20 mg/ml glycogen; and 300 µl 100% ethanol and vortex.
3. Place on dry ice for 15 minutes, then centrifuge at 12,000g for 15 minutes at +4°C.
4. Decant supernatant and add 800 µl cold 80% ethanol.
5. Centrifuge at 12,000g for 5 minutes at room temperature.
6. Carefully decant the ethanol and centrifuge again for 1 minute.
7. Carefully remove last traces of ethanol.

   Note: Avoid disturbing the pellet. It is better to leave a few microliters of ethanol behind rather than lose the pellet.
8. Air-dry or place in the speed-vac until all the liquid is removed. Be careful not to dry the pellet too much as it will be difficult to resuspend.
9. Resuspend the pellet in 10 µl deionized water and proceed to electroporation. Follow the manufacturer's recommendations for using the electroporator with *E. coli*.

Continued on next page

Electroporation of *E. coli*, Continued

Electroporation

1. Add 3.3 µl of the precipitated TOPO® Cloning reaction (from Step 9, previous page) into 50 µl of One Shot® TOP10 Electrocomp™ *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Carefully transfer the cells and DNA to a chilled 0.1 cm cuvette.
3. Electroporate your samples using your protocol.
4. Add 1 ml of room temperature SOC medium to the cuvette and transfer cells to a 15 ml snap-cap tube.
5. Cap the tube tightly and shake the tube (200 rpm) at 37°C for 1 hour.
6. Plate 10-25 µl from each transformation in duplicate and incubate overnight at 37°C. For the remaining transformation reaction, you may:
   - Plate all of the transformation reaction on additional plates, OR
   - Store the transformation reaction at +4°C and plate it all out the next day
7. An efficient TOPO® Cloning reaction will produce thousands of colonies. Pick ~10 white or light blue colonies for analysis (see page 12). **Do not pick dark blue colonies.**
8. If you plated out all of the transformation reaction you can pick white or light blue colonies into 96-well microtiter plates and either freeze them for later analysis or sequence plasmids using high-throughput techniques.

Low Transformation Efficiency

The most common cause of low transformation efficiency of electrocompetent *E. coli* is arcing. Arcing can be caused by any one of the following:

- Incomplete washing of precipitated TOPO® Cloning reactions with 80% ethanol.
- Presence of air bubbles in the sample.
- Partial lysis of cells because of the age of the cells, improper storage, or thawing the cells too fast.

Other reasons for low transformation efficiency are provided below and on page 9.

- Incorrect electroporation settings. Check the manufacturer's instructions for electroporating *E. coli*.

Analyzing and Sequencing Clones

Introduction

At this point you may analyze the colonies for insert or you may directly sequence recombinant plasmids.

Analysis of Positive Clones

1. Culture 10 transformants overnight in LB or SOB medium containing 50-100 µg/ml ampicillin or 25-50 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis (for insert size) or by sequencing. Please refer to the map on page 13 for sequence and restriction sites around the TOPO® Cloning site. Sequencing primers are included to help you sequence your insert.

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

Alternative Method of Analysis

You may wish to use PCR to directly analyze for inserts. You may use the T7 primer and the M13 reverse primer included in the kit. If this is the first time you have used this technique, we recommend that you perform restriction analysis in parallel to confirm that PCR gives you the correct result. Both false positive and false negative results can be obtained because of mispriming or contaminating template.

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

1. Prepare a PCR cocktail consisting of PCR buffer, dNTPs, primers, and *Taq* polymerase. Use a 20 µl reaction volume. Multiply by the number of colonies to be analyzed (e.g. 10).
2. Pick 10 colonies and resuspend them individually in 20 µl of the PCR cocktail. Don't forget to patch the colony to preserve it for further experiments.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles (94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute).
5. For the final extension, incubate at 72°C for 10 minutes. Hold at +4°C.
6. Visualize by agarose gel electrophoresis.

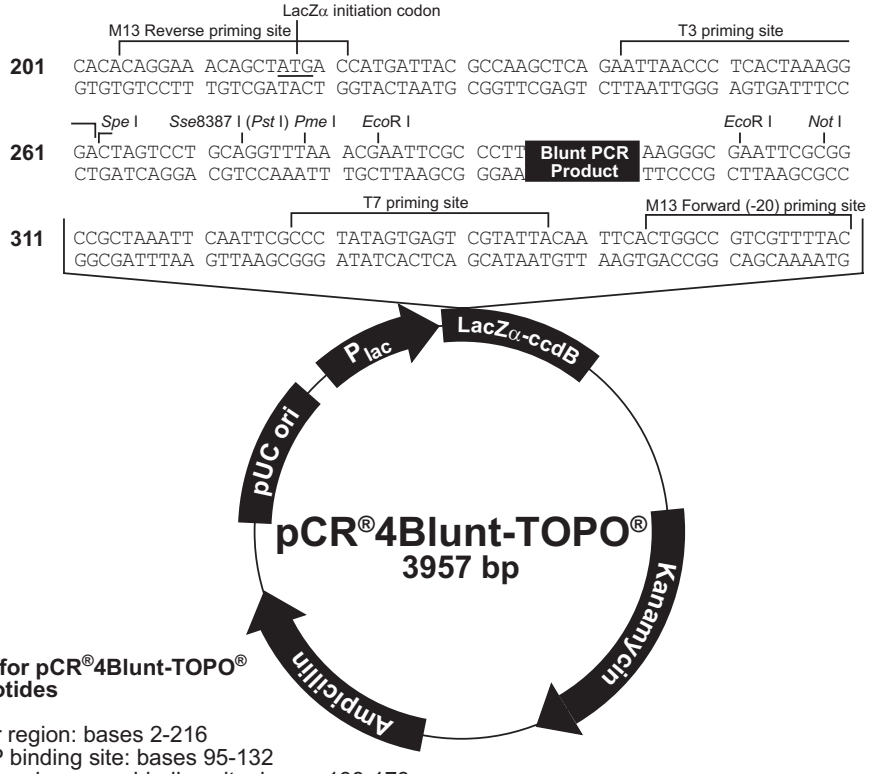
Important

If you have problems obtaining transformants, perform the reactions using the control DNA provided. These reactions will help you troubleshoot your experiment. Inserts should be between 200 and 1100 bp.

Map of pCR®4Blunt-TOPO®

pCR®4Blunt-TOPO® Map

The map below shows the features of pCR®4Blunt-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. **To obtain the full sequence of the vector, you may download it from our Web site (www.invitrogen.com) or call Technical Service (page 19).**



Comments for pCR®4Blunt-TOPO®
3957 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
- *ccdB* portion of fusion: bases 508-810

T3 priming site: bases 243-262
Polylinker: bases 262-312
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
neo (kanamycin) promoter region: bases 1021-1070
neo (kanamycin) resistance gene (ORF): bases 1159-1953
bla promoter region: bases 2062-2156
- RNA polymerase binding site: bases 2062-2143
- -35 region: bases 2087-2093
- *bla* promoter (P3): bases 2116-2122

Start of transcription: base 2122
Ribosome binding site: bases 2145-2149
bla (ampicillin) resistance gene (ORF): bases 2157-3017
pUC origin: bases 3162-3835

Appendix

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

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

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed (50-100 µg/ml of ampicillin or 25-50 µg/ml kanamycin).
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/ml of either ampicillin or kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

SOC Medium

SOC (per liter)

2% Tryptone
0.5% Yeast Extract
0.05% NaCl
2.5 mM KCl
20 mM glucose
10 mM $MgCl_2$

1. Dissolve 20 g Tryptone, 5 g Yeast Extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.0 with 5 M NaOH, then bring the volume to 1 L with deionized water.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$ and add 7.2 ml of 50% glucose.
5. Store at room temperature or +4°C.

Continued on next page

X-Gal Stock Solution

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

Purifying Sheared DNA

Introduction

If you elect to purify your sheared DNA, a procedure is supplied below. The procedure utilizes the purification reagents included in the kit. These reagents are available separately in the S.N.A.P.™ Gel Purification Kit (Catalog no. K1999-25). Note that other procedures are possible.

Materials Supplied by the User

You will need the following reagents and equipment for gel purification

- Apparatus for agarose mini-gel electrophoresis with 8-lane or 12-lane comb
- General purpose agarose
- 1X TAE buffer (50 mM Tris-acetate, pH 8, 1 mM EDTA)
- Clean glass flask
- Autoclaved water or TE buffer
- Sterile 15 ml bottle to prepare 1X Final Wash (see below)
- New razor blade
- 42°C to 50°C water bath
- Microcentrifuge
- Sterile microcentrifuge tubes

Before Starting

Bring the Sodium Iodide solution, Binding Buffer, and 4X Final Wash to room temperature. Mix well to re-dissolve salts before using. Please note that these solutions may be stored at room temperature after the first use.

To prepare 1X Final Wash, take a sterile 15 ml bottle and transfer all of the 4X Final Wash solution to the bottle. Add 7.5 ml of 100% **ethanol** to the 4X Final Wash solution to prepare the 1X Final Wash solution. Store at room temperature.

Nuclease Control

It is very important to minimize the presence of nucleases to ensure the highest cloning efficiencies. Please follow the guidelines listed below. While some guidelines may not appear as rigorous as others may, they are sufficient for purifying DNA.

- Wear gloves at all times
- Use sterile plasticware and glassware
- Autoclave TAE to use as the running buffer
- Rinse agarose gel apparatus and comb with autoclaved water or TE buffer
- Use a new razor to excise gel slice*
- Use new plastic wrap (i.e. Saran® Wrap) if needed

*The same razor may be used to excise different bands in the same gel if you are careful not to bring over pieces from an earlier excision.

Continued on next page

Purifying Sheared DNA, Continued

Preparation of Gel

Follow the instructions below to prepare a 1% agarose gel. The recipe will make one agarose gel with a volume of 50 ml.

1. Mix 0.5 g of general purpose agarose and 50 ml 1X TAE buffer in a clean glass flask.
2. Place flask in the microwave and heat until just boiling. Swirl to dissolve agarose and continue to heat in this fashion for 3 minutes to destroy nucleases.
3. Remove from the microwave and cool for 3 minutes.
4. Rinse the gel box and comb with autoclaved water or TE buffer. **Note**: Use a comb that will hold 1-5 µg sheared DNA in one well. Wells should be as small as possible.
5. Pour the gel and set the comb in the gel.
6. When the gel has solidified, cover the gel with 1X TAE buffer.
7. Load your sample (1-5 µg DNA) and run the gel. Do not run the gel to long. You want to keep the gel slice as small as possible (**100 µl total volume**).
8. Stain in 0.5-1 µg/µl ethidium bromide.

Excision of Sheared DNA

1. Pour off the stain (or transfer the gel to new Saran® Wrap). Visualize gel under UV light.
2. Using a new razor blade, carefully excise the DNA from the gel. **Note**: Razor blade may be rinsed with autoclaved water or TE prior to cutting the next band.
3. Cut up the excised piece of agarose into small chunks and transfer to a sterile 1.5 ml microcentrifuge tube. **Note**: Cutting up the agarose piece reduces the time and temperature required melting the agarose.
4. Estimate the volume of the agarose pieces (generally this is around 100 µl). Alternatively, you can weigh the gel slice and assume that 1 mg ~ 1 µl.
5. Add 2.5 times its volume of 6.6 M sodium iodide (i.e. 250 µl) and mix by shaking vigorously by hand or vortexing.
6. Incubate at 42 to 50°C until the agarose is **completely** melted (~2 minutes). Mix the solution periodically by shaking vigorously.
7. Place the tube at room temperature and add 1.5 volumes of Binding Buffer (i.e. 525 µl) and mix well. Proceed directly to **Isolation of Sheared DNA,** next page.

Continued on next page

Purifying Sheared DNA, Continued

Isolation of Sheared DNA

1. Assemble a S.N.A.P.™ purification column (A) and collection vial (B) and load all of the mixture from Step 7, above, onto the column (875 µl).
2. Centrifuge at 2,000 to 3,000 x g in a microcentrifuge for 30 seconds at room temperature.
3. Pour the liquid in the collection vial **back onto the column** and repeat Step 2.
4. Repeat Step 3 one more time to bind all the DNA to the column (i.e. load solution onto the column for a total of 3 times).
5. After the last centrifugation, discard the liquid in the collection tube.
6. Add 400 µl of 1X Final Wash to the S.N.A.P.™ column and centrifuge as in Step 2.
7. Repeat Step 6 and discard the liquid in the collection tube after the final centrifugation (800 µl).
8. Centrifuge the column again **at maximum speed (>10,000 x g) for at least 1 minute** to dry the column resin. Discard the collection vial.
9. Transfer the column to a new, sterile 1.5 ml microcentrifuge tube.
10. Add 40 µl of sterile water directly to the column material and incubate for 1 minute at room temperature to let the buffer absorb into the column.
11. Centrifuge the column at maximum speed (>10,000 x g) for 1 minute to elute the DNA into the microcentrifuge tube.
12. Place the tube on ice and discard the column.
13. Assay 10 µl by ethidium bromide agarose gel electrophoresis to estimate the DNA concentration. Concentration should be between 2 and 40 ng/µl. In most cases, there is no need to concentrate the DNA further.

Store the DNA at -20°C or proceed directly to blunting and dephosphorylating the DNA (page 5). For the highest efficiencies, it is recommended that you proceed directly to TOPO® Cloning.

Technical Service

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Product Qualification

Introduction

Invitrogen qualifies the TOPO® Shotgun Subcloning Kit using the procedures below.

Restriction Digest

The parent vector of pCR®4Blunt-TOPO® is supercoiled pCR®4, which is qualified by restriction digest prior to adaptation. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pCR®4
*Acc*65 I (linearizes)	3520 bp
Xba I (linearizes)	3520 bp
Drd I	339, 1086, 2095 bp
Pst I	1368, 2152 bp

Quality Control Procedure

The TOPO® Shotgun Subcloning Kit is functionally qualified using 2 µg sheared herring sperm DNA and the protocol in the manual (pages 5-12).

The sheared herring sperm DNA is blunt-end repaired and dephosphorylated using the reagents in the kit. Three TOPO® Cloning reactions using either 6 ng, 20 ng, or 60 ng of blunt-end, dephosphorylated sheared DNA and 10 ng each of pCR®4Blunt-TOPO® are performed and transformed into One Shot® TOP10 Chemically Competent *E. coli*. Fifty microliters of the transformation reaction are plated in duplicate on LB plates containing 100 µg/ml ampicillin and incubated overnight. Colonies are counted and the total colonies are calculated for one TOPO® Cloning reaction. Each TOPO® Cloning reaction should yield greater than 1200 colonies/reaction (approximately 1.2×10^5 cfu per µg plasmid DNA).

If the colonies/reaction is greater than 1200, then 20 transformants are selected, plasmid DNA isolated and digested with *Eco*R I. Restriction analysis should yield a variety of different sized inserts that are greater than 100 bp. Eighty percent (16/20) of the plasmids should contain insert.

Primers

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

One Shot® TOP10 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 ~1×10^9 cfu/µg DNA for chemically competent cells and >1×10^9 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.

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