Gateway® pENTR™ Dual Selection Vectors

Catalog nos. A10462, A10463, A10464, A10465, A10467

Version A
6 Jun 2008
A10609

A Limited Label License covers this product (see Purchaser Notification). By use of this product, you accept the terms and conditions of the Limited Label License.
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Important Information

**pENTR™ Dual Selection Vectors**

This manual is supplied with the following products.

<table>
<thead>
<tr>
<th>Product</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR™1A Dual Selection Vector</td>
<td>A10462</td>
</tr>
<tr>
<td>pENTR™2B Dual Selection Vector</td>
<td>A10463</td>
</tr>
<tr>
<td>pENTR™3C Dual Selection Vector</td>
<td>A10464</td>
</tr>
<tr>
<td>pENTR™4 Dual Selection Vector</td>
<td>A10465</td>
</tr>
<tr>
<td>pENTR™11 Dual Selection Vector</td>
<td>A10467</td>
</tr>
</tbody>
</table>

**Shipping and Storage**

pENTR™ Dual Selection Vectors are supplied in TE buffer and shipped on wet ice. Upon receipt, store at -20°C. Products are guaranteed for six months from date of shipment when properly stored.

**Contents**

20 µl of pENTR™ Dual Selection Vector at 0.5 µg/µl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

**Product Qualification**

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to [www.invitrogen.com/support](http://www.invitrogen.com/support) and search for the Certificate of Analysis by product lot number, which is printed on the box.
## Accessory Products

### Additional Products

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR Clonase™ II Enzyme Mix</td>
<td>20 reactions</td>
<td>11791-020</td>
</tr>
<tr>
<td></td>
<td>100 reactions</td>
<td>11791-100</td>
</tr>
<tr>
<td>One Shot® ccdB Survival™ 2 T1&lt;sup&gt;R&lt;/sup&gt; Chemically Competent Cells</td>
<td>10 reactions</td>
<td>A10460</td>
</tr>
<tr>
<td>One Shot® TOP10 Chemically Competent Cells</td>
<td>10 reactions</td>
<td>C4040-10</td>
</tr>
<tr>
<td></td>
<td>20 reactions</td>
<td>C4040-03</td>
</tr>
<tr>
<td>One Shot® TOP10 Electrocompetent Cells</td>
<td>10 reactions</td>
<td>C4040-50</td>
</tr>
<tr>
<td></td>
<td>20 reactions</td>
<td>C4040-52</td>
</tr>
<tr>
<td>One Shot® MAX Efficiency® DH5α™-T1&lt;sup&gt;®&lt;/sup&gt; Chemically Competent Cells</td>
<td>20 reactions</td>
<td>12297-016</td>
</tr>
<tr>
<td>Kanamycin Sulfate</td>
<td>5 g</td>
<td>11815-024</td>
</tr>
<tr>
<td>PureLink™ PCR Purification Kit</td>
<td>50 reactions</td>
<td>K3100-01</td>
</tr>
<tr>
<td>PureLink™ HQ Mini Plasmid Purification Kit</td>
<td>100 preps</td>
<td>K2100-01</td>
</tr>
</tbody>
</table>

### 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 the Gateway® Technology Central application portal on our website at [www.invitrogen.com/gateway](http://www.invitrogen.com/gateway) or contact Technical Support (see page 24).

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Methods

Overview

Introduction

The pENTR™ Dual Selection Vectors allow restriction cloning of a gene of interest into a vector for entry into the Gateway® System available from Invitrogen. A choice of pENTR™ Dual Selection 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 about the Gateway® Technology, see the next page.

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

1

continued on next page
Features of the pENTR™ Dual Selection Vectors

The pENTR™ Dual Selection Vectors contain the following elements:

- *rrnB* transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*
- *attL1* and *attL2* sites for site-specific recombination of the entry clone with a Gateway® destination vector (for more information, refer to the Gateway® Technology with Clonase™ II 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*)
- Dual selection cassette containing the Chloramphenicol resistance gene (*Cm*R*) and the ccdB gene located between the two *attL* sites for counterselection
- Kanamycin resistance gene for selection in *E. coli*
- pUC origin behaves as medium to low-copy origin of replication for maintenance of the plasmid in *E. coli*

The Gateway® Technology

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

1. Clone your gene of interest into one of the pENTR™ Dual Selection 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 the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual. You may download the manual from www.invitrogen.com or contact Technical Support (page 24).
Using the pENTR™ Dual Selection Vectors

Introduction

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

Propagating the pENTR™ Dual Selection Vectors

If you wish to propagate and maintain the pENTR™ Dual Selection Vectors, we recommend using One Shot® ccdB Survival™ 2 T1R Chemically Competent Cells (Catalog no. A10460) from Invitrogen for transformation. The ccdB Survival™ 2 T1R E. coli strain is resistant to CcdB effects and can support the propagation of plasmids containing the ccdB 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.

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 dual selection cassette located between the two attL sites. Before cloning your gene of interest into a pENTR™ Dual Selection Vector, we recommend that you:

- Digest the pENTR™ Dual Selection Vector on each side of the dual selection cassette
- Dephosphorylate and gel purify the pENTR™ Dual Selection Vector

This will minimize the competition between the ccdB 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.
Using the pENTR™ Dual Selection 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 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

(G/A)NNATGG

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™ Dual Selection 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.

continued on next page
## Cloning Considerations

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

<table>
<thead>
<tr>
<th>If you wish to...</th>
<th>Then your insert...</th>
</tr>
</thead>
</table>
| 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 |
Cloning PCR Products

If you include an N-terminal tag following recombination with a destination vector, and your insert contains an ATG initiation codon, note that translation initiation may also occur at this site. This may result in a small amount of native, untagged protein being expressed along with your tagged fusion protein.

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 PureLink™ PCR Purification Kit (Catalog no. K3100-01).
- Remove small DNA fragments such as primers, primer-dimers, and excess dNTP’s. Refer to the Gateway® Technology with Clonase™ II manual for a purification protocol using PEG/MgCl₂ 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 16.
Multiple Cloning Site for pENTR™ 1A Dual Selection Vector

Below is the multiple cloning site for pENTR™ 1A Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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.

Below is a table summarizing the restriction sites:

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dra I</td>
<td>GGGCCCAAAC TAAATGATTTT ATTATGACTG ATAGTACAGT GTTCGTCGCA ACCAAATTGAT CCCCAGGTATT ATTACTAAAA TAAAACAGAC TATCAGGGA CAAAGAAGCT TTTTTCAGT</td>
</tr>
<tr>
<td>Xmn I</td>
<td>AAG CAA TGG TTT TTT ATA ATG CCA ACT TTG TAC AAA AAA GCA GGC TTT</td>
</tr>
<tr>
<td>SaI</td>
<td>TTC GTT AGG AAA AGA TAG TGA AAC ATG ATG GTC TAA GCG CCG CCG TAA</td>
</tr>
<tr>
<td>BamHI</td>
<td>ACT GGT AGG ATT TCA GTC GAC TGG ATC CCG TAC CTA ATT CCG GGC GGC ATT</td>
</tr>
<tr>
<td>Kpn I</td>
<td>TTT CTT TGG TTA AGT CAG CTG ACC TAG GCC ATG GCT TAA GCG CCG CCG TAA</td>
</tr>
<tr>
<td>EcoR I</td>
<td>ATTAGCCAGC AGTACGAGT CGG CCG CAC TCG AGA TAT TCA GAC CCA GCT TTC TTG</td>
</tr>
<tr>
<td>Not I</td>
<td>TCC AGC TGT GGG TCC GAA ATG TAG AAT AAC AAG GCC GAG CAT ATT</td>
</tr>
<tr>
<td>Xho I</td>
<td>“dual selection cassette”</td>
</tr>
<tr>
<td>EcoR V</td>
<td>Sal I</td>
</tr>
<tr>
<td>412</td>
<td>AAG CAA TGG TTT TTT ATA ATG CCA ACT TTG TAC AAA AAA GCA GGC TTT</td>
</tr>
<tr>
<td>460</td>
<td>TTC GTT AGG AAA AGA TAG TGA AAC ATG ATG GTC TAA GCG CCG CCG TAA</td>
</tr>
<tr>
<td>511</td>
<td>ACT GGT AGG ATT TCA GTC GAC TGG ATC CCG TAC CTA ATT CCG GGC GGC ATT</td>
</tr>
<tr>
<td>1946</td>
<td>TTT CTT TGG TTA AGT CAG CTG ACC TAG GCC ATG GCT TAA GCG CCG CCG TAA</td>
</tr>
<tr>
<td>1997</td>
<td>ATTAGCCAGC AGTACGAGT CGG CCG CAC TCG AGA TAT TCA GAC CCA GCT TTC TTG</td>
</tr>
<tr>
<td>2048</td>
<td>TCC AGC TGT GGG TCC GAA ATG TAG AAT AAC AAG GCC GAG CAT ATT</td>
</tr>
<tr>
<td>2108</td>
<td>“reverse primer binding site”</td>
</tr>
</tbody>
</table>

**Features are indicated as follows:**

- **dual selection cassette**
- **reverse primer binding site**
- **attL sites**
- **restriction sites**

**Not:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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 Dual Selection Vector

Below is the multiple cloning site for pENTR™ 2B Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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).

```
<table>
<thead>
<tr>
<th>attL1</th>
</tr>
</thead>
<tbody>
<tr>
<td>352 GGGCCCGCAA TAATGATTTT ATTTTGA CAG ATAGTACCT GTTGGTGGCA ACAAATGGAT CCGGGTTTT ATTACTAAAA TAATACCGC TATCACCTGGA CAAGCAACGG TGGTAAACTA</td>
</tr>
<tr>
<td>412 AAG CAA TGC TTT TTT ATA ATG CCA ACT TTA TAC AAA AAA GCA GGC TGG TTC GGT ACG AAA AAA TAT TAC GGT TGA ATT AGT TTT TGG CCG ACC</td>
</tr>
<tr>
<td>460 EheI</td>
</tr>
<tr>
<td>GGC CGG AAG CAA TTC AGT CCA CTC GAT CCG GTA CCG AAT TCG CCG CCG CAA CCG GCC CTC GCT</td>
</tr>
<tr>
<td>511</td>
</tr>
<tr>
<td>1947</td>
</tr>
<tr>
<td>1998</td>
</tr>
<tr>
<td>2049</td>
</tr>
<tr>
<td>2109</td>
</tr>
</tbody>
</table>
| 2B Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:
- The attL 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 Dual Selection Vector

Below is the multiple cloning site for pENTR™ 3C Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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 Dual Selection Vector

Below is the multiple cloning site for pENTR™ 4 Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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

Below is the multiple cloning site for pENTR™ 4 Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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

**attL1**

<table>
<thead>
<tr>
<th>Restriction Site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NcoI</td>
<td>GGGCCCAAAA TAATGATTTT ATTTTGACGT ATAGTGACCT GTTCGTTGCA ACAATTTGAT CCGGGSTTT ATTACAAAA TAAACTGAC TATCAGTGG CAAGCAACGT TGTTTAAC'TA</td>
</tr>
<tr>
<td>XmnI</td>
<td>AAG CA A TGC TTT TTT ATA ATG CCA ACT TGC TAC AAA AAA GCA GGC TCC</td>
</tr>
<tr>
<td>SalI</td>
<td>TGG TAC AAA AAA AAT TAC GGT TGA AAC ATG TTT TTT GTG CCG AGT</td>
</tr>
<tr>
<td>BamHI</td>
<td>TGG TAC CCT TGG GTA AGT CAG CTG ACC TAG GCC ATG GTT TAA GGC CCG GCG</td>
</tr>
<tr>
<td>KpnI</td>
<td>ACC ATG GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC GGA ATT CGC GCC GGC TGG</td>
</tr>
<tr>
<td>EcoRI</td>
<td>GAT GC ATT CAC CCC AGG CTG TAC ACT TTA TGC TTC CGG CTC GTA</td>
</tr>
<tr>
<td>NotI</td>
<td>TAA TCC GTG GGG TCC GAA ATG TGA AAT AGC AAG GCC GAG CAT</td>
</tr>
</tbody>
</table>

**attL2**

<table>
<thead>
<tr>
<th>Restriction Site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SalI</td>
<td>ATT AGG CAC CCC AGG CTG TAC ACT TTA TGC TTC CGG CTC GTA</td>
</tr>
<tr>
<td>EcoRI</td>
<td>TAA TCC GTG GGG TCC GAA ATG TGA AAT AGC AAG GCC GAG CAT</td>
</tr>
<tr>
<td>NotI</td>
<td>ATT AGG CAC CCC AGG CTG TAC ACT TTA TGC TTC CGG CTC GTA</td>
</tr>
<tr>
<td>XhoI</td>
<td>TAA TCC GTG GGG TCC GAA ATG TGA AAT AGC AAG GCC GAG CAT</td>
</tr>
<tr>
<td>EcoRV</td>
<td>TAA TCC GTG GGG TCC GAA ATG TGA AAT AGC AAG GCC GAG CAT</td>
</tr>
</tbody>
</table>

**Reverse Primer Binding Site**

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTCTCAAAAT CTCTGATGTT ACATTTGCACA CAGAGTATTAA CAGACTCAA TGTAACGFTG</td>
</tr>
</tbody>
</table>
Multiple Cloning Site for pENTR™ 11 Dual Selection Vector

Below is the multiple cloning site for pENTR™ 11 Dual Selection Vector. Restriction sites are labeled to indicate the cleavage site. **Note:** Your gene of interest must replace the dual selection cassette located between the two attL sites. Features are indicated as follows:

- The attL 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.

**attL1**

<table>
<thead>
<tr>
<th>restriction site</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nsp V</td>
<td>GGGCCCGAAA TAAATGATTTT ATTTTGTACGT ATAGTGACCCT GTTCGTTGCA ACAAATGAT CCGGGGTTTT ATTACTAAAAA TAAAAGTACG TATCAGTGGA CAAGCAACGT TGTATTTACTA</td>
</tr>
<tr>
<td>Xmn I</td>
<td>AAG CAA TGC TTT TTT ATA ATG CCA ACT TGC TTC GGT ACG AAA AAA GCA GGC TTC TCC GAT ACC GTC GAG</td>
</tr>
<tr>
<td>Nco I</td>
<td>CTT CCT CTA TCT TGG TTA AGA GAT TCC TTT ATG AAT TGG TAC CAG CTG ACC</td>
</tr>
<tr>
<td>Sal I</td>
<td>Kpn I</td>
</tr>
<tr>
<td>BamH I</td>
<td></td>
</tr>
<tr>
<td>dual selection</td>
<td></td>
</tr>
<tr>
<td>cassette</td>
<td></td>
</tr>
</tbody>
</table>

**attL2**

<table>
<thead>
<tr>
<th>restriction site</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoR I</td>
<td>GCG GCC GCG GAT TCG CGG CCT GCA GAC TGG CTG TGT ATA TAG GCC ATG GCT TAA GCA GCT GGA CGT CTG ACC GAC ACA TAT</td>
</tr>
<tr>
<td>Not I</td>
<td>ACG CCG AGT CTA AGG AAT TAC TTA ACC ATG GTC GAC TGG CTT CCT CTA TCT TGG TTA AGA GAT TCC TTT ATG AAT TGG TAC CAG CTG ACC</td>
</tr>
<tr>
<td>Xho I</td>
<td></td>
</tr>
<tr>
<td>EcoR V</td>
<td></td>
</tr>
<tr>
<td>reverse primer binding site</td>
<td>CAGGTCTACTA TCGACTAAA TAAATCAT CTTTGTCATCGA CAAATTCGACG TCTGGGCGGT GTCCAGTGAT AGTCAAGTTT ATTTTAGTAA TTTTAGTAA CAGCT</td>
</tr>
</tbody>
</table>

**reverse primer binding site**

<table>
<thead>
<tr>
<th>restriction site</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoR I</td>
<td>GCTCTAAAAT CTTTGATGATG ACAATTCGACG CAGAGTTTTA GAGACTCAAAT TGTAAACGTT</td>
</tr>
</tbody>
</table>

**reverse primer binding site**
Transforming and Analyzing Entry Clones

Introduction

Once you have restriction cloned your gene of interest into your entry vector, you will transform the ligation reaction into competent *E. coli* and select for positive transformants. See below for general guidelines to transform and analyze 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 recA, endA *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 for culture in LB or SOB medium containing 50 µg/ml kanamycin. In parallel streak the same five clones on LB plates containing 30 µg/ml chloramphenicol. Check growth after overnight culture at 37°C. True positive clones will be kanamycin-resistant (KmR) and chloramphenicol-sensitive (CmS).

2. Isolate plasmid DNA from clones that are KmR and CmS using your method of choice. We recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01).

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.

*continued on next page*
Analyzing Transformants by PCR

You may also analyze positive transformants using PCR. Use a primer that hybridizes within the pENTR™ Dual Selection 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, 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 50 μl of the PCR SuperMix containing primers (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 using the recommended primer (see table below and the diagrams on pages 7-11) to confirm the presence and orientation of the insert. For your convenience, Invitrogen offers a custom primer synthesis service. See www.invitrogen.com/oligos or contact Technical Support (page 24) for more information.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse</td>
<td>5’-GTAACATCAGAGATTTTGAGACAC-3’</td>
</tr>
</tbody>
</table>
Guidelines to Perform 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.

Important

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

- Supercoiled attL-containing entry clone
- Supercoiled attR-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 with Clonase™ II 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 website (www.invitrogen.com) or contact Technical Support (page 24).

E. coli Host

Once you have performed the LR recombination reaction, you will transform the reaction mixture into competent E. coli and select for expression clones. You may use any recA, endA 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 ccdA gene and will prevent negative selection with the ccdB gene.

continued on next page
Performing the LR Reaction

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

- Purified plasmid DNA of your entry clone
- A destination vector of choice
- LR Clonase™ II enzyme mix (Catalog no. 11791-020; see below)
- 2 µg/µl proteinase K solution (supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate chemically competent E. coli host and growth media for expression
- Appropriate selective plates

For instructions to perform the LR recombination reaction, refer to the LR Clonase™ II enzyme mix manual or to the manual for the destination vector you are using.

LR Clonase™ II Enzyme Mix

To catalyze the LR recombination reaction, we recommend using Gateway® LR Clonase™ II enzyme mix. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Reaction Buffer previously supplied by Invitrogen as separate components in LR Clonase™ enzyme mix (Catalog no. 11791-019) into an optimized single-tube format for easier set-up of the LR recombination reaction.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the instructions included with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix as reaction conditions differ.
# Appendices

## Blunt Cloning of PCR Products

### Introduction

Use this protocol to clone blunt-end PCR products into your pENTR™ Dual Selection 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 MgCl₂; 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 MgCl₂
- T4 DNA ligase and buffer (1 unit/µl) (Catalog no. 15224-017)
- Entry vector (blunt, dephosphorylated, ~50 ng)

*continued on next page*
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 H₂O: 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 MgCl₂. 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.

**Transformation**

Refer to the Gateway® Technology with Clonase™ II manual for instructions to transform 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.
Maps and Features of the pENTR™ Dual Selection Vectors

Maps of the pENTR™ Dual Selection Vectors

The following maps show the features of the pENTR™ Dual Selection Vectors. Maps and a complete sequence for each pENTR™ Dual Selection Vector are available for downloading from our website (www.invitrogen.com) or by contacting Technical Support (page 24).

![Diagram of the pENTR™ 1A Vector]

pENTR™ 1A
Dual Selection Vector

3.8 kb

Comments for pENTR™ 1A
3754 nucleotides

*rrnB* T1 transcription termination sequence: bases 106-149
*rrnB* T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
Chloramphenicol resistance gene (*Cm*R): bases 608-1266
*ccdB* gene: bases 1608-1913
attL2: bases 1983-2082
Kanamycin resistance gene: bases 2205-3014
pUC origin: bases 3078-3751

continued on next page
Comments for pENTR™2B
3755 nucleotides

*rrmB* T1 transcription termination sequence: bases 106-149
*rrmB* T2 transcription termination sequence: bases 281-308
*attL1*: bases 358-457 (complementary strand)
Chloramphenicol resistance gene (*Cm*<sup>R</sup>): bases 609-1267
*ccdB* gene: bases 1609-1914
*attL2*: bases 1984-2083
Kanamycin resistance gene: bases 2206-3015
pUC origin: bases 3079-3752

*continued on next page*
Comments for pENTR™3C
3756 nucleotides

rmB T1 transcription termination sequence: bases 106-149
rmB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
Chloramphenicol resistance gene (CmR): bases 610-1268
ccdB gene: bases 1610-1915
attL2: bases 1985-2084
Kanamycin resistance gene: bases 2207-3016
pUC origin: bases 3080-3753

continued on next page
Comments for pENTR™4
3757 nucleotides

*rmb* T1 transcription termination sequence: bases 106-149
*rmb* T2 transcription termination sequence: bases 281-308
*attL1*: bases 358-457 (complementary strand)
Chloramphenicol resistance gene (Cm<sup>R</sup>): bases 611-1269
*ccdB* gene: bases 1611-1916
*attL2*: bases 1986-2085
Kanamycin resistance gene: bases 2208-3017
pUC origin: bases 3081-3754

*continued on next page*
Comments for pENTR™11
3781 nucleotides

rmB T1 transcription termination sequence: bases 106-149
rmB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
ccdB gene: bases 570-872 (complementary strand)
Chloramphenicol resistance gene (CmR): bases 1217-1872 (complementary strand)
attL2: bases 2010-2109
Kanamycin resistance gene: bases 2232-3041
pUC origin: bases 3105-3778

continued on next page
Maps and Features of the pENTR™ Dual Selection Vectors, Continued

### Features of the pENTR™ Dual Selection Vectors

PENTR™1A Dual Selection Vector (3754 bp), pENTR™2B Dual Selection Vector (3755 bp), pENTR™3C Dual Selection Vector (3756 bp), pENTR™4 Dual Selection Vector (3757 bp), and pENTR™11 Dual Selection Vector (3781 bp) contain the following elements. All features have been functionally tested.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rrnB</em> T1 and T2 transcription termination sequences</td>
<td>Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz et al., 1991)</td>
</tr>
<tr>
<td><em>attL1</em> and <em>attL2</em> sites</td>
<td>Allows site-specific recombination of the entry clone with a Gateway® destination vector (Landy, 1989)</td>
</tr>
<tr>
<td>Chloramphenicol resistance gene (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Allows counterselection of expression clones</td>
</tr>
<tr>
<td><em>ccdB</em> gene</td>
<td>Allows negative selection of expression clones</td>
</tr>
<tr>
<td>Kanamycin resistance gene</td>
<td>Allows selection of the plasmid in <em>E. coli</em></td>
</tr>
<tr>
<td>pUC origin</td>
<td>Allows maintenance of the plasmid in <em>E. coli</em></td>
</tr>
</tbody>
</table>
Visit the Invitrogen website 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

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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E-mail: eurotech@invitrogen.com

*continued on next page*
Technical Support, Continued

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Material Safety Data Sheets (MSDSs) are available on our website at [www.invitrogen.com/msds](http://www.invitrogen.com/msds).

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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, page 28.
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 attL1 and attL2 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 attB1 and attB2 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.
References


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Notes