USER GUIDE

invitrogen™
by life technologies™

pENTR™ Directional TOPO® Cloning Kits

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

Catalog numbers K2400-20, K2420-20, K2525-20, K2535-20, K2435-20, and K2635-20

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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>0.5–4 µL</td><td>0.5–4 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 in the following section.</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 5–30 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 and storage

Each pENTR™ Directional TOPO® Cloning Kit is shipped on dry ice. Each kit contains 2 boxes. Upon receipt, store the boxes as detailed in the following table.

Box	Item	Storage
1	pENTR™ TOPO® Reagents	–30°C to –10°C
2	One Shot® Chemically Competent *E. coli*	–85°C to –68°C

Product use

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

Continued on next page

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 –30°C to –10°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 following table 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

One Shot® reagents

The following reagents are included with the One Shot® TOP10 or Mach1™-T1^R Chemically Competent *E. coli* kit (Box 2). The transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store Box 2 at −85°C to −68°C.**

Reagent	Composition	Amount
S.O.C. Medium (may be stored at room temperature or at 2°C to 8°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 × 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.

Introduction

About the kit

System overview

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. 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 the following table).

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:

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 from **www.lifetechnologies.com/support** or by contacting Technical Support (see page 35).

Continued on next page

About the kit, Continued

MultiSite Gateway® Technology

The MultiSite Gateway® Technology uses modifications of the site-specific recombination reactions of the Gateway® Technology (see page 1) to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation. The MultiSite Gateway® Three-Fragment Vector Construction Kit (see page 22 for ordering information) 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 at **www.lifetechnologies.com/support** or by contacting Technical Support.

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

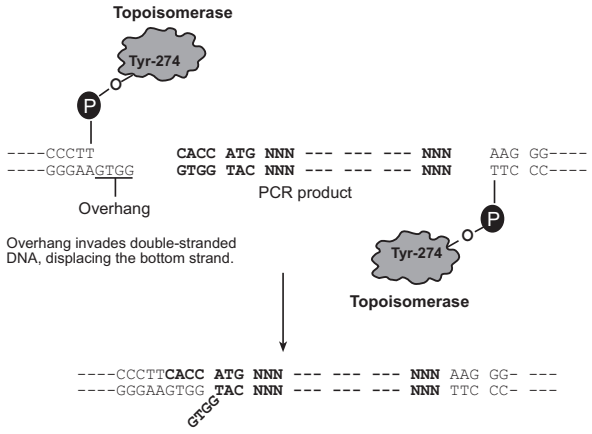
How Topoisomerase I works

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT; see the **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. In the TOPO® Cloning Kits, a 4 nucleotide overhang sequence has been added to the TOPO®-charged DNA, 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%.



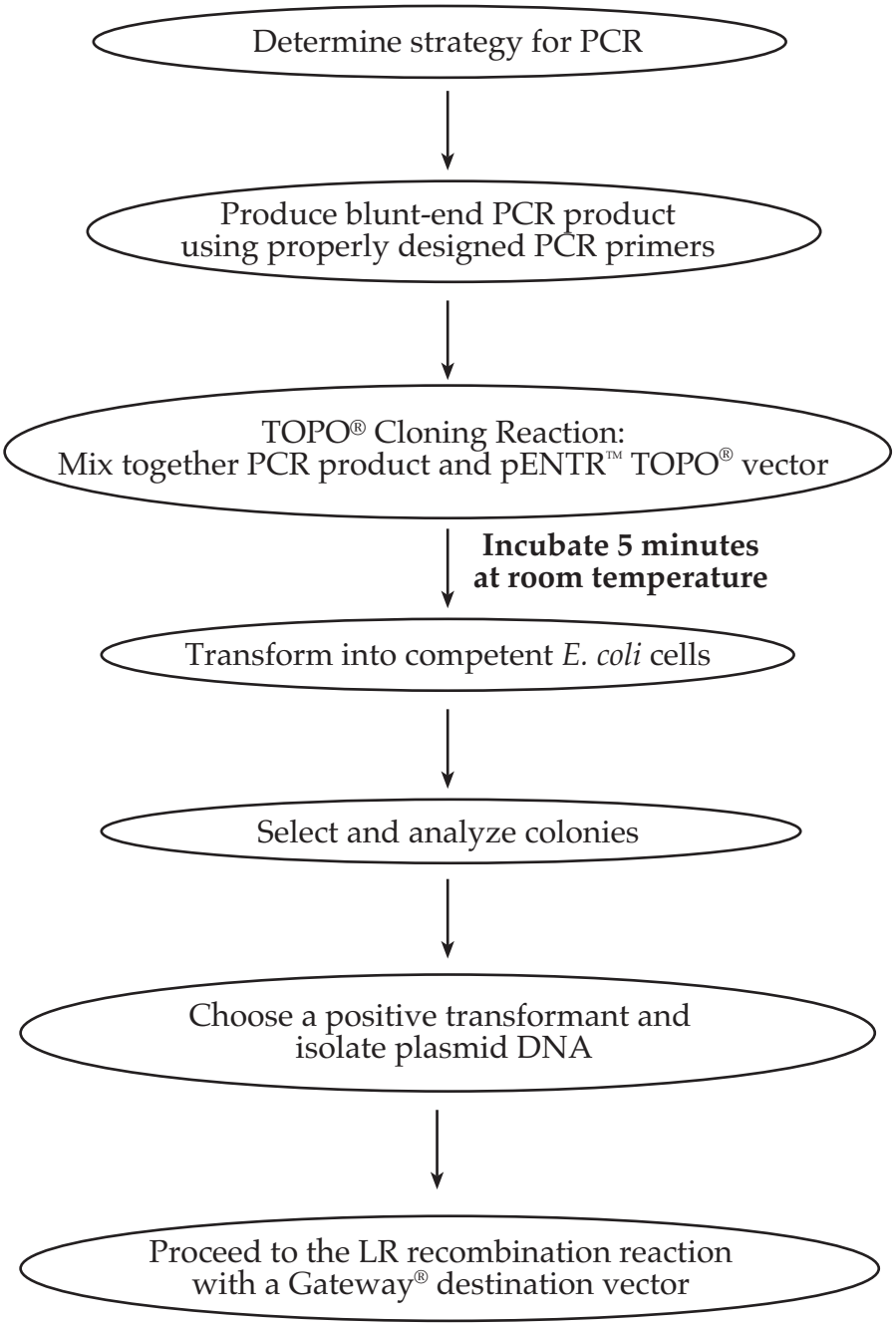
Note

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

Design PCR Primers

Design 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 intend 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 the forward PCR primer, consider the following points. (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 the **Example** on page 6).
- 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 the **Example** on page 6).

Continued on next page

Design PCR Primers, Continued

Example of forward primer design

The DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for your forward PCR primer are shown here: (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 3 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 1 base pair mismatch can reduce the directional cloning efficiency from 90%–50%, increasing the likelihood of your ORF cloning in the opposite orientation (see Example #1 on page 7).** We have not observed evidence of PCR products cloning in the opposite orientation from a 2 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 page 7).
- 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 page 7).

Continued on next page

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 2 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

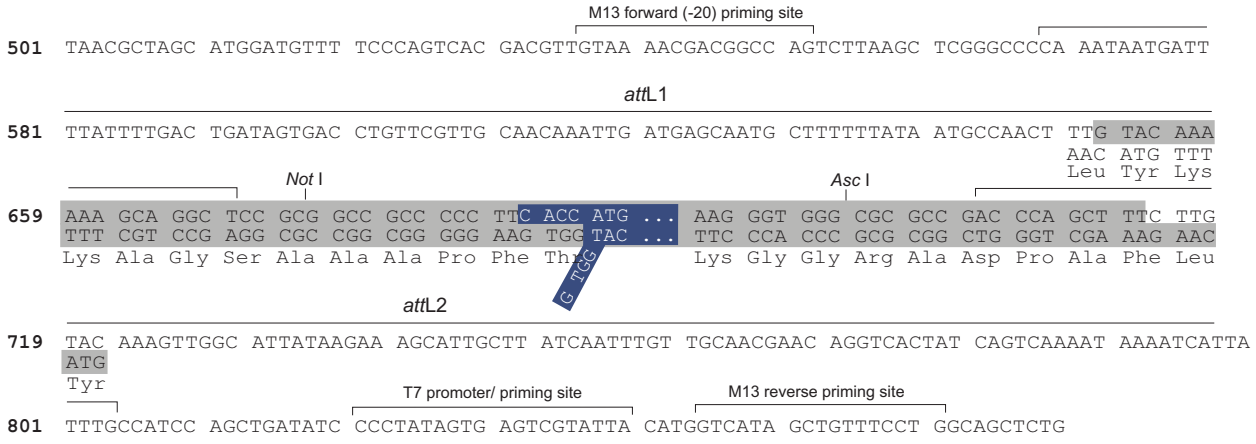
- Remember that the pENTR™ TOPO® vectors accept blunt-end PCR products.
- Do not add 5´ phosphates to your primers for PCR. This will prevent ligation into the pENTR™ TOPO® vectors.
- We recommend that you gel-purify your oligonucleotides, especially if they are long (> 30 nucleotides).

Continued on next page

Design PCR Primers, Continued

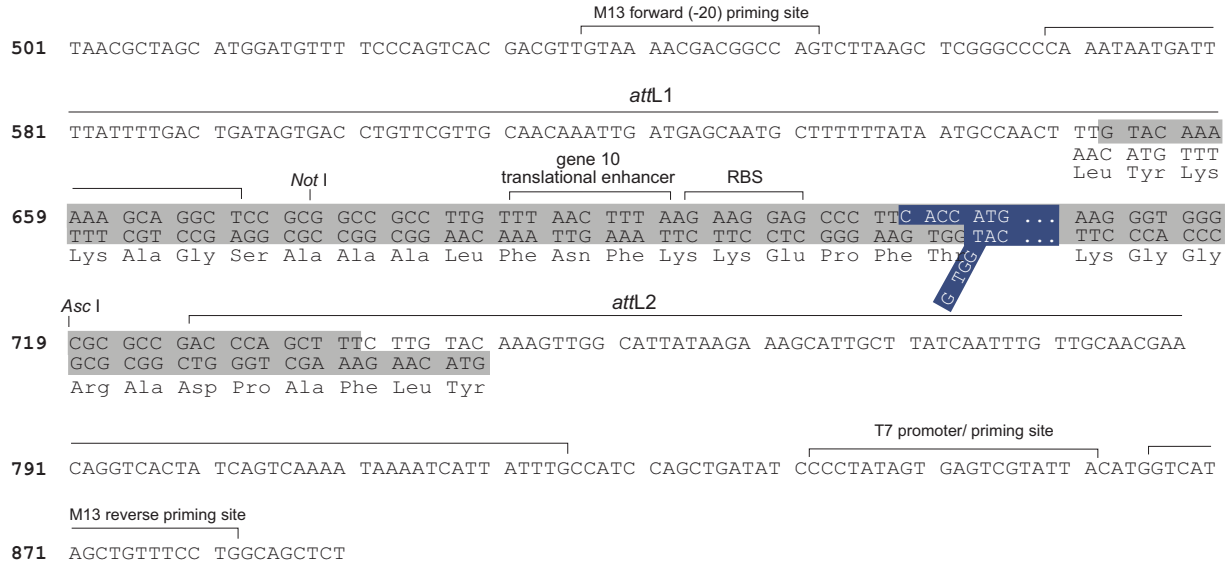
TOPO® Cloning Site for pENTR™/D-TOPO®

Use the following diagram 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 from www.lifetechnologies.com/support or by contacting Technical Support (page 35).** For more information about pENTR™/D-TOPO®, see pages 28–29.



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

Use the following diagram to help 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 from www.lifetechnologies.com/support or by contacting Technical Support (page 35).** For more information about pENTR™/SD/D-TOPO®, see pages 30–31.



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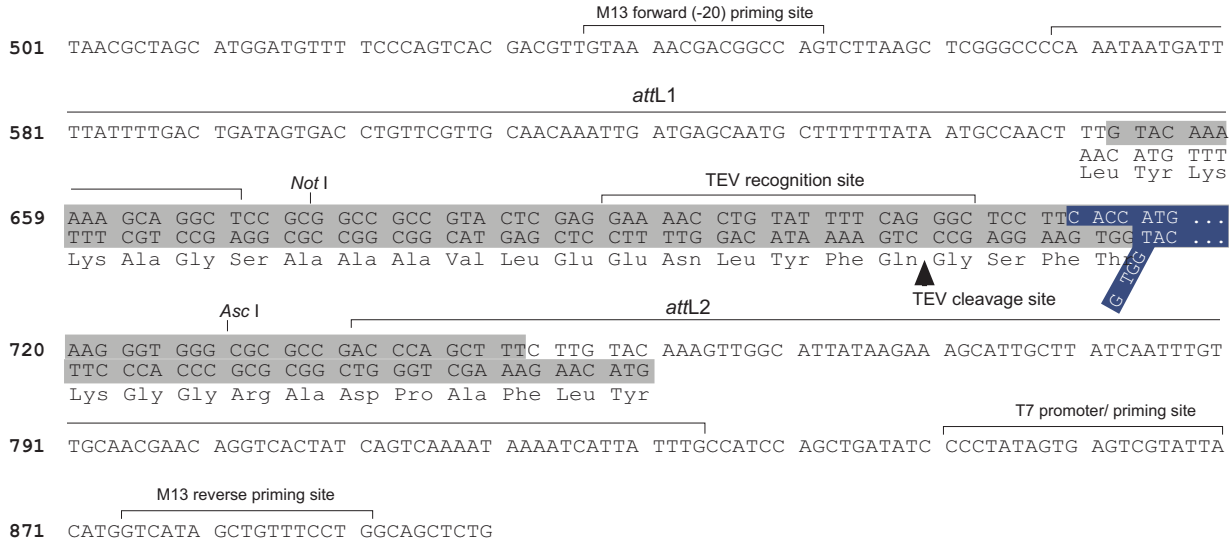
Design PCR Primers, Continued

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

Use the following diagram to help 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 from www.lifetechnologies.com/support or by contacting Technical Support (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.**



Produce Blunt-End PCR Products

Introduction

After deciding on a PCR strategy and synthesizing 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 using the following guidelines.

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

Check the PCR product

After producing your blunt-end PCR product, use agarose gel electrophoresis to verify the quality and quantity of your PCR product. Check 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** on page 11 for details).

Set Up the TOPO® Cloning Reaction

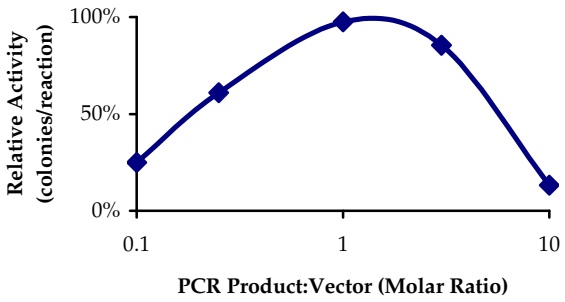
Introduction

After producing 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 **Transform 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 0.5:1–2:1 molar ratio of PCR product:TOPO® vector (see the following figure).** Note that the TOPO® Cloning efficiency decreases significantly if the ratio of PCR product: TOPO® vector is <0.1:1 or >5:1 (see the following figure). 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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Set Up the TOPO® Cloning Reaction, Continued

Use 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 22 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 in **Perform the TOPO® Cloning Reaction**.

Perform the TOPO® Cloning Reaction

Use the following procedure 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 0.5:1–2:1 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	0.5–4 µL	0.5–4 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 the 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***, page 13.

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

Transform One Shot® Competent *E. coli*

Introduction

After performing 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 22 for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Required materials

Components required but not supplied:

- TOPO® Cloning reaction (from step 2, page 12)
- 15-mL sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 50 µg/mL kanamycin (2 for each transformation)
- LB plates containing 100 µg/mL ampicillin (if transforming pUC19 control)
- 37°C shaking and non-shaking incubator
- general microbiological supplies (i.e. plates, spreaders)
- 42°C water bath (or electroporator with cuvettes, optional)

Components supplied with the kit:

- One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli* (Box 2)
- S. O.C. Medium (Box 2)
- *Optional:* pUC19 positive control (Box 2)

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.

Prepare for transformation

For each transformation, you will need 1 vial of One Shot® competent cells and 2 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** 1 vial of One Shot® cells from Box 2 for each transformation.

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 5–30 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.

Continued on next page

One Shot® chemical transformation protocol, Continued

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 2 different volumes to ensure that at least 1 plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5–10 colonies for analysis (see **Analyze Transformants**, page 15).

Transformation by electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1R 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 page 14.
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 2 different volumes to ensure that at least 1 plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5–10 colonies for analysis (see **Analyze Transformants**, page 15).

To prevent arcing of your samples during electroporation, the volume of cells should be between 50–80 µL (0.1-cm cuvettes) or 100–200 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

Analyze Transformants

Analyze positive clones

1. Pick 5–10 colonies and culture them overnight in LB or SOB medium containing 50–100 g/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 the PureLink® HQ Mini Plasmid Purification Kit (see page 22 for ordering information).
3. Analyze the plasmids by restriction analysis or PCR (see **Analyze transformants by PCR**) to confirm the presence and correct orientation of the insert.

Analyze transformants by PCR

Use the following protocol (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 (see page 22 for ordering information)
- 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 20–30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Continued on next page

Sequence

After identifying 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 in the kit to help you sequence the insert (see the diagrams on pages 8–9 for the location of the priming sites in each pENTR™ TOPO® vector). For the sequence of each pENTR™ TOPO® vector, see **www.lifetechnologies.com/support** or call Technical Support (see page 35).

Note: The M13 Forward (−20) and M13 Reverse primers are available separately (see page 22 for ordering information).

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 20, for tips to help you troubleshoot your experiment.

Long-Term Storage

After identifying 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

After obtaining your entry clone, you may:

- Perform an LR recombination reaction using Gateway® LR Clonase® II enzyme mix (see page 22 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 22 for ordering information) to generate an expression clone.

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 22 for ordering information). The LR Clonase® II enzyme mix combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied as separate components in LR Clonase® enzyme mix 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 you prefer.

Destination Vectors

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

E. coli Host

After performing 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.

Continued on next page

Guidelines to Perform the LR Recombination Reaction

Materials supplied by the user

- Purified plasmid DNA of the entry clone containing your gene of interest
- A destination vector of choice
- Gateway® LR Clonase® II enzyme mix (see page 22 for ordering information)
- 2 µg/µL Proteinase K solution (supplied with the Gateway® 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

Perform the MultiSite Gateway® LR recombination reaction

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.

Before performing the MultiSite Gateway® LR recombination reaction, you will first need to generate 5′ and 3′ entry clones using the MultiSite Gateway® Three-Fragment Vector Construction Kit (see page 22 for ordering information). After generating 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® II 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.

Obtain TEV protease

For highly efficient TEV protease-directed cleavage, we recommend using AcTEV™ Protease (see page 22 for ordering information). 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 in this section. 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; see page 22 for ordering information).

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 following table lists some potential problems and possible solutions that may help troubleshoot the TOPO® Cloning and transformation reactions. To help evaluate results, we recommend performing the control reactions (see pages 22–25) in parallel with your samples.

Observation	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 0.5:1–2:1 molar ratio of PCR product:TOPO® vector.
	Too much PCR product used in the TOPO® Cloning reaction	• Dilute the PCR product. • Use a 0.5:1–2:1 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 0.5:1–2:1 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

TOPO® Cloning Reaction and Transformation, Continued

Observation	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 0.5:1–2:1 molar ratio of PCR product:TOPO® vector.
	Incomplete extension during PCR	Include a final extension step of 7–30 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 7–30 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

Accessory Products

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. Ordering information for these reagents is provided below. For more information, refer to **www.lifetechnologies.com/support** or call Technical Support (see page 35).

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	5 g	11815-024
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
LR Clonase® II Plus Enzyme Mix	20 reactions	12538-120
MultiSite Gateway® Three-Fragment Vector Construction Kit	1 kit	12537-023
PCR Super Mix High Fidelity	100 reactions	10790-020
ProBond™ Resin	50 mL	R801-01
PureLink® Quick Gel Extraction Kit	50 reactions	K2100-12
AcTEV™ Protease	1,000 units	12575-015
	10,000 units	12575-023

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 this product directly in a TOPO® Cloning reaction.

Before starting

For each transformation, prepare 2 LB plates containing 50 µg/mL kanamycin.

Produce 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. 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. Make sure that you see a single, discrete 750-bp band.
4. 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. 0.5:1–2:1). Proceed to the **Control TOPO® Cloning Reactions**, page 24.

Continued on next page

Control TOPO® Cloning reactions

Using the control PCR product produced on page 23 and the pENTR™ TOPO® vector, set up two 6 µL TOPO® Cloning reactions as described in this section. If you plan to transform electrocompetent *E. coli*, use the 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 2 different volumes to ensure that at least 1 plate has well-spaced colonies.
5. Incubate overnight at 37°C.

Continued on next page

Perform the Control Reactions, Continued

Analyze 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 in the following table, which 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 1 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. The transformation efficiency should be $\geq 1 \times 10^9$ cfu/µg DNA.

Gel Purify 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. Two simple protocols are provided in this section.

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 **Produce Blunt-End PCR Products**, page 10).

Using the PureLink® Quick Gel Extraction Kit

The PureLink® Quick Gel Extraction Kit (see page 22 for ordering information) allows you to rapidly purify PCR products from regular agarose gels.

1. Electrophorese amplification reaction on a 1–5% regular TAE agarose gel.
2. Equilibrate a water bath or heat block to 50°C.
3. 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.
4. Weigh the gel slice.
5. 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.
6. 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.
7. Preheat an aliquot of TE Buffer (TE) to 65–70°C.
8. 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.
9. Centrifuge at > 12,000 × g for 1 minute. Discard the flow-through. Place the column back into the Wash Tube.
10. *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.
11. 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.

Procedure continued on the following page

Continued on next page

Using the PureLink® Quick Gel Extraction Kit, Continued

12. 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.
13. Add 50 µL *warm* (65–70°C) TE Buffer (TE) to the center of the cartridge. Incubate at room temperature for 1 minute.
14. Centrifuge at > 12,000 × g for 2 minutes. *The Recovery Tube contains the purified DNA.* Store DNA at –20°C. Discard the column.
15. Use 4 µL of the purified DNA for the TOPO® Cloning reaction and proceed as described on page 12.

Low-Melt Agarose Method

If you prefer to use low-melt agarose, use the following procedure. 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 (0.8–1.2%) 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 5–10 minutes**. This is to keep the agarose melted.
7. Transform 2–4 µL directly into One Shot® competent cells using the method on page 14.

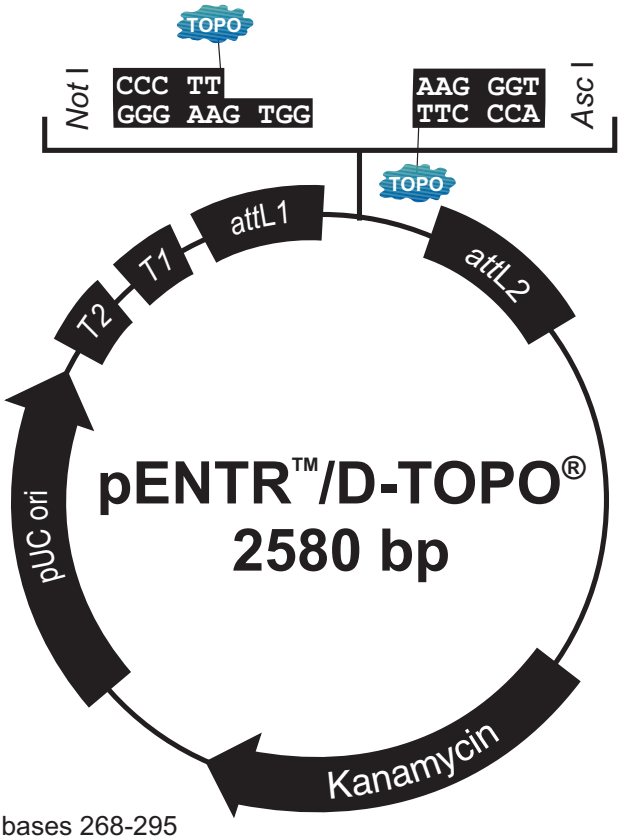
Note

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 following figure shows the features of pENTR™/D-TOPO® vector. **The sequence of pENTR™/D-TOPO® is available from www.lifetechnologies.com/support or by contacting Technical Support (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®

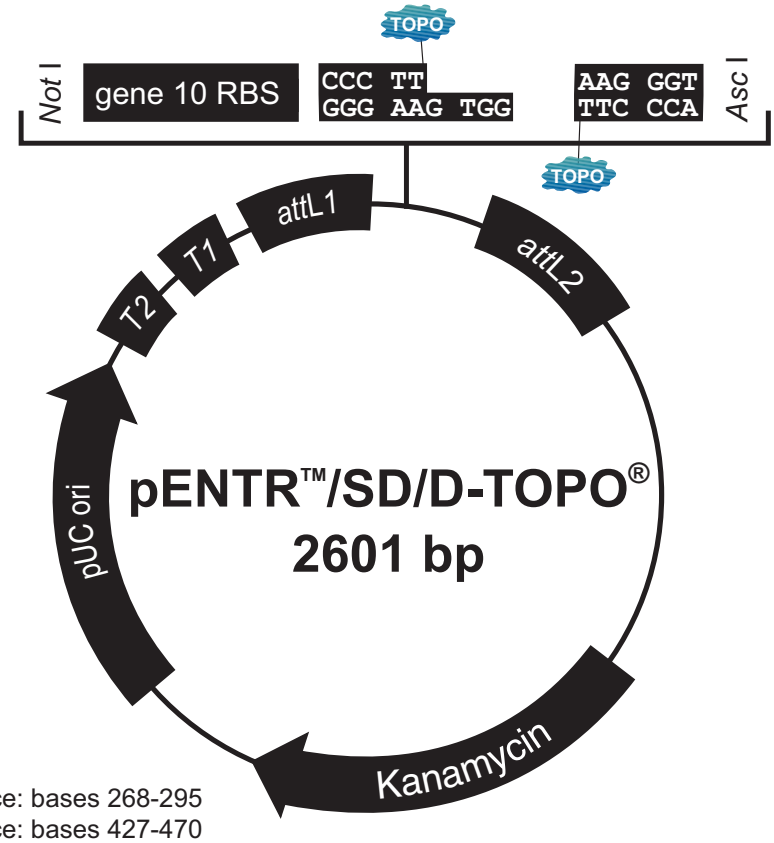
pENTR™/D-TOPO® (2580 bp) contains the following elements. The features have been functionally tested.

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 sequence of pENTR™/SD/D-TOPO® is available from www.lifetechnologies.com/support or by contacting Technical Support (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®

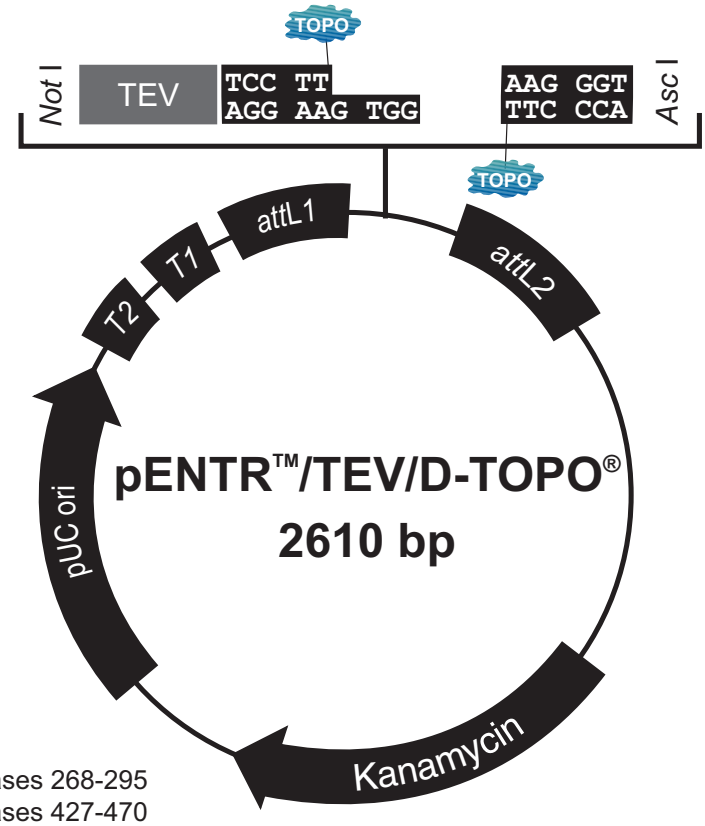
pENTR™/SD/D-TOPO® (2601 bp) contains the following elements. The features have been functionally tested.

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 sequence of pENTR™/TEV/D-TOPO® is available from www.lifetechnologies.com/support or by contacting Technical Support (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. The 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

Luria-Bertani (LB) 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 Support

Obtaining support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

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Information for European Customers

The Mach1™-T1^R *E. coli* strain is genetically modified to carry the *lac*Z M15 *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.

Gateway® Clone Distribution Policy

For additional information about Life Technologies' policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 38.

Continued on next page

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

Life Technologies 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. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway® Expression Clones

Life Technologies 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. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Life Technologies.

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 Life Technologies 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 Life Technologies' 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 Life Technologies' licensing department at 760-603-7200.

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