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pENTR™ 5′-TOPO® TA Cloning Kit

Five-minute TOPO® Cloning of *Taq* polymerase-amplified PCR products into an entry vector for the ViraPower™ Promoterless Lentiviral Gateway® and MultiSite Gateway® Systems

Catalog nos. K591-10, K591-20, and K5910-00

Version C
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User Manual

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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>Produce PCR product</td><td>Amplify DNA encoding a eukaryotic promoter of choice using Taq polymerase and your own protocol. End the PCR reaction with a final 7 to 10 minute extension step.</td></tr>
<tr><td>Perform the TOPO® Cloning Reaction</td><td>1. Set up one of the following TOPO® Cloning reactions using the reagents in the order shown. For electroporation, dilute Salt Solution 4-fold to prepare Dilute Salt Solution.
<table>
<tr><th>Reagent</th><th>For Chemical Transformation</th><th>For 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</td><td>--</td><td>1 µl</td></tr>
<tr><td>Sterile Water</td><td>to a final volume of 5 µl</td><td>to a final volume of 5 µl</td></tr>
<tr><td>pENTR™5′-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® TOP10 chemically competent E. coli, below.</td></tr>
<tr><td>Transform One Shot® TOP10 Chemically Competent E. coli</td><td>1. For each transformation, thaw one vial of One Shot® TOP10 E. coli cells on ice.
2. Add 2 µl of the TOPO® Cloning reaction into a vial of One Shot® TOP10 chemically competent E. coli and mix gently.
3. Incubate on ice for 5 to 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Incubate at 37°C for 1 hour with shaking.
7. Spread 10-50 µl of bacterial culture on a prewarmed LB agar plate containing 50 µg/ml kanamycin, 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 19-20 for instructions.

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Kit	Catalog No.
pENTR™5′-TOPO® TA Cloning® Kit	K591-20
ViraPower™ Promoterless Lentiviral Gateway® Vector Kit	K591-10
ViraPower™ Promoterless Lentiviral Gateway® Expression Kit	K5910-00

Note: Catalog nos. K591-10 and K5910-00 also include ViraPower™ Promoterless Lentiviral components required for production of a lentiviral expression construct. For more information about the ViraPower™ Promoterless Lentiviral components, refer to the ViraPower™ Promoterless Lentiviral Gateway® Expression System manual. This manual is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Kits, but is also available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (see page 27).

Shipping/Storage

Each pENTR™5′-TOPO® TA Cloning® Kit is shipped on dry ice, and contains two boxes as described below. Upon receipt, store the boxes as detailed below.

Box	Item	Storage
1	pENTR™5′-TOPO® TA Cloning® Reagents	-20°C
2	One Shot® TOP10 Chemically Competent *E. coli*	-80°C

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Kit Contents and Storage, continued

pENTR™5′-TOPO® Reagents

The following reagents are supplied with the pENTR™5′-TOPO® vector (Box 1). **Note that the user must supply *Taq* polymerase. Store at -20°C.**

Item	Concentration	Amount
pENTR™5′-TOPO® vector, TOPO®-adapted	5-10 ng/µl linearized plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/ml BSA 30 µM phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µl
dNTP Mix	12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
Sterile Water	---	1 ml
GW1 Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
GW3 Primer	0.1 µg/µl in TE Buffer, pH 8.0	20 µl
Control PCR Primers	0.1 µg/µl **each** in TE Buffer, pH 8.0	10 µl
Control PCR Template	0.05 µg/µl in TE Buffer, pH 8.0	10 µl

Primer Sequences

The table below provides the sequences of GW1 and GW3 primers.

Primer	Sequence	pMoles Supplied
GW1	5′-GTTGCAACAAATTGATGAGCAATGC-3′	260
GW3	5′-TTAATATATTGATATTTATATCATTTTACG-3′	219

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Kit Contents and Storage, continued

One Shot® TOP10 Reagents

The following reagents are included with the One Shot® TOP10 Chemically Competent *E. coli* kit. Transformation efficiency is ≥ 1 x 10^9 cfu/µg plasmid DNA. **Store 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 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 TOP10 Strain

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

Accessory Products

Introduction

The products listed in this section may be used with the pENTR™5′-TOPO® TA Cloning® Kit. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Support (see page 27).

Additional Products

Some of the reagents supplied in the pENTR™5′-TOPO® TA Cloning® Kit and other reagents suitable for use with the kit are available separately from Invitrogen. Ordering information for these reagents is provided below.

Note: Other reagent quantities may be available.

Item	Quantity	Catalog no.
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
	250 reactions	10966-026
	500 reactions	10966-034
Taq DNA Polymerase, Recombinant	100 units	10342-053
	250 units	10342-012
	500 units	10342-020
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
	500 units	11304-029
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
Kanamycin Sulfate	100 ml (10 mg/ml)	15160-054
PureLink™ HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Gateway® LR Clonase™ II Plus Enzyme Mix	20 reactions	12538-020
	100 reactions	12538-100
MultiSite Gateway® Three-Fragment Vector Construction Kit	1 kit	12537-023

Introduction

Overview

Introduction

The pENTR™5′-TOPO® TA Cloning® Kit combines Invitrogen's TOPO® Cloning and MultiSite Gateway® Technologies to facilitate five-minute, one-step cloning of a *Taq* polymerase-amplified PCR product encoding a eukaryotic promoter of interest into a MultiSite Gateway® entry vector with ≥ 85% efficiency. Once cloned, your eukaryotic promoter may be transferred from the pENTR™5′-TOPO® vector to a suitable MultiSite Gateway® destination vector in a MultiSite Gateway® LR recombination reaction with other suitable entry clone(s) to generate an expression construct.

For more information about how TOPO® Cloning works and the MultiSite Gateway® Technology, see the rest of this section.

The MultiSite Gateway® Technology

The Gateway® Technology is a universal cloning system that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move a single DNA sequence of interest into multiple vector systems. The MultiSite Gateway® Technology uses modifications of the Gateway® Technology to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation to facilitate creation of an expression construct that expresses a gene of interest from your promoter of choice. To express a gene of interest using the MultiSite Gateway® Technology, you will:

1. TOPO® Clone a *Taq*-amplified PCR product encoding a eukaryotic promoter of choice into the pENTR™5′-TOPO® vector to generate a 5′ entry clone.
2. Generate an entry clone containing your gene of interest using one of the standard Gateway® entry vectors available from Invitrogen.

   Note: If you are using the MultiSite Gateway® Three-Fragment Vector Construction Kit, also generate an entry clone containing a 3′ element of interest (*e.g.* polyadenylation signal) using the reagents supplied in the kit.
3. Perform a MultiSite Gateway® LR recombination reaction between your 5′ entry clone, other suitable entry clone(s), and the appropriate MultiSite Gateway® destination vector to generate an expression construct.
4. Introduce your expression construct into mammalian cells and express your recombinant protein.

For more information about the MultiSite Gateway® Technology, refer to the ViraPower™ Promoterless Lentiviral Gateway® Expression System or MultiSite Gateway® Three-Fragment Vector Construction Kit manuals, as appropriate. Both manuals are available for downloading from www.invitrogen.com or by contacting Technical Support (see page 27).

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Overview, continued

Features of the pENTR™5′-TOPO® Vector

Features of the pENTR™5′-TOPO® vector include:

- TOPO® Cloning site for rapid and efficient cloning of a *Taq*-amplified PCR product encoding a promoter of choice
- *att*L4 and *att*R1 sites to allow two-fragment or three-fragment recombination with appropriate entry clone(s) and a MultiSite Gateway® destination vector to generate an expression construct
- Primer binding sites within the *att*L4 and *att*R1 sites for sequencing using the GW1 and GW3 primers
- *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 of the plasmid in *E. coli*

att Sites in pENTR™5′-TOPO®

In the Gateway® Technology, recombinational cloning is mediated via optimized *att* sites. To accommodate simultaneous recombinational cloning of multiple DNA fragments in the MultiSite Gateway® Technology, these *att* sites have been further modified and optimized. Modifications include alterations to both the sequence and length of the *att* sites, resulting in the creation of "new" *att* sites exhibiting enhanced specificities and the improved efficiency required to permit cloning of multiple DNA fragments in a single reaction.

While traditional Gateway® entry vectors contain *att*L1 and *att*L2 sites and may be recombined with any standard Gateway® destination vector to generate an expression construct, the pENTR™5′-TOPO® vector contains ***att*L4** and ***att*R1** sites (refer to the diagram on page 7 for the sequence of the *att* sites). **Because of the different *att* sites, entry clones generated in pENTR™5′-TOPO® are only suitable for use in MultiSite Gateway® applications (see the next page) and not in other standard Gateway® applications.**

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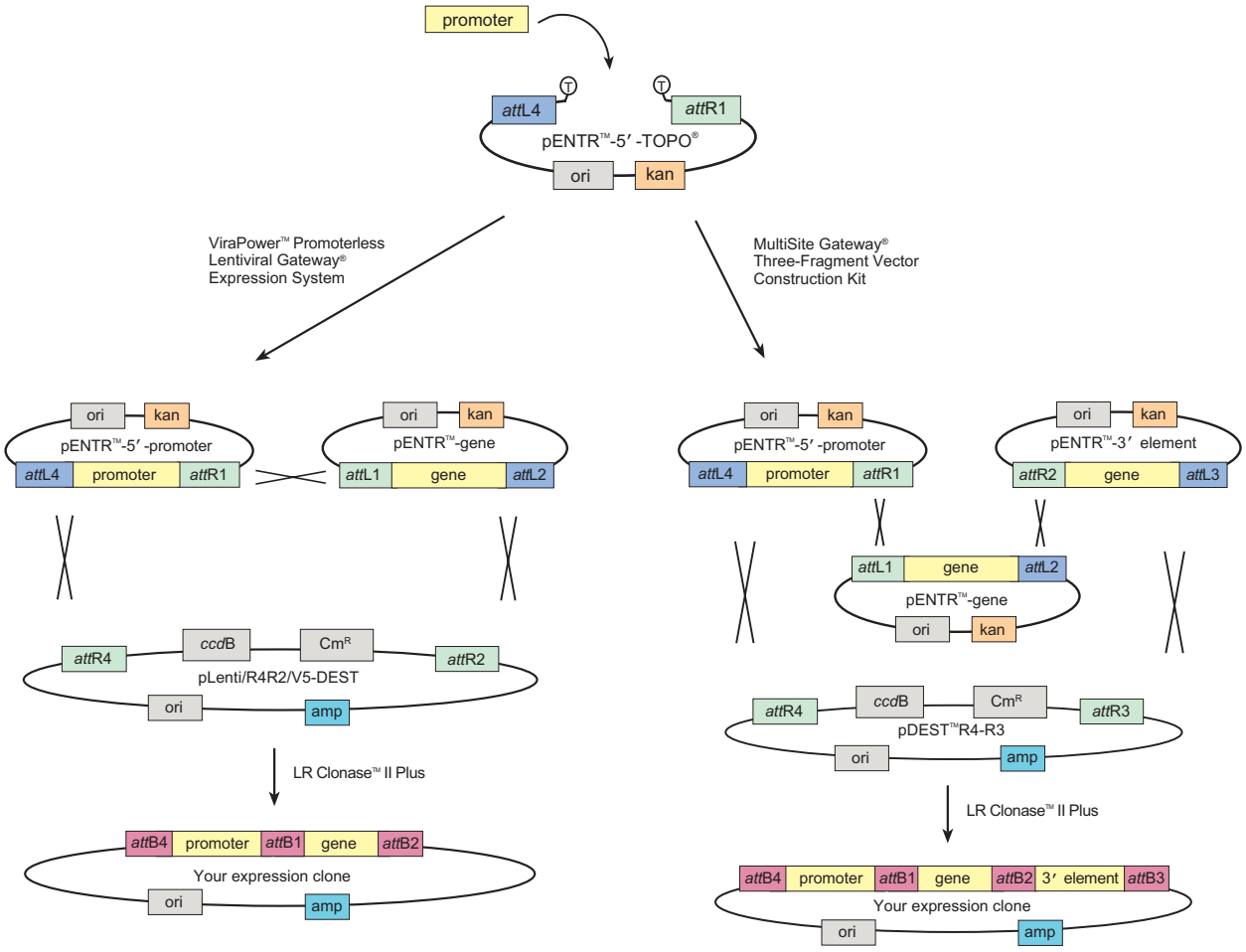
Overview, continued

Applications for pENTR™5′-TOPO® Entry Clones

Entry clones generated in the pENTR™5′-TOPO® vector are suitable for use in the following MultiSite Gateway® applications. For an illustration, see the figure below.

- Combine a pENTR™5′-TOPO® entry clone containing a promoter of interest with an *att*L1 and *att*L2-flanked entry clone containing a gene of interest and pLenti6/R4R2/V5-DEST to create a lentiviral expression construct. Use the lentiviral construct to facilitate expression of your recombinant protein in dividing or non-dividing mammalian cells. For more information, refer to the ViraPower™ Promoterless Lentiviral Gateway® Expression System manual.
- Combine a pENTR™5′-TOPO® entry clone containing a promoter of interest with an *att*L1 and *att*L2-flanked entry clone containing a gene of interest, an *att*R2 and *att*L3-flanked entry clone containing a 3′ element of interest, and pDEST™R4-R3 to create an expression construct. Use the expression construct to facilitate expression of your recombinant protein in mammalian cells. For more information, refer to the MultiSite Gateway® Three-Fragment Vector Construction Kit manual.



Overview, continued

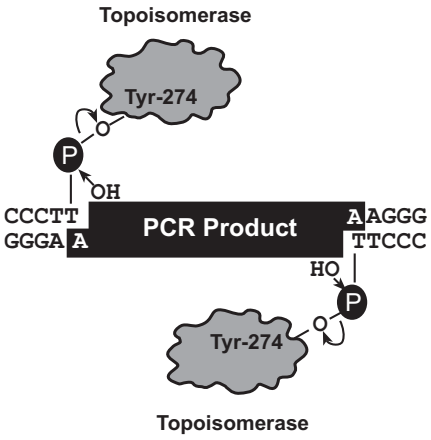
How Topoisomerase I Works

The pENTR™5′-TOPO® vector is supplied linearized with:

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

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

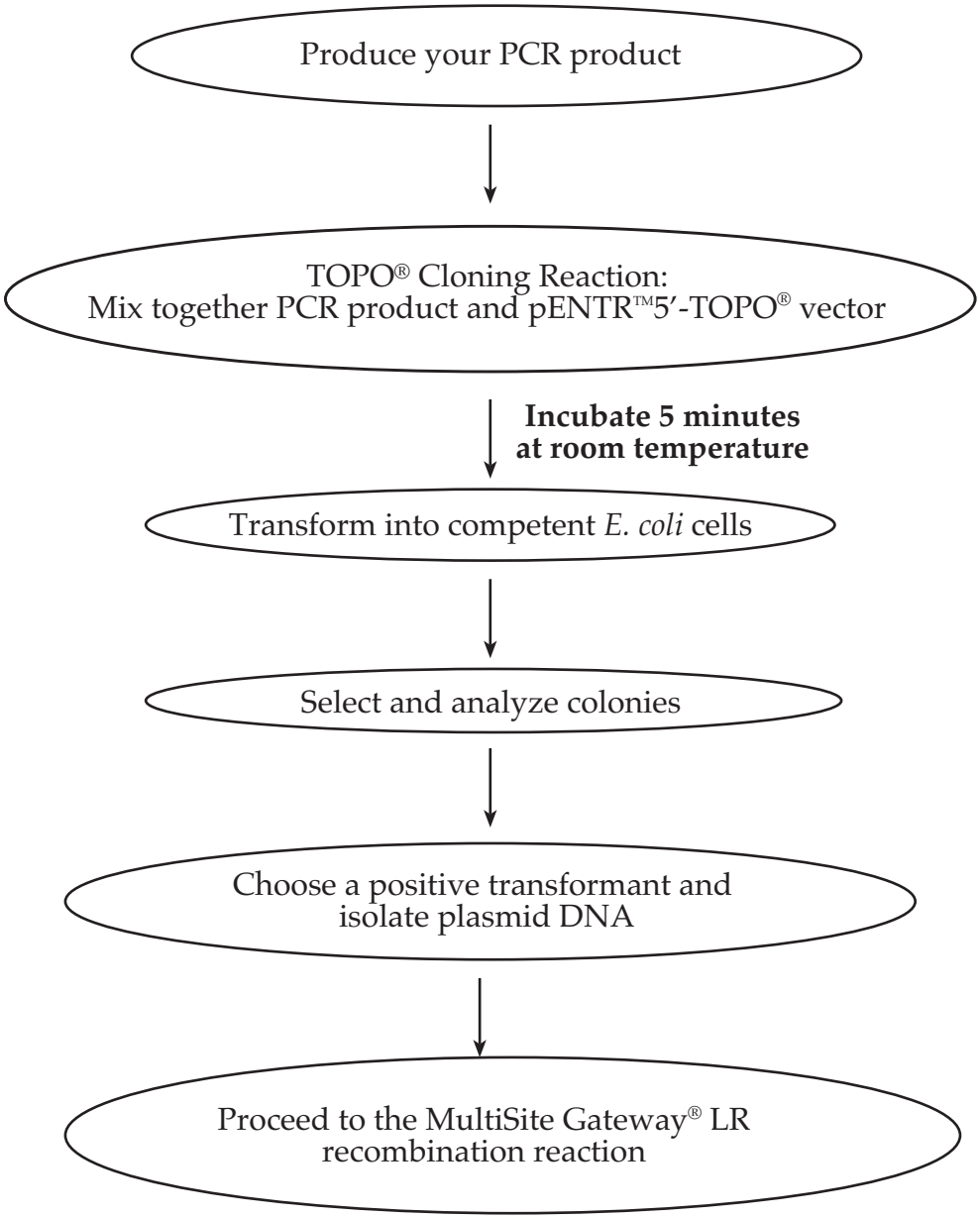
Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and 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.



Experimental Outline

Flow Chart

The flow chart below describes the general steps required to produce and TOPO® Clone your *Taq*-amplified PCR product. Remember that the PCR product should encode a eukaryotic promoter of interest.



Methods

Designing PCR Primers

Introduction

Before you use the pENTR™5′-TOPO® TA Cloning® Kit, you must first design PCR primers and produce your PCR product. Guidelines are provided in this section to help you design PCR primers.

Factors to Consider

It is important to properly design your PCR primers to ensure that you obtain the PCR product you need for your studies. Consider the following when designing your PCR primers:

- Remember that the pENTR™5′-TOPO® entry clone will be flanked by *att*L4 and *att*R1 sites, and can **only** be used in a MultiSite Gateway® LR recombination reaction with one or more types of entry clones and a suitable destination vector.

  Example: The pENTR™5′-TOPO® entry clone may be used in a MultiSite Gateway® LR recombination reaction with an *att*L1 and *att*L2-flanked entry clone containing the gene of interest and the pLenti6/R4R2/V5-DEST vector.
- Your PCR product should encode a eukaryotic promoter of interest suitable for controlling expression of the downstream gene of interest. Make sure that the PCR product contains all sequences (*e.g.* TATA box, transcription factor binding sites, etc.) necessary to regulate the downstream gene of interest (after MultiSite Gateway® LR recombination).
- Your PCR product should contain a transcriptional start site (if one is not included in the entry clone containing your gene of interest).
- Your PCR product should **not** contain an ATG for translation initiation (if one is included in the entry clone containing your gene of interest).

If you are planning to use your pENTR™5′-TOPO® entry clone in a MultiSite Gateway® LR reaction with an *att*L1 and *att*L2-flanked entry clone and the pLenti6/R4R2/V5-DEST vector to generate a lentiviral expression vector (*i.e.* ViraPower™ Promoterless Lentiviral Gateway® Kits), note that the size of your insert DNA (*i.e.* promoter + gene of interest) can influence the viral titer. **To obtain efficient lentiviral packaging, make sure that the combined size of your promoter + gene of interest does not exceed 4.5-5 kb.** Larger inserts may not be properly packaged and may result in lower lentiviral titers.

Important

When synthesizing PCR primers, **do not** add 5´ phosphates to the primers as this will prevent the synthesized PCR product from ligating into the pENTR™5′-TOPO® vector.

continued on next page

Designing PCR Primers, continued

TOPO® Cloning Site for pENTR™5′-TOPO®

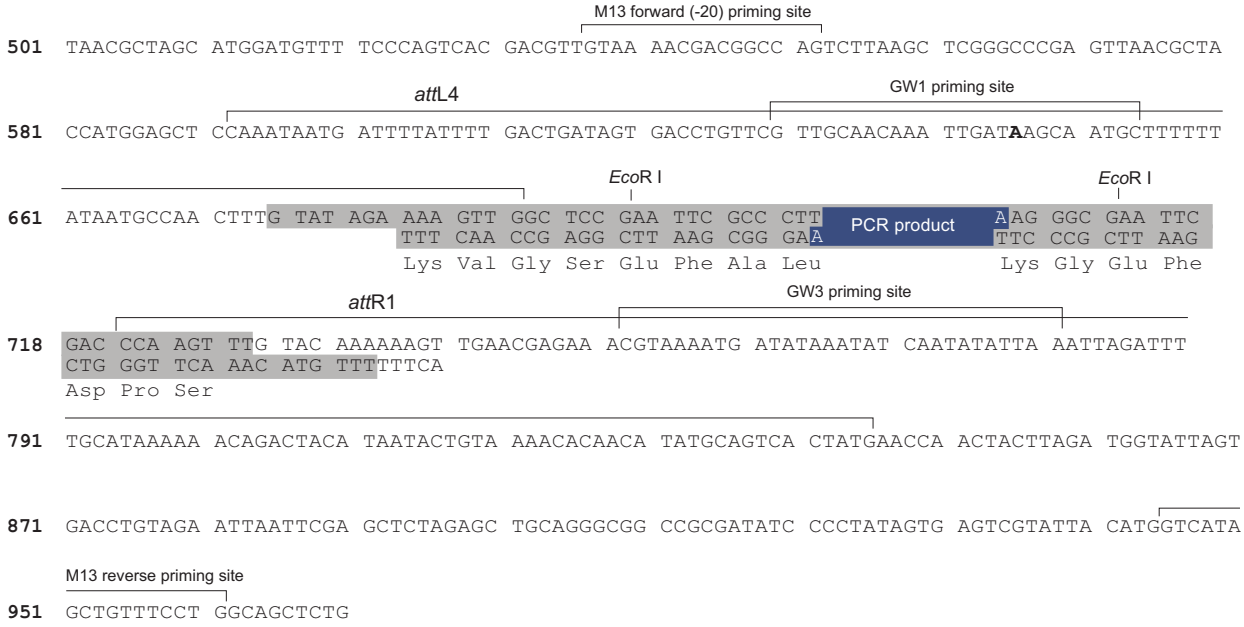
Use the diagram below to help you design PCR primers and produce your PCR product for TOPO® Cloning into the pENTR™5′-TOPO® vector. Note that the pENTR™5′-TOPO® vector is supplied linearized between nucleotides 705 and 706.

Features of the TOPO® Cloning Region:

- Restriction sites are labeled to indicate the actual cleavage site.
- The binding sites for the GW1 and GW3 primers included in the kit are labeled.

  Note: When propagating the pENTR™5′-TOPO® vector and entry clones, we have found that one nucleotide within the GW1 priming site can vary in sequence between G and A (highlighted in bold below). This single nucleotide change **does not** affect the efficiency of MultiSite Gateway® LR recombination or the functionality of the GW1 sequencing primer.
- The shaded region corresponds to the DNA sequences that will be transferred from the clone into the MultiSite Gateway® destination vector after MultiSite Gateway® LR recombination.

For a map and a description of the features of pENTR™5′-TOPO®, see page 24. **The sequence of pENTR™5′-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Support (see page 27).**



Note

If you have used other Gateway® entry vectors, note that the sequences within the *att* sites may vary slightly. The efficiency of LR recombination remains the same.

Producing PCR Products

Introduction

Once you have synthesized appropriate PCR primers, you may use the primers and a suitable DNA polymerase to produce your PCR product. **Remember that your PCR product must have single 3′ A-overhangs.**

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.

- *Taq* polymerase or other suitable DNA polymerase

  Note: For improved specificity and higher yields, we recommend using Platinum® *Taq* DNA Polymerase available from Invitrogen (see page x for ordering information) to generate your PCR product.
- Thermocycler
- DNA template (containing the desired promoter region)
- PCR primers suitable for amplification of the desired region

Polymerase Mixtures

You may use a polymerase mixture containing *Taq* polymerase and a proofreading polymerase to produce your PCR product; however, the mixture must contain a ratio of *Taq* polymerase:proofreading polymerase in excess of 10:1 to ensure the presence of 3′ A-overhangs on the PCR product. We recommend using Platinum® *Taq* DNA Polymerase High Fidelity available from Invitrogen (see page x for ordering information).

If you use polymerase mixtures that do not have enough *Taq* polymerase or a proofreading polymerase only, you may add 3′ A-overhangs to your PCR product using the method on page 23.

Producing PCR Products

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

DNA Template	10-100 ng
10X PCR Buffer	5 µl
dNTP Mix (50 mM)	0.5 µl
PCR primers (100-200 ng each)	1 µM each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 U/µl)	1 µl
Total volume	50 µl

2. Use agarose gel electrophoresis to verify the quality of your PCR product. You should see a single, discrete band of the correct size. If you do not see a single band, refer to the **Note** on the next page.

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Producing PCR Products, continued

Note

If you do not obtain a single, discrete band from your PCR, try the following:

- Optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit available from Invitrogen (Catalog no. K1220-01) incorporates many of the recommendations found in this reference. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Support (see page 27).
- Gel-purify your fragment using one of the methods on pages 21-22. Take special care to avoid sources of nuclease contamination.

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pENTR™5′-TOPO® vector and transform the recombinant vector into One Shot® TOP10 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 12-13) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 19-20 in parallel with your samples.

Note

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

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

Using Salt Solution in the TOPO® Cloning Reaction

You will perform TOPO® Cloning in a reaction buffer containing salt (*i.e.* using the stock salt solution provided in the kit). **Note that 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 on the next page.
- 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 this Dilute Salt Solution to set up the TOPO® Cloning reaction as directed on the next page.

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Setting Up the TOPO® Cloning Reaction, continued

Materials Needed

You should have the following materials on hand before beginning:

- Your PCR product (freshly prepared)
- The pENTR™5′-TOPO® vector (supplied with the kit, Box 1; keep at -20°C until use)
- Salt Solution (supplied with the kit, Box 1) or Dilute Salt Solution (see previous page), as appropriate
- Sterile water (supplied with the kit, Box 1)

Performing the TOPO® Cloning Reaction

Use the procedure below to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction using the reagents in the order shown, and depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli.*

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

Reagent*	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
pENTR™5′-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, a 5 minute incubation 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 (previous page), transform your pENTR™5′-TOPO® construct into competent *E. coli*. One Shot® TOP10 Chemically Competent *E. coli* are included with the kit to facilitate transformation. You may also transform electrocompetent cells, if desired (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 chemically competent *E. coli* (supplied with the kit, Box 2)
- S.O.C. Medium (supplied with the kit, Box 2)
- pUC19 positive control (to check transformation efficiency, if desired; supplied with the kit, 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; see page 26 for a recipe to prepare LB plates)
- 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 into the vector. The GW1 and GW3 primers are included in the kit to allow you to sequence across an insert in the TOPO® Cloning site to confirm orientation.

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 to room temperature.
- Warm LB plates containing 50 µg/ml kanamycin at 37°C for 30 minutes. If you are including the pUC19 positive control, prewarm LB plates containing 100 µg/ml ampicillin as well.
- Thaw **on ice** one vial of One Shot® cells for each transformation.

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

One Shot® Chemical Transformation Protocol

Use the following protocol to transform One Shot® TOP10 chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 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 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 15).

One Shot® Electroporation Protocol

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from **Performing the TOPO® Cloning Reaction**, Step 2, page 11 into a sterile microcentrifuge tube containing 50 µl of One Shot® TOP10 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 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
6. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick 10 colonies for analysis (see **Analyzing Transformants**, page 15).

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 10 colonies and culture them overnight in LB or SOB medium containing 50 µg/ml kanamycin.
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 to confirm the presence and correct orientation of the insert.

   Note: pENTR™5′-TOPO® contains *Eco*RI sites flanking the TOPO® Cloning site. You may use *Eco*RI digestion to check for the presence of insert, if desired.

Sequencing

Once you have identified the correct clone(s), you may sequence your construct to confirm that your promoter is cloned in the correct orientation. Use the GW1 and GW3 primers included in the kit to sequence your insert. The GW1 and GW3 primer binding sites are located within the *att*L4 and *att*R1 sites, respectively, and therefore minimize the amount of vector-encoded DNA that needs to be read to less than 55 base pairs. (see the diagram on page 7 for the location of the priming sites).

Alternatively, the pENTR™5′-TOPO® vector also includes M13 forward and reverse primer binding sites for sequencing using M13 forward (-20) and M13 reverse primers, if desired. Note that the M13 forward and reverse primer binding sites are located upstream and downstream of the of the *att*L4 and *att*R1 sites, respectively, requiring that at least 130 base pairs of vector-encoded DNA be read before reaching the insert DNA.

Reminder: When using the GW1 primer for sequencing, note that one nucleotide (position 646) within the primer binding site of the vector can vary in sequence between G and A. This variation does not affect the functionality of the GW1 sequencing primer or sequencing results.

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 colonies on an LB plate 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. Transfer to a cryovial.
5. Store at -80°C.

Guidelines to Perform the MultiSite Gateway® LR Recombination Reaction

Introduction

Once you have obtained your pENTR™5′-TOPO® entry clone, you may:

- Perform a two-fragment MultiSite Gateway® LR recombination reaction using the pENTR™5′-TOPO® entry clone, an *att*L1 and *att*L2-flanked entry clone containing your gene of interest, and the pLenti6/R4R2/V5-DEST destination vector to generate a lentiviral expression construct. For more information about pLenti6/R4R2/V5-DEST, refer to the ViraPower™ Promoterless Lentiviral Gateway® Expression System manual.
- Perform a three-fragment MultiSite Gateway® LR recombination reaction using the pENTR™5′-TOPO® entry clone, an *att*L1 and *att*L2-flanked entry clone containing your gene of interest, an *att*R2 and *att*L3-flanked entry clone containing a 3′ element of interest, and the pDEST™R4-R3 destination vector to generate an expression construct. For more information about pDEST™R4-R3 and how to generate an *att*R2 and *att*L3-flanked entry clone, refer to the MultiSite Gateway® Three Fragment Vector Construction Kit manual.

General guidelines to perform the MultiSite Gateway® LR recombination reaction are provided in this section. For detailed instructions, see the appropriate manual as recommended above.

Important

To perform a two-fragment or three-fragment MultiSite Gateway® LR recombination reaction, you **must** use the exact combination of entry clone and destination vector substrates listed above. Note that Gateway® destination vectors other than the ones listed above cannot be used.

For optimal results, we recommend performing the MultiSite Gateway® LR recombination reaction using:

- Supercoiled entry clones
- Supercoiled destination vector

LR Clonase™ II Plus Enzyme Mix

Use LR Clonase™ II Plus enzyme mix available from Invitrogen to catalyze the MultiSite Gateway® LR recombination reaction. Note that the LR Clonase™ enzyme mix used for standard Gateway® LR recombination reactions **cannot** be used for MultiSite Gateway® LR recombination reactions. See page x for ordering information.

E. coli Host

Once you have performed the MultiSite Gateway® LR recombination reaction, you will transform the reaction mixture into competent *E. coli* and select for expression clones. For pLenti6/R4R2/V5-DEST expression clones, use Stbl3™ *E. coli* for transformation. For pDEST™R4-R3 expression clones, use any *rec*A, *end*A *E. coli* strain including TOP10, Mach1™-T1^{R}, DH5α™, or equivalent for transformation. Do not transform the 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.

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 19-20) in parallel with your samples.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	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.
	Excess (or overly diluted) PCR product used in the TOPO® Cloning reaction	Reduce (or concentrate) the amount of PCR product.
	PCR primers contain 5′ phosphates	Do not add 5′ phosphates to your PCR primers.
	Used a proofreading polymerase or a *Taq*/proofreading polymerase mixture for PCR	• Use *Taq* polymerase or another DNA polymerase that leaves 3′ A-overhangs to produce your PCR product. • Add 3′ A-overhangs to your blunt PCR product by incubating with *Taq* polymerase (see page 23).
	Large PCR product	• Increase the amount of PCR product used in the TOPO® Cloning reaction. • Increase the incubation time of the TOPO® Cloning 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 band on an agarose gel)	• Optimize your PCR conditions. • 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	PCR product does not contain sufficient 3′ A-overhangs even though you used *Taq* polymerase	• Increase the final extension time to ensure that all 3′ ends are adenylated. • *Taq* polymerase is most efficient at adding a non-template 3′ A next to a C, and less efficient at adding a nontemplate 3′ A next to another A. You may have to re-design your primers so that they contain a 5′ G instead of a 5′ T (Brownstein *et al.*, 1996).
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 conditions. • Include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
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 containing the *lac* promoter and the LacZα fragment using the reagents included in the kit. Successful TOPO® Cloning of the control PCR product in either direction will yield blue colonies on LB agar plates containing kanamycin and X-gal.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin and X-gal (see page 26 for recipes).

Producing the Control PCR Product

Use the procedure below to produce the 500 bp control PCR product using *Taq* polymerase.

1. In a 0.5 ml microcentrifuge tube, set up the following 50 µl PCR:

Reagent	Amount
Control DNA Template (50 ng)	1 µl
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/µl each)	1 µl
Sterile water	41.5 µl
Taq polymerase (1 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	60°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. A discrete 500 bp band should be visible. 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™5′-TOPO® vector, set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Sterile Water	4 µl	3 µl
Salt Solution	1 µl	1 µl
Control PCR Product	--	1 µl
pENTR™5′-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 procedure on page 13.
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin and X-gal. When plating small volumes, add 20 µl of S.O.C. Medium to ensure even spreading. Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies.
5. Incubate overnight at 37°C.

What You Should See

The "vector + PCR insert" reaction should be produce hundreds of colonies. Greater than 85% of these will be blue.

The "vector only" reaction should yield very few colonies (< 15% of the vector + PCR insert plate) and these should be white.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® TOP10 competent cells. Transform one vial of One Shot® TOP10 cells with 10 pg of pUC19 using the protocol on page 13. 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 PCR Products**, page 8).

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 11.

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 (see page 11). 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 11.
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 13.

Note

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

Addition of 3′ A-Overhangs Post-Amplification

Introduction

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

Before Starting

You will need the following items:

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

Procedure

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

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

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

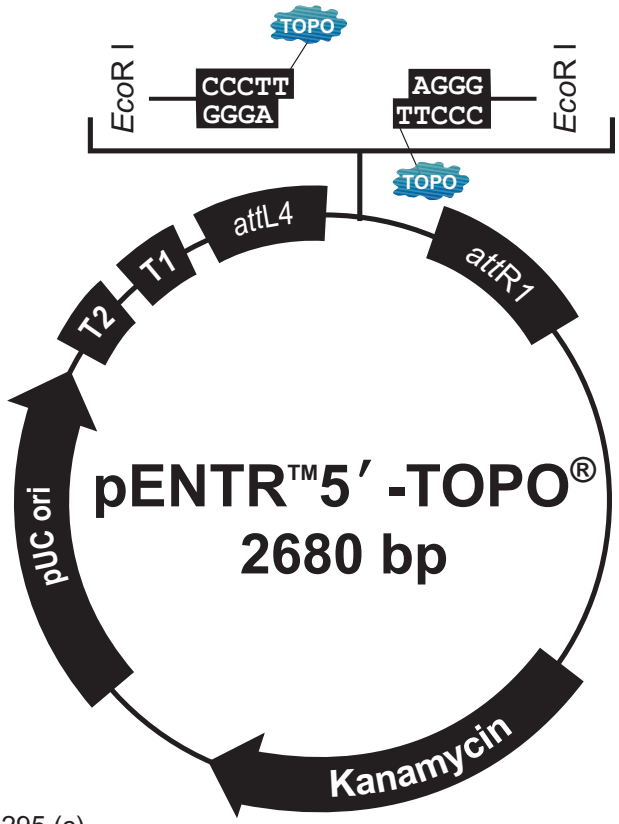
Note

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

Map and Features of pENTR™5′-TOPO®

pENTR™5′-TOPO® Map

The figure below shows the features of the pENTR™5′-TOPO® vector. Note that the pENTR™5′-TOPO® vector is supplied linearized between nucleotides 705 and 706. **The complete sequence of pENTR™5′-TOPO® is available for downloading from www.invitrogen.com or by contacting Technical Support (see page 27).**



Comments for pENTR™5′-TOPO®
2680 nucleotides

*rrn*B T2 transcription terminator: bases 268-295 (c)
*rrn*B T1 transcription terminator: bases 427-470 (c)
M13 forward (-20) priming site: bases 537-552
*att*L4: bases 592-691
GW1 priming site: bases 630-654
TOPO® recognition site 1: bases 701-705
TOPO® recognition site 2: bases 706-710
*att*R1: bases 721-845
GW3 priming site: bases 752-781
M13 reverse priming site: bases 945-961
Kanamycin resistance gene: 1074-1883
pUC origin: bases 2004-2677

(c) = complementary strand

continued on next page

Map and Features of pENTR™5′-TOPO®, continued

Features of pENTR™5′-TOPO®

pENTR™5′-TOPO® (2680 bp) contains the following elements. All features have been functionally tested and the vector fully sequenced.

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.
GW1 priming site	Allows sequencing of the insert.
*att*L4 and *att*R1 sites	Bacteriophage λ-derived recombination sequences that have been optimized to allow recombinational cloning of a DNA fragment in the entry construct with a suitable MultiSite Gateway® destination vector in conjunction with any *att*L1 and *att*L2-flanked entry clone (Landy, 1989).
TOPO® Cloning site	Allows rapid cloning of your *Taq*-amplified PCR product.
GW3 priming site	Allows 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.
5. To add X-gal to the plate, warm the plate to 37°C. Pipette 40 µl of the 40 mg/ml X-gal stock solution (see below), spread evenly, and let dry for 15 minutes. Protect plates from light.

X-Gal Stock Solution

1. Dissolve 400 mg of X-gal in 10 ml dimethylformamide to prepare a 40 mg/ml stock solution.
2. Store at -20°C, protected from light.

Technical Support

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 support 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 website (www.invitrogen.com).

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MSDS

MSDSs (Material Safety Data Sheets) are available on our Web site at www.invitrogen.com/msds.

Certificate of Analysis

Product qualification is described in the Certificate of Analysis (CofA), available on our Web site by product lot number at www.invitrogen.com/cofa.

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Purchaser Notification

Introduction

Use of the pENTR™5′-TOPO® TA Cloning® Kit is covered under the licenses detailed below.

Limited Use Label License No. 5: Invitrogen Technology

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Purchaser Notification, continued

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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.

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