invitrogen™

pENTR™ Directional TOPO® Cloning Kits

Five-minute, directional TOPO® Cloning of blunt-end PCR products into an entry vector for the Gateway® System

Catalog nos. K2400-20, K2420-20, K2525-20, K2535-20, K2435-20, and K2635-20

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User Manual

Table of Contents

TOPO® Cloning Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>Design PCR Primers</td><td>• Include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer.
• Design the primers such that your gene of interest will be optimally expressed and fused in frame with the TEV recognition site (in pENTR™/TEV/D-TOPO® only) or any N- or C-terminal tags, if desired (after recombination with the Gateway® destination vector).</td></tr>
<tr><td>Amplify Your Gene of Interest</td><td>1. Use a thermostable, proofreading DNA polymerase and the PCR primers above to produce your blunt-end PCR product.
2. Use agarose gel electrophoresis to check the integrity and determine the yield of your PCR product.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td>1. Set up the following TOPO® Cloning reaction. For optimal results, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
<table>
<tr><th>Reagent</th><th>Chemical Transformation</th><th>Electroporation</th></tr>
<tr><td>Fresh PCR product</td><td>0.5 to 4 µl</td><td>0.5 to 4 µl</td></tr>
<tr><td>Salt solution</td><td>1 µl</td><td>--</td></tr>
<tr><td>Dilute salt solution (1:4)</td><td>--</td><td>1 µl</td></tr>
<tr><td>Water</td><td>to a final volume of 5 µl</td><td>to a final volume of 5 µl</td></tr>
<tr><td>TOPO® vector</td><td>1 µl</td><td>1 µl</td></tr>
<tr><td>Total volume</td><td>6 µl</td><td>6 µl</td></tr>
</table>
2. Mix gently and incubate for 5 minutes at room temperature.
3. Place on ice and proceed to transform One Shot® chemically competent E. coli, below.</td></tr>
<tr><td>Transform One Shot® Chemically Competent E. coli</td><td>1. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® chemically competent E. coli cells and mix gently.
2. Incubate on ice for 5 to 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.
4. Add 250 µl of room temperature S.O.C. Medium.
5. Incubate at 37°C for 1 hour with shaking.
6. Spread 50-200 µl of bacterial culture on a prewarmed selective plate and incubate overnight at 37°C.</td></tr>
</table>

Control Reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 23-25 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Size	Catalog no.
pENTR™/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2400-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2435-20
pENTR™/SD/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2420-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2635-20
pENTR™/TEV/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2525-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2535-20

Shipping/Storage

Each pENTR™ Directional TOPO® Cloning Kit is shipped on dry ice. Each kit contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pENTR™ TOPO® Reagents	-20°C
2	One Shot® Chemically Competent *E. coli*	-80°C

continued on next page

Kit Contents and Storage, continued

pENTR™ TOPO® Reagents

The following reagents are supplied with each pENTR™ TOPO® vector (Box 1). **Note that the user must supply a thermostable, proofreading polymerase and the appropriate PCR buffer.**

Store Box 1 at -20°C.

Item	Concentration	Amount
pENTR™ TOPO® vector, TOPO®-adapted (pENTR™/D-TOPO® **or** pENTR™/SD/D-TOPO® **or** pENTR™/TEV/D-TOPO®)	15-20 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM bromophenol blue	20 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP in water, pH 8	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Water	---	1 ml
M13 Forward (-20) Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
M13 Reverse Sequencing Primer	0.1 µg/µl in TE Buffer, pH 8	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8	10 µl
Control PCR Template	0.1 µg/µl in TE Buffer, pH 8	10 µl

Sequences of the Primers

The table below provides the sequences of the M13 Forward (-20) and M13 Reverse sequencing primers.

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

continued on next page

Kit Contents and Storage, continued

One Shot® Reagents

The following reagents are included with the One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* kit (Box 2). Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store Box 2 at -80°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 or Mach1™-T1^R cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of *E. coli* Strains

TOP10: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)*7697 gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Mach1™-T1^R: F$^-$ Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(r_k^-, m_k^+) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

Information for Non-U.S. Customers Using Mach1™-T1^R Cells

The parental strain of Mach1™-T1^R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S.A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Accessory Products

Introduction

The products listed in this section may be used with the pENTR™ Directional TOPO® Cloning Kits. For more information, refer to www.invitrogen.com or call Technical Service (see page 35).

Additional Products

Many of the reagents supplied in the pENTR™ Directional TOPO® Cloning Kits and other reagents suitable for use with the kits are available separately from Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
One Shot® TOP10 **Chemically Competent** *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 **Electrocompetent** *E. coli*	10 reactions	C4040-50
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03
M13 Forward (-20) Primer	2 µg (407 pmoles)	N520-02
M13 Reverse Primer	2 µg (385 pmoles)	N530-02
Kanamycin Sulfate	1 g	11815-016
LB Broth	500 ml	10855-021
LB Agar	500 g	22700-025
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Gateway® LR Clonase™ II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
Gateway® LR Clonase™ Plus Enzyme Mix	20 reactions	12538-013
MultiSite Gateway® Three-Fragment Vector Construction Kit	1 kit	12537-023
AcTEV™ Protease	1,000 units	12575-015
	10,000 units	12575-023

Introduction

Overview

Introduction

The pENTR™ Directional TOPO® Cloning Kits utilize a highly efficient, 5-minute cloning strategy ("TOPO® Cloning") to directionally clone a blunt-end PCR product into a vector for entry into the Gateway® System or the MultiSite Gateway® System available from Invitrogen. Blunt-end PCR products clone directionally at greater than 90% efficiency, with no ligase, post-PCR procedures, or restriction enzymes required.

A choice of pENTR™ Directional TOPO® vectors is available for optimal expression of your PCR product after recombination with the Gateway® destination vector of interest (see table below).

Vector	Benefit
pENTR™/D-TOPO®	For efficient expression of your gene of interest after recombination with a Gateway® destination vector
pENTR™/SD/D-TOPO®	Contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) for optimal expression of native protein after recombination with a prokaryotic Gateway® destination vector **Note:** Also suitable for efficient expression of your gene of interest in other hosts after recombination with a Gateway® destination vector (*e.g.* mammalian, insect, yeast)
pENTR™/TEV/D-TOPO®	Contains a Tobacco Etch Virus (TEV) recognition site for efficient TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein after recombination and expression from a Gateway® destination vector

The Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest using the Gateway® Technology, simply:

1. TOPO® Clone your blunt-end PCR product into one of the pENTR™ TOPO® vectors to generate an entry clone.
2. Generate an expression construct by performing an LR recombination reaction between the entry clone and a Gateway® destination vector of choice.
3. Introduce your expression construct into the appropriate host (*e.g.* bacterial, mammalian, yeast, insect) and express your recombinant protein.

For more information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual which is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 35).

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Overview, continued

MultiSite Gateway® Technology

The MultiSite Gateway® Technology uses modifications of the site-specific recombination reactions of the Gateway® Technology (see the previous page) to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation. The MultiSite Gateway® Three-Fragment Vector Construction Kit available from Invitrogen (Catalog no. 12537-023) facilitates simultaneous cloning of DNA fragments in three entry vectors to create your own expression clone. For more information about the MultiSite Gateway® Technology and the MultiSite Gateway® Three-Fragment Vector Construction Kit, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual which is available for downloading from our Web site or by contacting Technical Service.

Features of the pENTR™ TOPO® Vectors

The pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO® vectors are designed to facilitate rapid, directional TOPO® Cloning of blunt-end PCR products for entry into the Gateway® System. Features of the vectors include:

- *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway® destination vector
- T7 gene 10 translation enhancer and ribosome binding site for efficient translation of the PCR product in prokaryotes **(pENTR™/SD/D-TOPO® only)**
- TEV recognition site for TEV protease-dependent cleavage of an N-terminal tag from your recombinant protein **(pENTR™/TEV/D-TOPO® only)**
- Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products (see page 3 for more information)
- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

How Directional TOPO® Cloning Works

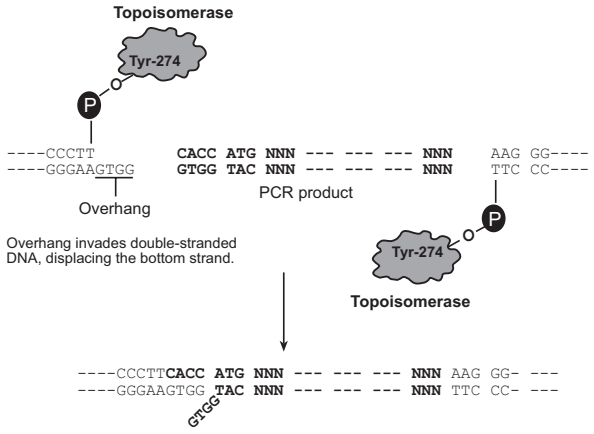
How Topoisomerase I Works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT; see **Note** below) and cleaves the phosphodiester backbone 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.

Directional TOPO® Cloning

Directional joining of double-strand DNA using TOPO®-charged oligonucleotides occurs by adding a 3′ single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5′ end of the TOPO®-charged DNA fragment. At Invitrogen, this idea has been modified by adding a 4 nucleotide overhang sequence to the TOPO®-charged DNA and adapting it to a 'whole vector' format.

In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.



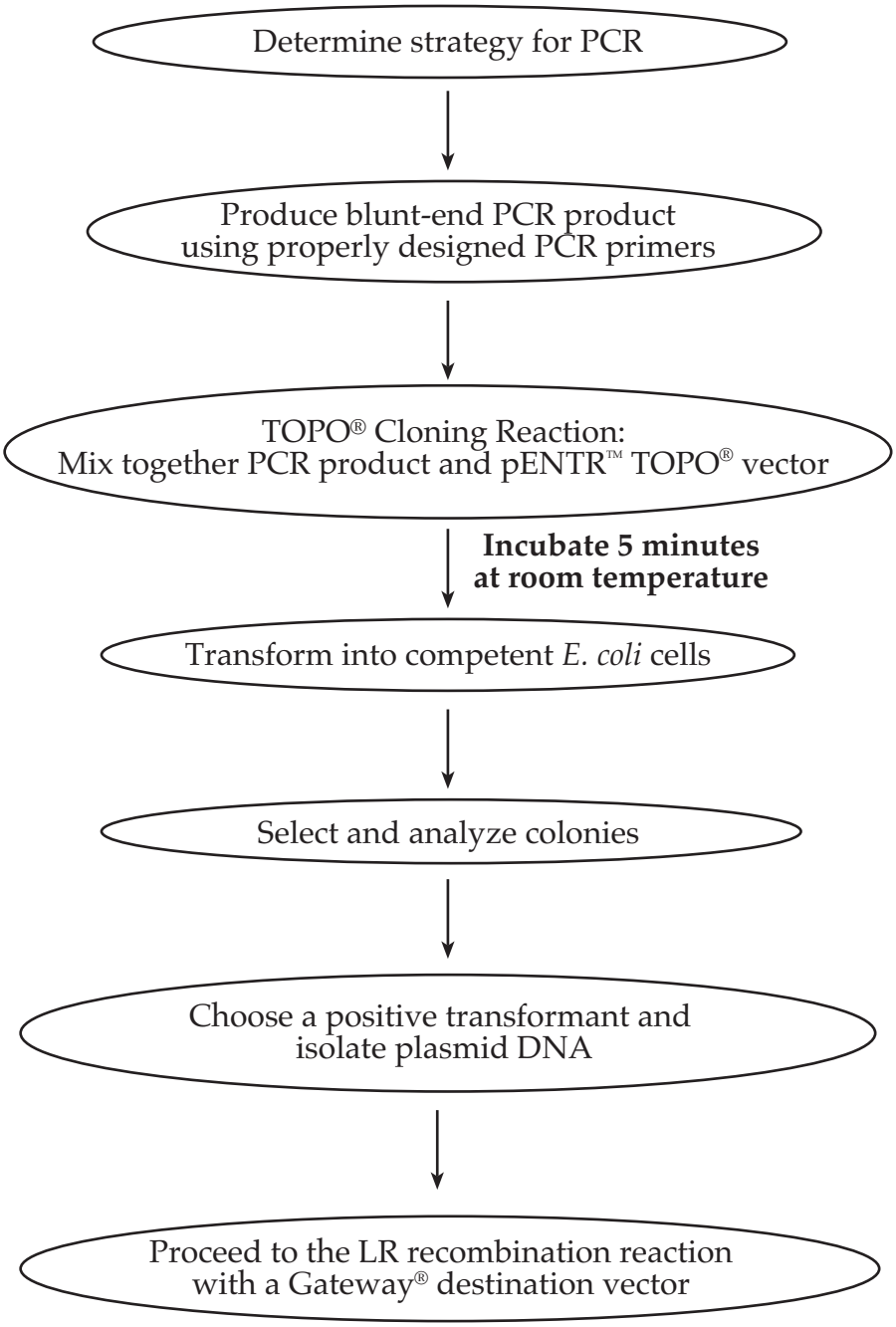
Note

The 5′ TOPO® recognition site in pENTR™/TEV/D-TOPO® is encoded by the sequence TCCTT rather than CCCTT. This is because the 5′ TOPO® recognition site directly follows the TEV recognition site, and studies have shown that TEV protease does not cleave efficiently if the first amino acid following the TEV recognition sequence is proline (Kapust *et al.*, 2002) as would be the case if the 5′ TOPO® recognition site was encoded by **CCC**TT. By changing the sequence of the 5′ TOPO® recognition site to **TCC**TT, the first amino acid following the TEV recognition site is now serine. **This change does not affect TOPO® Cloning efficiency and allows efficient TEV cleavage.**

Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and clone your blunt-end PCR product.



Methods

Designing PCR Primers

Designing Your PCR Primers

The design of the PCR primers to amplify your gene of interest is critical for expression. Depending on the pENTR™ TOPO® vector you are using, consider the following when designing your PCR primers.

- Sequences required to facilitate directional cloning
- Sequences required for proper translation initiation of your PCR product
- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal tag after recombination of your entry clone with a Gateway® destination vector

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- To enable directional cloning, the forward PCR primer **must** contain the sequence, CACC, at the 5′ end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in each pENTR™ TOPO® vector.
- If you plan to express your PCR product in mammalian cells as a native or C-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest should include a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1991; Kozak, 1990). An example of a Kozak consensus sequence is **(G/A)**NNATG**G**. Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold). The ATG initiation codon is underlined.

  Note: If your sequence of interest does not contain an initiation codon within the context of a Kozak sequence, design the forward PCR primer to contain a Kozak sequence at the 5′ end of the primer (see **Example** on the next page).
- If you plan to express your PCR product in mammalian cells as an N-terminal fusion-tagged protein (following recombination of the entry clone with a Gateway® destination vector), your sequence of interest does not need to contain a Kozak translation initiation sequence. A Kozak sequence is provided by the appropriate destination vector. **Note:** In this case, internal initiation may occur if your PCR product contains an endogenous Kozak sequence.
- If you plan to express your PCR product in **prokaryotic** cells without an N-terminal fusion tag (following recombination of the entry clone with a Gateway® destination vector), you should TOPO® Clone your PCR product into pENTR™/SD/D-TOPO®. pENTR™/SD/D-TOPO® contains a T7 gene 10 translational enhancer and a ribosome binding site (RBS) to enable efficient translation of the PCR product in *E. coli*. **To ensure optimal spacing for proper translation, design your forward PCR primer so that the ATG initiation codon of your PCR product directly follows the CACC necessary for directional cloning** (see **Example** on the next page).

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Designing PCR Primers, continued

Example of Forward Primer Design

Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer. The ATG initiation codon is underlined.

DNA sequence: 5′-ATG GGA TCT GAT AAA

Proposed Forward PCR primer: 5′-C ACC ATG GGA TCT GAT AAA

If you design the forward PCR primer as noted above, then:

- The ATG initiation codon falls within the context of a Kozak sequence (see boxed sequence), allowing proper translation initiation of the PCR product in mammalian cells.
- The ATG initiation codon is properly spaced from the RBS (in pENTR™/SD/D-TOPO® only), allowing proper translation of the PCR product in prokaryotic cells.

Note

The first three base pairs of the PCR product following the 5′ CACC overhang will constitute a functional codon.

Guidelines to Design the Reverse Primer

When designing your reverse PCR primer, consider the following points below. Refer to pages 8-9 for diagrams of the TOPO® Cloning site for pENTR™/D-TOPO®, pENTR™/SD/D-TOPO®, and pENTR™/TEV/D-TOPO®.

- **To ensure that your PCR product clones directionally with high efficiency, the reverse PCR primer MUST NOT be complementary to the overhang sequence GTGG at the 5′ end. A one base pair mismatch can reduce the directional cloning efficiency from 90% to 50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 on the next page).** We have not observed evidence of PCR products cloning in the opposite orientation from a two base pair mismatch.
- If you wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then design the reverse PCR primer to remove the native stop codon in the gene of interest (see **Example #2** on the next page).
- If you **do not** wish to fuse your PCR product in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector), then include the native sequence containing the stop codon in the reverse primer or make sure the stop codon is upstream from the reverse PCR primer binding site (see **Example #2** on the next page).

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Designing PCR Primers, continued

Example #1 of Reverse Primer Design

Below is the sequence of the C-terminus of a theoretical protein. You want to fuse the protein in frame with a C-terminal tag (following recombination of the entry clone with a Gateway® destination vector). The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. You want to avoid this situation.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3′
Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5′

Another solution is to design the reverse primer so that it hybridizes just downstream of the stop codon, but still includes the C-terminus of the ORF. Note that you will need to replace the stop codon with a codon for an innocuous amino acid such as glycine, alanine, or lysine.

Example #2 of Reverse Primer Design

Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

…**GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3′**

- To fuse the ORF in frame with a C-terminal tag (supplied by the destination vector after recombination), remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

  5′-TGC AGT CGT CGA GTG CTC CGA CTT-3′

  This will amplify the C-terminus without the stop codon and allow you to join the ORF in frame with a C-terminal tag.

- If you don't want to join the ORF in frame with a C-terminal tag, simply design the reverse primer to include the stop codon.

  5′-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3′

Important

- Remember that the pENTR™ TOPO® vectors accept blunt-end PCR products.
- Do not add 5´ phosphates to your primers for PCR. This will prevent ligation into the pENTR™ TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

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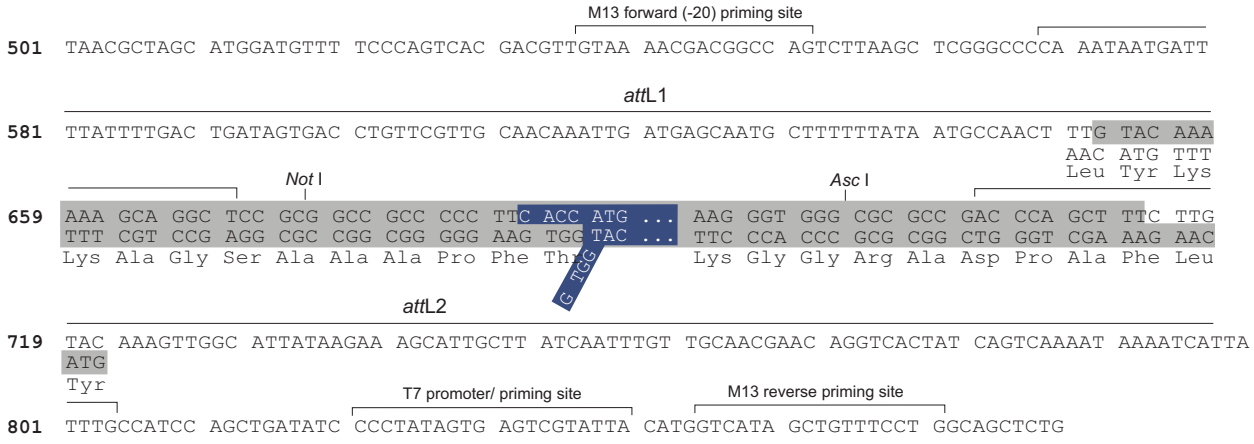
Designing PCR Primers, continued

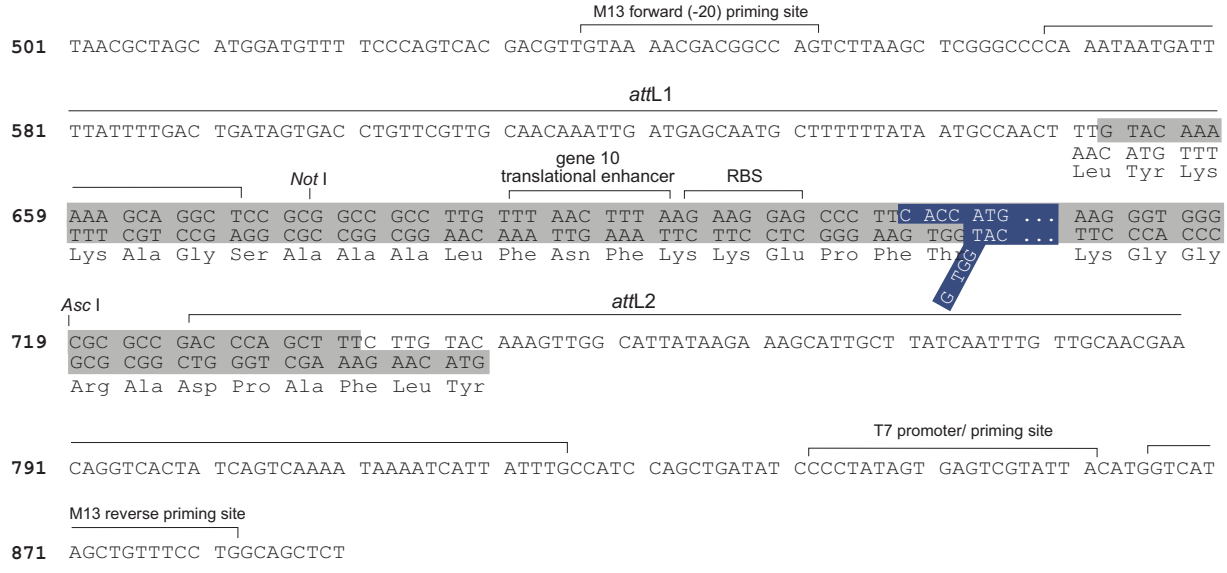
TOPO® Cloning Site for pENTR™/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/D-TOPO®, see pages 28-29.



TOPO® Cloning Site for pENTR™/SD/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/SD/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/SD/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/SD/D-TOPO®, see pages 30-31.



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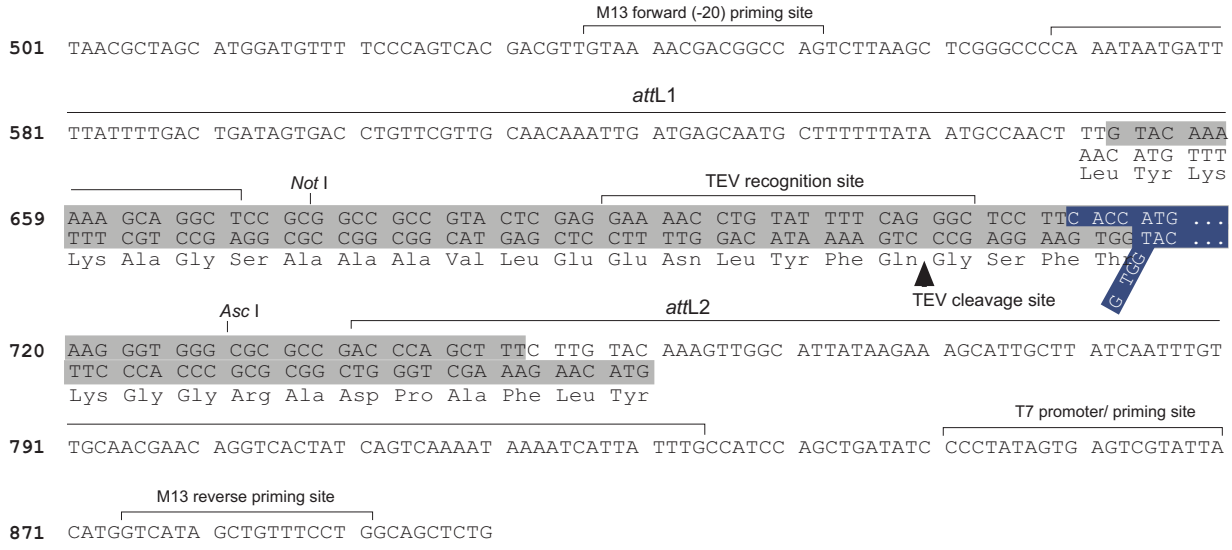
Designing PCR Primers, continued

TOPO® Cloning Site for pENTR™/TEV/D-TOPO®

Use the diagram below to help you design suitable PCR primers to clone your PCR product into pENTR™/TEV/D-TOPO®. Restriction sites are labeled to indicate the actual cleavage site. The shaded region corresponds to the DNA sequences that will be transferred from the entry clone into the destination vector following LR recombination. **The sequence of pENTR™/TEV/D-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 35).** For more information about pENTR™/TEV/D-TOPO®, see pages 32-33.

Note: The sequence of the 5′ TOPO® recognition site has been changed from **C**CCTT to **T**CCTT, resulting in an amino acid substitution of serine for proline**. This amino acid change increases the efficiency of TEV protease cleavage (Kapust *et al.*, 2002), but does not affect the efficiency of TOPO® Cloning.**



Producing Blunt-End PCR Products

Introduction

Once you have decided on a PCR strategy and have synthesized the primers, produce your blunt-end PCR product using any thermostable, proofreading polymerase. Follow the guidelines below to produce your blunt-end PCR product.

Materials Supplied by the User

You will need the following reagents and equipment for PCR. **Note:** dNTPs (adjusted to pH 8) are provided in the kit.

- Thermocycler and thermostable, proofreading polymerase
- 10X PCR buffer appropriate for your polymerase
- DNA template and primers to produce the PCR product

Producing Blunt-End PCR Products

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

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

Checking the PCR Product

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

- Be sure you have a single, discrete band of the correct size. If you do not have a single, discrete band, follow the manufacturer's recommendations to optimize your PCR with the polymerase of your choice. Alternatively, you may gel-purify the desired product (see pages 26-27).
- Estimate the concentration of your PCR product. You will use this information when setting up your TOPO® Cloning reaction (see **Amount of PCR Product to Use in the TOPO® Cloning Reaction**, next page for details).

Setting Up the TOPO® Cloning Reaction

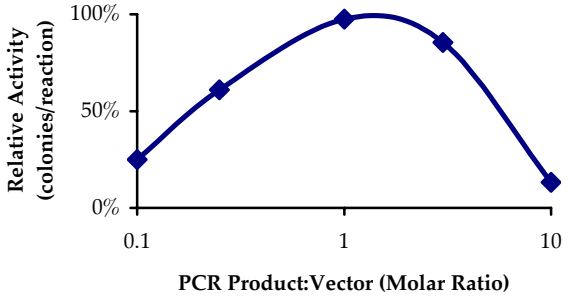
Introduction

Once you have produced the desired blunt-end PCR product, you are ready to TOPO® Clone it into the pENTR™ TOPO® vector and transform the recombinant vector into One Shot® competent *E. coli*. You should have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the section entitled **Transforming One Shot® Competent *E. coli*** (pages 13-14) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 23-25 in parallel with your samples.

Amount of PCR Product to Use in the TOPO® Cloning Reaction

When performing directional TOPO® Cloning, we have found that the molar ratio of PCR product:TOPO® vector used in the reaction is critical to its success. **To obtain the highest TOPO® Cloning efficiency, use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector (see figure below).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see figure below). These results are generally obtained if too little PCR product is used (*i.e.* PCR product is too dilute) or if too much PCR product is used in the TOPO® Cloning reaction. If you have quantitated the yield of your PCR product, you may need to adjust the concentration of your PCR product before proceeding to TOPO® Cloning.

Tip: For pENTR™ TOPO® vectors, using 1-5 ng of a 1 kb PCR product or 5-10 ng of a 2 kb PCR product in a TOPO® Cloning reaction generally results in a suitable number of colonies.



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Setting Up the TOPO® Cloning Reaction, continued

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells (provided) or electrocompetent cells (see page x for ordering information).**

- If you are transforming chemically competent *E. coli*, use the stock Salt Solution as supplied and set up the TOPO® Cloning reaction as directed below.
- If you are transforming electrocompetent *E. coli*, the amount of salt in the TOPO® Cloning reaction **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing during electroporation. Dilute the stock Salt Solution 4-fold with water to prepare a 300 mM NaCl, 15 mM $MgCl_2$ Dilute Salt Solution. Use the Dilute Salt Solution to set up the TOPO® Cloning reaction as directed below.

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.* **Reminder:** For optimal results, be sure to use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector in your TOPO® Cloning reaction.

Note: The blue color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution (1:4)	--	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 µl

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

1. Mix reaction gently and incubate for 5 minutes at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to **Transforming One Shot® Competent *E. coli***, next page.

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

Transforming One Shot® Competent *E. coli*

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pENTR™ TOPO® construct into competent *E. coli*. One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page x for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Materials Needed

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

- TOPO® Cloning reaction (from Step 2, previous page)
- One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* (supplied with the kit, Box 2)
- S. O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (to verify transformation efficiency, if desired, Box 2)
- 42°C water bath (or electroporator with cuvettes, optional)
- 15 ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 50 µg/ml kanamycin (two for each transformation)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator

Note

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Preparing for Transformation

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

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw **on ice** one vial of One Shot® cells from Box 2 for each transformation.

continued on next page

Transforming One Shot® Competent *E. coli*, continued

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.

   Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).

2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.

3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50-200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. 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 may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

Transformation by Electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1^R chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 12 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.** Transfer the cells to a 0.1 cm cuvette.
2. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see the next page.

3. Immediately add 250 µl of room temperature S.O.C. Medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the kanamycin resistance gene.
5. Spread 20-100 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.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5-10 colonies for analysis (see **Analyzing Transformants**, page 16).

continued on next page

Transforming One Shot® Competent *E. coli*, continued

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5-10 colonies and culture them overnight in LB or SOB medium containing 50-100 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 only 4 hours in pre-warmed LB medium containing 50 µg/ml kanamycin before isolating plasmid DNA. For optimal results, inoculate as much of a single colony as possible.

2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend using Invitrogen's PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

3. Analyze the plasmids by restriction analysis or PCR (see below) to confirm the presence and correct orientation of the insert.

Analyzing Transformants by PCR

Use the protocol below (or any other suitable protocol) to analyze positive transformants using PCR. For PCR primers, use a combination of the M13 Forward (-20) primer or the M13 Reverse primer and a primer that hybridizes within your insert. You will have to determine the amplification conditions. 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.

Materials Needed:

- PCR Super Mix High Fidelity (Invitrogen, Catalog no. 10790-020)
- Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5-10 colonies and resuspend them individually in 50 µl of the PCR SuperMix containing PCR primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your gene is cloned in the correct orientation. Use the M13 Forward (-20) and M13 Reverse included to help you sequence your insert (see the diagrams on pages 8-9 for the location of the priming sites in each pENTR™ TOPO® vector). For the complete sequence of each pENTR™ TOPO® vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 35).

Note: The M13 Forward (-20) and M13 Reverse primers are available separately from Invitrogen (see page x for ordering information).

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on page 23-25 or refer to the **Troubleshooting** section, page 21 for tips to help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to purify the colony and make 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 for single colony on LB plates containing 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Guidelines to Perform the LR Recombination Reaction

Introduction

Once you have obtained your entry clone, you may:

- Perform an LR recombination reaction using Gateway® LR Clonase™ II enzyme mix (see page x for ordering information) to transfer your gene of interest from the entry construct into any Gateway® destination vector of choice to generate an expression clone.
- Perform a MultiSite Gateway® LR recombination reaction with 5′ and 3′ entry clones, the appropriate MultiSite Gateway® destination vector, and LR Clonase™ Plus enzyme mix (see page x for ordering information) to generate an expression clone.

General guidelines are provided below.

Important

For most applications, we recommend performing the LR recombination reaction or the MultiSite Gateway® LR recombination reaction using a:

- Supercoiled entry clone(s) **and**
- Supercoiled destination vector

To catalyze the LR recombination reaction, we recommend using Gateway® LR Clonase™ II Enzyme Mix (see page x for ordering information). The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied by Invitrogen as separate components in LR Clonase™ enzyme mix (Catalog no. 11791-019) into an optimized single tube format to allow easier set-up of the LR recombination reaction. Follow the instructions included with the product to perform the LR recombination reaction.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired.

Destination Vectors

A large selection of Gateway® destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available, see our Web site (www.invitrogen.com) or call Technical Service (see page 35). Manuals supporting all of the destination vectors are available for downloading from our Web site or by contacting Technical Service.

E. coli Host

Once you have performed the LR recombination reaction or the MultiSite Gateway® LR recombination reaction, you will transform the reaction mixture into competent *E. coli* and select for expression clones. You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α™, or equivalent for transformation. Do not transform the Gateway® or MultiSite Gateway® LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

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Guidelines to Perform the LR Recombination Reaction

Performing the LR Recombination Reaction

To perform the Gateway® LR recombination reaction, you will need:

- Purified plasmid DNA of the entry clone containing your gene of interest
- A destination vector of choice
- LR Clonase™ II enzyme mix (see page x for ordering information)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate chemically competent *E. coli* host and growth media for expression
- Appropriate selective plates

For instructions to perform the LR recombination reaction, refer to the Gateway® Technology with Clonase™ II manual or to the manual for the destination vector you are using.

Performing the MultiSite Gateway® LR Recombination Reaction

Before you can perform the MultiSite Gateway® LR recombination reaction, you will first need to generate 5′ and 3′ entry clones using Invitrogen's MultiSite Gateway® Three-Fragment Vector Construction Kit (Catalog no. 12537-023). Once you have generated the 5′ and 3′ entry clones, you will use the 5′ and 3′ entry clones, the entry clone containing your gene of interest, and the other reagents supplied in the MultiSite Gateway® Three-Fragment Vector Construction Kit (including LR Clonase™ Plus enzyme mix and the pDEST™R4-R3 destination vector) in a MultiSite Gateway® LR recombination reaction to generate an expression clone.

For instructions to generate 5′ and 3′ entry clones and to perform the MultiSite Gateway® LR recombination reaction, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual.

Guidelines to Perform TEV Cleavage of Recombinant Proteins

Introduction

If you have cloned your PCR product into pENTR™/TEV/D-TOPO®, your gene of interest will be fused in frame with a TEV recognition sequence. Performing an LR recombination or MultiSite Gateway® LR recombination using the pENTR™/TEV/D-TOPO® entry clone and a suitable destination vector allows you to generate an expression clone containing a TEV cleavage site just upstream of the translation initiation site of your recombinant protein. Once expressed, you may generate nearly native protein by using TEV protease to cleave any N-terminal fusion tags or sequences (*e.g.* the *att*L site) from the recombinant protein. Guidelines to perform TEV cleavage are provided in this section.

Obtaining TEV Protease

For highly efficient TEV protease-directed cleavage, we recommend using AcTEV™ Protease available from Invitrogen (Catalog nos. 12575-015 and 12575-023). AcTEV™ Protease is an enhanced form of TEV protease that is highly site-specific, active, and more stable than native TEV protease (Nayak *et al.*, 2003). Following digestion, AcTEV™ Protease may be easily removed from the cleavage reaction by affinity chromatography using the polyhistidine (6xHis) tag at the N-terminus of the protease.

General guidelines to use AcTEV™ Protease for cleavage are provided below. For detailed instructions and recommendations to optimize cleavage, refer to the manual included with the product.

AcTEV™ Protease Unit Definition

One unit of AcTEV™ Protease cleaves ≥ 85% of 3 µg of a control substrate in 1 hour at 30°C.

General Guidelines to Use AcTEV™ Protease

Follow the guidelines below when using AcTEV™ Protease.

- For optimal yield of cleaved recombinant protein, partially purify or purify recombinant fusion protein before performing cleavage.
- Use the following digestion conditions as a starting point, and optimize the cleavage reaction as necessary by varying the amount of AcTEV™ Protease, incubation temperature, or reaction time.

  For a cleavage reaction using 20 µg of fusion protein, use 10 units of AcTEV™ Protease in a reaction volume of 150 µl. Incubate the reaction mixture at 30°C for 1 hour **or** at 4°C for 4 hours to overnight. For detailed instructions to set up the cleavage reaction, refer to the manual included with the product.
- After cleavage, remove AcTEV™ Protease from the reaction mixture using affinity chromatography on a nickel-chelating resin (*e.g.* ProBond™ Resin; Catalog no. K801-01).

Note

After digestion with TEV protease, four vector-encoded amino acids will remain at the N-terminus of your recombinant protein.

Troubleshooting

TOPO® Cloning Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate your results, we recommend that you perform the control reactions (see pages 23-25) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Suboptimal ratio of PCR product:TOPO® vector used in the TOPO® Cloning reaction	Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Incorrect PCR primer design	• Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end. • Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
	Used *Taq* polymerase or a *Taq*/proofreading polymerase mixture for PCR	Use a proofreading polymerase for PCR.
	Large PCR product	• Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector. • Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Gel-purify the PCR product to remove primer-dimers and other artifacts.
	PCR reaction contains artifacts (*i.e.* does not run as a single, discrete band on an agarose gel)	• Optimize your PCR using the proofreading polymerase of your choice. • Gel-purify your PCR product.

continued on next page

Troubleshooting, continued

TOPO® Cloning Reaction and Transformation, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies, continued	Cloning large pool of PCR products or a toxic gene	• Increase the incubation time of the TOPO® reaction from 5 minutes to 30 minutes. • Use a 0.5:1 to 2:1 molar ratio of PCR product:TOPO® vector.
	Incomplete extension during PCR	Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Large percentage of inserts cloned in the incorrect orientation	Incorrect PCR primer design	Make sure that the forward PCR primer contains the sequence, CACC, at the 5′ end.
	Reverse PCR primer is complementary to the GTGG overhang at the 5′ end	Make sure that the reverse PCR primer **does not** contain the sequence, CACC, at the 5′ end.
Large number of incorrect inserts cloned	PCR cloning artifacts	• Gel-purify your PCR product to remove primer-dimers and smaller PCR products. • Optimize your PCR. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
	Incorrect PCR primer design	Make sure that the forward and reverse PCR primers are designed correctly.
Few or no colonies obtained from sample reaction **and** the transformation control gave **no** colonies	One Shot® competent *E. coli* stored incorrectly	Store One Shot® competent *E. coli* at -80°C. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C before plating.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.
	Transformants plated on selective plates containing the wrong antibiotic	Use the appropriate antibiotic for selection.

Appendix

Performing the Control Reactions

Introduction

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

Before Starting

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

Producing the Control PCR Product

Use your thermostable, proofreading polymerase and the appropriate buffer to amplify the control PCR product. Follow the manufacturer's recommendations for the proofreading polymerase you are using.

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

Component	Amount
Control DNA Template (100 ng)	1 µl
10X PCR Buffer (appropriate for enzyme)	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile water	41.5 µl
Proofreading polymerase (1-2.5 U/µl)	1 µl
Total volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil, if required.
3. Amplify using the following cycling parameters:

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

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. Make sure that you see a single, discrete 750 bp band.
5. Estimate the concentration of the PCR product, and adjust as necessary such that the amount of PCR product used in the control TOPO® Cloning reaction results in an optimal molar ratio of PCR product:TOPO® vector (*i.e.* 0.5:1 to 2:1). Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Performing the Control Reactions, continued

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the pENTR™ TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below. If you plan to transform electrocompetent *E. coli*, use Dilute Salt Solution in place of the Salt Solution.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pENTR™/D-TOPO® vector	1 µl	1 µl
Total volume	6 µl	6 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® competent cells using the protocol on page 14.
4. Spread 50-200 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.
5. Incubate overnight at 37°C.

continued on next page

Performing the Control Reactions, continued

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. To analyze the transformations, isolate plasmid DNA and digest with the appropriate restriction enzyme as listed below. The table below lists the digestion patterns that you should see for inserts that are cloned in the correct orientation or in the reverse orientation.

Vector	Restriction Enzyme	Expected Digestion Patterns (bp)
pENTR™/D-TOPO®	*Not* I	Correct orientation: 127, 3203 Reverse orientation: 646, 2684 Empty vector: 2580
pENTR™/SD/D-TOPO®	*Not* I	Correct orientation: 148, 3203 Reverse orientation: 667, 2684 Empty vector: 2601
pENTR™/TEV/D-TOPO®	*Eco*R V/*Pst* I	Correct orientation: 757, 2602 Reverse orientation: 250, 3109 Empty vector: 2610

Greater than 90% of the colonies should contain the 750 bp insert in the correct orientation.

Relatively few colonies should be produced in the vector-only reaction.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform one vial of One Shot® competent cells with 10 pg of pUC19 using the protocol on page 14. Plate 10 µl of the transformation mixture plus 20 µl of S.O.C. Medium on LB plates containing 100 µg/ml ampicillin. Transformation efficiency should be ≥ 1 x 10^9 cfu/µg DNA.

Gel Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>3 kb) may necessitate gel purification. If you wish to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to *Current Protocols in Molecular Biology*, Unit 2.6 (Ausubel *et al.*, 1994) for the most common protocols. Three simple protocols are provided below.

Note

The cloning efficiency may decrease with purification of the PCR product (*e.g.* PCR product too dilute). You may wish to optimize your PCR to produce a single band (see **Producing Blunt-End PCR Products**, page 10).

Using the S.N.A.P.™ Gel Purification Kit

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.

   Note: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.
3. Add 1.5 volumes Binding Buffer.
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3000 x g in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer.
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.
8. Repeat Step 7.
9. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 12.

Quick S.N.A.P.™ Method

An even easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 12) Be sure to make the gel slice as small as possible for best results.

continued on next page

Gel Purifying PCR Products, continued

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the procedure below. Note that gel purification will result in a dilution of your PCR product and a potential loss of cloning efficiency.

1. Electrophorese as much as possible of your PCR reaction on a low-melt agarose gel (0.8 to 1.2%) in TAE buffer.
2. Visualize the band of interest and excise the band.
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. Add 4 µl of the melted agarose containing your PCR product to the TOPO® Cloning reaction as described on page 12.
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes**. This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into One Shot® competent cells using the method on page 14.

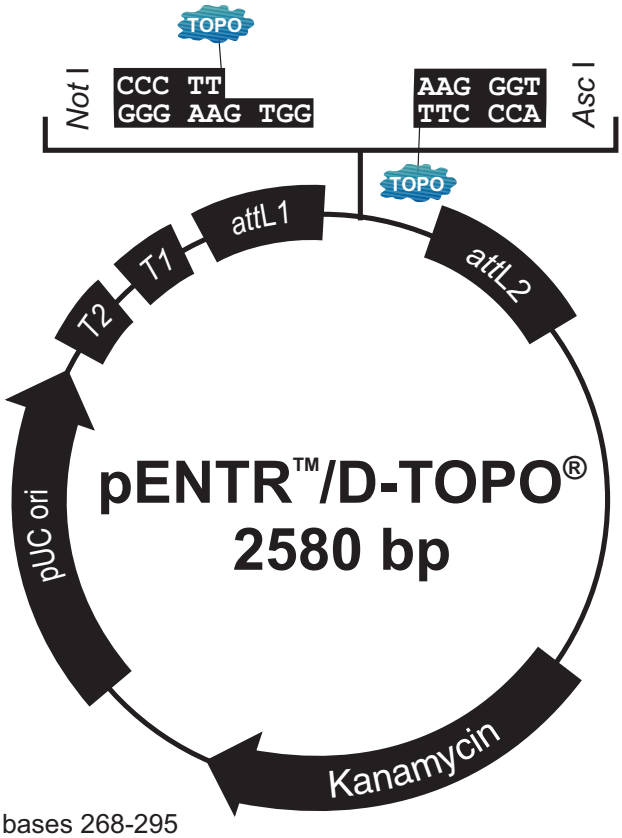
Note

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

Map and Features of pENTR™/D-TOPO®

pENTR™/D-TOPO® Map

The figure below shows the features of pENTR™/D-TOPO® vector. **The complete sequence of pENTR™/D-TOPO® is available for downloading from www.invitrogen.com)or by contacting Technical Service (see page 35).**



Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

continued on next page

Map and Features of pENTR™/D-TOPO®, continued

Features of pENTR™/D-TOPO®

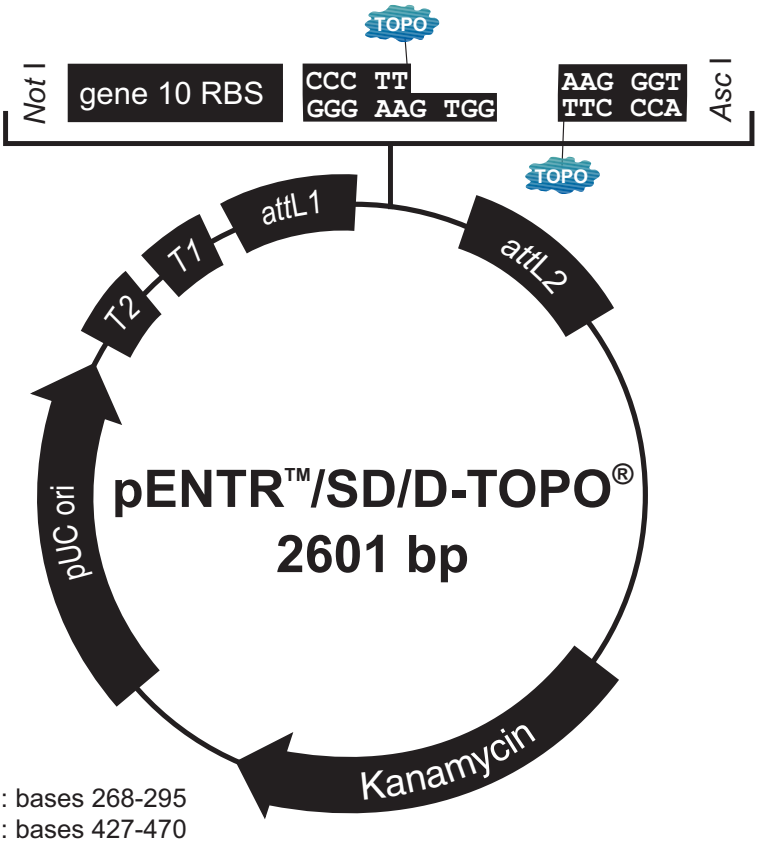
pENTR™/D-TOPO® (2580 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product.
M13 forward (-20) priming site	Allows sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Map and Features of pENTR™/SD/D-TOPO®

pENTR™/SD/D-TOPO® Map

The figure below shows the features of pENTR™/SD/D-TOPO® vector. **The complete sequence of pENTR™/SD/D-TOPO® is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 35).**



Comments for pENTR™/SD/D-TOPO®
2601 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
T7 gene 10 translational enhancer: bases 684-692
Ribosome binding site: bases 694-700
TOPO® recognition site 1: bases 701-705
Overhang: bases 706-709
TOPO® recognition site 2: bases 710-714
*att*L2: bases 726-825
T7 Promoter/priming site: bases 842-861 (c)
M13 reverse priming site: bases 866-882
Kanamycin resistance gene: bases 995-1804
pUC origin: bases 1925-2598

(c) = complementary sequence

continued on next page

Map and Features of pENTR™/SD/D-TOPO®, continued

Features of pENTR™/SD/D-TOPO®

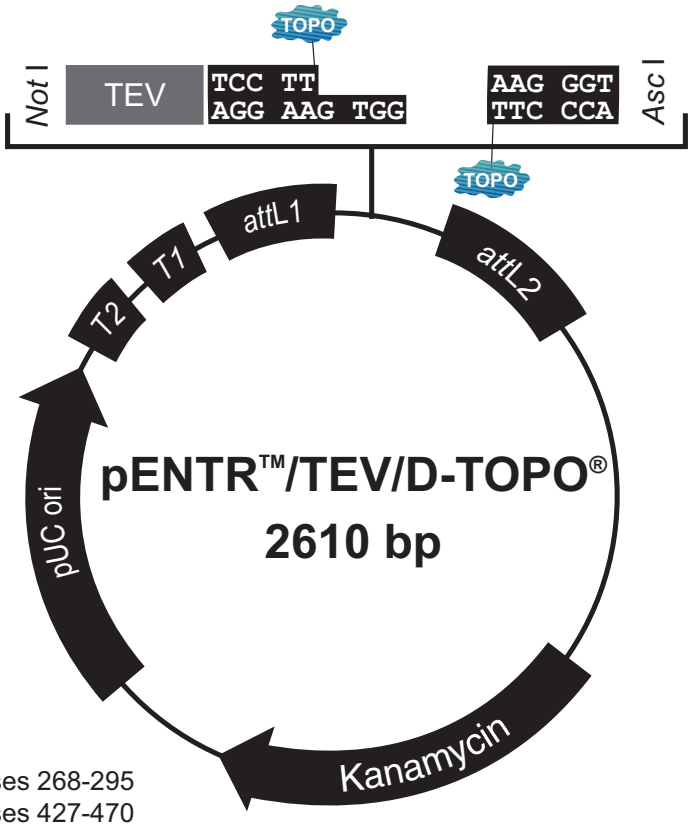
pENTR™/SD/D-TOPO® (2601 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product (Orosz *et al.*, 1991).
M13 forward (-20) priming site	Allows sequencing of the insert.
T7 gene 10 translational enhancer	Sequence from bacteriophage T7 gene 10 that optimizes translation initiation (Olins *et al.*, 1988).
Ribosome binding site (RBS)	Optimally spaced from the TOPO® Cloning site for efficient translation of the PCR product.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

Map and Features of pENTR™/TEV/D-TOPO®

pENTR™/TEV/D-TOPO® Map

The figure below shows the features of pENTR™/TEV/D-TOPO® vector. **The complete sequence of pENTR™/TEV/D-TOPO® is available for downloading from www.invitrogen.com or by contacting Technical Service (see page 35).**



Comments for pENTR™/TEV/D-TOPO®
2610 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TEV recognition site: bases 689-709
TOPO® recognition site: bases 710-714
Overhang: bases 715-718
TOPO® recognition site: bases 719-723
*att*L2: bases 735-834
T7 Promoter/priming site: bases 851-870 (c)
M13 reverse priming site: bases 875-891
Kanamycin resistance gene: bases 1004-1813
pUC origin: bases 1934-2607

(c) = complementary sequence

continued on next page

Map and Features of pENTR™/TEV/D-TOPO®, continued

Features of pENTR™/TEV/D-TOPO®

pENTR™/TEV/D-TOPO® (2610 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Reduces potential toxicity in *E. coli* by preventing basal expression of the PCR product (Orosz *et al.*, 1991).
M13 forward (-20) priming site	Allows sequencing of the insert.
*att*L1 and *att*L2 sites	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the entry construct with a Gateway® destination vector (Landy, 1989).
TEV recognition site	Allows removal of the N-terminal tag from your recombinant protein using AcTEV™ protease (Carrington and Dougherty, 1988; Dougherty *et al.*, 1988)
TOPO® Cloning site (directional)	Allows rapid, directional cloning of your PCR product.
T7 promoter/priming site	Allows *in vitro* transcription, and sequencing of the insert.
M13 reverse priming site	Allows sequencing of the insert.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Allows high-copy replication and maintenance in *E. coli*.

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. Allow solution to cool to ~55°C and add antibiotic, 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.
3. After autoclaving, cool to ~55°C, add antibiotic and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C, in the dark.

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

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Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

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Purchaser Notification

Introduction

Use of the pENTR™ Directional TOPO® Cloning Kits is covered under the licenses detailed below.

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, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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Purchaser Notification, continued

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This product and its use is the subject of one or more of U.S. Patent Nos. 5,888,732, 6,143,557, 6,171,861, 6,270,969, and 6,277,608 and/or other pending U.S. and foreign patent applications owned by Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Invitrogen Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase™ purchased from Invitrogen Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Invitrogen under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200.

Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, next page.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the pENTR™ TOPO® Cloning Kits.

Vectors

Prior to adaptation with topoisomerase I, each supercoiled pENTR™ vector is qualified by:

- Performing restriction enzyme digestion to verify its structure.
- Performing an LR recombination reaction with a Gateway® destination vector to confirm its functionality.

TOPO® Cloning Efficiency

After adaptation with topoisomerase I, each pENTR™ TOPO® vector is lot-qualified using the control reagents included in the kit. Under conditions described on pages 23-25, a 750 bp control PCR product is amplified using a forward primer containing CACC at its 5′ end and a reverse primer. The PCR product is TOPO® Cloned into the pENTR™ TOPO® vector and transformed into the One Shot® TOP10 chemically competent *E. coli* included with the kit.

Each lot of vector should yield greater than 90% cloning efficiency. At least 10 transformants are characterized using restriction digest. Of the transformants characterized, greater than 90% should contain an insert in the correct orientation.

Primers

Primers are lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Chemically Competent *E. coli*

One Shot® TOP10 and Mach1™-T1^R chemically competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA.

In addition, untransformed cells are tested for the appropriate antibiotic sensitivity and lack of phage contamination.

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