Invitrogen™
life technologies

Instruction Manual

Gateway™ pENTR™ Vectors

Catalog nos. 11813-011, 11816-014, 11817-012

11818-010, 11816-018

Version A
051602
25-0521

Table of Contents

Important Information

pENTR™ Vectors

This manual is supplied with the following products.

Product	Catalog no.
pENTR™1A Vector	11813-011
pENTR™2B Vector	11816-014
pENTR™3C Vector	11817-012
pENTR™4 Vector	11818-010
pENTR™11 Vector	11819-018

Shipping and Storage

pENTR™ vectors are shipped at room temperature. Upon receipt, store at -20°C. Products are guaranteed for six months from date of shipment when stored properly.

Contents

10 µg pENTR™ vector, lyophilized in TE, pH 8.0.

Quality Control

pENTR™ vectors are qualified by restriction enzyme digestion. pENTR™ vectors are further qualified in a recombination assay using Gateway™ LR Clonase™ enzyme mix. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

Accessory Products

Additional Products

Additional products that may be used with the pENTR™ vectors are available from Invitrogen. Ordering information is provided below.

Item	Quantity	Catalog no.
LR Clonase™ Enzyme Mix	20 reactions	11791-019
Library Efficiency® DB3.1™ Competent Cells	5 x 0.2 ml	11782-018
One Shot® TOP10 Chemically Competent Cells	10 reactions	C4040-10
	20 reactions	C4040-03
One Shot® TOP10 Electrocompetent Cells	10 reactions	C4040-50
	20 reactions	C4040-52
One Shot® MAX Efficiency® DH5α™-T1® Chemically Competent Cells	20 reactions	12297-016
Kanamycin Sulfate	1 g	11815-016

Gateway™ Destination Vectors

A large selection of Gateway™ destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 19).

Methods

Overview

Introduction

The pENTR™ vectors allow restriction cloning of the gene of interest for entry into the Gateway™ system available from Invitrogen. A choice of pENTR™ vectors is available (see table below) for optimal expression of your gene of interest after recombination with the Gateway™ destination vector of choice. For more information on the Gateway™ Technology, see the next page.

Product	Benefit
pENTR™1A Vector	• Three reading frames available • Kozak sequence for efficient initiation of translation in eukaryotic cells • *E. coli* ribosome binding site for efficient initiation of translation in prokaryotic cells **(pENTR™1A and pENTR™3C only)**
pENTR™2B Vector	
pENTR™3C Vector	
pENTR™4 Vector	• Same multiple cloning site as pENTR™1A except that first restriction enzyme site is *Nco* I • Kozak sequence for efficient initiation of translation in eukaryotic cells
pENTR™11 Vector	• Kozak sequence for efficient initiation of translation in eukaryotic cells • Two *E. coli* ribosome binding sites for efficient initiation of translation in prokaryotic cells

continued on next page

Overview, continued

Features of the pENTR™ Vectors

The pENTR™ vectors contain the following elements:

- *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway™ destination vector (for more information, refer to the Gateway™ Technology manual or Landy, 1989)
- Kozak consensus sequence for efficient translation initiation in eukaryotic systems
- Ribosome binding site for efficient translation initiation in prokaryotic systems **(pENTR™1A, pENTR™3C, and pENTR™11 only)**
- The *ccd*B gene located between the two *att*L sites for negative selection
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

The Gateway™ Technology

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

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

For more information about Gateway™, refer to the Gateway™ Technology manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 19).

Using the pENTR™ Vectors

Introduction

This section provides general guidelines for using the pENTR™ vectors. Diagrams are provided on pages 7-11 to help you ligate your gene of interest into the appropriate pENTR™ vector.

Propagating the pENTR™ Vectors

If you wish to propagate and maintain the pENTR™ vectors, we recommend using Library Efficiency® DB3.1™ Competent Cells (Catalog no. 11782-018) from Invitrogen for transformation. The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

Note: DO NOT use general *E. coli* cloning strains including TOP10 or DH5α for propagation and maintenance as these strains are sensitive to CcdB effects.

Resuspension

Before using, resuspend your pENTR™ plasmid DNA in 40 µl of sterile water to a final concentration of 150 ng/µl.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Important

Your gene of interest must replace the *ccd*B gene located between the two *att*L sites. Before cloning your gene of interest into a pENTR™ vector, we recommend that you:

- digest the pENTR™ vector on each side of the *ccd*B gene
- dephosphorylate and gel purify the pENTR™ vector

This will minimize the competition between the *ccd*B fragment and your gene of interest during the ligation process.

For more guidelines to help you develop your cloning strategy, see **Cloning Considerations** on page 5.

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Using the pENTR™ Vectors, continued

Kozak Sequence for Mammalian Expression

If you will be recombining your entry clone with a destination vector for mammalian expression, your insert should contain a Kozak consensus 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 provided below. 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 shown underlined.

(G/A)NNATG**G**

Note: Cloning a blunt-ended fragment containing a 5′ ATGG (where ATG is the initiation codon) into the *Xmn* I site of any of the pENTR™ vectors will constitute a Kozak consensus sequence (see diagrams on pages 7-11).

Ribosome Binding Site for Prokaryotic Expression

If you will be recombining your entry clone with a destination vector for prokaryotic expression, your insert should contain an *E. coli* ribosome binding site [AAGGA(A/G)] approximately 9-10 base pairs upstream of the ATG initiation codon (Gold, 1988; Miller, 1992). This will ensure the optimal spacing for proper translation.

Note: Ribosome binding sites are provided in pENTR™1A, pENTR™3C, and pENTR™11 (see diagrams on pages 7-11). If your insert will not be properly spaced from the vector-encoded ribosome binding site, you will need to include your own ribosome binding site for proper initiation of translation.

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Using the pENTR™ Vectors, continued

Cloning Considerations

Consider the following factors when cloning into the pENTR™ vectors.

If you wish to....	Then your insert...
express your native protein without an N-terminal or C-terminal tag	• should contain a Kozak consensus sequence for mammalian expression or an *E. coli* ribosome binding site for prokaryotic expression (see previous page for more information) • should contain a stop codon if one is not provided in the destination vector
include an N-terminal tag (following recombination of the entry clone with a Gateway™ destination vector)	• does not need a Kozak consensus sequence, *E. coli* ribosome binding site, or an ATG initiation codon (these will be provided by the appropriate destination vector) • should be in frame with the tag after recombination (see diagrams on pages 7-11) • should contain a stop codon if one is not provided in the destination vector
include a C-terminal tag (following recombination of the entry clone with a Gateway™ destination vector)	• should contain a Kozak consensus sequence for mammalian expression or an *E. coli* ribosome binding site for prokaryotic expression (see previous page for more information) • should be in frame with the tag after recombination (see diagrams on pages 7-11) • should not contain a stop codon
include an N-terminal and C-terminal tag (following recombination of the entry clone with a Gateway™ destination vector)	• does not need a Kozak consensus sequence, *E. coli* ribosome binding site, or an ATG initiation codon (these will be provided by the appropriate destination vector) • should be in frame with both the N-terminal and C-terminal tags after recombination (see diagrams on pages 7-11) • should not contain a stop codon

Note

If you wish to include an N-terminal tag following recombination and your insert contains an ATG initiation codon, initiation of translation may also occur at this site. As a result, a small amount of native, untagged protein may be expressed along with your tagged fusion protein.

Cloning PCR Products

If you wish to clone a PCR product made using primers containing restriction enzyme sites, we recommend the following to ensure efficient cloning:

- Inactivate or remove the DNA polymerase (*Taq* DNA polymerase can fill in sticky ends and add bases to blunt ends of PCR products) using phenol extraction or the S.N.A.P.™ MiniPrep Kit available from Invitrogen (Catalog no. K1900-01).
- Remove small DNA fragments such as primers, primer-dimers, and excess dNTP's. Refer to the Gateway™ Technology manual for a purification protocol using PEG/$MgCl_2$ precipitation.

Cloning Blunt PCR Products

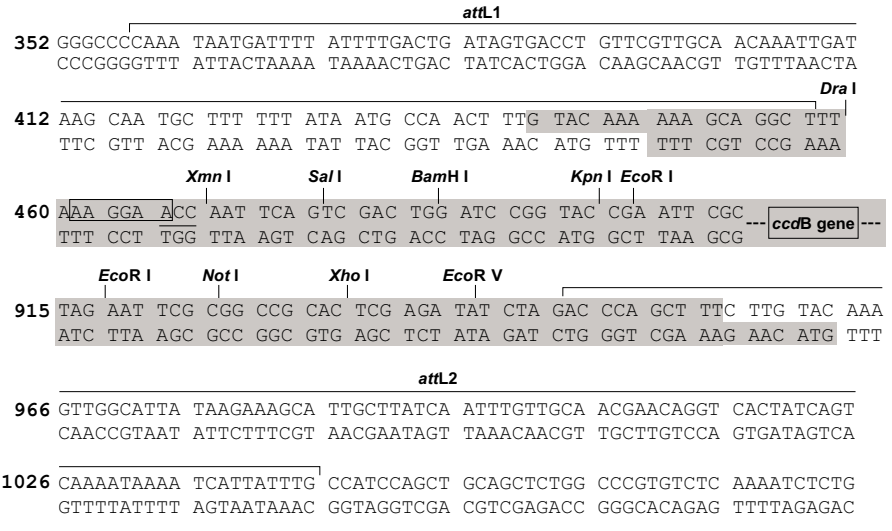
Because primers usually contain a 5′ hydroxy group, PCR products generally do not have 5′ phosphates and are not necessarily blunt. If you wish to clone a blunt PCR product into your entry vector, we recommend you perform the **Blunt Cloning of PCR Products** protocol provided in the Appendix, page 15.

Multiple Cloning Site for pENTR™1A

Multiple Cloning Site

Below is the multiple cloning site for pENTR™1A. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the *ccd*B gene located between the two *att*L sites. Features are indicated as follows:

- The *att*L sites are properly spaced to indicate the correct reading frame for fusion of your gene of interest to an N-terminal or C-terminal tag following recombination with a destination vector.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following recombination.
- Boxed and underlined sequences correspond to the *E. coli* ribosome binding site [AAGGA(A/G)] and the 5′ end of the Kozak consensus sequence (ACC), respectively.

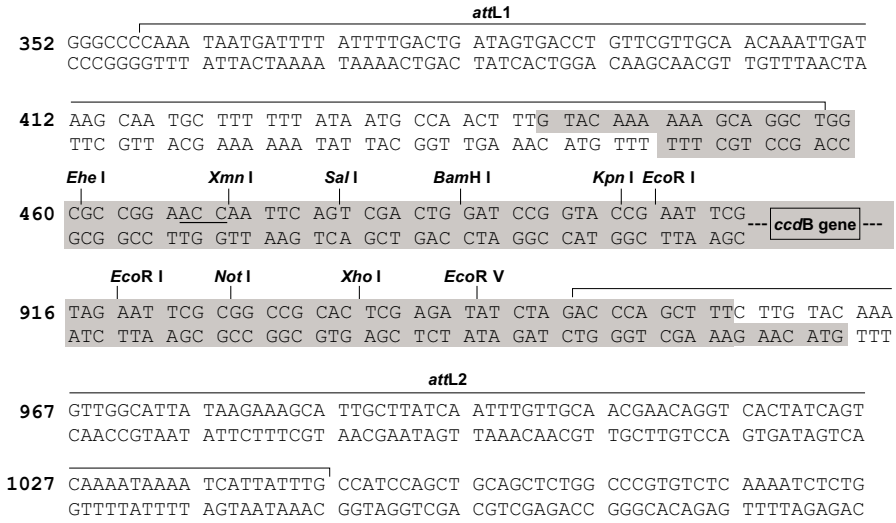


Multiple Cloning Site for pENTR™2B

Multiple Cloning Site

Below is the multiple cloning site for pENTR™2B. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the *ccd*B gene located between the two *att*L sites. Features are indicated as follows:

- The *att*L sites are properly spaced to indicate the correct reading frame for fusion of your gene of interest to an N-terminal or C-terminal tag following recombination with a destination vector.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following recombination.
- Underlined sequence corresponds to the 5′ end of the Kozak consensus sequence (ACC).

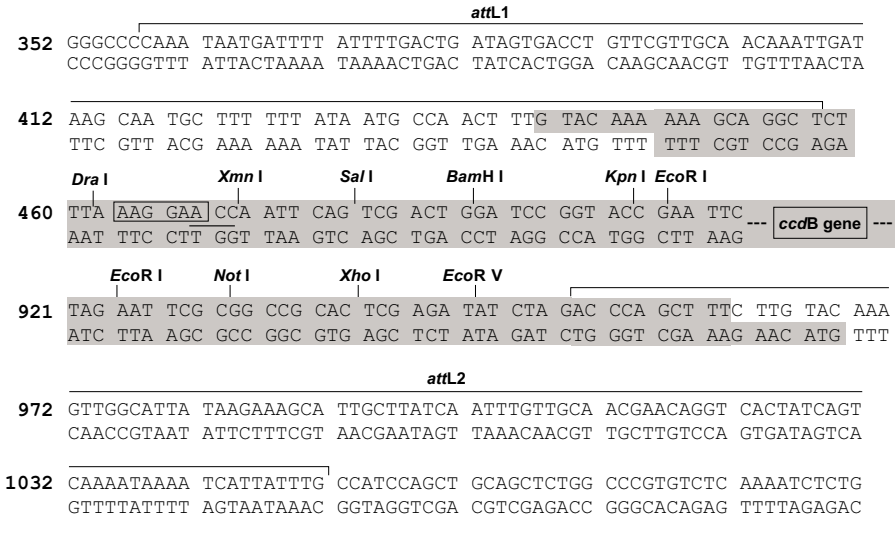


Multiple Cloning Site for pENTR™3C

Multiple Cloning Site

Below is the multiple cloning site for pENTR™3C. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the *ccd*B gene located between the two *att*L sites. Features are indicated as follows:

- The *att*L sites are properly spaced to indicate the correct reading frame for fusion of your gene of interest to an N-terminal or C-terminal tag following recombination with a destination vector.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following recombination.
- Boxed and underlined sequences correspond to the *E. coli* ribosome binding site [AAGGA(A/G)] and the 5′ end of the Kozak consensus sequence (ACC), respectively.

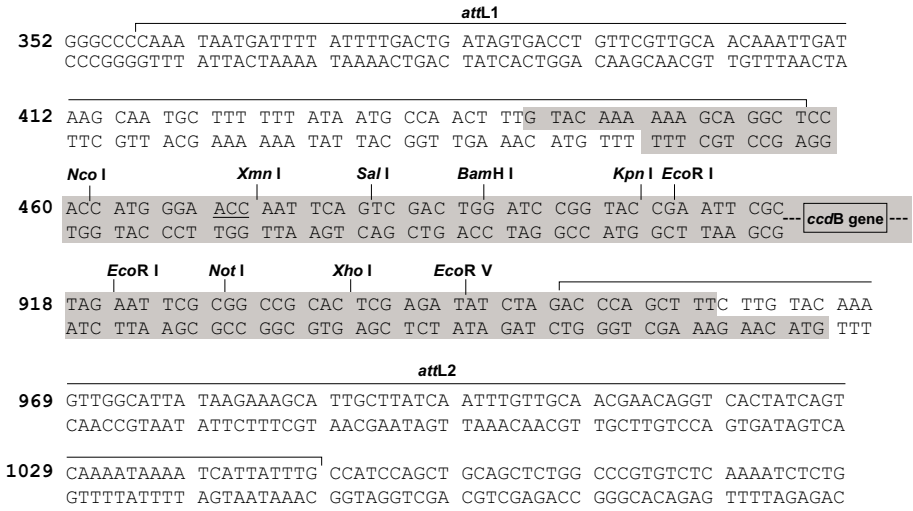


Multiple Cloning Site for pENTR™4

Multiple Cloning Site

Below is the multiple cloning site for pENTR™4. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the *ccd*B gene located between the two *att*L sites. Features are indicated as follows:

- The *att*L sites are properly spaced to indicate the correct reading frame for fusion of your gene of interest to an N-terminal or C-terminal tag following recombination with a destination vector.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following recombination.
- Underlined sequence corresponds to the 5′ end of the Kozak consensus sequence (ACC).

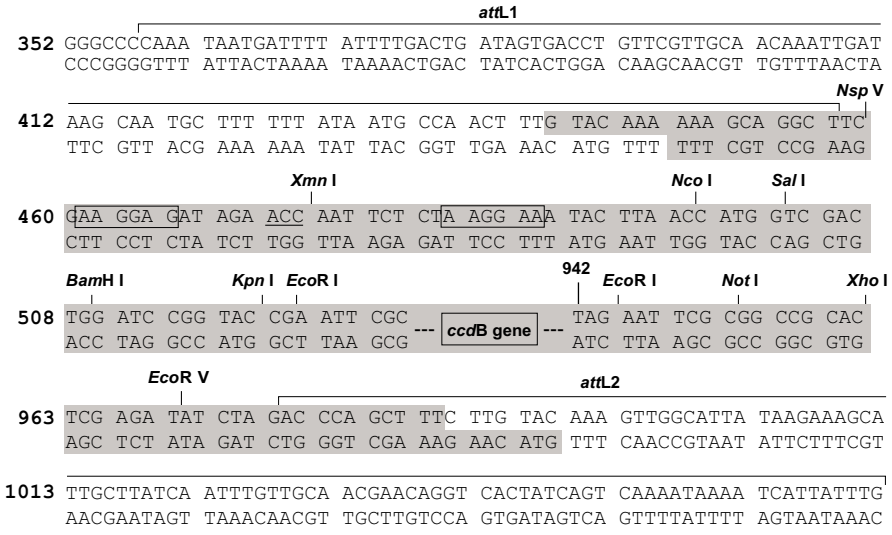


Multiple Cloning Site for pENTR™11

Multiple Cloning Site

Below is the multiple cloning site for pENTR™11. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the *ccd*B gene located between the two *att*L sites. Features are indicated as follows:

- The *att*L sites are properly spaced to indicate the correct reading frame for fusion of your gene of interest to an N-terminal or C-terminal tag following recombination with a destination vector.
- Shaded regions correspond to those DNA sequences transferred from the entry clone into the destination vector following recombination.
- Boxed and underlined sequences correspond to the two available *E. coli* ribosome binding site [AAGGA(A/G)] and the 5′ end of the Kozak consensus sequence (ACC), respectively.



Transforming and Analyzing Entry Clones

Introduction

Once you have restriction cloned your gene of interest into your entry vector, you will transform it into competent *E. coli* and select for positive transformants. See below for general guidelines for transforming and analyzing your entry clones.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

E. coli Transformation

Transform your ligation mixture into a competent *rec*A, *end*A *E. coli* strain (*e.g.* TOP10, DH5α) and select on LB plates containing 50 µg/ml kanamycin. For your convenience, competent TOP10 and DH5α *E. coli* are available from Invitrogen in a One Shot® format (see page vi for ordering information).

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB or SOB medium containing 50 µg/ml kanamycin.
2. Isolate plasmid DNA using your method of choice. We recommend using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the entry clones by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

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Transforming and Analyzing Entry Clones, continued

Analyzing Transformants by PCR

You may also analyze positive transformants using PCR. Use a primer that hybridizes within the pENTR™ vector and one that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, you may want to perform restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed:

PCR SuperMix High Fidelity (Catalog no. 10790-020)

Appropriate forward and reverse PCR primers, 20 µM each

Protocol:

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 colonies and resuspend them individually in 48 µl of the PCR SuperMix (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

Sequencing

You may sequence your entry clone to confirm the presence and orientation of the insert. For your convenience, Invitrogen offers a custom primer synthesis service. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 19).

Guidelines for Performing the LR Recombination Reaction

Introduction

Once you have obtained an entry clone containing your gene of interest, you may perform an LR recombination reaction between the entry clone and a destination vector of choice. General guidelines are provided below. Refer to the Gateway™ Technology manual for detailed information on performing the LR reaction, transforming competent cells, and selecting expression clones.

Important

For most applications, we recommend performing the LR recombination reaction using a:

- Supercoiled *att*L-containing entry clone
- Supercoiled *att*R-containing destination vector

Note: If your destination vector or entry clone is large (>10 kb), you may linearize either vector to increase recombinational efficiency. You may also relax the destination vector using topoisomerase I to increase efficiency. For more details, refer to the Gateway™ Technology manual.

Destination Vectors

A large selection of Gateway™ destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 19).

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, DH10B™or equivalent for transformation. **DO NOT** transform the 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.

Appendix

Blunt Cloning of PCR Products

Introduction

Use this protocol to clone blunt-end PCR products into your pENTR™ vector.

Materials Needed

You should have the following materials on hand before beginning:

- PCR product (~40 ng as judged from an agarose gel)
- 3 M sodium acetate
- 100% ethanol
- 10 mM ATP
- 2 mM dNTP's
- 5X T4 forward reaction buffer (350 mM Tris-HCl, pH 7.6; 50 mM $MgCl_2$; 500 mM KCl; 5 mM 2-mercaptoethanol)
- T4 polynucleotide kinase and buffer (10 units/µl) (Catalog no. 18004-010)
- T4 DNA polymerase (5 units/µl) (Catalog no. 18005-017)
- 30% PEG 8000/30 mM $MgCl_2$
- T4 DNA ligase and buffer (1 unit/µl) (Catalog no. 15224-017)
- Entry vector (blunt, dephosphorylated, ~50 ng)

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Blunt Cloning of PCR Products, continued

Protocol

1. In a 0.5 ml tube, precipitate approximately 40 ng of PCR product by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
2. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
3. Add the following reagents to the DNA:

Distilled H_2O	4 µl
10 mM ATP	1 µl
2 mM of each dNTP (*i.e.* dATP, dCTP, dTTP, dGTP)	1 µl
5X T4 Forward Reaction Buffer	2 µl
T4 polynucleotide kinase	1 µl
T4 DNA polymerase	1 µl
Total Volume	10 µl

4. Incubate at 37°C for 10 minutes, then at 65°C for 15 minutes. Cool on ice for 5 minutes. Centrifuge briefly to bring any condensate to the bottom of the tube.
5. Add 5 µl of 30% PEG 8000/30 mM $MgCl_2$. Mix and centrifuge immediately at room temperature for 10 minutes.
6. Carefully remove and discard supernatant.
7. Dissolve the invisible pellet in a 10 µl cocktail containing:
   - 2 µl of 5X T4 DNA ligase buffer
   - 0.5 units T4 DNA ligase
   - ~ 50 ng of blunt, dephosphorylated entry vector
   - Sterile water up to 10 µl
8. Incubate at 25°C for 1 hour, then at 65°C for 10 minutes. Add 40 µl TE.
9. Transform competent *E. coli* using your method of choice.

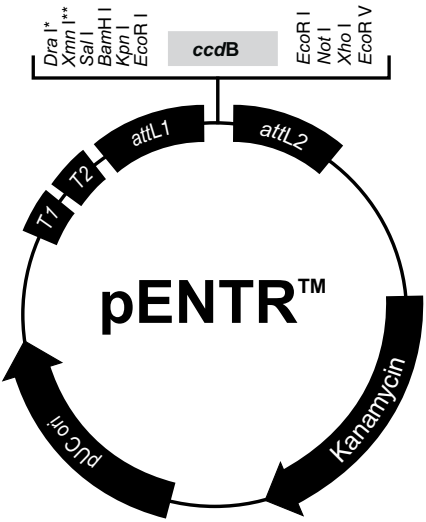
Transformation

Refer to the Gateway™ Technology manual for instructions on transforming the appropriate competent *E. coli* host. Make sure to digest isolated DNA from positive clones with the appropriate restriction enzymes to determine the orientation of the PCR fragment.

Map and Features of the pENTR™ Vectors

Map of the pENTR™ Vectors

The map below shows the elements of the pENTR™ vectors. **Maps and complete sequences for each pENTR™ vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 19).**



Comments for pENTR™1A
2717 nucleotides

*rrn*B T1 transcription termination sequence: bases 106-149
*rrn*B T2 transcription termination sequence: bases 281-308
*att*L1: bases 358-457 (complementary strand)
*ccd*B gene: bases 612-917
*att*L2: bases 946-1045
Kanamycin resistance gene: bases 1168-1977
pUC origin: bases 2041-2714

* There is a unique *Ehe* I site but no *Dra* I site in pENTR™2B.
There is a unique *Nco* I site but no *Dra* I site in pENTR™4.
There is a unique *Nsp* V site but no *Dra* I site in pENTR™11.

** There is a unique *Nco* I site between the *Xmn* I site and the *Sal* I site in pENTR™11 only.

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Map and Features of the pENTR™ Vectors, continued

Features of the pENTR™ Vectors

pENTR™1A (2717 bp), pENTR™2B (2718 bp), pENTR™3C (2723 bp), pENTR™4 (2720 bp), and pENTR™11 (2744 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription termination sequences	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz *et al.*, 1991)
*att*L1 and *att*L2 sites	Allows site-specific recombination of the entry clone with a Gateway™ destination vector (Landy, 1989)
*ccd*B gene	Allows negative selection of expression clones
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*
pUC origin	Allows high-copy number replication and growth in *E. coli*

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

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

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Technical Service, continued

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To request an MSDS, visit our Web site (www.invitrogen.com).

1. On the home page, go to the left-hand column under 'Technical Resources' and select 'MSDS Requests'.
2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

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