invitrogen™

Zero Blunt® TOPO® PCR Cloning Kit

Five-minute cloning of blunt-end PCR products

Catalog nos. K2800-20, K2800-40, K2820-20, K2820-40, K2830-20, K2860-20, K2860-40, and K2800-02

Version N
10 April 2006
25-0215

User Manual

Table of Contents

Kit Contents and Storage

Shipping and Storage

Zero Blunt® TOPO® PCR Cloning Kits are shipped on dry ice. Each kit contains a box with Zero Blunt® TOPO® PCR Cloning reagents (Box 1) and a box with One Shot® *E. coli* (Box 2).

Zero Blunt® TOPO® PCR Cloning Kit supplied with the PureLink™ Quick Plasmid Miniprep (cat. no.K2800-02) 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.

Zero Blunt® TOPO® PCR Cloning Kits

Zero Blunt® TOPO® PCR Cloning Kits are available with either One Shot® TOP10 Chemically Competent, One Shot® DH5α™-T1^R Chemically Competent, One Shot® Mach1™- T1^R Chemically Competent, or One Shot® TOP10 Electrocomp™ *E. coli* (see page vii for the genotypes of the strains). Catalog no. K2800-02 also includes PureLink™ Quick Plasmid Miniprep Kit.

Catalog no.	Reactions	One Shot® Cells	Type of Cells
K2800-20	20	TOP10	Chemically competent
K2800-40	40	TOP10	Chemically competent
K2820-20	20	DH5α™-T1^R	Chemically competent
K2820-40	40	DH5α™-T1^R	Chemically competent
K2830-20	20	Mach1™- T1^R	Chemically competent
K2860-20	20	TOP10	Electrocompetent
K2860-40	40	TOP10	Electrocompetent
K2800-02*	20	TOP10	Chemically competent

*Includes PureLink™ Quick Plasmid Miniprep Kit

continued on next page

Zero Blunt® TOPO® PCR Cloning Reagents

Zero Blunt® TOPO® PCR Cloning reagents (Box 1) are listed below. **Note that the user must supply the proofreading polymerase.** Store Box 1 at -20°C.

Item	Concentration	Amount
pCR®-Blunt II-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
M13 Reverse Primer	0.1 µg/µl in TE Buffer	20 µl
M13 Forward (-20) Primer	0.1 µg/µl in TE Buffer	20 µl
Control Template	0.1 µg/µl in TE Buffer	10 µl
Water	--	1 ml

Sequence of Primers

The table below lists the sequence of the M13 Sequencing primers included in the kit.

Primer	Sequence	pMoles Supplied
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385
M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´	407

PureLink™ Quick Plasmid Miniprep Kit

For kit components of the PureLink™ Quick Plasmid Miniprep Kit (Box 3) supplied with cat. no K2800-02, refer to the manual supplied with the miniprep kit.

continued on next page

One Shot® Reagents

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

Item	Composition	Amount
S.O.C. Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10, Mach1™-T1^R, **OR** DH5α-T1^R **OR** TOP10	Chemically Competent Electrocomp™	21 x 50 µl
pUC19 Control DNA	10 pg/µl	50 µl

Genotypes of *E. coli* Strains

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)

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

F$^-$ ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R(r_k^-, m_k^+) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

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

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.

Accessory Products

Additional Products

The table below lists additional products that may be used with Zero Blunt® TOPO® PCR Cloning Kits. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 19).

Product	Amount	Catalog no.
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
	250 units	11708-021
	500 units	11708-039
AccuPrime™ *Pfx* DNA Polymerase	200 reactions	12344-024
	1000 reactions	12344-032
AccuPrime™ *Pfx* SuperMix	200 reactions	12344-040
Pfx50™ DNA Polymerase	100 reactions	12355-012
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
Kanamycin	5 g	11815-024
	25 g	11815-032
	100 ml (10 mg/ml)	18160-054
Zeocin™	1 g	R250-01
	5 g	R250-05
S.O.C. Medium	10 x 10 ml	15544-034

Introduction

Overview

Introduction

Zero Blunt® TOPO® PCR Cloning provides a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of blunt-end PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

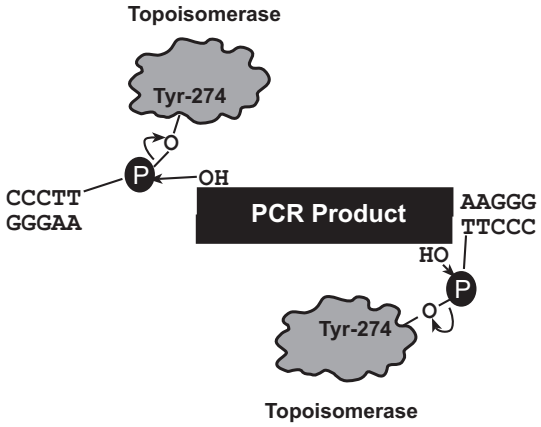
How Topoisomerase I Works

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 diagram below).

TOPO® Cloning

The plasmid vector (pCR®-Blunt II-TOPO®) is supplied linearized with *Vaccinia* virus DNA topoisomerase I covalently bound to the 3´ end of each DNA strand (referred to as "TOPO®-activated" vector). The TOPO® Cloning Reaction can be transformed into chemically competent cells or electroporated directly into electrocompetent cells.

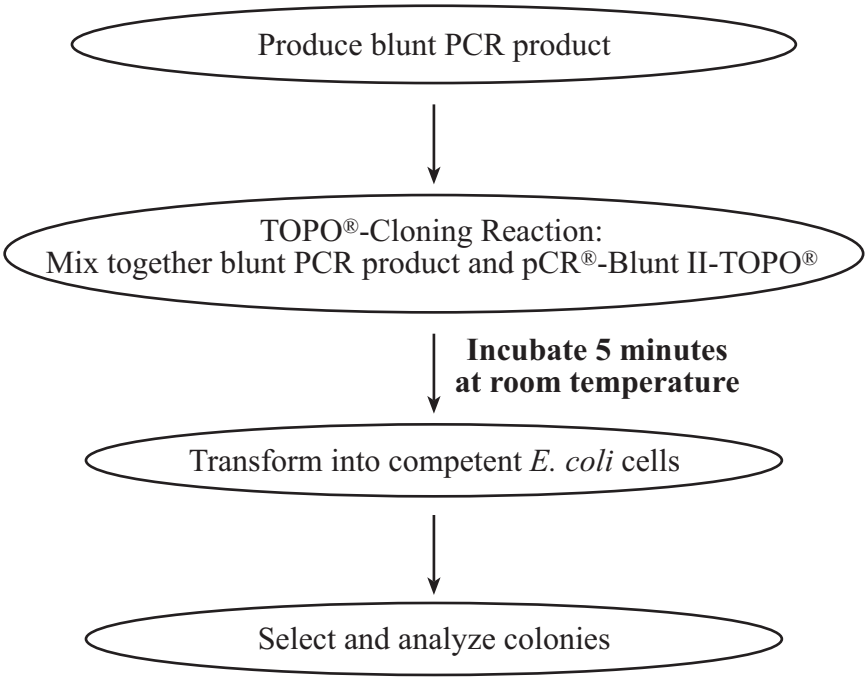
In addition, pCR®-Blunt II-TOPO® allows direct selection of recombinants via disruption of the lethal *E. coli* gene, *ccd*B (Bernard *et al.*, 1994). The vector contains the *ccd*B gene fused to the C-terminus of the LacZα fragment. Ligation of a blunt-end PCR product disrupts expression of the *lac*Zα-*ccd*B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



continued on next page

Experimental Outline

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



Methods

Producing Blunt-End PCR Products

Introduction

This kit is specifically designed to clone blunt-end PCR products generated by thermostable proofreading polymerases such as Platinum® *Pfx* DNA Polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Note

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

Materials Supplied by the User

You will need the following reagents and equipment for PCR.

Note: dNTPs (adjusted to pH 8) are provided in the kit.

- Thermostable proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- Thermocycler
- DNA template and primers for your PCR product

Producing PCR Products

Set up a 25 µl or 50 µl PCR reaction using the guidelines below:

- Follow the instructions and recommendations provided by the manufacturer of your thermostable, proofreading polymerase to produce blunt-end PCR products.
- Use the cycling parameters suitable for your primers and template. Make sure to optimize PCR conditions to produce a single, discrete PCR product.
- Use a 7 to 30 minute final extension to ensure that all PCR products are completely extended.
- After cycling, place the tube on ice or store at -20ºC for up to 2 weeks. Proceed to **Checking the PCR Product**, below.

Checking the PCR Product

After you have produced your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check for the following outcomes below.

Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations for optimizing your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see page 16).

Performing the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pCR®-Blunt II-TOPO® vector and transform the recombinant vector into competent *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent Cells** (pages 6-8) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 13-15 in parallel with your samples.

Note

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

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

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 below.
- 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 blue color of the pCR®II-Blunt-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 (1:4)	--	1 µl
Water	add to a final volume of 5 µl	add to a final volume of 5 µl
pCR®II-Blunt-TOPO®	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 **Transforming One Shot® Competent Cells,** next page.

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

Transforming One Shot® Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pCR®-Blunt II-TOPO® construct into competent *E. coli* provided with your kit. Protocols to transform chemically competent and electrocompetent *E. coli* are provided below.

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)
- 42°C water bath or electroporator with 0.1 cm cuvettes
- 15 ml snap-cap plastic tubes (sterile) (electroporation only)
- LB plates containing 50 µg/ml kanamycin or Low Salt LB plates containing 25 µg/ml Zeocin™ (use two plates per transformation; see page 18 for recipes)
- 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 or set up electroporator
- Bring the vial of S.O.C. medium to room temperature.
- Warm LB plates containing 50 µg/ml kanamycin or 25 µg/ml Zeocin™ at 37°C for 30 minutes.
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

Important

If you are transforming One Shot® Mach1™-T1^R Chemically Competent *E. coli*, it is essential that selective plates are prewarmed to 37° prior to spreading for optimal growth of cells.

continued on next page

Transforming One Shot® Competent Cells, Continued

One Shot® Chemical Transformation

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. Incubate plates over night at 37°C.
8. An efficient TOPO® Cloning reaction will produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing Transformants**, page 9).

One Shot® Electroporation

1. Add 2 µl of the TOPO® Cloning reaction to a vial (50 µl) of One Shot® Electrocomp™ *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Carefully transfer cells and DNA to a chilled 0.1 cm cuvette.
3. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.
4. Immediately add 250 µl of room temperature S.O.C. medium to the cuvette.
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. Incubates plates over night at 37°C.
7. An efficient TOPO® Cloning reaction will produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing Transformants**, page 9).

continued on next page

Transforming One Shot® Competent Cells, Continued

Note

Addition of the Dilute Salt Solution in the **TOPO® Cloning Reaction** brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, 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 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 medium containing 50 µg/ml kanamycin or 25 µg/ml Zeocin™. Be sure to save the original colony by patching to a fresh plate, if needed.

   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 kanamycin or 25 µg/ml Zeocin™ 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. K2800-02 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 by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation. The M13 Forward (-20) and M13 Reverse primers are included to help you sequence your insert. Refer to the map on page 12 for sequence surrounding the TOPO Cloning® site. For the full sequence of pCR®-Blunt II-TOPO®, visit www.invitrogen.com or contact Technical Service (page 19).

Alternative Method of Analysis

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use either the M13 Forward (-20) or the M13 Reverse primer 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 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.

continued on next page

Long-Term Storage

Once you have identified the correct clone, be sure to prepare a glycerol stock for long-term storage.

1. Streak the original colony out on LB plates containing 50 μg/ml kanamycin (or 25 μg/ml Zeocin™).
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 μg/ml kanamycin (or 25 μg/ml Zeocin™).
3. Grow with shaking to log phase (OD_{600} = ~0.5)
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 cloning efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

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

More Transformants

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

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

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

Cloning Dilute PCR Products

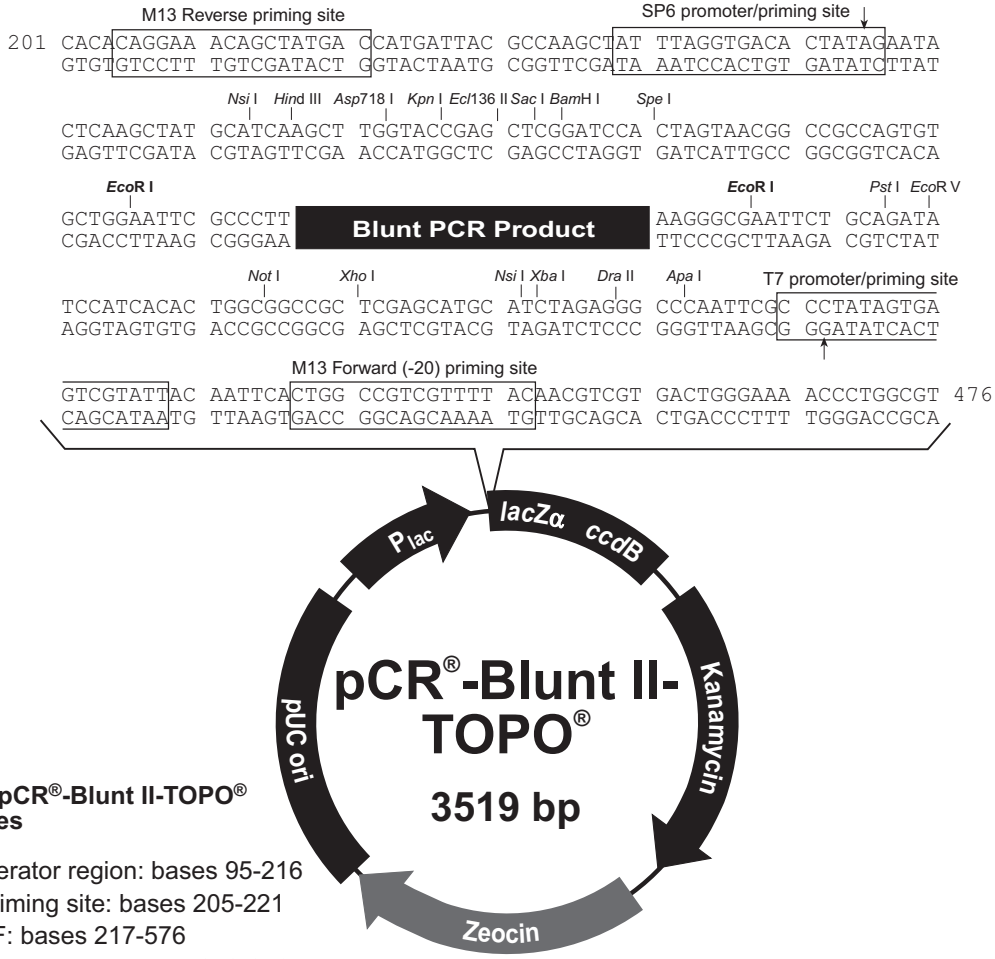
To clone dilute PCR products, you may:

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

Map of pCR®-Blunt II-TOPO®

pCR®-Blunt II-TOPO® Map

The map below shows the features of pCR®-Blunt II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for the T7 and SP6 polymerases. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 19).**



Comments for pCR®-Blunt II-TOPO® 3519 nucleotides

lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-576
SP6 promoter priming site: bases 239-256
Multiple Cloning Site: bases 269-399
TOPO®-Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 (-20) Forward priming site: bases 433-448
Fusion joint: bases 577-585
ccdB lethal gene ORF: bases 586-888
kan gene: bases 1099-2031
  kan promoter: bases 1099-1236
  Kanamycin resistance gene ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2724-3397

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 an 800 bp blunt-end PCR product utilizing the reagents included in the kit and using it directly in a TOPO® Cloning reaction.

Producing Control PCR Product

1. To produce the 800 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng/µl)	1.0 µl
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
M13 Reverse primer (0.1 µg/µl)	1 µl
M13 Forward (-20) primer (0.1 µg/µl)	1 µl
Water	40.5 µl
Thermostable proofreading polymerase (1-2.5 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 minutes	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 800 bp band should be visible. You may quantify the amount of the blunt-end PCR product by running a known standard of the gel. The concentration of the 800 bp, blunt-end PCR product should be about 20 ng/µl. Proceed to **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®II-Blunt-TOPO®, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pCR®II-Blunt-TOPO®	1 µl	1 µl
Final Volume	**6 µl**	**6 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® competent cells (pages 6-8).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of S.O.C. medium to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

There should >100 colonies on the vector + PCR insert plate. Ninety-five percent of these colonies should contain the 800 bp insert when analyzed by *Eco*R I digestion and agarose gel electrophoresis.

Relatively few colonies (less than 5% of foreground) will be produced in the vector-only reaction.

Transformation Control

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 6-8.

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)	Gel-purify your insert as described on page 16. For cloning long PCR products (3-10 kb) generated with enzyme mixtures designed to produce long PCR products (*e.g.* Platinum® *Taq* DNA Polymerase High Fidelity), we recommend the TOPO XL PCR Cloning™ Kit (Catalog no. K4700-10)
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning fragments generated using *Taq* polymerase	Remove 3´ A-overhangs by incubating with either a proofreading polymerase or T4 DNA polymerase in the presence of dNTPs. Alternatively, you may use the TOPO TA Cloning® Kit (Catalog no. K4500-01).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 16).

Appendix

Purifying PCR Products

Introduction

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

Low-Melt Agarose Method

Note that gel purification will result in a dilution of your PCR product and a less efficient TOPO® Cloning reaction. 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. Minimize exposure to UV to prevent damage to your DNA.
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 One Shot® competent *E. coli* using the protocols on pages 6-8.

Note

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

Recipes

LB (Luria-Bertani) Medium and Plates

Do not use Luria-Bertani Medium with Zeocin™. See Low Salt LB medium below or imMedia™.

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 (50 µg/ml of kanamycin) if needed.
4. Store at room temperature or at +4°C.

LB agar plates

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

Low Salt LB Medium

Reduce the salt in LB medium if you are using Zeocin™ for selection.

Composition:

1.0% Tryptone
0.5% Yeast Extract
0.5% NaCl
pH 7.5

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and **5 g NaCl** in 950 ml deionized water. For plates, be sure to add 15 g/l agar.
2. Adjust the pH of the solution to 7.5 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 Zeocin™ to a final concentration of 25 µg/ml.
4. Store at room temperature or at +4°C.

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 Zero Blunt® TOPO® PCR Cloning Kit as described below.

Restriction Digest

The parental supercoiled pCR®II-Blunt vector is qualified by restriction digest before adaptation with topoisomerase. Restriction digests must demonstrate the correct banding pattern when electrophoresed on an agarose gel.

TOPO® Cloning Efficiency

Once the pCR®II-Blunt-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 13-14, a 800 bp control PCR product is 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, 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.

Limited Use Label License No. 86: Zeocin™ Selection Marker

The *ble* resistance gene is the subject of one or more of U.S. Patent Nos. 5,021,344 and 5,118,620 and foreign equivalents owned by Cayla and licensed to Invitrogen. This product is sold for research purposes only. For commercial license information, please contact: Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008. Phone: 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., Gabant, P., Bahassi, E. M., and Couturier, M. (1994). Positive Selection Vectors Using the F Plasmid *ccd*B Killer Gene. Gene *148*, 71-74.

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

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

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**