USER GUIDE

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Zero Blunt® TOPO® PCR Cloning Kit

Five-minute cloning of blunt-end PCR products

Catalog numbers K2800-20, K2800-40, K2800-J10, K2820-20, K2820-40, K2830-20, K2860-20, K2860-40, K2800-02, 450245

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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.

TRADEMARKS

Contents

About this guide

IMPORTANT!

Before using this product, read and understand the information in the "Safety" appendix in this document.

Changes from previous version

Revision	Date	Description
B.0	14 February 2014	• Increase from 20 to 25 reaction kit size. • Include Cat. no. K2800-J10
A.0	15 November 2013	• Correction to the TOPO® Cloning diagram on page 8. • Version numbering changed to alphanumeric format and reset to A in conformance with internal document control procedures.

Product information

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 7 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	25	TOP10	Chemically competent
K2800-40	50	TOP10	Chemically competent
K2800-J10	10	TOP10	Chemically competent
K2820-20	25	DH5α™-T1^R	Chemically competent
K2820-40	50	DH5α™-T1^R	Chemically competent
K2830-20	25	Mach1™- T1^R	Chemically competent
K2860-20	25	TOP10	Electrocompetent
K2860-40	50	TOP10	Electrocompetent
K2800-02*	25	TOP10	Chemically competent
450245	25	Not supplied	NA

*Includes PureLink® Quick Plasmid Miniprep Kit

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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		
		10 Rxns	25 Rxns	50 Rxns
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	10 µL	25 µL	2 × 25 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	10 µL	2 × 10 µL
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µL	50 µL	2 × 50 µL
M13 Forward (–20) Primer	0.1 µg/µL in TE Buffer	20 µL	20 µL	2 × 20 µL
M13 Reverse Primer	0.1 µg/µL in TE Buffer	20 µL	20 µL	2 × 20 µL
Control Template	0.1 µg/µL in TE Buffer	10 µL	10 µL	2 × 10 µL
Water	—	1 mL	1 mL	2 × 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

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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.

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		
		10 Rxns	25 Rxns	50 Rxns
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	6 mL	2 × 6 mL
TOP10, Mach1™-T1^R, DH5α™-T1^R cells ***or*** TOP10 cells	Chemically competent Electrocomp™	11 × 50 µL	26 × 50 µL	2 × (26 × 50 µL)
pUC19 Control DNA	10 pg/µL	50 µL	50 µL	2 × 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.

Description of the system

Zero Blunt® TOPO® PCR Cloning

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.

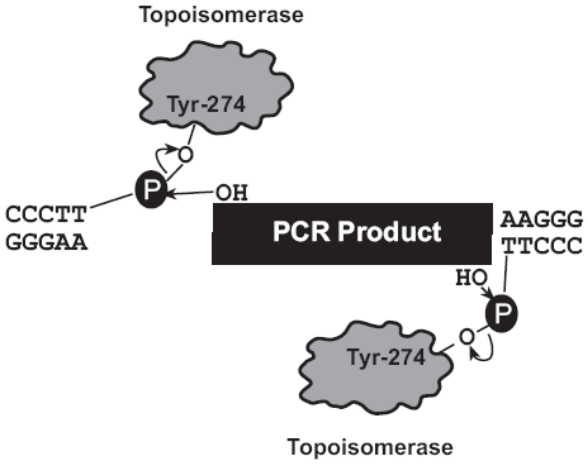
How Topoisomerase I works

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, *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.



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Experimental Outline

- Produce your blunt PCR product
- Set up the TOPO® cloning reaction (mix together the PCR Product and pCR®-Blunt II-TOPO® vector)
- Incubate for 5 minutes at room temperature
- Transform the TOPO® cloning reaction into One Shot® Competent Cells or equivalent
- Select and analyze 10 white or light blue colonies for insert

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.

Note

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-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 7–30 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 **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 22).

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 13–15) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 19–21 in parallel with your samples.

Note

We have found that including salt (200 mM NaCl; 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. 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 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 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 $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.
- 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 $MgCl_2$ 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.

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 µL
Salt Solution	1 µL
Water	add to a total volume of 5 µL
pCR™II-Blunt-TOPO®	1 µL
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°C–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 (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 13.

   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. 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 12)
- 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 24 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.

IMPORTANT!

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.

Continued on next page

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 12 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 16).

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 12. 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 15.
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 16).

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Note

Diluting 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 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 26). 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 25 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 29).

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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 26)

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 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 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL Kanamycin or 25 µg/mL Zeocin™ selective antibiotic.
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.

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 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 20–30 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 µL PCR:

Control DNA Template (100 ng)	1 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. 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 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 the **Control TOPO® Cloning reactions** on page 20.

Continued on next page

Perform the control reactions, continued

Control TOPO® Cloning reactions

Using the control PCR product produced on page 19 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 13–15) 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 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 *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

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 13–15.

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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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: • 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 (see page 26). • Gel-purify the insert (see page 22).
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 (see page 26).
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 22).

Appendix A: Support protocols

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 26) 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 ≤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°C –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 × *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 × *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 × *g* for 1 minute. Discard the flow-through.
11. Centrifuge the column at >12,000 × *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 × *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

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 12).
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 13–15.

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 26 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

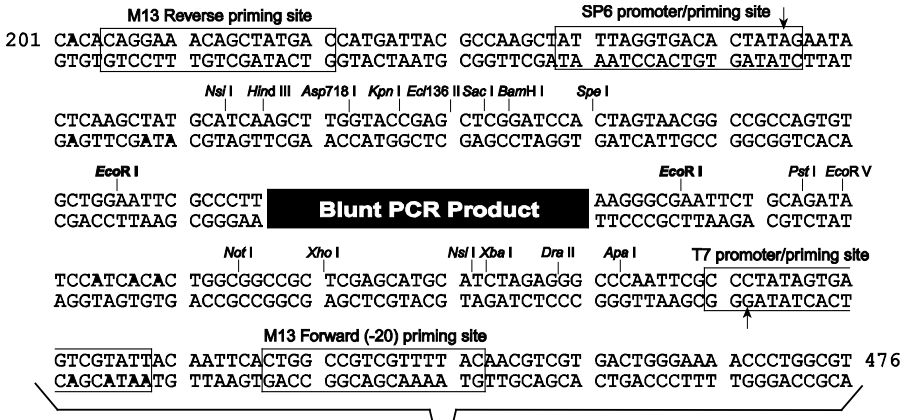
1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and **5 g NaCl** in 950 mL deionized water. For plates, be sure to add 15 g/L agar.
2. Adjust the pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to 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.

Appendix B: Vectors

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 sequence of the vector** is available from www.lifetechnologies.com/support or by contacting Technical Support (page 29).



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

Appendix C: Ordering information

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 29).

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 *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
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

Appendix D: Safety

Chemical safety

WARNING!

GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING!

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and 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

Obtaining SDS

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

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited product warranty

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

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