Gateway® Technology

A universal technology to clone DNA sequences for functional analysis and expression in multiple systems

Catalog Numbers 12535-019 and 12535-027

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Design your MultiSite Gateway® Pro experiments with Vector NTI Advance® Software- Go to www.lifetechnologies.com/vectornti for detailed instructions to get started using Vector NTI Advance® sequence analysis software.

For Research Use Only. Not for use in diagnostic procedures.
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# BP and LR Recombination Reaction Protocols for Experienced Users

## Introduction

This quick reference sheet is provided for experienced users of the Gateway® Technology. If you are performing the BP or LR recombination reactions for the first time, we recommend that you follow the detailed protocols provided in the manual.

## BP Recombination Reaction

Perform a BP recombination reaction between an attB-flanked DNA fragment and an attP-containing donor vector to generate an entry clone.

Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:

- attB-PCR product or linearized attB expression clone (40-100 fmol) 1-10 µl
- pDONR™ vector (supercoiled, 150 ng/µl) 2 µl
- 5X BP Clonase™ reaction buffer 4 µl
- TE Buffer, pH 8.0 to 16 µl

Vortex BP Clonase™ enzyme mix briefly. Add 4 µl to the components above and mix well by vortexing briefly twice.

Incubate reaction at 25°C for 1 hour.

Add 2 µl of 2 µg/µl Proteinase K solution and incubate at 37°C for 10 minutes.

Transform competent E. coli and select for the appropriate antibiotic-resistant entry clones.

## LR Recombination Reaction

Perform an LR recombination reaction between an attL-containing entry clone and an attR-containing destination vector to generate an expression clone.

Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:

- Entry clone (supercoiled, 100-300 ng) 1-10 µl
- Destination vector (supercoiled, 150 ng/µl) 2 µl
- 5X LR Clonase™ reaction buffer 4 µl
- TE Buffer, pH 8.0 to 16 µl

Vortex LR Clonase™ enzyme mix briefly. Add 4 µl to the components above and mix well by vortexing briefly twice.

Incubate reaction at 25°C for 1 hour.

Add 2 µl of 2 µg/µl Proteinase K solution and incubate at 37°C for 10 minutes.

Transform competent E. coli and select for the appropriate antibiotic-resistant expression clones.
Kit Contents and Storage

Types of Products
This manual is supplied with the products listed below. For a description of the reagents supplied with the *E. coli*, Baculovirus, and Mammalian Expression Systems and their usage, refer to the individual Expression System manual supplied with each kit.

<table>
<thead>
<tr>
<th>Product</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Cloning System with Gateway® Technology</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>with pDONR™221</em></td>
<td>1 kit</td>
<td>12535-019</td>
</tr>
<tr>
<td><em>with pDONR™/Zeo</em></td>
<td>1 kit</td>
<td>12535-027</td>
</tr>
<tr>
<td><em>E. coli</em> Expression System with Gateway® Technology</td>
<td>1 kit</td>
<td>11824-026</td>
</tr>
<tr>
<td><em>Baculovirus Expression System with Gateway® Technology</em></td>
<td>1 kit</td>
<td>11827-011</td>
</tr>
<tr>
<td><em>Mammalian Expression System with Gateway® Technology</em></td>
<td>1 kit</td>
<td>11826-021</td>
</tr>
</tbody>
</table>

Shipping/Storage
The PCR Cloning System with Gateway® Technology is shipped on dry ice as described below. Upon receipt, store each box as detailed below.

<table>
<thead>
<tr>
<th>Box</th>
<th>Item</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Donor Vector <em>(pDONR™221 or pDONR™/Zeo)</em></td>
<td>Vector: -20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zeocin™ (supplied with pDONR™/Zeo): -20°C, protected from light</td>
</tr>
<tr>
<td>2</td>
<td>BP Clonase™ Enzyme Mix and Reagents</td>
<td>BP Clonase™ Enzyme Mix: -80°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BP Clonase™ Reaction Buffer and all other reagents: -20°C</td>
</tr>
<tr>
<td>3-4</td>
<td>M13 Sequencing Primers</td>
<td>-20°C</td>
</tr>
<tr>
<td>5</td>
<td>Library Efficiency® DH5α™ Chemically Competent <em>E. coli</em></td>
<td>-80°C</td>
</tr>
</tbody>
</table>

continued on next page
Kit Contents and Storage, continued

Contents

The Donor Vector box, the BP Clonase™ Enzyme Mix and Reagents box, and the M13 Sequencing Primers box (Boxes 1-4) contain the following items. Store the BP Clonase™ enzyme mix at -80°C. Store all other components at -20°C. Store Zeocin™ at -20°C, protected from light.

Note: For a description of the reagents supplied with Catalog nos. 11824-026, 11827-011, and 11826-013, refer to the manuals for the E. coli, Baculovirus, and Mammalian Expression System with Gateway® Technology, respectively.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR™ Vector (pDONR™221 or pDONR™/Zeo)</td>
<td>Lyophilized in TE Buffer, pH 8.0</td>
<td>6 µg</td>
</tr>
<tr>
<td>Zeocin™ (supplied with pDONR™/Zeo)</td>
<td>100 mg/ml in deionized, sterile water</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>BP Clonase™ Enzyme Mix</td>
<td>Proprietary</td>
<td>80 µl</td>
</tr>
<tr>
<td>5X BP Clonase™ Reaction Buffer</td>
<td>Proprietary</td>
<td>100 µl</td>
</tr>
<tr>
<td>Proteinase K solution</td>
<td>2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM CaCl2 50% glycerol</td>
<td>40 µl</td>
</tr>
<tr>
<td>30% PEG/Mg solution</td>
<td>30% PEG 8000/30 mM MgCl₂</td>
<td>1 ml</td>
</tr>
<tr>
<td>pEXP7-tet positive control</td>
<td>50 ng/µl in TE Buffer, pH 8.0</td>
<td>20 µl</td>
</tr>
<tr>
<td>M13 Forward (-20) Primer</td>
<td>Lyophilized in TE Buffer, pH 8.0</td>
<td>2 µg</td>
</tr>
<tr>
<td>M13 Reverse Primer</td>
<td>Lyophilized in TE Buffer, pH 8.0</td>
<td>2 µg</td>
</tr>
</tbody>
</table>

Sequence of Primers

The table below lists the sequence of the M13 Sequencing Primers included in the kit.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>pMoles Supplied</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 Forward (-20)</td>
<td>5’-GTAAAAACGACGGCCAG-3’</td>
<td>407</td>
</tr>
<tr>
<td>M13 Reverse</td>
<td>5’-CAGGAAACAGCTATGAC-3’</td>
<td>385</td>
</tr>
</tbody>
</table>

continued on next page
Kit Contents and Storage, continued

DH5α™ Competent E. coli Reagents

The Library Efficiency® DH5α™ Chemically Competent E. coli box (Box 5) includes the following items. Transformation efficiency is 1 x 10⁸ cfu/µg DNA. Store Box 5 at -80°C.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Amount</th>
</tr>
</thead>
</table>
| 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 MgCl₂  
10 mM MgSO₄  
20 mM glucose | 2 x 6 ml |
| Library Efficiency® Chemically Competent DH5α™ | --                                      | 5 x 200 µl   |
| pUC19 Control DNA                         | 10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8 | 50 µl        |

Genotype of DH5α™: F recA1 endA1 hsdR17(rK-, mK+) supE44 λ- thi-1 gyrA96 relA1
**Accessory Products**

### Introduction
The products listed in this section may be used with the PCR Cloning System with Gateway® Technology. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 52).

### Additional Products
Many of the reagents supplied in the PCR Cloning System with Gateway® Technology as well as other products suitable for use with the kit are available separately 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>BP Clonase™ Enzyme Mix</td>
<td>20 reactions</td>
<td>11789-013</td>
</tr>
<tr>
<td></td>
<td>100 reactions</td>
<td>11789-021</td>
</tr>
<tr>
<td>LR Clonase™ Enzyme Mix</td>
<td>20 reactions</td>
<td>11791-019</td>
</tr>
<tr>
<td></td>
<td>100 reactions</td>
<td>11791-043</td>
</tr>
<tr>
<td>Library Efficiency DH5α™ Chemically Competent Cells</td>
<td>5 x 0.2 ml</td>
<td>18263-012</td>
</tr>
<tr>
<td>One Shot® TOP10 Chemically Competent E. coli</td>
<td>20 x 50 µl</td>
<td>C4040-03</td>
</tr>
<tr>
<td>Library Efficiency DB3.1™ Competent Cells</td>
<td>5 x 0.2 ml</td>
<td>11782-018</td>
</tr>
<tr>
<td>pDONR™201</td>
<td>6 µg</td>
<td>11798-014</td>
</tr>
<tr>
<td>pDONR™221</td>
<td>6 µg</td>
<td>12536-017</td>
</tr>
<tr>
<td>pDONR™/Zeo</td>
<td>6 µg</td>
<td>12535-035</td>
</tr>
<tr>
<td>Gateway® Vector Conversion System</td>
<td>20 reactions</td>
<td>11828-019</td>
</tr>
<tr>
<td>S.N.A.P.™ MiniPrep Kit</td>
<td>100 reactions</td>
<td>K1900-01</td>
</tr>
<tr>
<td>S.N.A.P.™ MidiPrep Kit</td>
<td>20 reactions</td>
<td>K1910-01</td>
</tr>
<tr>
<td>S.N.A.P.™ Gel Purification Kit</td>
<td>25 reactions</td>
<td>K1999-25</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>20 ml (10 mg/ml)</td>
<td>11593-019</td>
</tr>
<tr>
<td>Kanamycin Sulfate</td>
<td>100 ml (10 mg/ml)</td>
<td>15160-054</td>
</tr>
<tr>
<td>Zeocin™</td>
<td>1 g</td>
<td>R250-01</td>
</tr>
<tr>
<td></td>
<td>5 g</td>
<td>R250-05</td>
</tr>
<tr>
<td>Platinum® Pfx DNA Polymerase</td>
<td>100 reactions</td>
<td>11708-013</td>
</tr>
<tr>
<td></td>
<td>250 reactions</td>
<td>11708-021</td>
</tr>
<tr>
<td>Platinum® Taq DNA Polymerase High Fidelity</td>
<td>100 reactions</td>
<td>11304-011</td>
</tr>
<tr>
<td></td>
<td>500 reactions</td>
<td>11304-029</td>
</tr>
<tr>
<td>Dpn I</td>
<td>100 units</td>
<td>15242-019</td>
</tr>
<tr>
<td>REaCT® 4 Buffer</td>
<td>2 x 1 ml</td>
<td>16304-016</td>
</tr>
</tbody>
</table>

*continued on next page*
Accessory Products, continued

Gateway® Entry Vectors

A variety of Gateway® entry vectors are available from Invitrogen to facilitate creation of entry clones. For rapid TOPO® Cloning of PCR products, we recommend using the pENTR/D-TOPO® or pENTR/SD/D-TOPO® Cloning Kits. For traditional restriction enzyme digestion and ligase-mediated cloning, use one of the other pENTR™ vectors. For more information about the features of the entry vectors, see our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR/D-TOPO® Cloning Kit</td>
<td>20 reactions</td>
<td>K2400-20</td>
</tr>
<tr>
<td></td>
<td>480 reactions</td>
<td>K2400-480</td>
</tr>
<tr>
<td></td>
<td>500 reaction</td>
<td>K2400-500</td>
</tr>
<tr>
<td>pENTR/SD/D-TOPO® Cloning Kit</td>
<td>20 reactions</td>
<td>K2420-20</td>
</tr>
<tr>
<td></td>
<td>480 reactions</td>
<td>K2420-480</td>
</tr>
<tr>
<td></td>
<td>500 reactions</td>
<td>K2420-500</td>
</tr>
<tr>
<td>pENTR™1A</td>
<td>10 µg</td>
<td>11813-011</td>
</tr>
<tr>
<td>pENTR™2B</td>
<td>10 µg</td>
<td>11816-014</td>
</tr>
<tr>
<td>pENTR™3C</td>
<td>10 µg</td>
<td>11817-012</td>
</tr>
<tr>
<td>pENTR™4</td>
<td>10 µg</td>
<td>11818-010</td>
</tr>
<tr>
<td>pENTR™11</td>
<td>10 µg</td>
<td>11819-018</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 and their features, see our Web site (www.invitrogen.com) or contact Technical Service (see page 52).
Introduction

Overview

Introduction

The Gateway® Technology is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). The Gateway® Technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression (Hartley et al., 2000) (see diagram below).

Advantages of the Gateway® Technology

Using the Gateway® Technology provides the following advantages:

- Enables rapid and highly efficient transfer of DNA sequences into multiple vector systems for protein expression and functional analysis while maintaining orientation and reading frame
- Permits use and expression from multiple types of DNA sequences (e.g. PCR products, cDNA clones, restriction fragments)
- Easily accommodates the transfer of a large number of DNA sequences into multiple destination vectors
- Suitable for adaptation to high-throughput (HTP) formats
- Allows easy conversion of your favorite vector into a Gateway® destination vector

continued on next page
Overview, continued

**Purpose of This Manual**

This manual provides an overview of the Gateway® Technology and provides instructions and guidelines to:

- Design attB PCR primers and amplify your sequence of interest.
- Perform a BP recombination reaction with your attB-PCR product and a donor vector to generate an entry clone.
- Perform an LR recombination reaction with your entry clone and a Gateway® destination vector of choice to generate an expression clone which may then be used in the appropriate application or expression system.
- Convert your own vector to a destination vector.

For details about a particular Invitrogen destination vector or expression system, refer to the manual for the specific destination vector or system. All Gateway® product manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).

**Glossary of Terms**

To help you understand the terminology used in the Gateway® Technology, a glossary of terms is provided in the Appendix, page 57.
## The Gateway® Technology

### The Basis of Gateway®

The Gateway® Technology is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). In the Gateway® Technology, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman *et al.*, 1985). This section provides a brief overview of lambda recombination and the reactions that constitute the Gateway® Technology.

### Recombination Components

Lambda-based recombination involves two major components:

- The DNA recombination sequences (*att* sites)
- The proteins that mediate the recombination reaction (*i.e.* Clonase™ enzyme mix)

These components are discussed below.

### Characteristics of the Recombination Reactions

Lambda integration into the *E. coli* chromosome occurs via intermolecular DNA recombination that is mediated by a mixture of lambda and *E. coli*-encoded recombination proteins (*i.e.* Clonase™ enzyme mix). The hallmarks of lambda recombination are listed below.

- Recombination occurs between specific attachment (*att*) sites on the interacting DNA molecules.
- Recombination is conservative (*i.e.* there is no net gain or loss of nucleotides) and requires no DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector. For example, *att*L sites are comprised of sequences from *att*B and *att*P sites.
- Strand exchange occurs within a core region that is common to all *att* sites (see below).
- The recombination can occur between DNAs of any topology (*i.e.* supercoiled, linear, or relaxed), although efficiency varies.

For more detailed information about lambda recombination, see published references and reviews (Landy, 1989; Ptashne, 1992).

### *att* Sites

Lambda recombination occurs between site-specific attachment (*att*) sites: *att*B on the *E. coli* chromosome and *att*P on the lambda chromosome. The *att* sites serve as the binding site for recombination proteins and have been well-characterized (Weisberg and Landy, 1983). Upon lambda integration, recombination occurs between *att*B and *att*P sites to give rise to *att*L and *att*R sites. The actual crossover occurs between homologous 15 bp core regions on the two sites, but surrounding sequences are required as they contain the binding sites for the recombination proteins (Landy, 1989).

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Recombination Proteins

Lambda recombination is catalyzed by a mixture of enzymes that bind to specific sequences (att sites), bring together the target sites, cleave them, and covalently attach the DNA. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel form. The recombination proteins involved in the reaction differ depending upon whether lambda utilizes the lytic or lysogenic pathway (see table below).

The lysogenic pathway is catalyzed by the bacteriophage λ. Integrase (Int) and E. coli Integration Host Factor (IHF) proteins (BP Clonase™ enzyme mix) while the lytic pathway is catalyzed by the bacteriophage λ. Int and Excisionase (Xis) proteins, and the E. coli Integration Host Factor (IHF) protein (LR Clonase™ enzyme mix). For more information about the recombination enzymes, see published references and reviews (Landy, 1989; Ptashne, 1992).

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Reaction</th>
<th>Catalyzed by…</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysogenic</td>
<td>attB x attP → attL x attR</td>
<td>BP Clonase™ (Int, IHF)</td>
</tr>
<tr>
<td>Lytic</td>
<td>attL x attR → attB x attP</td>
<td>LR Clonase™ (Int, Xis, IHF)</td>
</tr>
</tbody>
</table>

Gateway® Recombination Reactions

The Gateway® Technology uses the lambda recombination system to facilitate transfer of heterologous DNA sequences (flanked by modified att sites) between vectors (Hartley et al., 2000). Two recombination reactions constitute the basis of the Gateway® Technology:

BP Reaction: Facilitates recombination of an attB substrate (attB-PCR product or a linearized attB expression clone) with an attP substrate (donor vector) to create an attL-containing entry clone (see diagram below). This reaction is catalyzed by BP Clonase™ enzyme mix.

LR Reaction: Facilitates recombination of an attL substrate (entry clone) with an attR substrate (destination vector) to create an attB-containing expression clone (see diagram below). This reaction is catalyzed by LR Clonase™ enzyme mix.
Gateway® BP and LR Recombination Reactions

Introduction

The wild-type λ att recombination sites have been modified to improve the efficiency and specificity of the Gateway® BP and LR recombination reactions. This section describes the modifications and provides examples of the Gateway® recombination reactions between the attB x attP and attL x attR sites.

Modifications to the att Sites

In the Gateway® System, the wild-type λ att recombination sites have been modified in the following ways to improve the efficiency and specificity of the Gateway® BP and LR recombination reactions:

- Mutations have been made to the core regions of the att sites to eliminate stop codons and to ensure specificity of the recombination reactions to maintain orientation and reading frame.
- Mutations have been introduced into the short (5 bp) regions flanking the 15-bp core regions of the attB sites to minimize secondary structure formation in single-stranded forms of attB plasmids (e.g. phagemid ssDNA or mRNA).
- A 43 bp portion of the attR site has been removed to make the in vitro attL x attR reaction irreversible and more efficient (Bushman et al., 1985).

In addition to the modifications described above, site-specific point mutations have been made to some att sites to increase recombination efficiency. As a result, sequence variations may exist among the att sites. For example, the pDONR™201 attP1 sequence varies slightly from the pDONR™221 attP1 sequence. These sequence variations do not affect the specificity of the recombination reactions or the functionality of the vectors.

Characteristics of the Modified att Sites

The modified att sites have the following characteristics and specificity. Refer to the diagrams on pages 6 and 7 for more information.

<table>
<thead>
<tr>
<th>Site</th>
<th>Length</th>
<th>Found in…</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB</td>
<td>25 bp</td>
<td>Expression vector</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expression clone</td>
</tr>
<tr>
<td>attP</td>
<td>200 bp</td>
<td>Donor vector</td>
</tr>
<tr>
<td>attL</td>
<td>100 bp</td>
<td>Entry vector</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entry clone</td>
</tr>
<tr>
<td>attR</td>
<td>125 bp</td>
<td>Destination vector</td>
</tr>
</tbody>
</table>

Specificity:

attB1 sites react only with attP1 sites
attB2 sites react only with attP2 sites
attL1 sites react only with attR1 sites
attL2 sites react only with attR2 sites

continued on next page
Example of an attB x attP Recombination Reaction

The diagram below depicts a BP recombination reaction between an attB-PCR product and the pDONR™221 or pDONR™/Zeo vector to create an entry clone and a by-product.

Note: If you are performing a BP recombination reaction using a donor vector other than pDONR™221 or pDONR™/Zeo, note that the sequences of the recombination regions may vary slightly but the mechanism of recombination remains the same.

Features of the Recombination Region:

Shaded regions correspond to those sequences transferred from the attB-PCR product into the entry clone following recombination. Note that the attL sites are composed of sequences from attB and attP.

Boxed regions correspond to those sequences transferred from pDONR™221 or pDONR™/Zeo into the by-product following recombination.
Example of an \textit{attL} x \textit{attR} Recombination Reaction

The diagram below depicts an LR recombination reaction between a pENTR/D-TOPO\textsuperscript{®} entry clone and the pcDNA\textsuperscript{™}6.2/V5-DEST destination vector to create an expression clone and a by-product.

\textbf{Note:} If you are performing an LR recombination reaction using different vectors, note that the sequences of the recombination regions may vary slightly but the mechanism of recombination remains the same.

\textbf{Features of the Recombination Region:}

Shaded regions correspond to those sequences transferred from the pENTR/D-TOPO\textsuperscript{®} entry clone into the expression clone following recombination. Note that the \textit{attB} sites are composed of sequences from \textit{attL} and \textit{attR} sites.

Boxed regions correspond to those sequences transferred from pcDNA\textsuperscript{™}6.2/V5-DEST into the by-product following recombination.
Features of the Gateway® Vectors

Gateway® Vectors

Three different types of Gateway®-adapted vectors are available from Invitrogen:

<table>
<thead>
<tr>
<th>Gateway® Vector</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor vector (pDONR™)</td>
<td>Contains attP sites&lt;br&gt;Used to clone attB-flanked PCR products and genes of interest to generate entry clones</td>
</tr>
<tr>
<td>Entry vector (pENTR™)</td>
<td>Contains attL sites&lt;br&gt;Used to clone PCR products or restriction fragments that do not contain att sites to generate entry clones</td>
</tr>
<tr>
<td>Destination vector</td>
<td>Contains attR sites&lt;br&gt;Recombines with the entry clone in an LR reaction to generate an expression clone&lt;br&gt;Contains elements necessary to express the gene of interest in the appropriate system (i.e. E. coli, mammalian, yeast, insect)</td>
</tr>
</tbody>
</table>

Common Features of the Gateway® Vectors

To enable recombinational cloning and efficient selection of entry or expression clones, most Gateway® vectors contain two att sites flanking a cassette containing:

- The ccdB gene (see below) for negative selection (present in donor, destination, and supercoiled entry vectors)
- Chloramphenicol resistance gene (CmR) for counterselection (present in donor and destination vectors)

After a BP or LR recombination reaction, this cassette is replaced by the gene of interest to generate the entry clone and expression clone, respectively.

ccdB Gene

The presence of the ccdB gene allows negative selection of the donor and destination (and some entry) vectors in E. coli following recombination and transformation. The CcdB protein interferes with E. coli DNA gyrase (Bernard and Couturier, 1992), thereby inhibiting growth of most E. coli strains (e.g. DH5α™, TOP10). When recombination occurs (i.e. between a destination vector and an entry clone or between a donor vector and an attB-PCR product), the ccdB gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the ccdB gene or by-product molecules retaining the ccdB gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Propagating Gateway® Vectors

Because of the lethal effects of the CcdB protein, all Gateway® vectors containing the ccdB gene must be propagated in an E. coli strain that is resistant to CcdB effects. We recommend using the DB3.1™ E. coli strain which contains a gyrase mutation (gyrA462) that renders it resistant to the CcdB effects (Bernard and Couturier, 1992; Bernard et al., 1993; Miki et al., 1992).

Library Efficiency® DB3.1™ Competent Cells are available from Invitrogen (Catalog no. 11782-018) for transformation. See page 18 for the genotype of DB3.1™.
**Gateway® Nomenclature**

**Suggested Naming Convention**

For your convenience, we suggest using the following nomenclature to catalog your Gateway® vectors and clones. Other naming conventions are suitable.

<table>
<thead>
<tr>
<th>Plasmid Type</th>
<th>Description</th>
<th>Individual Vector or Clone Names</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>attL Vector</strong></td>
<td>Entry Vector</td>
<td>pENTR1, 2,...</td>
</tr>
<tr>
<td><strong>attL Subclone</strong></td>
<td>Entry Clone</td>
<td>pENTR3-gus,..; pENTR221-gus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The number 3 refers to the entry vector</td>
</tr>
<tr>
<td></td>
<td></td>
<td>221 refers to the donor vector used to make the entry clone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gus is the subcloned gene</td>
</tr>
<tr>
<td><strong>attR Vector</strong></td>
<td>Destination Vector</td>
<td>pDEST1, 2, 3; p...-DEST</td>
</tr>
<tr>
<td><strong>attB Vector</strong></td>
<td>Expression Vector</td>
<td>pEXP501, 502,...</td>
</tr>
<tr>
<td></td>
<td></td>
<td>This vector is used to prepare expression cDNA libraries</td>
</tr>
<tr>
<td><strong>attB Subclone</strong></td>
<td>Expression Clone</td>
<td>pEXP14-cat,..; pcDNA/GW-47/cat</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 and 47 refers to the destination vector (i.e. pDEST™14 and pcDNA-DEST47™, respectively) used to make the expression clone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cat is the subcloned gene</td>
</tr>
<tr>
<td><strong>attP Vector</strong></td>
<td>Donor Vector</td>
<td>pDONR201, 221,...</td>
</tr>
</tbody>
</table>

**Example:**

**LR Reaction**

pENTR201-tet x pDEST14 → pEXP14-tet

pENTR221-cat x pcDNA-DEST47 → pcDNA/GW-47/cat

**Examples:**

**BP Reaction**

attB-p53 PCR product x pDONR221 → pENTR221-p53

pEXP14-lacZ x pDONR201 → pENTR201-lacZ
Methods

Options to Create Entry Clones

Introduction

To create entry clones containing your gene of interest, you may:

Clone a PCR product or a restriction enzyme fragment into an entry (pENTR™) vector (see the next page for more information). For an alternative, see below.

Generate a PCR product containing attB sites and use this attB-PCR product in a BP recombination reaction with a donor (pDONR™) vector. To use this method, refer to the guidelines and instructions provided in this manual.

Generate or obtain a cDNA library cloned into a Gateway®-compatible vector (i.e. attB-containing pCMV SPORT6 or pEXP-AD502 vectors), and use the cDNA clones in a BP recombination reaction with a donor vector (see the Appendix, page 47 for more information).

If you wish to express a particular human or murine gene, we recommend using an Ultimate™ ORF Human or Mouse Clone available from Invitrogen. Each Ultimate™ hORF or mORF Clone is a fully-sequenced clone provided in a Gateway® entry vector that is ready-to-use in an LR recombination reaction with a Gateway® destination vector. For more information about the Ultimate™ ORF Clones available, refer to our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

continued on next page
Options to Create Entry Clones, continued

Entry Vectors

Many entry vectors are available from Invitrogen to facilitate generation of entry clones. The pENTR/D-TOPO® and pENTR/SD/D-TOPO® vectors allow rapid TOPO® Cloning of PCR products while the pENTR™ vectors allow ligase-mediated cloning of restriction enzyme fragments. All entry vectors include:

- **attL1** and **attL2** sites to allow recombinational cloning of the gene of interest with a destination vector to produce an expression clone.

- A Kozak consensus sequence for efficient translation initiation in eukaryotic cells.

- Some entry vectors include a Shine-Dalgarno sequence (Shine and Dalgarno, 1975) for initiation in *E. coli* (see table below).

- Kanamycin resistance gene for selection of plasmid in *E. coli*.

- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*.

For more information about the features of each pENTR™ vector, see our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

<table>
<thead>
<tr>
<th>Entry Vector</th>
<th>Kozak</th>
<th>Shine-Dalgarno</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR/D-TOPO®</td>
<td>•</td>
<td></td>
<td>K2400-20</td>
</tr>
<tr>
<td>pENTR/SD/D-TOPO®</td>
<td>•</td>
<td>•</td>
<td>K2420-20</td>
</tr>
<tr>
<td>pENTR™1A</td>
<td>•</td>
<td>•</td>
<td>11813-011</td>
</tr>
<tr>
<td>pENTR™2B</td>
<td>•</td>
<td></td>
<td>11816-014</td>
</tr>
<tr>
<td>pENTR™3C</td>
<td>•</td>
<td>•</td>
<td>11817-012</td>
</tr>
<tr>
<td>pENTR™4</td>
<td>•</td>
<td></td>
<td>11818-010</td>
</tr>
<tr>
<td>pENTR™11</td>
<td>•</td>
<td>•</td>
<td>11819-018</td>
</tr>
</tbody>
</table>

Constructing Entry Clones

To construct an entry clone, refer to the manual for the specific entry vector you are using. All entry vector manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).
Designing *attB* PCR Primers

**Introduction**

To generate PCR products suitable for use as substrates in a Gateway® BP recombination reaction with a donor vector, you will need to incorporate *attB* sites into your PCR products. Guidelines are provided below to help you design your PCR primers.

**Designing Your PCR Primers**

The design of the PCR primers to amplify your gene of interest is critical for recombinational cloning using Gateway®. Consider the following when designing your PCR primers:

- **Sequences required to facilitate Gateway® cloning**
- **Sequence required for efficient expression of the native protein** (*i.e.* Shine-Dalgarno or Kozak consensus), if necessary
- **Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal fusion tag**

**Guidelines to Design the Forward PCR Primer**

When designing your forward PCR primer, consider the points below. Refer to the diagram below and **Examples 1 and 2**, next page for more help.

To enable efficient Gateway® cloning, the forward primer **MUST** contain the following structure:

- Four guanine (G) residues at the 5′ end followed by
- The 25 bp *attB*1 site followed by
- At least 18-25 bp of template- or gene-specific sequences

**Note:** If you plan to express native protein in *E. coli* or mammalian cells, you may want to include a Shine-Dalgarno (Shine and Dalgarno, 1975) or Kozak consensus sequence (Kozak, 1987; Kozak, 1991; Kozak, 1990), respectively, in your PCR primer (see **Example 1**, next page).

The *attB*1 site ends with a thymidine (T). If you wish to fuse your PCR product in frame with an N-terminal tag, the primer must include two additional nucleotides to maintain the proper reading frame with the *attB*1 region (see diagram below and **Example 2**, next page). These two nucleotides **cannot** be AA, AG, or GA, because these additions will create a translation termination codon.

*attB1* Forward Primer:

\[
5' - GGGG-ACA-AGT-TT-G-TAC-BAB-BAB-GCA-GGC-TNN---(\text{template-specific sequence})-3' \]

*attB1*

*continued on next page*
Designing attB PCR Primers, continued

Example 1: Forward Primer Design for Native Expression

In this example, we design the following forward attB PCR primer to allow expression of native protein of interest. The attB1 site is indicated in bold and the ATG initiation codon for the protein of interest is underlined. Inclusion of the Shine-Dalgalno and Kozak consensus sequence allows protein expression in both E. coli and mammalian cells.

Note: The ATG initiation codon in this example is in frame with the attB1 sequence, so the PCR product can also be expressed from an N-terminal fusion destination vector.

```
Shine-Dalgalno       Kozak
5' - GGGGCRACAGTTTGTACRARRRGRCAAGCTCAGAGATAGAACATGG(18-25 gene-specific nucleotides) - 3' 
```

Example 2: Forward Primer Design for N-terminal Fusions

In this example, we design the following forward attB PCR primer to allow expression of an N-terminal fusion protein of interest. The attB1 site is indicated in bold. Remember that the gene-specific nucleotides need to be in frame with the attB1 sequence and that no stop codons should be introduced.

Tip: Keep the -AAA-AAA- triplets in the attR1 site in frame with the translation reading frame of the fusion protein.

```
Lys Lys
5' - GGGG ACAR AGT TTG TAR AAA AAA GCR GGC TTC(18-25 gene-specific nucleotides) - 3' 
```

Guidelines to Design the Reverse PCR Primer

When designing your reverse PCR primer, consider the points below. Refer to the diagram below and Examples 1 and 2, next page for more help.

To enable efficient Gateway® cloning, the reverse primer MUST contain the following structure:

- Four guanine (G) residues at the 5' end followed by
- The 25 bp attB2 site followed by
- 18-25 bp of template- or gene-specific sequences

If you wish to fuse your PCR product in frame with a C-terminal tag:

- The primer must include one additional nucleotide to maintain the proper reading frame with the attB2 region (see diagram below and Example 2, next page)
- Any in-frame stop codons between the attB2 site and your gene of interest must be removed

If you do not wish to fuse your PCR product in frame with a C-terminal tag, your gene of interest or the primer must include a stop codon (see Example 1, next page)

```
attB2 Reverse Primer:
5' - GGGG-AC-CAC-TCG-GTA-CAA-GAA-AGC-TGG-GTN--(template-specific sequence) - 3'
```

continued on next page
Designing attB PCR Primers, continued

Example 1: Reverse Primer Design
In this example, we design the following reverse attB PCR primer to allow expression of a protein of interest with no C-terminal fusion tag. The attB2 site is indicated in bold and the stop codon for the protein of interest is underlined. Remember that the gene-specific nucleotides need to be in frame with the stop codon.

5′-GGGAGCCACTTTGTAAGAAGGTGGCTCCTA(18-25 gene-specific nucleotides)-3′

Example 2: Reverse Primer Design for C-terminal Fusions
In this example, we design the following reverse attB PCR primer to allow expression of a C-terminal fusion protein of interest. The attB2 site is indicated in bold. Remember that the gene-specific nucleotides need to be in frame with the attB2 sequence and that stop codons should be removed.

**Tip:** Keep the -TTT-GTA (TAC-AAA on the complementary strand) triplets in the attR2 site in frame with the translation reading frame of the fusion protein.

Lys Tyr
5′-GGGGRACCRCTTTGTRACGAGGAGGTCGTC(18-25 gene-specific nucleotides)-3′

Note
If desired, you may incorporate a protease cleavage sequence into your PCR product to allow removal of N-terminal or C-terminal fusion tags from your recombinant fusion protein. When designing your forward or reverse PCR primer, include this sequence between the gene-specific and the attB sequences of the primer, as appropriate.

Important
50 nmol of standard purity, desalted oligonucleotides is sufficient for most applications.

Dissolve oligonucleotides to 20-50 mM in water or TE Buffer and verify the concentration before use.

For more efficient cloning of large PCR products (greater than 5 kb), we recommend using HPLC or PAGE-purified oligonucleotides.

The Next Step
Proceed to the next section for guidelines to produce your attB-PCR products.
If you are performing high throughput applications or are using long PCR primers (greater than 70 nucleotides) to generate your PCR products, we recommend using the attB adapter protocol provided in the Appendix, pages 44-45.
**Producing attB-PCR Products**

### DNA Templates

The following DNA templates can be used for amplification with attB-containing PCR primers:
- Genomic DNA
- mRNA
- cDNA libraries
- Plasmids containing cloned DNA sequences

### Recommended Polymerases

We recommend using the following DNA polymerases available from Invitrogen to produce your attB-PCR products. Other DNA polymerases are suitable.

To generate PCR products less than 5-6 kb for use in protein expression, use Platinum® Pfx DNA Polymerase (Catalog no. 11708-013)

To generate PCR products for use in other applications (e.g. functional analysis), use Platinum® Taq DNA Polymerase High Fidelity (Catalog no. 11304-011)

### Producing PCR Products

Standard PCR conditions can be used to prepare attB-PCR products. Follow the manufacturer's instructions for the DNA polymerase you are using, and use the cycling parameters suitable for your primers and template.

**Note:** In general, attB sequences do not affect PCR product yield or specificity.

### Checking the PCR Product

Remove 1-2 µl from each PCR reaction and use agarose gel electrophoresis to verify the quality and yield of your PCR product. If the PCR product is of the appropriate quality and quantity, proceed to Purifying attB-PCR Products, next section.

**Note**

If your PCR template is a plasmid that contains the kanamycin resistance gene, we suggest treating your PCR reaction mixture with Dpn I before purifying the attB-PCR product. This treatment degrades the plasmid (i.e. Dpn I recognizes methylated GATC sites) and helps to reduce background in the BP recombination reaction associated with template contamination.

**Materials Needed:**
- 10X REact® 4 Buffer (Invitrogen, Catalog no. 16304-016)
- Dpn I (Invitrogen, Catalog no. 15242-019)

**Protocol:**

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer and ≥5 units of Dpn I.
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the Dpn I at 65°C for 15 minutes.
4. Proceed to Purifying attB-PCR Products, next page.
After you have generated your attB-PCR product, we recommend purifying the PCR product to remove attB primers and any attB primer-dimers. Primers and primer-dimers can recombine efficiently with the donor vector in the BP reaction and may increase background after transformation into E. coli. A protocol is provided below to purify your PCR product.

Standard PCR product purification protocols using phenol/chloroform extraction followed by sodium acetate and ethanol or isopropanol precipitation are not recommended for use in purifying attB-PCR products. These protocols generally have exclusion limits of less than 100 bp and do not efficiently remove large primer-dimer products.

You should have the following materials on hand before beginning:

- attB-PCR product (in a 50 µl volume)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 30% PEG 8000/30 mM MgCl₂ Solution (supplied with the PCR Cloning System with Gateway® Technology)
- Agarose gel of the appropriate percentage to resolve your attB-PCR product

Use the protocol below to purify attB-PCR products. Note that this procedure removes DNA less than 300 bp in size.

Add 150 µl of TE, pH 8.0 to a 50 µl amplification reaction containing your attB-PCR product.

Add 100 µl of 30% PEG 8000/30 mM MgCl₂. Vortex to mix thoroughly and centrifuge immediately at 10,000 x g for 15 minutes at room temperature.

Note: In most cases, centrifugation at 10,000 x g for 15 minutes results in efficient recovery of PCR products. To increase the amount of PCR product recovered, the centrifugation time may be extended or the speed of centrifugation increased.

Carefully remove the supernatant. The pellet will be clear and nearly invisible.

Dissolve the pellet in 50 µl of TE, pH 8.0 (to concentration > 10 ng/µl).

Check the quality and quantity of the recovered attB-PCR product on an agarose gel.

If the PCR product is suitably purified, proceed to Creating Entry Clones Using the BP Recombination Reaction, page 17. If the PCR product is not suitably purified (e.g. attB primer-dimers are still detectable), see below.

If you use the procedure above and your attB-PCR product is not suitably purified, you may gel purify your attB-PCR product. We recommend using the S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25).
Creating Entry Clones Using the BP Recombination Reaction

**Introduction**

The BP recombination reaction facilitates transfer of a gene of interest in an attB expression clone or attB-PCR product to an attP-containing donor vector to create an entry clone. Once you have created an entry clone, your gene of interest may then be easily shuttled into a large selection of destination vectors using the LR recombination reaction. To ensure that you obtain the best possible results, we suggest that you read this section and the ones entitled **Performing the BP Recombination Reaction** (pages 20-22) and **Transforming Competent Cells** (pages 23-25) before beginning.

**Note:** If you wish to go directly from an attB-PCR product or attB expression clone into a destination vector, see the **Appendix**, page 43 for a one-tube protocol.

**Experimental Outline**

To generate an entry clone, you will:

Perform a BP recombination reaction using the appropriate attB and attP-containing substrates (see below)

Transform the reaction mixture into a suitable E. coli host (see page 23)

Select for entry clones

**Substrates for the BP Recombination Reaction**

To perform a BP recombination reaction, you need to have the following substrates:

attB-flanked PCR products or attB-containing expression clones

attP-containing donor (pDONR™) vector (see below)

**Important**

For optimal efficiency, perform the BP recombination reaction using:

Linear attB substrates (see the next page for guidelines to linearize attB expression clones)

Supercoiled attP-containing donor vector

**Note:** Supercoiled or relaxed attB substrates may be used, but will react less efficiently than linear attB substrates.

**Donor Vectors**

The PCR Cloning System with Gateway® Technology includes a choice of donor (pDONR™221 or pDONR™/Zeo) vectors. Other donor vectors are available from Invitrogen (see page x for ordering information). For a map and a description of the features of pDONR™221 and pDONR™/Zeo, see the **Appendix**, pages 50-51.

**Resuspending the Donor Vectors**

All donor vectors are supplied as 6 μg of supercoiled plasmid, lyophilized in TE Buffer, pH 8.0. To use, simply resuspend the pDONR™ plasmid DNA in 40 μl of sterile water to a final concentration of 150 ng/μl. To propagate donor vectors, see the next page.

*continued on next page*
Propagating Donor Vectors

If you wish to propagate and maintain the pDONR™ 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 ccdB gene. To maintain the integrity of the vector, select for transformants in media containing the appropriate antibiotic and 15 µg/ml chloramphenicol. If you are using pDONR™/Zeo, you will need to select transformants in Low Salt LB medium containing Zeocin™ and 15 µg/ml chloramphenicol (see page 23 for more information).

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.

Genotype of DB3.1

F: gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20(rB-, mB-) supE44 ara14 galK2 lacY1 proA2 rpsL20(SmR) xyl5 Δleu mtl1

Linearizing Expression Clones

If you wish to perform a BP recombination reaction using an attB expression clone, we recommend that you linearize the expression clone using a suitable restriction enzyme (see the guidelines below).

Linearize 1 to 2 µg of the expression clone with a unique restriction enzyme that does not digest within the gene of interest and is located outside the attB region.

Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.

Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.

Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50-150 ng/µl.
Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of pDONR™221 and pDONR™/Zeo

The recombination region of the expression clone resulting from pDONR™221 × entry clone or pDONR™/Zeo × entry clone is shown below.

Features of the Recombination Region:

Shaded regions correspond to DNA sequences transferred from the \textit{attB} substrate into pDONR™221 or pDONR™/Zeo by recombination. Non-shaded regions are derived from the pDONR™221 or pDONR™/Zeo vector.

Bases 651 and 2897 of the pDONR™221 or pDONR™/Zeo vector sequence are marked.

M13 Forward (20) priming site

\begin{verbatim}
531  GACGTTGAAA AAGCAGCGCC AGTCTTAACG TCGGCCCCCA AAATAATGTT TTATTTGAC
     AGCCGGGGGT TTATTACAA AATAAAACTG

591  TGATAGTGAC CTGTTGCTTG CAAACACATTG ATGAGCAATG CTTTTTTATA ATG CCA ACT
     ACTATGACTG GACAAGCAAC GGTGCTGAACT TACTCGTTAC GAAAAATAT TAC GGT TGA

\end{verbatim}

\begin{verbatim}
651  |||

650  TTG TAC AAA AAA GCA GGC TNN --- --- NAC CCA GCT TTC TTG TAC AAA
     AAC ATG TTT TTT GGT CCG ANN --- GGN --- NTG GGT CGA AAG AAC ATG TTT

2907  GTC GGC ATT ATAGAAAAGC ATGCTTATTC ATTGTGTTGC AAGAACAGGC TCACTATTACG
       CAA CCG TAA TATTTTTCC TACAGATAAG TAAACACCG TTGCTTGGCC AGTGAATAGC

\end{verbatim}

\begin{verbatim}
2966  TCAAAATTAAA ATCATTTTT GCCATCCAGC TGATATCCCC TATAGTGACT CGTATACAT
     AGTTTTTTTT TACTAATAAA GCGTACGTCG

M13 Reverse priming site

3026  GTCATAGCT GTTTTCTGGC AGCTCTGCCC CGTGCTCTCAA AATCTCTGAT GTTACATTGC
\end{verbatim}
Performing the BP Recombination Reaction

Introduction

General guidelines and instructions are provided below and in the next section to perform a BP recombination reaction using an appropriate attB substrate and a donor vector, and to transform the reaction mixture into a suitable \textit{E. coli} host to select for entry clones. We recommend that you include a positive control (see below) and a negative control (no attB substrate) in your experiment to help you evaluate your results.

Positive Control

pEXP7-tet is provided as a positive control for the BP reaction. pEXP7-tet is an approximately 1.4 kb linear fragment and contains attB sites flanking the tetracycline resistance gene and its promoter (Tc'). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene. The efficiency of the BP recombination reaction can easily be determined by streaking entry clones onto LB plates containing 20 \( \mu \)g/ml tetracycline.

Determining How Much attB DNA and Donor Vector to Use in the Reaction

For optimal efficiency, we recommend using the following amounts of attB-PCR product (or linearized attB expression clone) and donor vector in a 20 \( \mu \)l BP recombination reaction:

- An equimolar amount of attB-PCR product (or linearized attB expression clone) and the donor vector
- 100 femtomoles (fmol) of attB-PCR product (or linearized attB expression clone) and donor vector is preferred, but the amount of attB-PCR product used may range from 40-100 fmol

\textbf{Note:} 100 fmol of donor vector (pDONR™201, pDONR™221, or pDONR™/Zeo) is approximately 300 ng

For large PCR products (>4 kb), use at least 100 fmol of attB-PCR product, but no more than 500 ng

For a formula to convert fmol of DNA to nanograms (ng), see below. For an example, see the next page.

\textbf{Caution}

Do not use more than 500 ng of donor vector in a 20 \( \mu \)l BP reaction as this will affect the efficiency of the reaction.

Do not exceed more than 1 \( \mu \)g of total DNA (donor vector plus attB-PCR product) in a 20 \( \mu \)l BP reaction as excess DNA will inhibit the reaction.

Converting Femtomoles (fmol) to Nanograms (ng)

Use the following formula to convert femtomoles (fmol) of DNA to nanograms (ng) of DNA:

\[ \text{ng} = (\text{fmol})(\frac{660 \text{ fg}}{\text{fmol}})(\frac{1 \text{ ng}}{10^6 \text{ fg}}) \]

where \( N \) is the size of the DNA in bp. For an example, see the next page.

\( \text{continued on next page} \)
Performing the BP Recombination Reaction, continued

Example of fmol to ng Conversion

In this example, you need to use 100 fmol of an attB-PCR product in the BP reaction. The attB-PCR product is 2.5 kb in size. Calculate the amount of attB-PCR product required for the reaction (in ng) by using the equation on the previous page:

\[
(100 \text{ fmol})(2500 \text{ bp})(\frac{660 \text{ fg}}{\text{fmol}})(\frac{1 \text{ ng}}{10^6 \text{ fg}}) = 165 \text{ ng of PCR product required}
\]

Materials Needed

You should have the following materials on hand before beginning:

- attB-PCR product or linearized attB expression clone (see the previous page to determine the amount of DNA to use)
- pDONR™ vector (supplied with the kit; resuspend to 150 ng/µl with water)
- BP Clonase™ enzyme mix (supplied with the PCR Cloning System; keep at -80°C until immediately before use)
- 5X BP Clonase™ Reaction Buffer (supplied with the BP Clonase™ enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the BP Clonase™ enzyme mix; thaw and keep on ice until use)
- pEXP7-tet positive control (50 ng/µl; supplied with the BP Clonase™ enzyme mix)

continued on next page
Performing the BP Recombination Reaction, continued

Setting Up the BP Recombination Reaction

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.
   
   **Note:** To include a negative control, set up a second sample reaction and omit the BP Clonase™ enzyme mix (see Step 4).

<table>
<thead>
<tr>
<th>Components</th>
<th>Sample</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB-PCR product or linearized attB expression clone (40-100 fmol)</td>
<td>1-10 µl</td>
<td>--</td>
</tr>
<tr>
<td>pDONR™ vector (150 ng/µl)</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>pEXP7-tet positive control (50 ng/µl)</td>
<td>--</td>
<td>2 µl</td>
</tr>
<tr>
<td>5X BP Clonase™ Reaction Buffer</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>TE Buffer, pH 8.0</td>
<td>to 16 µl</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

2. Remove the BP Clonase™ enzyme mix from -80°C and thaw on ice (~ 2 minutes).

3. Vortex the BP Clonase™ enzyme mix briefly twice (2 seconds each time).

4. To each sample above, add 4 µl of BP Clonase™ enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).
   
   **Reminder:** Return BP Clonase™ enzyme mix to -80°C immediately after use.

5. Incubate reactions at 25°C for 1 hour.
   
   **Note:** For most applications, a 1 hour incubation will yield a sufficient number of entry clones. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. An overnight incubation typically yields 5-10 times more colonies than a 1 hour incubation. For large PCR products (≥5 kb), longer incubations (*i.e.* overnight incubation) will increase the yield of colonies and are recommended.

6. Add 2 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.

7. Proceed to Transforming Competent Cells, next page.
   
   **Note:** You may store the BP reaction at -20°C for up to 1 week before transformation, if desired.
TransformingCompetent Cells

Introduction
Once you have performed the BP recombination reaction, you will transform competent *E. coli* and select for entry clones using the appropriate antibiotic. If you are using the PCR Cloning System with Gateway® Technology, Library Efficiency® DH5α™ chemically competent *E. coli* are included with the kit for use in transformation, however, you may also transform electrocompetent cells. Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

*E. coli* Host Strain
You may use any recA, endA *E. coli* strain including TOP10, DH5α™, DH10B™ or equivalent for transformation. Other strains are suitable. **Do not** use *E. coli* strains that contain the F’ episome (e.g. TOP10F’) for transformation. These strains contain the ccdA gene and will prevent negative selection with the ccdB gene.

For your convenience, TOP10, DH5α™, and DH10B™ *E. coli* are available as chemically competent or electrocompetent cells from Invitrogen (see table below).

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Library Efficiency® DH5α™</td>
<td>5 x 200 µl</td>
<td>18263-012</td>
</tr>
<tr>
<td>One Shot® TOP10 Chemically Competent <em>E. coli</em></td>
<td>20 x 50 µl</td>
<td>C4040-03</td>
</tr>
<tr>
<td>One Shot® Max Efficiency® DH10B™ T1 Phage Resistant Chemically Competent <em>E. coli</em></td>
<td>20 x 50 µl</td>
<td>12331-013</td>
</tr>
<tr>
<td>One Shot® TOP10 Electrocomp <em>E. coli</em></td>
<td>20 x 50 µl</td>
<td>C4040-52</td>
</tr>
<tr>
<td>ElectroMax™ DH10B™</td>
<td>5 x 100 µl</td>
<td>18290-015</td>
</tr>
</tbody>
</table>

Selection Media
Refer to the table below for the appropriate selection medium to use to select for entry clones. You will need two LB plates containing the appropriate antibiotic for each transformation. Pre-warm plates at 37°C for 30 minutes.

**Important:** If you are using pDONR™ / Zeo, you will need to use Low Salt LB agar for selection (see Note below).

<table>
<thead>
<tr>
<th>Donor Vector</th>
<th>Selection Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR™201</td>
<td>LB + 50 µg/ml kanamycin</td>
</tr>
<tr>
<td>pDONR™221</td>
<td>LB + 50 µg/ml kanamycin</td>
</tr>
<tr>
<td>pDONR™ / Zeo</td>
<td>Low Salt LB + 50 µg/ml Zeocin™</td>
</tr>
</tbody>
</table>

The Zeocin™ resistance gene in pDONR™ / Zeo allows selection of *E. coli* transformants using Zeocin™ antibiotic. For selection, use Low Salt LB agar plates containing 50 µg/ml Zeocin™ (see page 49 for a recipe). Note that for Zeocin™ to be active, the salt concentration of the bacterial medium must remain low (<90mM) and the pH must be 7.5. For more information on storing and handling Zeocin™, refer to page 48.

*continued on next page*
Transforming Competent Cells, continued

Materials Needed

You should have the following materials on hand before beginning:

- BP recombination reaction (from Step 7, page 22)
- Library Efficiency® DH5α™ chemically competent E. coli (supplied with the PCR Cloning System; thaw on ice before use) or another suitable E. coli strain
- S.O.C. medium (supplied with the PCR Cloning System; warm to room temperature)
- Positive control (e.g. pUC19 supplied with the PCR Cloning System; use as a control for transformation if desired)
- LB plates containing the appropriate antibiotic, refer to table on the previous page (two for each transformation; warm at 37°C for 30 minutes)
- 42°C water bath (for chemical transformation)
- 37°C shaking and non-shaking incubator

Note

Library Efficiency® DH5α™ competent cells are supplied in 5 tubes containing 0.2 ml of competent cells each. Each tube contains enough competent cells to perform 4 transformations using 50 µl of cells per transformation. Once you have thawed a tube of competent cells, discard any unused cells. Do not re-freeze cells as repeated freezing/thawing of cells may result in loss of transformation efficiency.

Chemical Transformation Protocol

For each transformation, aliquot 50 µl of Library Efficiency® DH5α™ competent cells into a sterile 1.5 ml microcentrifuge tube.

Add 1 µl of the BP recombination reaction (from Performing the BP Recombination Reaction, Step 7, page 22) into the tube containing 50 µl of Library Efficiency® DH5α™ competent cells and mix gently. Do not mix by pipetting up and down.

Incubate on ice for 30 minutes.

Heat-shock the cells for 30 seconds at 42°C without shaking.

Immediately transfer the tubes to ice.

Add 450 µl of room temperature S.O.C. medium.

Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.

Spread 20 µl and 100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

An efficient BP recombination reaction may produce hundreds of colonies (>1500 colonies if the entire transformation is plated).

continued on next page
Transforming Competent Cells, continued

Verifying pEXP7-tet Entry Clones

If you included the pEXP7-tet control in your BP recombination reaction, you may transform Library Efficiency® DH5α™ competent cells using the protocol on the previous page. The efficiency of the BP reaction may then be assessed by streaking entry clones onto LB agar plates containing 20 µg/ml tetracycline. True entry clones should be tetracycline-resistant.

Transformation by Electroporation

Use only electrocompetent cells for electroporation to avoid arcing. Do not use the Library Efficiency® DH5α™ chemically competent cells for electroporation.

Add 1 µl of the BP recombination reaction (from Performing the BP Recombination Reaction, Step 7, page 22) into a 0.1 cuvette containing 50 µl of electrocompetent E. coli and mix gently. Do not mix by pipetting up and down. Avoid formation of bubbles.

Electroporate your samples using an electroporator and the manufacturer’s suggested protocol.

Note: If you have problems with arcing, see below.

Immediately add 450 µl of room temperature S.O.C. medium.

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 antibiotic resistance marker.

Spread 50-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

An efficient BP recombination reaction may produce hundreds of colonies.

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:

Reduce the voltage normally used to charge your electroporator by 10%
Reduce the pulse length by reducing the load resistance to 100 ohms
Dilute the BP reaction 5-10 fold with sterile water, then transform 1 µl into cells
Sequencing Entry Clones

Introduction
You may sequence entry clones generated by BP recombination using dye-labeled terminator chemistries including DYEnamic™ energy transfer or BigDye™ reaction chemistries.

Sequencing Primers
You may use the M13 Sequencing Primers included with the PCR Cloning System with Gateway® Technology kits to sequence entry clones derived from BP recombination with pDONR™/221 or pDONR™/Zeo. Refer to the diagram on page 19 for the location of the primer binding sites.

The M13 Sequencing Primers are supplied as 2 µg of primer, lyophilized in TE Buffer, pH 8.0. To use, simply resuspend each primer in 20 µl of water to a final concentration of 0.1 µg/µl.

Sequencing Using BigDye™ Chemistry
To sequence entry clones using the BigDye™ chemistry, we recommend the following:

Use at least 500 ng of DNA
Use 5-50 pmoles of primers
For entry clones derived from recombination with pDONR™/221 or pDONR™/Zeo, use 1/4 reaction and the PCR conditions listed below

PCR Conditions
For entry clones derived from recombination with pDONR™/221 or pDONR™/Zeo, use the following PCR conditions. These conditions are suitable for most inserts, including small inserts.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>5 minutes</td>
<td>95°C</td>
<td>1X</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10-30 seconds</td>
<td>96°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>5-15 seconds</td>
<td>50°C</td>
<td>30X</td>
</tr>
<tr>
<td>Extension</td>
<td>4 minutes</td>
<td>60°C</td>
<td></td>
</tr>
</tbody>
</table>

DYEnamic™ is a trademark of Amersham Biosciences.

BigDye™ is a trademark of Applied Biosystems.
Creating Expression Clones Using the LR Recombination Reaction

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into an attR-containing destination vector to create an attB-containing expression clone. To ensure that you obtain the best possible results, we suggest that you read this section and the next section entitled Performing the LR Recombination Reaction (pages 29-30) before beginning.

Experimental Outline

To generate an expression clone, you will:
- Perform an LR recombination reaction using the appropriate attL and attR-containing substrates (see below)
- Transform the reaction mixture into a suitable E. coli host (see page 23)
- Select for expression clones

Substrates for the LR Recombination Reaction

To perform an LR recombination reaction, you need to have the following substrates:
- attL-containing entry clone
- An Invitrogen destination vector or your converted destination vector (see below)

Important

For most applications, we recommend performing the LR recombination reaction using a:
- Supercoiled attL-containing entry clone
- Supercoiled attR-containing destination vector

Exception: If your destination vector or entry clone is large (>10 kb), you may do the following to increase recombinational efficiency by up to 2-fold:
- Linearize either the destination vector or the entry clone. To linearize the destination vector, choose a unique restriction site that cuts within the attR cassette but does not disrupt the attR sites or the ccdB gene. To linearize the entry clone, choose a unique restriction site that does not cut within the attL sites or the gene of interest.
- Relax the destination vector using topoisomerase I if suitable restriction sites are unavailable. Refer to the Appendix, page 46 for a protocol to perform a modified LR reaction using a relaxed destination vector.

Note

Although the Gateway® Technology manual has previously recommended using a linearized destination vector and entry clone for more efficient LR recombination, further testing at Invitrogen has found that linearization of destination vectors and entry clones is generally NOT required to obtain optimal results for any downstream application.

continued on next page
## Destination Vectors

A large selection of destination vectors is available from Invitrogen to allow expression of your gene of interest in virtually any protein expression system. For more information about the options available, see our Web site (www.invitrogen.com) or call Technical Service (see page 52).

## Converting Your Vector to a Destination Vector

You may convert any vector to a destination vector using the Gateway® Vector Conversion System available from Invitrogen. For guidelines and instructions, see Constructing a Gateway® Destination Vector, pages 31-36.
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, and transform the reaction mixture into a suitable *E. coli* host (see below) to select for an expression clone. We recommend that you include the pENTR™-gus positive control (see below) in your experiments to help you evaluate your results.

**E. coli Host**
You may use any recA, endA *E. coli* strain including TOP10, DH5α™, DH10B™ or equivalent for transformation (see page 23 for ordering information). **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.

**Note:** If you plan to use Library Efficiency® DH5α™ cells for transformation, see the section entitled **Transforming Competent Cells**, pages 23-25.

**Positive Control**
The pENTR™-gus plasmid is provided with the LR Clonase™ Enzyme Mix for use as a positive control for recombination and expression. Using the pENTR™-gus entry clone in an LR recombination reaction with a destination vector will allow you to generate an expression clone containing the gene encoding β-glucuronidase (gus) (Kertbundit et al., 1991).

**Materials Needed**
You should have the following materials on hand before beginning:
- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- Destination vector of choice (150 ng/µl in TE, pH 8.0)
- LR Clonase™ enzyme mix (Catalog no. 11791-019; keep at -80°C until immediately before use)
- 5X LR Clonase™ Reaction Buffer (supplied with the LR Clonase™ enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ enzyme mix; thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µl; supplied with the LR Clonase™ enzyme mix)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates with the appropriate antibiotic (e.g. ampicillin) to select for expression clones

*continued on next page*
Performing the LR Recombination Reaction, continued

Setting Up the LR Recombination Reaction

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.
   
   **Note:** To include a negative control, set up a second sample reaction and omit the LR Clonase™ enzyme mix (see Step 4).

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>Negative Control</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry clone (100-300 ng/reaction)</td>
<td>1-10 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Destination vector (300 ng/reaction)</td>
<td>2 µl</td>
<td>2µl</td>
<td>2µl</td>
</tr>
<tr>
<td>pENTR™-gus (50 ng/µl)</td>
<td>--</td>
<td>--</td>
<td>2µl</td>
</tr>
<tr>
<td>5X LR Clonase™ Reaction Buffer</td>
<td>4 µl</td>
<td>4 µl</td>
<td>4 µl</td>
</tr>
<tr>
<td>TE Buffer, pH 8.0</td>
<td>16 µl</td>
<td>10 µl</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

2. Remove the LR Clonase™ enzyme mix from -80°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 4 µl of LR Clonase™ enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).
   
   **Reminder:** Return LR Clonase™ enzyme mix to -80°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.
   
   **Note:** For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation times (i.e. overnight incubation) will yield more colonies and are recommended.
6. Add 2 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. If you are transforming Library Efficiency® DH5α™ competent *E. coli*, follow the protocol on page 24.
   
   **Note:** You may store the LR reaction at -20°C for up to 1 week before transformation, if desired.

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1 x 10⁸ cfu/µg, the LR reaction should give >5000 colonies if the entire transformation is plated.

Expressing Your Recombinant Protein

Once you have obtained an expression clone, you are ready to express your recombinant protein. Refer to the manual for the destination vector you are using for guidelines and instructions to express your recombinant protein in the appropriate system. Manuals for all Gateway® destination vectors are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).
Constructing a Gateway® Destination Vector

Introduction

You may easily convert any vector of choice to a Gateway® destination vector by ligating a blunt-ended cassette containing attR sites flanking the ccdB gene and the chloramphenicol resistance gene into the multiple cloning site (MCS) of the vector. The Gateway® Vector Conversion System is available from Invitrogen (see page x for ordering information) to facilitate conversion of your vector into a destination vector (see below) for expression of native, N-, or C-terminally-tagged proteins.

Most entry vectors contain the kanamycin resistance gene for selection. For maximal compatibility within the Gateway® Technology, we recommend that your vector not contain a kanamycin resistance marker. If this is unavoidable, you will need to perform the LR recombination reaction with an entry clone that carries a selection marker other than the kanamycin resistance gene.

You may use pDONR™/Zeo and your attB-flanked gene of interest in a BP recombination reaction to generate an entry clone that confers Zeocin™ resistance. See page x for ordering information.

Gateway® Vector Conversion System

The Gateway® Vector Conversion System includes three conversion cassettes:
- Reading Frame (Rf) Cassette A (RfA)
- Reading Frame (Rf) Cassette B (RfB)
- Reading Frame (Rf) Cassette C.1 (RfC.1)

Each reading frame cassette contains the chloramphenicol resistance gene (CmR) and the ccdB gene flanked by attR1 and attR2 sites (see below). Each reading frame cassette also differs by one nucleotide to allow generation of attR sites in all three reading frames.

<table>
<thead>
<tr>
<th>Cassette</th>
<th>Restriction Site</th>
<th>Location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RfA</td>
<td>MluI</td>
<td>898</td>
</tr>
<tr>
<td>RfB</td>
<td>BglII</td>
<td>899</td>
</tr>
<tr>
<td>RfC.1</td>
<td>XbaI</td>
<td>899</td>
</tr>
</tbody>
</table>

Ligating the Reading Frame Cassette to Your Vector

Each reading frame cassette is supplied as a blunt-ended DNA fragment that can be ligated into any blunt-ended restriction site. It is possible to linearize your vector using a restriction enzyme that generates 5’ overhangs, however, the ends of the vector must first be made blunt (using a Klenow fill-in reaction) before the blunt-ended reading frame cassette may be ligated into the vector.

continued on next page
Constructing a Gateway® Destination Vector, continued

**Experimental Outline**
To convert your vector to a destination vector, you will:
- Choose an appropriate reading frame cassette depending on your needs.
- Linearize the vector with a restriction enzyme of choice. If you use a restriction enzyme that generates an overhang, you will need to blunt the ends.
- Remove the 5’ phosphates using calf intestinal alkaline phosphatase.
- Ligate the reading frame cassette into your vector using T4 DNA ligase.
- Transform the ligation reaction into DB3.1™ E. coli and select for transformants.
- Analyze transformants.

**Factors to Consider**
To determine which Gateway® reading frame cassette to use when converting your vector, you should consider the following:
- If you plan to express a fusion protein from the destination vector, use a reading frame cassette with the correct translation reading frame.
- If you plan to linearize your vector using a restriction enzyme that generates an overhang, choose the correct reading frame cassette based on what the sequence of the ends will be after the vector has been made blunt (*i.e.* after filling in a protruding 5’ end or polishing a protruding 3’ end).

**N-terminal Fusions**
If you wish to create a destination vector to express N-terminal fusion proteins, use the table below and the diagram on the next page to help you determine which reading frame cassette to use.

**Tip:** Keep the -AAA-AAA- triplets in the attR1 site in frame with the translation reading frame of the fusion protein.

<table>
<thead>
<tr>
<th>If the coding sequence of the blunt end…</th>
<th>Then use…</th>
</tr>
</thead>
<tbody>
<tr>
<td>terminates after a complete codon triplet</td>
<td>RfA</td>
</tr>
<tr>
<td>encodes two bases of a complete codon triplet</td>
<td>RfB</td>
</tr>
<tr>
<td>encodes one base of a complete codon triplet</td>
<td>RfC.1</td>
</tr>
</tbody>
</table>

**C-terminal Fusions**
If you wish to create a destination vector to express C-terminal fusion proteins, use the table below and the diagram on the next page to help you determine which reading frame cassette to use.

**Tip:** Keep the -TAC-AAA- triplets in the attR2 site in frame with the translation reading frame of the fusion protein.

<table>
<thead>
<tr>
<th>If the coding sequence of the blunt end…</th>
<th>Then use…</th>
</tr>
</thead>
<tbody>
<tr>
<td>terminates after a complete codon triplet</td>
<td>RfB</td>
</tr>
<tr>
<td>encodes two bases of a complete codon triplet</td>
<td>RfC.1</td>
</tr>
<tr>
<td>encodes one base of a complete codon triplet</td>
<td>RfA</td>
</tr>
</tbody>
</table>

*continued on next page*
Constructing a Gateway® Destination Vector, continued

If you wish to create a destination vector to express both N-terminal and C-terminal fusions, choose a restriction enzyme that will produce blunt-ends that allow in-frame cloning with the attR sites in one of the three cassettes.

The sequences of the ends of each reading frame cassette are shown below. The complete sequence of each reading frame cassette is available from our Web site (www.invitrogen.com) or by calling Technical Service (see page 52).

Features of the reading frame cassettes:

Non-shaded regions correspond to those DNA sequences that are transferred into the attB expression clone following the LR recombination reaction.

The EcoRV half-site present on the 5’ and 3’ ends of each cassette is labeled.

Sequences contributed by your vector are denoted by Ns.

Note: If you are using RfB to create an N-terminal fusion vector, the two nucleotides next to the 5’ EcoRV half-site cannot be TG or TA otherwise this will generate a stop codon. Similarly, if you are using RfC.1 to create a C-terminal fusion vector, the two nucleotides next to the 3’ EcoRV half-site cannot be GA, AA, or AG.

The BsrGI restriction site common to all att1 and att2 sites is indicated.

continued on next page
Constructing a Gateway® Destination Vector, continued

**E. coli Host**

To propagate and maintain your destination vector, you must use DB3.1™ *E. coli*. The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the ccdB gene. Library Efficiency® DB3.1™ Competent Cells are provided with the Gateway® Vector Conversion System and are also available separately from Invitrogen (Catalog no. 11782-018).

**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.

To linearize your vector, we recommend that you choose restriction enzymes that will remove as many of the MCS restriction sites as possible. This will minimize the number of additional amino acids added to the fusion and will increase the number of unique restriction sites in the destination vector, which is important if you wish to linearize the vector for the LR recombination reaction.

**Materials Needed**

Your vector of choice

Appropriate restriction enzymes to linearize your vector at the position where you wish your gene (flanked by *att* sites) to be after recombination (see Recommendation above)

T4 DNA polymerase or Klenow fragment (if necessary to create blunt ends in your vector)

Calf intestinal alkaline phosphatase (CIAP; Invitrogen, Catalog no. 18009-019)

10X CIAP Buffer (supplied with Catalog no. 18009-019)

Sterile water (autoclaved, distilled)

TE Buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA)

T4 DNA ligase (Invitrogen, Catalog no. 15224-017)

5X T4 DNA ligase buffer (supplied with Catalog no. 15224-017)

Appropriate Gateway® reading frame cassette (5 ng/µl)

Library Efficiency® DB3.1™ competent cells (supplied with the Gateway® Vector Conversion System)

S.O.C. Medium (supplied with the Gateway® Vector Conversion System)

LB agar plates containing the appropriate antibiotic to select for your vector and 30 µg/ml chloramphenicol

*continued on next page*
Construction a Gateway® Destination Vector, continued

**Conversion Procedure**

Digest 1-5 μg of your plasmid vector with the appropriate restriction enzyme(s). If necessary, convert the ends of the vector to blunt double-stranded DNA using T4 DNA polymerase or Klenow fragment according to the manufacturer’s recommendations.

Remove the 5’ phosphates with calf intestinal alkaline phosphatase (CIAP) to decrease the background associated with self-ligation of the vector.

Determine the mass of DNA required for 1 pmol of the DNA 5’ end.

Add the following reagents to a 1.5 ml microcentrifuge tube:

10X CIAP Buffer 4 μl
DNA 1 pmol of 5’ ends
Sterile water to a final volume of 39 μl

Dilute the CIAP in dilution buffer such that 1 μl contains the amount of enzyme required to dephosphorylate the appropriate 5’ end (*i.e.* 1 unit for blunt ends). Add 1 μl of CIAP and incubate for 1 hour at 50°C.

Heat-inactivate CIAP for 15 minutes at 65°C.

Adjust the DNA to a final concentration of 20-50 ng/μl in TE Buffer, pH 8.0. Run 20-100 ng of DNA on an agarose gel to verify digestion and recovery.

To set up the ligation reaction, add the following reagents to a 1.5 ml microcentrifuge tube:

Dephosphorylated vector (20-50 ng) 1-5 μl
5X T4 DNA ligase buffer 2 μl
Gateway® reading frame cassette (10 ng) 2 μl
T4 DNA ligase 1 unit (in 1 μl)
Sterile water to a final volume of 10 μl

Incubate at room temperature for 1 hour.

**Note:** Overnight incubation at 16°C is also suitable.

Transform the ligation reaction into competent DB3.1™ E. coli. Follow the instructions provided with the cells.

After expression in S.O.C. medium, spread 20 μl and 100 μl from each transformation on a prewarmed selective plate containing the appropriate antibiotic to select for your vector and 30 μg/ml chloramphenicol. Incubate plates overnight at 37°C.

Because the reading frame cassettes are blunt-ended, they will ligate into your vector in both orientations. You will need to screen transformants to identify plasmids containing the reading frame cassette in the proper orientation.

*continued on next page*
Constructing a Gateway® Destination Vector, continued

**Analyzing Transformants**

Pick 10 colonies and culture them overnight in 3-5 ml of LB medium containing 30 µg/ml chloramphenicol.

Isolate plasmid DNA using your method of choice (Ausubel et al., 1994; Sambrook et al., 1989). We recommend the S.N.A.P.™ MiniPrep Kit (see page x for ordering information).

Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the cassette. You can use the BsrGI restriction enzyme to identify clones containing the reading frame cassette (see page 3/33).

**Verifying the Function of the ccdB Gene in the Destination Vector**

It is important to verify the functionality of the ccdB gene and check for the presence of contaminating antibiotic-resistant plasmids (e.g., no containing ampicillin-resistant plasmids if your destination vector is ampicillin-resistant). The presence of an inactive ccdB gene or contamination with other antibiotic-resistant plasmids can result in high backgrounds in the LR reaction.

**Materials Needed:**

- DB3.1™ competent E. coli
- DH5α™ or TOP10 competent E. coli (or any other strain sensitive to CcdB effects)
- Positive control plasmid (e.g., pUC 19) to verify success of transformation
- Selective plates (e.g., LB + ampicillin)

**Procedure:**

Transform equal amounts (10-50 pg) of your destination vector into competent DH5α™ and DB3.1™ cells using the protocol provided with the cells. Also transform each strain with 50 pg of the positive control plasmid.

Plate onto selective plates and incubate overnight at 37ºC.

Use the pUC19 control DNA to verify that the transformation efficiency is as expected for each strain.

Determine the number of colonies obtained in both strains transformed with the destination vector.

**What You Should See:**

The destination vector should give 10,000 times more colonies in DB3.1™ cells than in DH5α™ cells. Any ratio less than 10,000 indicates either an inactive ccdB gene or contamination of the plasmid prep with another antibiotic-resistant plasmid.

**Preparing the Destination Vector**

Once purified, you may use your supercoiled destination vector directly in the LR recombination reaction. If your destination vector is large (>10 kb), you may increase the efficiency of the LR reaction by linearizing the destination vector with a restriction enzyme or relaxing the DNA with topoisomerase I (see protocol on page 46.), if desired.

To linearize the destination vector, use a unique restriction enzyme that cuts within the Gateway® reading frame cassette but not within the ccdB gene (see table below for a list of possible restriction enzymes). Be sure to choose a restriction enzyme that does not cut within your vector sequence.

- AltI
- EcoRI
- NotI
- SalI
- SfcI
- BssHII
- NcoI
- PvuII
- ScaI
**Troubleshooting**

**LR and BP Reactions**

The table below lists some potential problems and possible solutions that may help you troubleshoot the BP or LR recombination reactions.

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Few or no colonies obtained from sample reaction and the transformation control gave colonies</td>
<td>Incorrect antibiotic used to select for transformants</td>
<td>Check the antibiotic resistance marker and use the correct antibiotic to select for entry clones or expression clones</td>
</tr>
<tr>
<td>Recombination reactions were not treated with proteinase K</td>
<td>Treat reactions with proteinase K before transformation</td>
<td></td>
</tr>
<tr>
<td>Used incorrect att sites for the reaction</td>
<td>Use an entry clone (attL) and a destination vector (attR) for the LR reaction</td>
<td>Use an expression clone (or attB-PCR product) and a donor vector (attP) for the BP reaction</td>
</tr>
<tr>
<td>Clonase™ enzyme mix is inactive or didn’t use suggested amount of Clonase™ enzyme mix</td>
<td>Test another aliquot of the Clonase™ enzyme mix</td>
<td>Make sure that you store the Clonase™ enzyme mix at -80°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Do not freeze/thaw the Clonase™ enzyme mix more than 10 times</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Use the recommended amount of Clonase™ enzyme mix (see page 22 or 30 as appropriate)</td>
</tr>
<tr>
<td>Used incorrect Clonase™ enzyme mix</td>
<td>Use the LR Clonase™ enzyme mix for the LR reaction and the BP Clonase™ enzyme mix for the BP reaction</td>
<td></td>
</tr>
<tr>
<td>Too much attB-PCR product was used in a BP reaction</td>
<td>Reduce the amount of attB-PCR product used. Remember to use an equimolar ratio of attB-PCR product and donor vector (i.e. ~100 fmol each)</td>
<td></td>
</tr>
<tr>
<td>Long attB-PCR product or linear attB expression clone (≥5 kb)</td>
<td>Incubate the BP reaction overnight</td>
<td></td>
</tr>
<tr>
<td>Too much entry clone was used in an LR reaction</td>
<td>Use equal fmol of destination vector and entry clone</td>
<td></td>
</tr>
<tr>
<td>Large destination vector or entry clone (&gt;10 kb)</td>
<td>Incubate the LR reaction overnight</td>
<td>Linearize the destination vector or the entry clone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relax the destination vector with topoisomerase I</td>
</tr>
</tbody>
</table>

*continued on next page*
## Troubleshooting

### LR and BP Reactions, continued

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
</table>
| Two distinct types of colonies (large and small) appear | **LR reaction:** Small colonies can be unreacted entry clone that cotransforms with expression clone  
**Note:** When small colonies are restreaked onto selective plates to select for unreacted entry clones (e.g. LB + kanamycin) and expression clones (e.g. LB + ampicillin), small colonies often only grow on the selective plates used to select for unreacted entry clones. | Reduce the amount of entry clone to 100 ng per 20 µl reaction  
Reduce the volume of sample used for transformation to 1 µl  
If you are using a destination vector that contains the ampicillin resistance gene for selection, increase the ampicillin concentration to 300 µg/ml |
| | **BP reaction:** The pDONR™ vector contains deletions or point mutations in the ccdB gene  
**Note:** The negative control will give a similar number of colonies. | Obtain a new pDONR™ vector |
| Loss of plasmid during culture (generally those containing large genes or toxic genes) | Incubate selective plates at 30°C instead of 37°C  
Confirm whether a deletion has occurred by analyzing the DNA derived from the colonies  
Use Stbl2™ *E. coli* (Invitrogen, Catalog no. 10268-019) to help stabilize plasmids containing large genes during propagation (Trinh *et al.*, 1994) | |
| High background of Zeocin™-resistant transformants that do not contain the entry clone | Selection of entry clones derived from pDONR™/Zeo not performed on Low Salt LB agar plates | Use Low Salt LB agar plates with 50 µg/ml Zeocin™ to select entry clones derived from pDONR™/Zeo. See page 23 for more information and page 49 for a recipe. |

*continued on next page*
## Troubleshooting

### LR and BP Reactions, continued

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LR Reaction: High background in the absence of the entry clone</strong></td>
<td>LR reaction transformed into an <em>E. coli</em> strain containing the F′ episome and the <em>ccdA</em> gene</td>
<td>Use an <em>E. coli</em> strain that does not contain the F′ episome for transformation (e.g. DH5α™, TOP10)</td>
</tr>
<tr>
<td></td>
<td>Deletions (full or partial) of the <em>ccdB</em> gene from the destination vector</td>
<td>To maintain the integrity of the vector, propagate in media containing the appropriate antibiotic (e.g. ampicillin) and 15-30 µg/ml chloramphenicol Prepare plasmid DNA from one or more colonies and verify the integrity of the vector before use If you have converted your own vector to a destination vector, try using a different vector backbone to reduce instability of the plasmid</td>
</tr>
<tr>
<td>Contamination of solution(s) with another plasmid carrying the same antibiotic resistance, or by bacteria carrying a resistance plasmid</td>
<td>Test for plasmid contamination by transforming <em>E. coli</em> with aliquots of each of the separate solutions used in the LR reaction Test for bacterial contamination by plating an aliquot of each solution directly onto LB plates containing ampicillin</td>
<td></td>
</tr>
<tr>
<td>Few or no colonies obtained from the transformation control</td>
<td>Competent cells stored incorrectly</td>
<td>Store competent cells at -80°C</td>
</tr>
<tr>
<td>Transformation performed incorrectly</td>
<td></td>
<td>If you are using Library Efficiency® DH5α™, follow the protocol on page 24 to transform cells If you are using another <em>E. coli</em> strain, follow the manufacturer’s instructions</td>
</tr>
<tr>
<td>Loss of transformation efficiency due to repeated freeze/thawing</td>
<td></td>
<td>Once you have thawed a tube of competent cells, discard any unused cells</td>
</tr>
<tr>
<td>Insufficient amount of <em>E. coli</em> plated</td>
<td></td>
<td>Increase the amount of <em>E. coli</em> plated</td>
</tr>
</tbody>
</table>

*continued on next page*
# Troubleshooting

## attB-PCR Cloning

The table below lists some potential problems and possible solutions that may help you troubleshoot the BP recombination reaction when using an attB-PCR product as a substrate. These potential problems are in addition to those encountered in the general BP reaction (see page 37).

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low yield of attB-PCR product obtained after PEG purification</td>
<td>attB-PCR product not diluted with TE</td>
<td>Dilute with 150 µl of 1X TE, pH 8.0 before adding the PEG/MgCl₂ solution</td>
</tr>
<tr>
<td>Centrifugation step too short or centrifugation speed too low</td>
<td></td>
<td>Increase time and speed of the centrifugation step to 30 minutes and 15,000 x g</td>
</tr>
<tr>
<td>Lost PEG pellet</td>
<td>When removing the tube from the microcentrifuge, keep track of the orientation of the outer edge of the tube where the pellet is located When removing the supernatant from the tube, take care not to disturb the pellet</td>
<td></td>
</tr>
<tr>
<td>Few or no colonies obtained from a BP reaction with attB-PCR product and both attB positive control and transformation control gave expected number of colonies</td>
<td>attB PCR primers incorrectly designed</td>
<td>Make sure that the attB PCR primers include four 5′ terminal Gs and the 25 bp attB1 or attB2 site (see page 12)</td>
</tr>
<tr>
<td></td>
<td>attB PCR primers contaminated with incomplete sequences</td>
<td>Use HPLC or PAGE-purified oligonucleotides to generate your attB-PCR product Use the attB adapter PCR protocol to generate your attB-PCR product</td>
</tr>
<tr>
<td></td>
<td>attB-PCR product not purified sufficiently</td>
<td>Gel purify your attB-PCR product to remove attB primers and attB primer-dimers</td>
</tr>
<tr>
<td></td>
<td>For large PCR products (&gt;5 kb), too few attB-PCR molecules added to the BP reaction</td>
<td>Increase the amount of attB-PCR product to 40-100 fmol per 20 µl reaction Note: Do not exceed 500 ng DNA per 20 µl reaction Incubate the BP reaction overnight</td>
</tr>
<tr>
<td></td>
<td>Insufficient incubation time</td>
<td>Increase the incubation time of the BP reaction up to 18 hours</td>
</tr>
</tbody>
</table>

*continued on next page*
## Troubleshooting

**attB PCR Cloning, continued**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry clones migrate as 2.2 kb supercoiled plasmids</td>
<td>BP reaction may have cloned attB primer-dimers</td>
<td>Purify attB-PCR product using the PEG/MgCl₂ purification protocol on page 16 or gel-purify the attB-PCR product. Use a Platinum® DNA polymerase with automatic hot-start capability for higher specificity amplification. Redesign attB PCR primers to minimize potential mutual priming sites leading to primer-dimers</td>
</tr>
</tbody>
</table>
Appendix

“One-Tube” Protocol for Cloning \textit{attB}-PCR Products Directly into Destination Vectors

\textbf{Introduction}

Use this one-tube protocol to:

Move \textit{attB}-PCR products into a destination vector in 2 steps - a BP reaction followed by an LR reaction without purification of the intermediate entry clone. See page 15 for guidelines to generate \textit{attB}-PCR products.

Transfer a gene from one expression clone into another destination vector.

\textbf{Note}: Using this protocol allows you to generate expression clones more rapidly than the protocols provided on pages 20-30; however, fewer expression clones will be obtained (generally 10-20\% of the total number of entry clones). If you wish to maximize the number of expression clones generated, do not use this protocol. Use the protocols on pages 20-30 instead.

\textbf{Expression Clones Containing PCR Products}

If you use the one-tube protocol to clone \textit{attB}-PCR products into a destination vector, note that expression clones obtained using this protocol will be derived from entry clones that are not unique. You will need to sequence your expression clone to confirm its identity.

\textbf{Note}

If you plan to transfer a gene from one expression clone into another destination vector, make sure that you linearize the expression clone before performing the one-tube protocol. Linearization ensures an optimal BP reaction and eliminates background due to co-transformation of your supercoiled expression plasmid.

\textbf{Materials Needed}

You should have the following materials on hand before beginning:

\textit{attB}-PCR product (100-200 ng)

\textit{attP} DNA (\textit{i.e.} pDONR™ vector; 150 ng/\(\mu\)l in 1X TE, pH 8.0)

BP Clonase™ enzyme mix (keep at \(-80\)°C until immediately before use)

5X BP Reaction Buffer (supplied with the BP Clonase™ enzyme mix)

TE Buffer, pH 8.0

Proteinase K solution (supplied with the BP and LR Clonase™ enzyme mixes)

Destination vector (supercoiled; 150 ng/\(\mu\)l in TE Buffer, pH 8.0)

0.75 M NaCl

LR Clonase™ enzyme mix (keep at \(-80\)°C until immediately before use)

Competent \textit{E. coli} cells (see page 23 to choose an appropriate host strain)

LB agar plates containing the appropriate antibiotic to select for entry clones (\textit{e.g.} kanamycin or Zeocin™)

LB agar plates containing the appropriate antibiotic to select for expression clones (\textit{e.g.} ampicillin)

\(\text{continued on next page}\)
“One-Tube” Protocol for Cloning attB-PCR Products Directly into Destination Vectors, continued

“One-Tube” Protocol

In a 1.5 ml microcentrifuge tube, prepare the following 25 µl BP reaction:

- attB DNA (100-200 ng)  5.0 µl
- attP DNA (pDONR™ vector, 150 ng/µl)  2.5 µl
- 5X BP Reaction Buffer  5.0 µl
- BP Clonase™ enzyme mix  5.0 µl
- TE Buffer, pH 8.0 add to a final volume of 20 µl

Final volume  25 µl

Mix well by vortexing briefly and incubate at 25°C for 4 hours.

**Note:** Depending on your needs, the length of the recombination reaction can be extended up to 20 hours. An overnight incubation typically yields 5 times more colonies than a 1 hour incubation. Longer incubation times are recommended for large plasmids (≥10 kb) and PCR products (≥5 kb).

Remove 5 µl of the reaction to a separate tube and use this aliquot to assess the efficiency of the BP reaction (see below).

To the remaining 20 µl reaction, add:

- 0.75 M NaCl  1.0 µl
- Destination vector (150 ng/µl)  3.0 µl
- LR Clonase™ enzyme mix  6.0 µl

Final volume  30 µl

Mix well by vortexing briefly and incubate at 25°C for 2 hours.

**Note:** Depending on your needs, the length of the recombination reaction can be extended up to 18 hours.

Add 3 µl of proteinase K solution. Incubate at 37°C for 10 minutes.

Transform 100 µl of the appropriate competent *E. coli* with 1 µl of the reaction.

Plate on LB plates containing the appropriate antibiotic to select for expression clones.

Assessing the Efficiency of the BP Reaction

To the 5 µl aliquot obtained from “One-Tube” Protocol, Step 3, above, add 0.5 µl of proteinase K solution. Incubate at 37°C for 10 minutes.

Transform 100 µl of the appropriate competent *E. coli* with 1 µl of the reaction.

Plate on LB plates containing the appropriate antibiotic to select for entry clones.
Preparing \textit{attB}-PCR Products Using \textit{attB} Adapter PCR

\section*{Introduction}
We recommend using this protocol to produce \textit{attB}-PCR products if your PCR primers are greater than 70 bp. To use this protocol, you will need to have 2 sets of PCR primers, one set for the gene-specific amplification and a second set to install the complete \textit{attB} sequences (adapter-primers \textit{attB1} and \textit{attB2}).

\section*{Template-Specific Primers}
Design the following template-specific primers. Include 12 bases of the \textit{attB1} or \textit{attB2} site on the 5' end of each primer, as appropriate.

\textit{attB1} forward: 5'--AA AAA GCA GGC TNN - template-specific sequences-3'
\textit{attB2} reverse: 5'--A GAA AGC TGG GTN - template-specific sequences-3'

\section*{Adapter Primers}
Design the following adapter primers which are required to install the complete \textit{attB} sequences.

\textit{attB1} adapter: 5'--GGG ACA AGT TTG TAC AAA AAA GCA GGC T -3'
\textit{attB2} adapter: 5'--GGG GAC CAC TTT GTA CAA GAA AGC TGG GT -3'

\section*{\textit{attB} Adapter PCR Protocol}
Set up a 50 \(\mu\)l PCR reaction containing 10 pmoles of each template-specific primer and the appropriate amount of template DNA.

\textbf{Note:} Do not use more than 10 pmoles of each template-specific primer as this can lead to reduced yield of clonable full-length \textit{attB}-PCR product.

Amplify using the following cycling parameters:

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|}
\hline
\textbf{Step} & \textbf{Time} & \textbf{Temperature} & \textbf{Cycles} \\
\hline
Initial Denaturation & 2 minutes & 95°C & 1X \\
\hline
Denaturation & 15 seconds & 94°C & \\
\hline
Annealing & 30 seconds & 50-60°C & 10X \\
\hline
Extension & 1 minute/kb & 68°C & \\
\hline
\end{tabular}
\caption{PCR cycling parameters for template-specific primers.}
\end{table}

Transfer 10 \(\mu\)l of the PCR reaction to a 40 \(\mu\)l PCR mixture containing 40 pmoles each of the \textit{attB1} and \textit{attB2} adapter primers.

Amplify using the following cycling parameters:

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|}
\hline
\textbf{Step} & \textbf{Time} & \textbf{Temperature} & \textbf{Cycles} \\
\hline
Initial Denaturation & 1 minutes & 95°C & 1X \\
\hline
Denaturation & 15 seconds & 94°C & 5X \\
\hline
Annealing & 30 seconds & 45°C & \\
\hline
Extension & 1 minute/kb & 68°C & \\
\hline
\end{tabular}
\caption{PCR cycling parameters for adapter primers.}
\end{table}

\textit{continued on next page}
Preparing *attB*-PCR Products Using *attB* Adapter PCR, continued

*attB* Adapter PCR Protocol, continued

Adjust cycling parameters and amplify for 15-20 cycles using the following parameters:

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>15 seconds</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>30 seconds</td>
<td>55°C</td>
<td>15-20X</td>
</tr>
<tr>
<td>Extension</td>
<td>1 minute/kb</td>
<td>68°C</td>
<td></td>
</tr>
</tbody>
</table>

Use agarose gel electrophoresis to check quality and yield of the *attB*-PCR product.

Proceed to page 16 to purify the *attB*-PCR product.
Relaxing Destination Vectors Using Topoisomerase I

Introduction
Use this protocol to perform a modified LR recombination reaction with a relaxed destination vector. Relaxing a destination vector with topoisomerase I may increase the efficiency of the LR reaction, and is useful when suitable restriction sites are unavailable to linearize the vector or if the destination vector is large (>10 kb).

Materials Needed
- Destination vector (supercoiled; 300 ng per reaction)
- Entry clone (supercoiled, 100-300 ng per reaction)
- Topoisomerase I (Invitrogen, Catalog no. 38042-024; use 15 units/µg of total DNA)
- TE Buffer, pH 8.0
- LR Clonase™ enzyme mix (Invitrogen, Catalog no. 11791-019; keep at -80°C until immediately before use)
- 5X LR Reaction Buffer (supplied with the LR Clonase™ enzyme mix)
- Proteinase K solution (supplied with the LR Clonase™; thaw and keep on ice until use)

Protocol
- Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix. Add 1-9 µl of LR Clonase™ enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).
- Incubate reactions at 25°C for 1 hour.
- Add 2 µl of the Proteinase K solution to the reaction. Incubate for 10 minutes at 37°C.
- Proceed to transform a suitable E. coli host and select for expression clones. If you are transforming Library Efficiency DH5α™ competent E. coli, follow the protocol on page 24.

Reminder: Return LR Clonase™ enzyme mix to -80°C immediately after use.
Transferring Clones from cDNA Libraries Made in Gateway® Vectors

Introduction

If you have obtained or generated a cDNA library in a Gateway®-compatible vector (i.e. pCMV SPORT6 or pEXP-AD502), you may create entry clones by performing a BP recombination with a donor vector. You will need to consider the following:

- Whether the cDNAs are full-length
- What expression system you want to use
- Whether you want to express native proteins or fusion proteins

Expressing Full-Length vs. Other cDNAs

Most cDNA libraries typically contain a mixture of:

- Full-length open reading frames (ORFs)
- Partial ORFs
- Full-length ORF plus 5' untranslated sequence (UTR)

Depending on which expression system you want to use, your clones may need to contain specialized sequences to permit efficient expression (e.g. Kozak consensus sequence for mammalian expression or Shine-Dalgarno sequence for *E. coli* expression). Those cDNAs which contain the full-length ORF plus 5' untranslated sequence may already contain the necessary sequences. In the other cases, you may incorporate the requisite sequence into attB PCR primers, amplify the cDNAs, and perform a BP recombination reaction with the attB-PCR products. Alternatively, if you plan to express the cDNAs in *E. coli*, you may also clone the cDNAs into an entry vector that contains a Shine-Dalgarno sequence (i.e. pENTR/SD/D-TOPO®).

Expressing Fusion Proteins

If you wish to express your cDNAs as N- or C-terminal fusions, keep the following in mind:

For full-length cDNAs containing 5' untranslated sequence, the 5' UTR will be translated as part of the fusion protein. This may present problems as the additional codons may interfere with expression or function of the protein, or may include stop codons.

N-terminal fusions: To express any cDNA as an N-terminal fusion protein, the reading frame of the gene must be in frame with the reading frame of the attB1 site. If the identity of the cDNAs is unknown, there is a one in three chance that the cDNA will be in frame with the N-terminal tag. You may construct three destination vectors, each allowing expression of the fusion protein in a different reading frame or alternatively, you may amplify the cDNA using attB primers designed to be in frame with the ORF.

C-terminal fusions: Stop codons present in full-length cDNAs must be removed to permit expression of a C-terminal fusion protein. This may be done by amplifying the gene using attB PCR primers in which the stop codon has been eliminated from the gene-specific sequence. Alternatively, the gene may be subcloned into any entry vector in such a way that no stop codon is present.
Zeocin™

Introduction
Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. It shows strong toxicity against bacteria, fungi (including yeast), plants and mammalian cell lines (Calmels et al., 1991; Drocourt et al., 1990; Gatignol et al., 1987; Mulsant et al., 1988; Perez et al., 1989).

A Zeocin™ resistance protein has been isolated and characterized (Calmels et al., 1991; Drocourt et al., 1990). This 13,665 Da protein, the product of the *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene), binds stoichiometrically to Zeocin™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure
The formula for Zeocin™ is C_{60}H_{89}N_{21}O_{21}S_{3} and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.

Handling Zeocin™
- High ionic strength and acidity or basicity inhibit the activity of Zeocin™. Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see page 49 for a recipe).
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ is light sensitive. Store the drug and plates or medium containing the drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses when handling Zeocin™-containing solutions.
- Do not ingest or inhale solutions containing the drug.
- Be sure to bandage any cuts on your fingers to avoid exposure to the drug.
Recipes

Low Salt LB Medium with Zeocin™

10 g Tryptone
5 g NaCl
5 g Yeast Extract

Combine the dry reagents above and add deionized, distilled water to 950 ml. Adjust the pH to 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.

Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.

Thaw Zeocin™ on ice and vortex before removing an aliquot.

Allow the medium to cool to at least 55°C before adding the Zeocin™ to 50 µg/ml final concentration.

Store plates at +4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.
The map below shows the elements of pDONR™221 and pDONR™/Zeo. The complete sequences of pDONR™221 and pDONR™/Zeo are available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).
Map and Features of pDONR™ 221 and pDONR™/Zeo, continued

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>rrnB T1 and T2 transcription terminators</strong></td>
<td>Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz et al., 1991).</td>
</tr>
<tr>
<td><strong>M13 forward (-20) priming site</strong></td>
<td>Allows sequencing in the sense orientation.</td>
</tr>
<tr>
<td><em>attP1 and attP2 sites</em></td>
<td>Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from an <em>attB</em>-containing expression clone or <em>attB</em>-PCR product (Landy, 1989).</td>
</tr>
<tr>
<td><strong>ccdB gene</strong></td>
<td>Allows negative selection of the plasmid.</td>
</tr>
<tr>
<td>Chloramphenicol resistance gene (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Allows counterselection of the plasmid.</td>
</tr>
<tr>
<td><strong>M13 reverse priming site</strong></td>
<td>Allows sequencing in the anti-sense orientation.</td>
</tr>
<tr>
<td>Kanamycin resistance gene</td>
<td>Allows selection of the plasmid in <em>E. coli</em>.</td>
</tr>
<tr>
<td>(pDONR™ 221 only)</td>
<td></td>
</tr>
<tr>
<td><strong>EM7 promoter</strong></td>
<td>Allows expression of the Zeocin™ resistance gene in <em>E. coli</em>.</td>
</tr>
<tr>
<td>(pDONR™/Zeo only)</td>
<td></td>
</tr>
<tr>
<td><strong>Zeocin™ resistance gene</strong></td>
<td>Allows selection of the plasmid in <em>E. coli</em>.</td>
</tr>
<tr>
<td>(pDONR™/Zeo only)</td>
<td></td>
</tr>
<tr>
<td>pUC origin and replisome assembly site</td>
<td>Allows high-copy replication and maintenance of the plasmid in <em>E. coli</em>.</td>
</tr>
</tbody>
</table>
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# Product Qualification

## Introduction

This section describes the criteria used to qualify the components of the PCR Cloning System with Gateway® Technology.

## pDONR™ Vectors

The structure of each pDONR™ vector is verified by restriction enzyme digestion. In addition, the functionality of each vector is tested in a recombination assay using Gateway® BP Clonase™ enzyme mix. The *codB* gene is assayed by transformation using an appropriate *E. coli* strain.

## Zeocin™

Zeocin™ is lot qualified by demonstrating the LB media containing 25 µg/ml Zeocin™ prevents growth of the TOP10 *E. coli* strain.

## BP Clonase™ Enzyme Mix

Gateway® BP Clonase™ enzyme mix is functionally tested in a 1 hour recombination reaction followed by a transformation assay.

## Chemically Competent *E. coli*

Library Efficiency® DH5α™ competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1 x 10^8 cfu/µg plasmid DNA.

To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.

Untransformed cells are plated on LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.
Glossary of Terms

**att**<sub>L</sub>, **att**<sub>R</sub>, **att**<sub>B</sub>, and **att**<sub>P**

The recombination sites from bacteriophage lambda that are utilized in the Gateway<sup>®</sup> Technology.

**att**<sub>L</sub> always recombines with **att**<sub>R</sub> in a reaction mediated by the LR Clonase<sup>™</sup> enzyme mix. The LR reaction is the basis for the entry clone x destination vector reaction. Recombination between **att**<sub>L</sub> and **att**<sub>R</sub> sites yields **att**<sub>B</sub> and **att**<sub>P</sub> sites on the resulting plasmids.

**att**<sub>B</sub> sites always recombine with **att**<sub>P</sub> sites in a reaction mediated by the BP Clonase<sup>™</sup> enzyme mix. The BP reaction is the basis for the reaction between the PCR cloning vector (pDONR™) and PCR products, source clones, or cDNA library clones containing **att**<sub>B</sub> sites. Recombination between **att**<sub>B</sub> and **att**<sub>P</sub> sites yields **att**<sub>L</sub> and **att**<sub>R</sub> sites on the resulting plasmids.

**BP Clonase<sup>™</sup> Enzyme Mix**

A proprietary mix of lambda recombination proteins that mediates the **att**<sub>B</sub> x **att**<sub>P</sub> recombination reaction.

**ccdB Gene**

A gene which encodes a protein that interferes with *E. coli* DNA gyrase, thereby inhibiting the growth of standard *E. coli* hosts. This gene is present on Gateway<sup>®</sup> destination, donor, and supercoiled entry vectors. When recombination occurs between a destination vector and an entry clone, the **ccdB** gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the **ccdB** gene, or by-product molecules that retain the **ccdB** gene, will fail to grow. This allows high-efficiency recovery of only the desired clones.

**DB3.1™ Competent Cells**

These cells are resistant to the effects of the **ccdB** gene product and are used to propagate vectors that contain the **ccdB** gene (i.e. donor, supercoiled entry, and destination vectors).

**Destination Vector**

Gateway<sup>®</sup>-adapted expression vectors which contain **att**<sub>R</sub> sites and allow recombination with entry clones.

**Donor Vector (pDONR™)**

A Gateway<sup>®</sup> vector containing **att**<sub>P</sub> sites. This vector is used for cloning PCR products and genes of interest flanked by **att**<sub>B</sub> sites (expression clones) to generate entry clones. When PCR fragments modified with **att**<sub>B</sub> sites are recombined with the pDONR™ vector in a BP reaction, they yield an entry clone.

\[ \text{PCR fragment (attB sites) + pDONR™ vector (attP sites) \rightarrow entry clone} \]

**Entry Clone**

The result of cloning a DNA segment into an entry vector or donor vector. The entry clone contains the gene of interest flanked by **att**<sub>L</sub> sites. It can be used for subsequent transfers into destination vectors.

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### Glossary of Terms, continued

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td><strong>Entry Vector</strong> (pENTR™)</td>
<td>A Gateway® vector containing attL sites used for cloning DNA fragments using either TOPO® Cloning or conventional restriction enzymes and ligase.</td>
</tr>
<tr>
<td><strong>Expression Clone</strong></td>
<td>The result of subcloning the DNA of interest from an entry clone into a destination vector of choice by LR recombination. The gene or DNA of interest in the expression clone is flanked by attB sites. Expression clones can be either fusion or native proteins. Entry clone + destination vector → expression clone</td>
</tr>
<tr>
<td><strong>LR Clonase™ Enzyme Mix</strong></td>
<td>A proprietary mix of lambda recombination proteins that mediates the attL x attR recombination reaction.</td>
</tr>
</tbody>
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References


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