USER GUIDE

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TOPO® TA Cloning® Kit for Sequencing

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

Catalog numbers K4530-20, K4575-J10, K4575-01, K4575-40, K4580-01, K4580-40, K4595-01, K4595-40, K4575-02, 450030

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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

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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
A.0	24 February 2014	• Increase from 20 to 25 reaction kit size. • 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

The TOPO® TA Cloning® Kits for Sequencing are shipped on dry ice. Kits containing competent cells include box with TOPO® TA Cloning® Reagents (Box 1) and a box with One Shot® competent *E. coli* (Box 2).

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

TOPO® TA Cloning® Kit for Sequencing (Cat. no. 450030) is shipped with only the TOPO® TA 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)

Types of kits

TOPO® TA Cloning® Kits for Sequencing are available with Mach1™-T1^R, TOP10, or DH5α™ -T1^R One Shot® Chemically Competent cells or TOP10 One Shot® Electrocomp™ cells (see page 8 for the genotypes of the strains).

Note: Cat. no. 450030 is not supplied with competent cells.

Cat. no. K4575-02 also includes the PureLink® Quick Plasmid Miniprep Kit.

Cat. no.	Reactions	One Shot® Cells	Type of Cells
K4530-20	25	Mach1™-T1^R	chem. competent
K4575-J10	10	TOP10	chem. competent
K4575-01	25	TOP10	chem. competent
K4575-40	50	TOP10	chem. competent
K4595-01	25	DH5α™ -T1^R	chem. competent
K4595-40	50	DH5α™-T1^R	chem. competent
K4580-01	25	TOP10 Electrocomp™	electrocompetent
K4580-40	50	TOP10 Electrocomp™	Electrocompetent
K4575-02*	25	TOP10	chem. competent
450030	25	Not supplied	NA

*Includes PureLink® Quick Plasmid Miniprep Kit

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TOPO® TA Cloning® reagents

TOPO® TA Cloning® reagents (Box 1) are listed in the following table. **Note that the user must supply *Taq* polymerase.** Store Box 1 at –30°C to –10°C.

Item	Concentration	Amount		
		10 Rxns	25 Rxns	50 Rxns
pCR™4-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 phenol red	10 µL	25 µL	2 × 25 µL
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µL	100 µL	2 × 100 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
Water	—	1 mL	1 mL	2 × 1 mL
M13 Forward (–20) Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL	20 µL	2 × 20 µL
M13 Reverse Primer	0.1 µg/µL in TE Buffer, pH 8	20 µL	20 µL	2 × 20 µL
T3 primer	0.1 µg/µL in TE Buffer, pH 8	20 µL	20 µL	2 × 20 µL
T7 primer	0.1 µg/µL in TE Buffer, pH 8	20 µL	20 µL	2 × 20 µL
Control PCR Template	0.1 µg/µL in TE Buffer, pH 8	10 µL	10 µL	2 × 10 µL
Control PCR Primers	0.1 µg/µL *each* in TE Buffer, pH 8	10 µL	10 µL	2 × 10 µL

Continued on next page

Sequence of primers

The following table lists the sequence 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
T3	5′-ATTAACCCTCACTAAAGGGA-3′	329
T7	5′-TAATACGACTCACTATAGGG-3′	328

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

Continued on next page

Contents and storage, continued

Genotypes

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*recA*1398 *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.

PureLink® Quick Plasmid Miniprep Kit

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

Description of the system

TOPO® TA Cloning

The TOPO® TA Cloning® Kits for Sequencing provide a highly efficient, 5 minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for sequencing. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

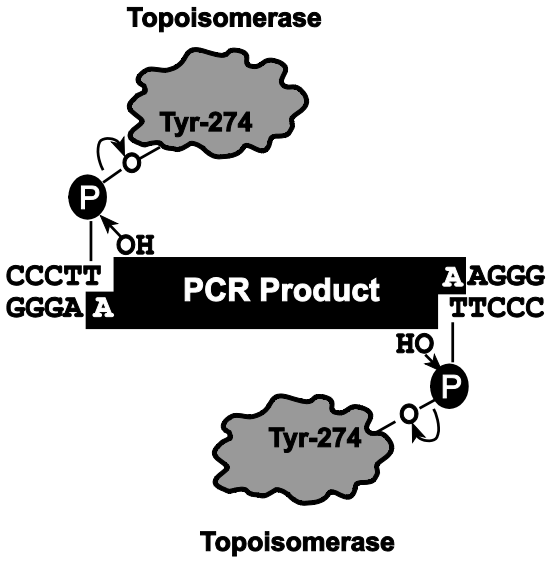
How Topoisomerase I works

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

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

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® Cloning exploits this reaction to efficiently clone PCR products.



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Positive selection

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

Experimental outline

- Produce your PCR product
- Set up the TOPO® cloning reaction (mix together the PCR Product and 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
- Isolate plasmid DNA and sequence.

Methods

Produce PCR Products

Introduction

The TOPO® TA Cloning® Kits for Sequencing are specifically designed to clone *Taq* polymerase-generated PCR products for sequencing. The first time you use the kit, we recommend performing the control TOPO® Cloning reaction on page 23 to evaluate your results.

Note

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

Materials supplied by the user

In addition to general microbiological supplies (e.g., plates, spreaders), you need:

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase mixtures

If you wish to use a mixture containing *Taq* polymerase and a proofreading polymerase, *Taq* must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proof-reading polymerase only, you can add 3' A-overhangs using the method on page 28.

Produce PCR products

1. Set up the following 50 µL PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7–30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

DNA Template	10–100 ng
10X PCR Buffer	5 µL
50 mM dNTPs	0.5 µL
Primers (~200 ng each)	1 µM each
Water	add to a final volume of 49 µL
Taq Polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, refer to the following **Note**.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before TOPO® Cloning® (see page 26). Take special care to avoid sources of nuclease contamination. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Cat. no. K1220-01) incorporates many of the recommendations found in this reference.

Perform the TOPO® Cloning Reaction

Introduction

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

Note

We have found that including salt (200 mM NaCl, 10 mM $MgCl_2$) in the TOPO® Cloning reaction can increase the number of transformants 2- to 3-fold. In addition, incubating the reaction mixture for greater than 5 minutes in the presence of salt can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Including salt in the TOPO® Cloning reaction allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules, leading to higher transformation efficiencies.

Using salt solution in the TOPO® Cloning reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (i.e. using the stock salt solution provided in the kit).

Note that 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 red or yellow color of the 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
TOPO® vector	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 to 23°C).

   Note: For most applications, 5 minutes will yield sufficient 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 14.

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

Note

TOPO® TA 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, you will transform your pCR™4-TOPO® construct into the competent *E. coli*.

General guidelines for transformation are provided below.

For transformation into competent *E. coli* supplied with your kit, refer to **Transform One Shot® Mach1™-T1^R competent cells** (pages 15–16) or **Transform One Shot® TOP10 and DH5α™-T1^R competent cells** (pages 17–19) depending on the competent *E. coli* you wish to transform.

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

Select a One Shot® Chemical Transformation protocol

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

If you wish to...	Then use the...
maximize the number of transformants	regular chemical transformation protocol
clone large PCR products (greater than 1000 bp)	
use kanamycin as the selective agent (see the following IMPORTANT!)	
obtain transformants as quickly as possible	rapid chemical transformation protocol

IMPORTANT!

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

Recommendation

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

Transform One Shot® Mach1™-T1^R competent cells

Introduction

Protocols to transform One Shot® Mach1™-T1^R chemically competent *E. coli* are provided in this section. If you are transforming cells other than Mach1™-T1^R cells, refer to the section entitled **Transform One Shot® TOP10 and DH5α™-T1R competent cells** (pages 17–19). If using other competent cells, follow manufacturer's instructions.

Note

The Mach1™-T1^R strain allows you to visualize colonies 8 hours after plating on ampicillin selective plates. If you are using kanamycin selection, you will need to incubate plates overnight in order to visualize colonies.

With the Mach1™-T1^R strain, you may also prepare plasmid DNA 4 hours after inoculating a single, overnight-grown colony. Note that you will get sufficient growth of transformed cells within 4 hours in either ampicillin or kanamycin selective media.

Required materials

Components required but not supplied:

- The TOPO® Cloning reaction from **Set up the TOPO® Cloning reaction**, step 2 on page 13
- LB plates containing 50 µg/mL ampicillin or 50 µg/mL kanamycin
- 42°C water bath
- 37°C shaking and non-shaking incubator
- General microbiological supplies (e.g., plates, spreaders)

Components supplied with the kit:

- S.O.C. medium

Prepare for transformation

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

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

IMPORTANT!

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

Continued on next page

One Shot® chemical transformation protocol

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

1. Add 2 µL of the TOPO® Cloning reaction from **Perform the TOPO® Cloning reaction**, step 2 on page 13 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 *pre-warmed* selective plate. To ensure even spreading of small volumes, add 20 µL of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Incubate plates at 37°C. If you are using ampicillin selection, visible colonies should appear within 8 hours. For kanamycin selection, incubate plates overnight.
9. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze positive clones** on page 20).

Rapid One Shot® chemical transformation protocol

The following alternative protocol is provided for rapid transformation of One Shot® Mach1™-T1R cells. This protocol is **only** recommended for transformations using **ampicillin** selection. For more information on selecting a transformation protocol, refer to page 14.

Note: Warm LB plates containing ampicillin to 37°C prior to spreading.

1. Add **4 µL** of the TOPO® Cloning reaction from **Perform the TOPO® Cloning reaction**, step 2, page 13 into a vial of One Shot® chemically competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µL** of cells on a prewarmed LB plate containing 50–100 µg/mL ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze positive clones**, page 20).

Transform One Shot® TOP10 and DH5α™-T1^R competent cells

Introduction

Protocols to transform One Shot® TOP10 and DH5α™-T1^R competent *E. coli* are provided in this section. Both chemical transformation and electroporation protocols are provided. If you are transforming Mach1™-T1^R cells, refer to the section entitled T**ransform One Shot® Mach1™-T1^R competent cells** (pages 15–16). If using other competent cells, follow manufacturer's instructions.

Required materials

Components required but not supplied:

- The TOPO® Cloning reaction from **Set up the TOPO® Cloning reaction**, step 2 on page 13
- LB plates containing 50 µg/mL ampicillin or 50 µg/mL kanamycin
- 15-mL snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath
- 37°C shaking and non-shaking incubator
- General microbiological supplies (e.g., plates, spreaders)

Components supplied with the kit:

- S.O.C. medium

Prepare for transformation

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

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

IMPORTANT!

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

Continued on next page

Transform One Shot® TOP10 and DH5α™-T1^R competent cells, continued

One Shot® chemical transformation protocol

1. Add 2 µL of the TOPO® Cloning reaction from **Set up the TOPO® Cloning reaction**, **s**tep 2 on page 13 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 pre-warmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µL of S.O.C. medium We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze positive clones** on page 20).

Rapid One Shot® chemical transformation protocol

An alternative protocol is provided below for rapid transformation of One Shot® chemically competent *E. coli*. This protocol is **only** recommended for transformations using **ampicillin** selection. For more information on selecting a transformation protocol, see page 14.

Note: It is essential that LB plates containing ampicillin are pre-warmed prior to spreading.

1. Add **4 µL** of the TOPO® Cloning reaction from **Set up the TOPO® Cloning reaction**, step 2 on page 13, into a vial of One Shot® chemically competent *E coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µL** of cells on a prewarmed LB plate containing 50–100 µg/mL ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyze positive clones** on page 20).

Continued on next page

Transform One Shot® TOP10 and DH5α™-T1^R competent cells, continued

One Shot® electroporation protocol

1. Add 18 µL of water to 6 µL of the TOPO® Cloning reaction from **Perform the TOPO® Cloning reaction**, step 2 on page 13. Mix gently.

   Note: The TOPO® Cloning reaction must be diluted in this step to prevent arcing.
2. Add 2 µL of the diluted TOPO® Cloning reaction (from step 1 of this procedure) into a vial of One Shot® electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
3. Carefully transfer the solution into a 0.1-cm cuvette to avoid formation of bubbles.
4. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the following **Note.**
5. Immediately add 250 µL of room temperature S.O.C. medium.
6. Transfer the solution into 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 onto a pre-warmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µL of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 colonies for analysis (see **Analyzing positive clones** on page 20).

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 (for 0.1-cm cuvettes) or 100–200 µL (for 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. Take 2–6 colonies and culture them overnight in LB or SOB medium containing 50 µg/mL ampicillin or 50 µg/mL kanamycin.

   Note: If you transformed One Shot® Mach1™-T1^R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for 4 hours in **pre-warmed** LB medium containing 50 µg/mL ampicillin or 50 µg/mL kanamycin before isolating the plasmid. For optimal results, we recommend inoculating as much of a single colony as possible.
2. Isolate plasmid DNA using the PureLink® Quick Plasmid Miniprep Kit (supplied with Cat. no. K4575-02 or available separately, see page 36). 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/support**. Other kits for plasmid DNA purification are also suitable for use.
3. Analyze the plasmids for inserts by restriction analysis (digest with *Eco*R I or refer to the vector map on page 35) or by PCR screening (see page 21). You may also proceed directly to sequencing.

Sequence

You may sequence your construct to confirm that your gene is cloned in the correct orientation. Four primers (M13 Forward (–20), M13 Reverse, T3, and T7) are included to help you sequence your insert. Refer to the map on page 35 for the sequence surrounding the TOPO® Cloning site. For the full sequence of the vector, refer to **www.lifetechnologies.com/support** or contact Technical Support (page 37).

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

- Synthesize additional primers to sequence into the insert
- Prepare a set of nested deletions (refer to the protocol on page 29)

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

Continued on next page

Analyze transformants by PCR

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

Materials Needed

- PCR SuperMix High Fidelity (see page 36)
- 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 100 µg/mL ampicillin.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 100 µg/mL ampicillin.
3. Grow overnight until culture is saturated.
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

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. 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.

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 a control PCR product using the reagents included in the kit and using the PCR product directly in a TOPO® Cloning reaction.

Before starting

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

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

Produce the control PCR product

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

Control DNA Template (100 ng)	1 µL
10X PCR Buffer	5 µL
50 mM dNTP Mix	0.5 µL
Control PCR Primers (0.1 µg/µL each)	1 µL
Water	41.5 µL
Taq Polymerase (1 unit/µL)	1 µL
Total Volume	50 µL

2. 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

3. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the **Control TOPO® cloning reactions** on page 24.

Continued on next page

Control TOPO® Cloning reactions

Using the control PCR product produced on page 23 and pCR™4-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™4-TOPO®	1 µL	1 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® TOP10 cells (pages 17–19) or equivalent.
5. Spread 10–100 µL of each transformation mix onto LB plates containing 50 µg/mL kanamycin. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes (less than 20 µL), add 20 µL of S.O.C. medium to allow even spreading.

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

Analyze results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than 90% (or more) of these will contain the 750-bp insert when analyzed by EcoR I digestion and agarose gel electrophoresis. Relatively few colonies will be produced in the vector-only reaction. These colonies usually result from frame shift events (usually T-T mismatches) and results in disruption of the LacZα-*ccdB* reading frame.

Transformation control

Kits containing competent cells include 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 15–19.

Use LB plates with 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

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% (+/– 4%) 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 both of the following: • Increase amount of insert. • Gel-purify the insert (see page 26).
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning blunt-ended fragments	Add 3´ A-overhangs to your blunt PCR product by incubating with *Taq* polymerase (page 28).
	Use the Zero Blunt® PCR Cloning Kit to clone blunt PCR products (see page 36).
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 26).
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	Increase the final extension time to ensure all 3´ ends are adenylated. *Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).
Cloning small PCR products (less than 100 bp)	Small PCR products may not completely disrupt the *lacZα-ccdB* gene fusion to allow growth of positive recombinants. Try TOP10F´ cells, which express the Lac repressor to repress expression of the fusion. Pick transformants and characterize.

Note

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

Appendix A: Support protocols

Purify PCR products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (greater than 3 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Two simple protocols are described in this section.

Using the PureLink® Quick Gel Extraction Kit

The PureLink® Quick Gel Extraction Kit (page 36) 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 65–70°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 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. Use only chemically competent cells for transformation.

1. Electrophorese all 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.
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 13).
6. Incubate the TOPO® Cloning reaction **at 37°C for 5–10 minutes.** This is to keep the agarose melted.
7. Transform 2–4 µL directly into competent One Shot® cells using the method described on pages 15–19.

Note

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

Adding 3´ A-overhangs post-amplification

Introduction

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

Required materials

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplifying with a proofreading polymerase, place vials on ice and add 0.7–1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer. A sufficient number of PCR products will retain the 3´ A-overhangs.
2. Incubate the vials at 72°C for 8–10 minutes (do not cycle).
3. Place the vials on ice and use immediately in the TOPO® Cloning reaction.

Note: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

Note

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

Recipes

LB (Luria-Bertani) medium and plates

Composition:

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

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

LB agar plates

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

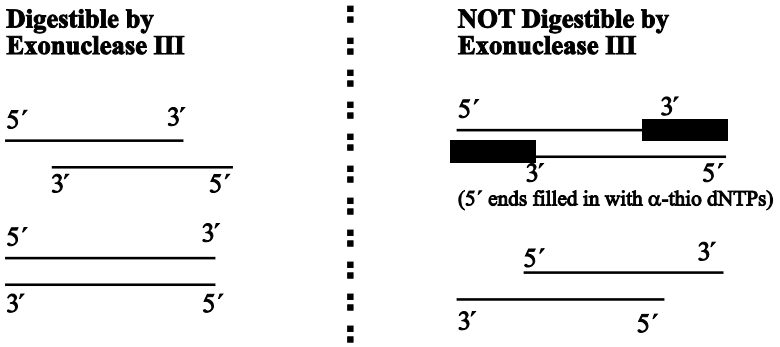
Generate nested deletions

Introduction

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

Background

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



Strategy

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

Note that the multiple cloning site in this vector contains an Sse8387 I site, a rare site that leaves a 3′ overhang after digestion. In addition, there is also a *Pme* I site that leaves a blunt end when digested.

Continued on next page

Generate nested deletions, continued

General outline

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

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

Required materials

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

Continued on next page

Recommendation

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

IMPORTANT!

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

Other considerations

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

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

Example

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

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

Plasmid preparation

You will need at least ~30 µg of DNA for restriction digestion and subsequent exonuclease III/mung bean nuclease digestion. Isolate DNA using the PureLink® HQ Mini Plasmid Purification Kit (see page 36) or CsCl gradient centrifugation.

First restriction digest

Digest ~30 µg DNA in a 500 µL reaction volume with an enzyme that leaves a 3´ overhang (e.g., *Sse*8387 I). Use 5 U of enzyme per µg DNA and digest the DNA to completion. Remember to inactivate the restriction enzyme and check the digest (1 µL) on an agarose gel to ensure that the reaction went to completion. (If you used an enzyme that leaves a 5´ overhang, see the **Important** note on page 33).

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

Continued on next page

IMPORTANT!

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

1. Add 2 µL of a 1 mM stock of α-thio-dNTPs and 5 U of Klenow polymerase to the restriction digest (from the **First restriction digest**, page 32) and incubate the reaction at room temperature for 10 minutes.
2. Extract with phenol:chloroform and ethanol precipitate. Resuspend DNA in a volume of 200 µL.

Incubate 1 µg of the filled-in DNA with 20 U of exonuclease III for 15 minutes at 37°C to check for protection against deletion. Analyze by agarose gel electrophoresis.

Second restriction digest

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

Exonuclease III/ Mung Bean Nuclease digestion

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

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

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

3. Add 5 µL of exonuclease III (100 U/µL) and incubate at the desired temperature (see page 32).
4. Remove 25 µL from the reaction for each time point (1–2 minutes per time point) and add to one of the tubes containing the stop solution. Place tubes on dry ice.
5. When all time points have been collected, heat the tubes at 68°C for 15 minutes to inactivate exonuclease III. Place the tubes on ice.
6. Dilute mung bean nuclease to 15 U/µL in 1X Mung Bean Nuclease buffer.
7. Add 1 µL of diluted mung bean nuclease to each time point tube. Incubate at 30°C for 30 minutes.
8. Extract each time point with phenol:chloroform and precipitate with ethanol. Resuspend each DNA pellet in 15 µL TE, pH 8.

Continued on next page

What you should see

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

Ligation and transformation

Use the DNA from step 8 of the **Exonuclease III/ Mung Bean Nuclease** digestion on page 33, to set up ligation reactions for each time point.

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

1. Set up the following 20 µL ligation reaction:

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

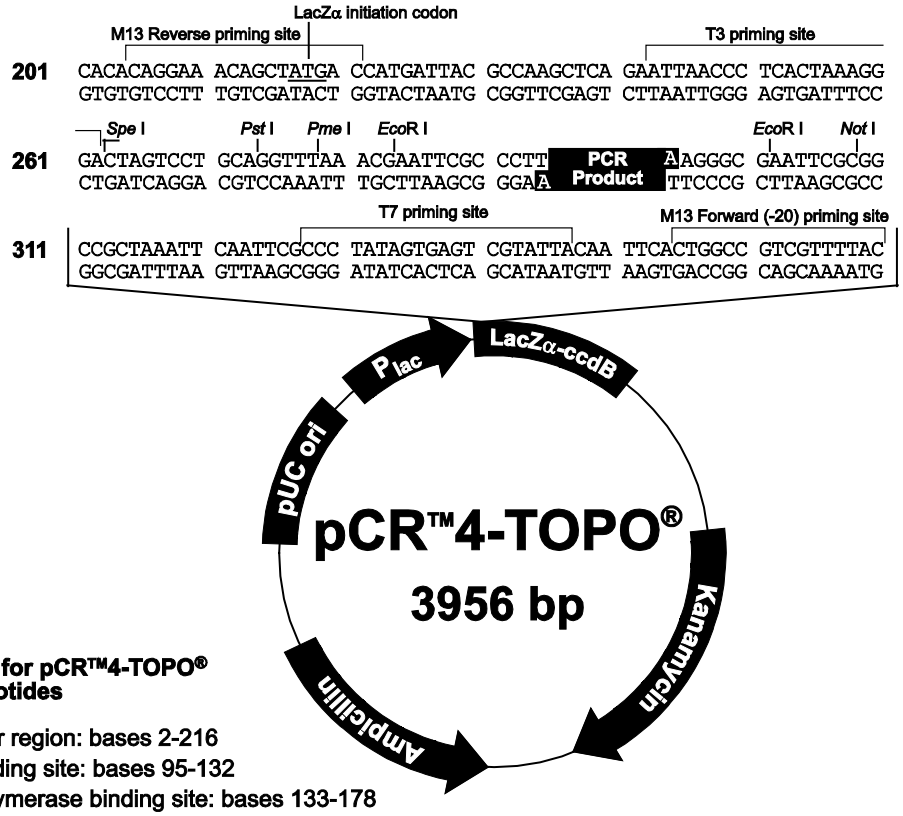
2. Incubate at room temperature for 4 hours or at 4°C overnight.
3. Transform 1 µL of the ligation reaction into competent *E. coli* and select on LB plates containing 50–100 µg/mL ampicillin.
4. Choose ~5 colonies per time point and isolate DNA using the PureLink® HQ Mini Plasmid Purification Kit (see page 36) or similar kit.
5. Analyze for deleted inserts. Order the deletions by descending size and proceed to sequencing. Clones can be sequenced using the M13 Reverse or the T3 primer.

Appendix B: Vectors

Map of pCR™4-TOPO®

pCR™4-TOPO® Map

The following map shows the features of pCR™4-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The sequence of pCR™4-TOPO® is available from **www.lifetechnologies.com** or by contacting Technical Support (page 39).



Comments for pCR™4-TOPO®
3956 nucleotides

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

Appendix C: Ordering information

Additional products

The following table lists additional products that may be used with TOPO® TA Cloning Kits for Sequencing. For more information, visit **www.lifetechnologies.com** or contact Technical Support (page 39).

Item	Quantity	Cat. no.
Taq DNA Polymerase, Native	100 units	18038-018
	500 units	18038-042
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
PCR SuperMix High Fidelity	100 reactions	10790-020
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
Ampicillin	200 mg	11593-027
Kanamycin	5 g	11815-024
	25 g	11815-032
	100 mL (10 mg/mL)	15160-054
S.O.C. Medium	10 × 10 mL	15544-034
PureLink® Quick Plasmid Miniprep Kit	50 reactions	K2100-10
PureLink® Quick Gel Extraction Kit	50 reactions	K2100-12
Zero Blunt® PCR Cloning Kit	20 reactions	K2700-20
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01

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/**

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