



Zero Blunt® TOPO® PCR Cloning Kit

Five-minute cloning of blunt-end PCR products

Catalog numbers K2800-20, K2800-40, K2820-20, K2820-40, K2830-20,
K2860-20, K2860-40, K2800-02, 450245

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

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Kit Contents and Storage

Shipping and Storage

Zero Blunt® TOPO® PCR Cloning Kits are shipped on dry ice. Kits supplied with competent cells contain 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 Kit (Cat. no.K2800-02) is shipped with an additional box containing reagents for plasmid purification (Box 3).

Zero Blunt® TOPO® PCR Cloning® Kit (Cat. no. 450245) is shipped with only the Zero Blunt® TOPO® PCR Cloning reagents (Box 1).

Box	Store at
1	-30°C to -10°C in a non-frost-free freezer
2	-85°C to -68°C
3	Room temperature (15°C to 30°C)

Zero Blunt® TOPO® PCR Cloning Kits

Zero Blunt® TOPO® PCR Cloning Kits are available with 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 vi for the genotypes of the strains).

Notes: Catalog no. 450245 is not supplied with competent cells.

Catalog no. K2800-02 also includes the PureLink® Quick Plasmid Miniprep Kit.

Cat. 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
450245	20	Not supplied	NA

*Includes PureLink® Quick Plasmid Miniprep Kit

Product Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

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Kit Contents and Storage, Continued

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 -30°C to -10°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
Salt Solution	1.2 M NaCl 0.06 M MgCl ₂	50 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
M13 Forward (-20) Primer	0.1 μg/μL in TE Buffer	20 μL
M13 Reverse 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 following table describes the sequence and pmoles supplied of the sequencing primers included in this kit.

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

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.

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Kit Contents and Storage, Continued

One Shot® Reagents

The following table describes the items included in each One Shot® Competent Cells Kit. Store at -85°C to -68°C .

Item	Composition	Amount
S.O.C. Medium (may be stored in a cold room at 2°C to 8°C , or at room temperature, 15°C to 30°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, Mach1™-T1 ^R , <i>or</i> DH5α™-T1 ^R <i>or</i> TOP10 cells	Chemically Competent Electrocomp™	21 × 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⁻ $\phi 80lacZ\Delta M15 \Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1 tonA$ (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⁻ $\phi 80(lacZ)\Delta M15 \Delta lacX74 hsdR(r_k^-, m_k^+) \Delta recA1398 endA1 tonA$ (confers resistance to phage T1)

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

F⁻ $mcrA \Delta(mrr-hsdRMS-mcrBC) \Phi 80lacZ\Delta M15 \Delta lacX74 recA1 araD139 \Delta(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG$

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.

Introduction

Product Overview

Description of the System

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.

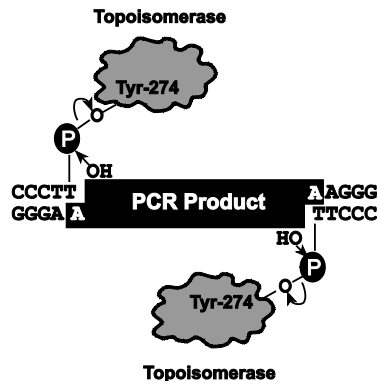
How Topoisomerase I Works

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 the following diagram).

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, *ccdB* (Bernard *et al.*, 1994). The vector contains the *ccdB* gene fused to the C-terminus of the LacZ α fragment. Ligation of a blunt-end PCR product disrupts expression of the *lacZ α -ccdB* 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.

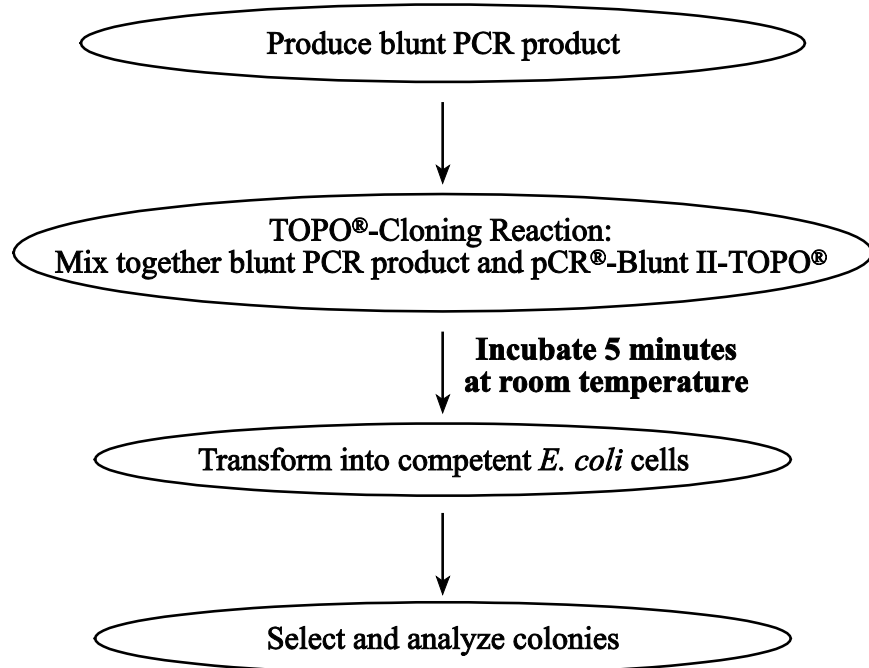


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Product Overview, Continued

Experimental Outline

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



Methods

Produce 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.



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

Required Materials

Components required but not supplied:

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

Components supplied with the kit:

- dNTPs (adjusted to pH 8)
-

Produce PCR Products

Set up a 25- or 50- μ L 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 7–30 minute final extension to ensure that all PCR products are completely extended.
 - After cycling, place the tube on ice or store at -20°C for up to 2 weeks. Proceed to **Check the PCR Product**.
-

Check the PCR Product

After producing your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check to be sure that 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 15).

Perform the TOPO[®] Cloning Reaction

Introduction

After producing 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 **Transform 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 12–14 in parallel with your samples.



Note

We have found that including salt (200 mM NaCl; 10 mM MgCl₂) 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.

Including salt allows for longer incubation times because it prevents topoisomerase I from re-binding 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 you must dilute the TOPO[®] Cloning reaction before transforming electrocompetent cells.

- For TOPO[®] Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM MgCl₂ in the TOPO[®] Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M MgCl₂) is provided to adjust the TOPO[®] Cloning reaction to the recommended concentration of NaCl and MgCl₂.
 - For TOPO[®] Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO[®] Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM MgCl₂ in order to prevent arcing. After performing the TOPO[®] Cloning reaction, and prior to electroporation, dilute the reaction 4-fold to achieve the proper salt concentration.
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Continued on next page

Perform the TOPO[®] Cloning Reaction, Continued

Set Up the TOPO[®] Cloning Reaction

Use the following procedure to perform the TOPO[®] Cloning reaction. Set up the TOPO[®] Cloning reaction using the reagents in the order shown.

Note: The blue color of the pCR[™]II-Blunt-TOPO[®] vector solution is normal and is used to visualize the solution.

Reagent*	Volume
Fresh PCR product	0.5–4 μ L
Salt Solution	1 μ L
Water	add to a total volume of 5 μ L
pCR [™] II-Blunt-TOPO [®]	1 μ L
Final Volume	6 μL

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

Perform the TOPO[®] Cloning Reaction

1. Mix the reaction gently and incubate for **5 minutes** at room temperature ($22\text{--}23^{\circ}\text{C}$).

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 (greater than 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 **Transform One Shot[®] Competent Cells**, on page 6.

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



Note

Zero Blunt[®] TOPO[®] PCR Cloning Kits are optimized to work with One Shot[®] Competent *E. coli* available from Life Technologies Corporation. Use of other competent cells may require further optimization.

Performing the control TOPO[®] Cloning reaction is recommended as this control when used with the supplied protocol will demonstrate high cloning efficiencies.

Additionally, transforming a control plasmid is highly recommended to confirm transformation efficiencies when using alternative competent cells not supplied by Life Technologies.

Transform One Shot[®] Competent Cells

Introduction

After performing the TOPO[®] Cloning reaction, transform your pCR[™]-Blunt II-TOPO[®] construct into competent *E. coli*.

Protocols to transform One Shot[®] chemically competent and electrocompetent *E. coli* supplied with the Zero Blunt[®] TOPO[®] PCR Cloning Kits are provided below.

To transform another competent strain, refer to the manufacturer's instructions.

Required Materials

Components required but not supplied:

- The TOPO[®] Cloning reaction from **Perform the TOPO[®] Cloning Reaction** (step 2 on page 5)
- LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin[™] selective antibiotic (use two plates per transformation; see page 17 for recipes)
- 15-mL snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath or electroporator with 0.1-cm cuvettes
- 37°C shaking and non-shaking incubator
- General microbiological supplies (e.g. plates, spreaders)

Components supplied with the kit:

- S.O.C. medium (included with the kit)
-

Prepare for Transformation

For each transformation, you will need 1 vial of competent cells and 2 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 to room temperature.
 - Warm LB plates containing 50 µg/mL kanamycin or 25 µg/mL Zeocin[™] selective antibiotic at 37°C for 30 minutes.
 - Thaw *on ice* 1 vial of One Shot[®] cells for each transformation.
-



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

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Transform One Shot[®] Competent Cells, Continued

One Shot[®] Chemical Transformation

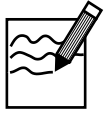
1. Add 2 μL of the TOPO[®] Cloning reaction from **Perform the TOPO[®] Cloning Reaction**, step 2 on page 5 into a vial of One Shot[®] Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
 2. Incubate on ice for 5–30 minutes.
Note: Longer incubations on ice do not seem to affect 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 will produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze Transformants** on page 9).
-

One Shot[®] Electroporation

1. Add 18 μL of water to 6 μL of the TOPO[®] Cloning reaction from **Perform the TOPO[®] Cloning Reaction**, step 2 on page 5. Mix gently.
Note: The TOPO[®] Cloning reaction must be diluted in this step to prevent arcing.
 2. Add 2 μL of the dilute TOPO[®] Cloning reaction (from step 1 of this procedure) 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.**
 3. Carefully transfer cells and DNA to a chilled 0.1-cm cuvette.
 4. Electroporate your samples using your own protocol and your electroporator.
Note: If you have problems with arcing, see page 8.
 5. Immediately add 250 μL of room temperature S.O.C. medium to the cuvette.
 6. 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.
 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. Incubates plates over night at 37°C.
 8. An efficient TOPO[®] Cloning reaction will produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze Transformants** on page 9).
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Transform One Shot[®] Competent Cells, Continued



Note

Diluting the TOPO[®] Cloning Reaction brings the final concentration of NaCl and MgCl₂ 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 50–80 μL (0.1-cm cuvettes) or 100–200 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
-

Analyze Transformants

Analyze Positive Clones

1. Culture 2–6 colonies overnight in LB medium containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic. 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™ selective antibiotic before isolating the 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, see page 19). The plasmid isolation protocol is included in the manual supplied with the PureLink® Quick Plasmid Miniprep Kit and is also available from www.lifetechnologies.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.

Sequence

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 maps on page 18 for sequence surrounding the TOPO Cloning® site. For the full sequence of pCR™-Blunt II-TOPO®, refer to www.lifetechnologies.com/support or contact Technical Support (page 20).

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Analyzing Transformants, Continued

Analyze Transformants by PCR

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 (see page 19)

Appropriate forward and reverse PCR primers (20 μ M each)

Procedure

1. For each sample, aliquot 48 μ L of PCR SuperMix High Fidelity into a 0.5-mL microcentrifuge tube. Add 1 μ L each of the forward and reverse PCR primer.
2. Pick 10 colonies and resuspend them individually in 50 μ L of the PCR cocktail from step 1 of this procedure. 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 20–30 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

After identifying the correct clone, be sure to prepare a glycerol stock for long term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out on LB plates containing 50 μ g/mL Kanamycin or 25 μ g/mL Zeocin™ selective antibiotic.
 2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 μ g/mL Kanamycin or 25 μ g/mL Zeocin™ selective antibiotic.
 3. Grow with shaking to log phase ($OD_{600} = \sim 0.5$).
 4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
 5. Store at -80°C.
-

Optimize the TOPO[®] Cloning Reaction

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 μ L 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 20–30 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. Adding salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Clone Dilute PCR Products

To clone dilute PCR products, you may:

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

Perform the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate results. Performing the control reactions involves producing 800-bp blunt-end PCR product using the reagents included in the kit and using the PCR product directly in a TOPO® Cloning reaction.

Producing the Control PCR Product

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

Control DNA Template (100 ng)	1 μ L
10X PCR Buffer	5 μ L
dNTP Mix	0.5 μ L
M13 Reverse primer (0.1 μ g/ μ L)	1 μ L
M13 Forward (-20) primer (0.1 μ g/ μ L)	1 μ L
Water	40.5 μ L
<u>Thermostable proofreading polymerase (1–2.5 unit/μL)</u>	<u>1 μL</u>
Total Volume	50 μ L

2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	94°C	1X
Denature	1 minute	94°C	25X
Anneal	1 minute	55°C	
Extend	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

3. Remove 10 μ L 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/ μ L. Proceed to the **Control TOPO® Cloning Reactions** on page 13.
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Perform the Control Reactions, Continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on page 12 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"
Control PCR Product	—	1 μL
Water	4 μL	3 μL
Salt Solution	1 μL	1 μL
pCR™II-Blunt-TOPO®	1 μL	1 μL
Final Volume	6 μL	6 μL

2. Incubate the reactions at room temperature for **5 minutes** and place on ice.
3. Prepare the samples for transformation:
 - For chemical transformation protocols, proceed directly to step 4.
 - For **electroporation protocols only**, dilute the TOPO® Cloning reaction 4-fold (e.g. add 18 μL of water to the 6 μL TOPO® Cloning reaction) before proceeding to step 4.
4. Transform 2 μL of each reaction into separate vials of One Shot® competent cells (pages 6–8) or equivalent competent cells.
5. Spread 10–50 μL of each transformation mix onto LB plates containing 50 μg/mL kanamycin. Be sure to plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies. For plating small volumes, add 20 μL of S.O.C. medium to allow even spreading.
6. Incubate overnight at 37°C.

Analyze Results

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

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

Transformation Control

Kit with competent cells includes pUC19 plasmid 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 S.O.C. medium.

Type of Cells	Volume to Plate	Transformation Efficiency
Chemically Competent	10 μL + 20 μL S.O.C.	~1 × 10 ⁹ cfu/μg DNA
Electrocompetent	20 μL (1:10 dilution)	> 1 × 10 ⁹ cfu/μg DNA

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Perform the Control Reactions, Continued

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 7–30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (greater than 1 kb)	Try one or all of the following: <ul style="list-style-type: none"> For cloning long PCR products (3–10 kb) generated with enzyme mixtures designed to produce long PCR products (e.g. Platinum[®] <i>Taq</i> DNA Polymerase High Fidelity), we recommend the TOPO[®] XL PCR Cloning[™] Kit (see page 19). Gel-purify the insert (see page 15).
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning fragments generated using <i>Taq</i> 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 (see page 19).
PCR cloning artifacts ("false positives")	TOPO [®] Cloning is very efficient for small fragments (less than 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 15).

Appendix

Purify PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (greater than 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. Two simple protocols are described in this section.

Using the PureLink® Quick Gel Extraction Kit

The PureLink® Quick Gel Extraction Kit (page 19) allows you to rapidly purify PCR products from regular agarose gels.

1. Equilibrate a water bath or heat block to 50°C.
 2. Excise 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.
 3. Weigh the gel slice.
 4. Add Gel Solubilization Buffer (GS1) supplied in the kit as follows:
 - For $\leq 2\%$ 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.
 5. Incubate the tube at 50°C for 15 minutes. Mix every 3 minutes to ensure gel dissolution. After the gel slice appears dissolved, incubate the tube for an **additional** 5 minutes.
 6. Preheat an aliquot of TE Buffer (TE) to 65–70°C
 7. Place a Quick Gel Extraction Column into a Wash Tube. Pipet the mixture from step 5 of this procedure onto the column. Use 1 column per 400 mg agarose.
 8. Centrifuge at $> 12,000 \times g$ for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
 9. **Optional:** Add 500 μL Gel Solubilization Buffer (GS1) to the column. Incubate at room temperature for 1 minute. Centrifuge at $> 12,000 \times g$ for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
 10. 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 $> 12,000 \times g$ for 1 minute. Discard the flow-through.
 11. Centrifuge the column at $> 12,000 \times g$ for 1 minute to remove any residual buffer. Place the column into a 1.5-mL Recovery Tube.
 12. Add 50 μL **warm** (65–70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
 13. Centrifuge at $> 12,000 \times g$ for 2 minutes. *The Recovery Tube contains the purified DNA.* Store DNA at -20°C . Discard the column.
 14. 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 dilute your PCR product resulting in 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 TAE agarose gel (0.8–1.2%).
 2. Visualize the band of interest and excise the band. Minimize exposure to UV to prevent damaging the 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 5–10 minutes to keep the agarose melted.
 7. Transform 2–4 µL directly into competent One Shot[®] cells using the methods described 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™ selective antibiotic. See Low Salt LB medium below or use imMedia™ (see page 19 for ordering information).

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 the solution to cool to 55°C and add antibiotic (50 µg/mL 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 kanamycin), and pour into 10-cm plates.
 4. Let the plates 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™ selective antibiotic for selection.

Composition:

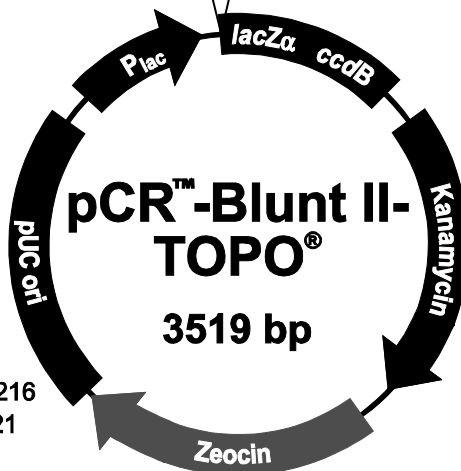
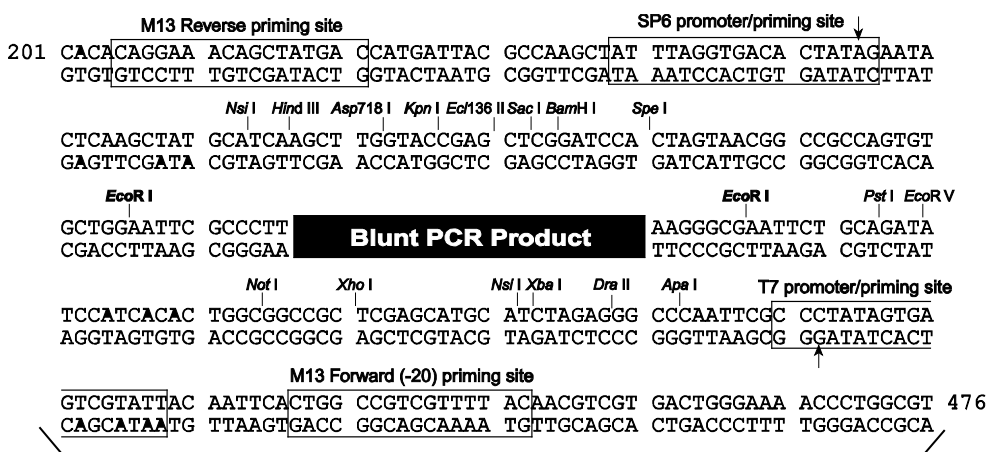
1.0% Tryptone
0.5% Yeast Extract
0.5% NaCl
pH 7.5

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™ selective antibiotic to a final concentration of 25 µg/mL.
 4. Store at room temperature or at 4°C.
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Map of pCR™-Blunt II-TOPO®

pCR™-Blunt II-TOPO® Map

The following map 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 from www.lifetechnologies.com/support or by contacting Technical Support (page 20).



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

Accessory Products

Additional Products

The following table lists additional products that may be used with TOPO® TA Cloning Kits. For more information, visit www.lifetechnologies.com/support or contact Technical Support (page 20).

Item	Quantity	Cat. 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 <i>E. coli</i>	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® TOP10 Electrocompetent <i>E. coli</i>	10 reactions	C4040-50
	20 reactions	C4040-52
One Shot® Mach1™-T1 ^R Chemically Competent <i>E. coli</i>	20 reactions	C8620-03
One Shot® MAX Efficiency® DH5α™-T1 ^R Chemically Competent <i>E. coli</i>	20 reactions	12297-016
PureLink® Quick Plasmid Miniprep Kit	50 reactions	K2100-10
PureLink® Quick Gel Extraction Kit	50 reactions	K2100-12
TOPO® XL PCR Cloning Kit	10 reactions	K4700-10
Zero Blunt® PCR Cloning Kit	20 reactions	K2700-20
TOPO® TA Cloning® Kit	20 reactions	K4500-01
Kanamycin	5 g	11815-024
	25 g	11815-032
	100 mL (10 mg/mL)	15160-054
Zeocin™ Selection Reagent	8 × 1.25 mL	R250-01
	50 mL	R250-05
S.O.C. Medium	10 × 10 mL	15544-034
imMedia™ Zeo Liquid	20 each	Q62020

Technical Support

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

At the website, you can:

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

Safety data sheets (SDS) Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

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Purchaser Notification

Information for European Customers

The Mach1™-T1^R *E. coli* strain is genetically modified to carry the *lacZ*ΔM15 *hsdR lacX74 recA endA tonA* 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.

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