MultiSite Gateway® Three-Fragment Vector Construction Kit

Using Gateway® Technology to simultaneously clone multiple DNA fragments

Catalog number 12537-023

Revision date 3 January 2012
Publication Part number 25-0541

MAN0000299

For Research Use Only. Not for diagnostic procedures.
Table of Contents

Table of Contents ............................................................................................................................................................ iii
Experienced Users Guide ............................................................................................................................................... v
Kit Contents and Storage .............................................................................................................................................. vii
Accessory Products ........................................................................................................................................................ ix

Introduction .................................................................................................................................................................. 1
Overview ..................................................................................................................................................................... 1
Gateway® Technology ...................................................................................................................................................... 2
MultiSite Gateway® Components .................................................................................................................................. 4
Experimental Overview .................................................................................................................................................. 8

Methods ...................................................................................................................................................................... 9
Propagating the MultiSite Gateway® Vectors .............................................................................................................. 9
General Information for Entry Clones ........................................................................................................................ 10
Making Entry Clones .................................................................................................................................................... 12
Designing attB PCR Primers ........................................................................................................................................ 15
Purifying attB PCR Products ........................................................................................................................................ 19
Creating Entry Clones Using the BP Recombination Reaction ............................................................................... 20
Transforming One Shot® TOP10 Competent Cells .................................................................................................... 27
Sequencing Entry Clones .............................................................................................................................................. 30
MultiSite Gateway® LR Recombination Reaction ..................................................................................................... 31
Performing the LR Recombination Reaction ............................................................................................................. 33
Troubleshooting ............................................................................................................................................................. 36

Appendix .................................................................................................................................................................. 40
Map of pDONR™ P4-P1R .............................................................................................................................................. 40
Map of pDONR™ 221 ..................................................................................................................................................... 41
Map of pDONR™ P2R-P3 .............................................................................................................................................. 42
Features of pDONR™ Vectors .................................................................................................................................. 43
Map of pDEST™ R4-R3 Vector II .................................................................................................................................. 44
Map of pMS/GW ........................................................................................................................................................... 46
Technical Support .......................................................................................................................................................... 47
Purchaser Notification .................................................................................................................................................. 48
Gateway® Clone Distribution Policy ........................................................................................................................... 49
References ....................................................................................................................................................................... 50
Experience Users Guide

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

BP Recombination Reaction
Perform a BP recombination reaction between each attB-flanked DNA fragment and the appropriate attP-containing donor vector to generate an entry clone (see page 26 for details).

1. Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:
   - attB PCR product (20–50 fmoles) 1–7 μl
   - pDONR™ vector (supercoiled, 150 ng/μl) 1 μl
   - 1x TE Buffer, pH 8.0 to 8 μl

2. Vortex BP Clonase® II enzyme mix briefly. Add 2 μl to the components above and mix well by vortexing briefly twice.

3. Incubate reaction at 25°C for 1 hour.

4. Add 1 μl of 2 μg/μl Proteinase K solution and incubate at 37°C for 10 minutes.

5. Transform 1 μl of the reaction into competent E. coli and select for kanamycin-resistant entry clones.

MultiSite Gateway® LR Recombination Reaction
IMPORTANT: This kit contains a new enzyme (LR Clonase II Plus) Please follow the protocol below and on pages 31-35.
Perform a MultiSite Gateway® LR recombination reaction between multiple entry clones (attL4-5’ element-attR1 + attL1-gene of interest-attL2 + attR2-3’ element-attL3) and the pDEST™ R4-R3 Vector II vector to generate an expression clone (attB4-5’ element-attB1-gene of interest-attB2-3’ element-attB3).

1. Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:
   - Entry clones (supercoiled, 10 fmoles each) 1–7 μl
   - pDEST™ R4-R3 Vector II (supercoiled, 20 fmol) 1 μl
   - 1x TE Buffer, pH 8.0 to 8 μl

2. Vortex LR Clonase® II Plus enzyme mix briefly. Add 2 μl to the components above and mix well by vortexing briefly twice.

3. Incubate reaction at 25°C for 16 hours (or overnight).

4. Add 1 μl of 2 μg/μl Proteinase K solution and incubate at 37°C for 10 minutes.

5. Transform 2 μl of the reaction into competent E. coli and select for ampicillin-resistant expression clones.

Continued on next page
Primer Sequences

To perform the three-fragment recombination, your PCR products will be flanked by different attB or attBr sites. Each DONR vector and the recommended primer sequences are shown below. For more information about primer design, see page 11.

<table>
<thead>
<tr>
<th>DONR Vector</th>
<th>att sites Flanking Insert</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR™ P4-P1R</td>
<td>attB4 attB1r</td>
<td>Fwd: 5’-GGGG ACA ACT TTG TAT AGA AAA GTT GNN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: 5’-GGGG AC TGC TTT TTT GTA CAA ACT TGN</td>
</tr>
<tr>
<td>pDONR™ 221</td>
<td>attB1 attB2</td>
<td>Fwd: 5’-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TNN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: 5’-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTN</td>
</tr>
<tr>
<td>pDONR™ P2R-P3</td>
<td>attB2r attB3</td>
<td>Fwd: 5’-GGGG ACA GCT TTC TTG TAC AAA GTG GNN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: 5’-GGGG AC AAC TTT GTA TAA TAA AGT TGN</td>
</tr>
</tbody>
</table>
Kit Contents and Storage

Shipping/Storage
The MultiSite Gateway® Three-Fragment Vector Construction Kit is shipped on dry ice in four boxes 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>Vectors</td>
<td>–20°C</td>
</tr>
<tr>
<td>2</td>
<td>BP Clonase® II Enzyme Mix</td>
<td>–20°C (6 months)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–80°C (long term)</td>
</tr>
<tr>
<td>3</td>
<td>LR Clonase® II Plus Enzyme Mix</td>
<td>–20°C (6 months)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–80°C (long term)</td>
</tr>
<tr>
<td>4</td>
<td>One Shot® TOP10 Chemically Competent E. coli</td>
<td>–80°C</td>
</tr>
</tbody>
</table>

Vectors
The Vectors box (Box 1) contains the following plasmids. Store Box 1 at –20°C.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR™ P4-P1R</td>
<td>6 μg at 150 ng/μl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).</td>
<td>40 μl</td>
</tr>
<tr>
<td>pDONR™ P2R-P3</td>
<td>6 μg at 150 ng/μl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).</td>
<td>40 μl</td>
</tr>
<tr>
<td>pDONR™ 221</td>
<td>6 μg at 150 ng/μl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).</td>
<td>40 μl</td>
</tr>
<tr>
<td>pDEST™ R4-R3 Vector II</td>
<td>6 μg at 150 ng/μl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).</td>
<td>40 μl</td>
</tr>
<tr>
<td>pMS/GW control plasmid</td>
<td>10 μg at 0.5 μg/μl, in TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

BP Clonase® II Enzyme Mix
The BP Clonase® II enzyme mix (Box 2) contains the following reagents. Store Box 2 at –20°C for up to 6 months. For long-term storage, store at –80°C.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP Clonase® II Enzyme Mix</td>
<td>Proprietary</td>
<td>40 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>
</tbody>
</table>

Continued on next page
Kit Contents and Storage, continued

**LR Clonase® II Plus Enzyme Mix**

The LR Clonase® II Plus enzyme mix (Box 3) contains the following reagents. 
*Store Box 3 at −20°C for up to 6 months. For long-term storage, store at −80°C.*

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR Clonase® II Plus Enzyme Mix</td>
<td>Proprietary</td>
<td>40 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>
</tbody>
</table>

**One Shot® TOP10 Reagents**

The One Shot® TOP10 Chemically Competent *E. coli* kit (Box 4) contains the following reagents. Transformation efficiency is 1 x 10⁹ cfu/μg DNA.
*Store Box 4 at −80°C.*

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.O.C. Medium (may be stored at room temperature or +4°C)</td>
<td>2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose</td>
<td>6 ml</td>
</tr>
<tr>
<td>TOP10 chemically competent cells</td>
<td>--</td>
<td>21 x 50 μl</td>
</tr>
<tr>
<td>pUC19 Control DNA</td>
<td>10 pg/μl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

**Genotype of TOP10 E. coli**

F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG

*Note: This strain cannot be used for single-strand rescue of DNA.*

**Product Use**

*For research use only.* Not intended for any human or animal diagnostic or therapeutic uses.
Many of the reagents supplied in the MultiSite Gateway® Three-Fragment Vector Construction Kit as well as other products suitable for use with the kit are available separately from Life Technologies. Ordering information for these reagents is provided below.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP Clonase® II Enzyme Mix</td>
<td>20 reactions</td>
<td>11789-020</td>
</tr>
<tr>
<td></td>
<td>100 reactions</td>
<td>11789-100</td>
</tr>
<tr>
<td>LR Clonase® II Plus Enzyme Mix</td>
<td>20 reactions</td>
<td>12538-120</td>
</tr>
<tr>
<td></td>
<td>100 reactions</td>
<td>12538-200</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>One Shot® ccdB Survival™ 2 T1® Chemically Competent cells</td>
<td>10 x 50 μl</td>
<td>A10460</td>
</tr>
<tr>
<td>pDONR™ 221</td>
<td>6 μg</td>
<td>12536-017</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>M13 Forward (−20) Sequencing Primer</td>
<td>2 μg</td>
<td>N520-02</td>
</tr>
<tr>
<td>M13 Reverse Sequencing Primer</td>
<td>2 μg</td>
<td>N530-02</td>
</tr>
<tr>
<td>Dpn I</td>
<td>100 units</td>
<td>15242-019</td>
</tr>
<tr>
<td>PureLink® HiPure Plasmid MidiPrep Kit</td>
<td>25 reactions</td>
<td>K2100-04</td>
</tr>
<tr>
<td>PureLink® HQ Mini Plasmid Purification Kit</td>
<td>100 reactions</td>
<td>K2100-01</td>
</tr>
<tr>
<td>PureLink® Gel Extraction Kit</td>
<td>50 reactions</td>
<td>K2100-12</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>MultiSite Gateway® Pro 2.0 Kit</td>
<td>20 reactions</td>
<td>12537-102</td>
</tr>
<tr>
<td>MultiSite Gateway® Pro 3.0 Kit</td>
<td>20 reactions</td>
<td>12537-102</td>
</tr>
<tr>
<td>MultiSite Gateway® Pro 4.0 Kit</td>
<td>20 reactions</td>
<td>12537-104</td>
</tr>
<tr>
<td>MultiSite Gateway® Pro Plus Kit</td>
<td>20 reactions</td>
<td>12537-100</td>
</tr>
</tbody>
</table>
Accessory Products, continued

**Gateway® Entry Vectors**

The MultiSite Gateway® Three-Fragment kit provides the pDONR™ 221 vector to facilitate creation of attL1 and attL2-flanked entry clones. Alternatively, a variety of Gateway® entry vectors are available from Life Technologies to allow creation of entry clones using TOPO® Cloning or restriction digestion and ligation. For more information about the various entry vectors and their features go to **www.lifetechologies.com** or contact Technical Support (see page 47).

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR®8/GW/TOPO® Cloning Kit</td>
<td>20 reactions</td>
<td>K2500-20</td>
</tr>
<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 reactions</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 Dual Selection Vector</td>
<td>10 μg</td>
<td>A10462</td>
</tr>
<tr>
<td>pENTR™/2B Dual Selection Vector</td>
<td>10 μg</td>
<td>A10463</td>
</tr>
<tr>
<td>pENTR™/3C Dual Selection Vector</td>
<td>10 μg</td>
<td>A10464</td>
</tr>
<tr>
<td>pENTR™/4 Dual Selection Vector</td>
<td>10 μg</td>
<td>A10465</td>
</tr>
<tr>
<td>pENTR™/11 Dual Selection Vector</td>
<td>10 μg</td>
<td>A10467</td>
</tr>
</tbody>
</table>

**Ultimate™ ORF Clones**

The Ultimate™ ORF (Open Reading Frame) Clones are fully sequenced human or mouse ORFs provided in the pENTR™ 221 Gateway® entry vector, allowing you to rapidly and efficiently transfer the ORF into any Gateway® destination vector. You may use an Ultimate™ ORF Clone in place of cloning the gene of interest into pDONR™ 221. For more information about using Ultimate ORF Clones in the MultiSite Gateway® Three-Fragment kit, go to **www.lifetechologies.com** or contact Technical Support (see page 47).

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate™ Human ORF Clones</td>
<td>1 clone</td>
<td>HORF01</td>
</tr>
<tr>
<td>Ultimate™ Mouse ORF Clones</td>
<td>1 clone</td>
<td>MORF01</td>
</tr>
</tbody>
</table>
Introduction

Overview

Introduction

The MultiSite Gateway® Three-Fragment Vector Construction Kit facilitates rapid and highly efficient construction of an expression clone containing your choice of promoter, gene of interest, and termination or polyadenylation sequence. Other sequences of interest may be easily substituted or incorporated, providing added flexibility for your vector construction needs. Based on the Gateway® Technology (Hartley et al., 2000), the MultiSite Gateway® Technology uses site-specific recombinational cloning to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation.

For more information about the Gateway® Technology, see the next page.

Important

The MultiSite Gateway® Three-Fragment Vector Construction Kit is designed to help you create a multiple-fragment clone or an expression clone using the MultiSite Gateway® Technology. Although the kit has been designed to help you produce your expression clone in the simplest, most direct fashion, use of the kit is geared towards those users who are familiar with the concepts of the Gateway® Technology and site-specific recombination. A working knowledge of the Gateway® Technology is recommended.

Purpose of This Manual

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

1. Design three sets of forward and reverse attB PCR primers, and PCR-amplify your DNA sequences of interest to generate PCR products that are flanked by attB or attBr sites for BP recombination.

2. Use each PCR product in separate BP recombination reactions with the appropriate donor vectors to generate entry clones containing your DNA sequences of interest.

3. Perform a MultiSite Gateway® LR recombination reaction with your three entry clones and the provided pDEST™ R4-R3 Vector II destination vector to generate an expression clone which may then be used in the appropriate application or expression system.
Gateway® Technology

Introduction

The Gateway® Technology is a universal cloning method based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the <i>E. coli</i> chromosome and the switch between the lytic and lysogenic pathways (Landy, 1989; Ptashne, 1992). In Gateway® Technology, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman et al., 1985), providing a rapid and highly efficient way to transfer heterologous DNA sequences into multiple vector systems for functional analysis and protein expression (Hartley et al., 2000). This section provides a brief overview of lambda recombination and the reactions that constitute the Gateway® Technology.

Lambda Recombination Reactions

In phage lambda, recombination occurs between phage and <i>E. coli</i> DNA via specific recombination sequences denoted as <i>att</i> sites. Recombination occurs following two pairs of strand exchanges and ligation of the DNAs in a novel form.

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 <i>att</i> sites are hybrid sequences comprised of sequences donated by each parental vector.

Recombination reactions are catalyzed by a mixture of enzymes that bind to the <i>att</i> sites, bring together the target sites, cleave them, and covalently attach the DNA. A different mixture of recombination proteins (Clonase® II enzyme mixes) is used depending upon whether lambda utilizes the lytic or lysogenic pathway.

Recombination Enzymes

The lysogenic pathway is catalyzed by phage lambda Integrase (Int) and <i>E. coli</i> Integration Host Factor (IHF) proteins (BP Clonase® II enzyme mix) while the lytic pathway is catalyzed by the phage lambda Int and Excisionase (Xis) proteins, and the <i>E. coli</i> Integration Host Factor (IHF) protein (LR Clonase® II Plus enzyme mix). For more information about the recombination enzymes, see published references and reviews (Landy, 1989; Ptashne, 1992).

Continued on next page
**Gateway® Technology, continued**

**attB, attP, attL, and attR**

*attB, attP, attL and attR are recombination sites that are utilized in the Gateway® Technology.*

*attB sites always recombine with attP sites in a reaction mediated by the BP Clonase® II enzyme mix:*

\[ \text{attB x attP} \rightarrow \text{attL x attR} \]

The BP reaction is the basis for the reaction between the donor vectors (pDONR™) and PCR products or other clones containing attB sites. Recombination between attB and attP sites yields attL and attR sites on the resulting plasmids. The entry clone containing the PCR product is used in the LR recombination reaction.

*attL sites always recombine with attR in a reaction mediated by LR Clonase® II or LR Clonase® II Plus enzyme mix:*

\[ \text{attL x attR} \rightarrow \text{attB x attP} \]

The LR reaction is the basis for the entry clones x destination vector reaction. Recombination between attL and attR sites yields attB and attP sites on the resulting plasmids. The expression clone containing the PCR product is used in your expression system. The by-product plasmid contains the ccdB gene and prevents growth if taken up by competent cells after transformation.

---

**For More Information**

For additional details about the Gateway® Technology, lambda DNA recombination, att sites, and the BP and LR recombination reactions, refer to the Gateway® Technology with Clonase® II manual. This manual is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 47).
MultiSite Gateway® Components

Introduction

The MultiSite Gateway® 3-Fragment Recombination Kit contains enzymes that catalyze the Gateway® recombination reactions (BP Clonase™ II and LR Clonase™ II Plus), three donor vectors, a destination vector, a control vector for the BP reaction, and One Shot® TOP10 Chemically Competent cells. More details about each component can be found below.

Vector NTI Advance® Software Users

The MultiSite Gateway® 3-Fragment Recombination Kit is compatible with Vector NTI Advance® sequence analysis software version 10.2 and higher. To begin using Vector NTI Advance® software go to www.lifetechnologies.com for detailed instructions.

MultiSite Gateway® Donor Vectors

The MultiSite Gateway® donor vectors are used to clone attB- or attBr-flanked PCR products to generate entry clones, and contain similar elements as other Gateway® donor vectors. However, because different attB sites will flank your PCR products, different donor vectors are required to facilitate generation of the entry clones. See the next section for detailed information.

See page 6 for more information about the general features of the donor vectors, and see pages 40-43 for maps and descriptions of the features of each MultiSite Gateway® Donor vector.

BP Clonase® II Enzyme Mix

BP Clonase® II enzyme mix is supplied with the kit to catalyze the BP recombination reaction. The BP Clonase® II enzyme mix combines the proprietary enzyme formulation and 5X BP Clonase® Reaction Buffer into an optimized single-tube format to allow easy set-up of the BP recombination reaction. Use the protocol provided on page 26 to perform the BP recombination reaction using BP Clonase® II enzyme mix.

LR Clonase® II Plus Enzyme Mix

The MultiSite Gateway® LR recombination reaction is catalyzed by LR Clonase® II Plus enzyme mix, which contains a proprietary combination of recombination proteins and reaction buffer provided in a single tube for convenient reaction set up. Gateway® LR Clonase® II Plus enzyme mix promotes in vitro recombination between attL- and attR-flanked regions on entry clones and destination vectors to generate attB-containing expression clones consisting of multiple DNA fragments.

Note: LR Clonase® or LR Clonase™ II enzyme mixes are not recommended for use in the MultiSite Gateway® LR recombination reaction. Use LR Clonase™ II Plus included in the kit.

Continued on next page
MultiSite Gateway® Components, continued

**MultiSite Gateway® Destination Vector**

The MultiSite Gateway® destination vector, pDEST™ R4-R3 Vector II, is designed for use in the MultiSite Gateway® three-fragment LR recombination reaction with the three entry clones. The pDEST™ R4-R3 Vector II vector contains attR4 and attR3 sites flanking a selection cassette and allows generation of the expression clone of interest.

See page 44 for a map and a description of the features of pDEST™ R4-R3 Vector II.

*Note:* Other Gateway® destination vectors are not suitable for use in the MultiSite Gateway® LR reaction.

---

**pMS/GW Control Vector**

The pMS/GW vector is included with the MultiSite Gateway® Three-Fragment Vector Construction Kit and contains multiple DNA fragments that have been joined using MultiSite Gateway® Technology. This expression clone is designed for use as a control for each BP recombination reaction (see page 24 for details). See page 46 for a map of pMS/GW.

---

**One Shot® TOP10 Chemically Competent E. coli**

One Shot® TOP10 Chemically Competent cells are included in the MultiSite Gateway® 3-Fragment Recombination Kit for transforming the BP and LR reactions. These cells should NOT be used to propagate destination or donor vectors, see page 9.
MultiSite Gateway® Donor Vectors

Introduction

The MultiSite Gateway® Donor vectors are used in a BP recombination reaction to clone attB or attBr-flanked PCR products to generate entry clones. The vectors contain similar elements as other Gateway® donor vectors.

Your PCR products will be flanked by different attB or attBr sites. Three different donor vectors facilitate generation of entry clones:

<table>
<thead>
<tr>
<th>Vector Insert</th>
<th>Insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDONR™ 221 P4-P1r</td>
<td>attB4 and attB1r-flanked PCR products</td>
</tr>
<tr>
<td>pDONR™ 221</td>
<td>attB1 and attB2-flanked PCR products</td>
</tr>
<tr>
<td>pDONR™ 221 P2r-P3</td>
<td>attB2r and attB3-flanked PCR products</td>
</tr>
</tbody>
</table>

Common Features of the MultiSite Gateway® Donor Vectors

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

- The ccdB gene (see below) for counter selection
- Chloramphenicol resistance gene (CmR) for counterscreening

After a BP recombination reaction, this cassette is replaced by the DNA element of interest to generate an entry clone.

ccdB Gene

The presence of the ccdB gene allows negative selection of the donor and destination vectors in E. coli following recombination and transformation. The CcdB protein interferes with E. coli DNA gyrase (Bernard & Couturier, 1992), thereby inhibiting growth of most E. coli strains (e.g. Mach1™, TOP10, DH5α™). When recombination occurs (i.e. between a destination vector and an entry clone or between a donor vector and an attB PCR product), the gene of interest replaces the ccdB gene. 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.

Modifications to the att Sites

To permit recombinational cloning using the Gateway® Technology, the wild-type λ att sites have been modified to improve the efficiency and specificity of the Gateway® BP and LR recombination reactions (see the Gateway® Technology manual for details).

In the MultiSite Gateway® System, the att sites have been optimized further to accommodate simultaneous, recombinational cloning of multiple DNA fragments. These modifications include alterations to both the sequence and length of the att sites, resulting in the creation of “new” att sites exhibiting enhanced specificities and the improved efficiency required to clone multiple DNA fragments in a single reaction.

Various combinations of these attB sites will flank each PCR product containing your DNA fragments of interest, depending on the number of fragments and their orientation.

Continued on next page
In general, the modified att sites in the MultiSite Gateway® Technology demonstrate the same specificity as in the Gateway® Technology. That is:

- attB sites react only with attP sites; for example attB1 sites react only with attP1 sites to generate attL1 sites
- attL sites react only with attR sites; for example attL1 sites react only with attR1 sites to generate attB1 sites

att sites are not palindromic and have an orientation. The direction of the arrow designates two possible orientations of the att sites in relation to the insert. When the arrow does not point towards the insert, the attP or attB site is designated with an “r”. In the example below, the attB1r site flanks the PCR product and an attP1r site resides on the donor vector generating an attR1 site in the entry clone:

Performing the BP recombination reaction with an attBr and attPr site will result in creation of an attR site instead of an attL site in the entry clone.

In the BP recombination reactions:

- attB1r sites react with attP1r sites to generate attR1 sites in the entry clone
- attB2r sites react with attP2r sites to generate attR2 sites in the entry clone

Example

In this example, an attB4 and attB1r-flanked PCR product is used in a BP recombination reaction with pDONR™ P4-P1r:

attB4-PCR product-attB1r × pDONR™ P4-P1r → pENTR™ attL4-PCR product-attR1

Because of the orientation and position of the attB1r site in the PCR product and the attP1r site in the donor vector, the resulting entry clone contains the PCR product flanked by an attL4 site and an attR1 site rather than two attL sites.
In the MultiSite Gateway® 3-Fragment recombination reaction, three PCR products (5' element, gene of interest, and 3' element) flanked by specific attB or attBr sites and three MultiSite Gateway® Donor vectors are used in separate BP recombination reactions to generate three entry clones. The three entry clones and the destination vector, pDEST™ R4-R3 Vector II are used together in a MultiSite Gateway® LR recombination reaction to create your expression clone.
Methods

Propagating the MultiSite Gateway® Vectors

Introduction

The MultiSite Gateway® Three-Fragment Vector Construction Kit includes the pDONR™ P4-P1R, pDONR™ 221, and pDONR™ P2R-P3 Donor vectors, the pDEST™ R4-R3 Vector II destination vector, and the pMS/GW control vector. See the guidelines below to propagate and maintain these vectors.

Propagating Donor and Destination Vectors

If you wish to propagate and maintain the MultiSite Gateway® donor and destination vectors prior to recombination, we recommend using One Shot® ccdB Survival™ 2 T1R Chemically Competent Cells for transformation. These cells are available separately from Life Technologies (page ix).

The ccdB Survival 2 T1R 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 as follows:

- For pDONR™ vectors, use LB plates containing 50 μg/ml kanamycin and 15–30 μg/ml chloramphenicol.
- For pDEST™ R4-R3 Vector II, use LB plates containing 100 μg/ml ampicillin and 15–30 μg/ml chloramphenicol.

Important

Do not use general E. coli cloning strains including One Shot® TOP10 (included with the kit) or strains such as DH5α™ for propagation and maintenance of the donor and destination vectors, as these strains are sensitive to ccdB effects.

Propagating the pMS/GW Vector

To propagate and maintain the pMS/GW plasmid, you may use any recA, endA E. coli strain including TOP10, DH5α™, or DH10B™ for transformation. We recommend using the One Shot® TOP10 Chemically Competent E. coli included with the kit for transformation. Select for transformants in media containing 50–100 μg/ml ampicillin.
General Information for Entry Clones

Introduction

To use the MultiSite Gateway® Three-Fragment kit to construct your own expression clone, you will create 3 types of entry clones, then use these entry clones in a MultiSite Gateway® LR recombination reaction with a MultiSite Gateway® destination vector to generate your expression clone. For proper expression of the gene of interest, these entry clones should, at a minimum, contain the sequences described below.

- An attL4 and attR1-flanked entry clone containing your 5′ element of interest. The 5′ element typically contains promoter sequences required to control expression of your gene of interest. Other additional sequences including an N-terminal fusion tag may be added.

- An attL1 and attL2-flanked entry clone containing your DNA fragment of interest. This DNA fragment generally encodes the gene of interest. To obtain proper expression in the system of choice, remember to include sequences necessary for efficient translation initiation (i.e. Shine-Dalgarno, Kozak consensus sequence, yeast consensus sequence).

- An attR2 and attL3-flanked entry clone containing your 3′ element of interest. The 3′ element typically contains transcription termination sequences or polyadenylation sequences required for efficient transcription termination and polyadenylation of mRNA. Other additional sequences including a C-terminal fusion tag may be added.

For more information about how to generate each type of entry clone, see pages 12-14.

Important

If you construct an expression clone containing the elements described above (i.e. promoter of choice + gene of interest + termination or polyadenylation sequence of choice), remember that this expression clone will be expressed transiently in mammalian, yeast, and insect systems, but may be expressed stably in prokaryotic systems. To perform stable expression studies in mammalian, yeast, or insect systems, include a resistance marker in one of the entry clones (generally the attR2 and attL3-flanked entry clone).

Continued on next page
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. The design of the PCR primers to amplify your DNA sequences of interest is critical for recombinational cloning using MultiSite Gateway® Technology. Your primer design must incorporate:

- Sequences required to facilitate MultiSite Gateway® cloning (att sites).
- Sequences required for efficient expression of the protein of interest (i.e. promoter sequences, termination or polyadenylation sequences, Shine-Dalgarno or Kozak consensus sequences) (Kozak, 1987; Kozak, 1990; Kozak, 1991; Shine & Dalgarno, 1975).

Each PCR product must be flanked by a different combination of attB or attBr sites:

<table>
<thead>
<tr>
<th>DNA Element</th>
<th>Flanking att sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ element</td>
<td>attB4, attB1r</td>
</tr>
<tr>
<td>Gene of interest</td>
<td>attB1, attB2</td>
</tr>
<tr>
<td>3’ element</td>
<td>attB2r, attB3</td>
</tr>
</tbody>
</table>

For more information on designing attB and attBr-flanked primers, see the following sections on page 16-17.

The MultiSite Gateway® 3 Fragment Vector Construction Kit is compatible with Vector NTI Advance® software version 10 and above. Go to [www.lifetechnologies.com](http://www.lifetechnologies.com) for detailed instructions to use the Vector NTI Advance® software to design attB and attBr primers for your DNA elements of choice.

**Primer Concentration**

- 50 nmoles of standard purity DNA is recommended.
- Dissolve oligonucleotides to 20–50 mM in water or TE Buffer and verify the concentration before use.
- For more efficient cloning when primer length is >50 bp, we recommend using HPLC or PAGE-purified oligonucleotides.

For your convenience, Life Technologies offers a custom primer synthesis service. Go to [www.lifetechnologies.com](http://www.lifetechnologies.com) for more information.
Making Entry Clones

Generating Entry Clone for 5’ Element

To generate an attL4 and attR1-flanked entry clone containing your 5’ element of interest:

1. Design appropriate PCR primers and produce your attB4 and attB1r-flanked PCR product (see pages 16-17).

2. Perform a BP recombination reaction between the attB4 and attB1r-flanked PCR product and pDONR™ P4-P1R to generate the entry clone (see figure below).

Generating Entry Clone for 3’ Element

To generate an attR2 and attL3-flanked entry clone containing your 3’ element of interest:

1. Design appropriate PCR primers and produce your attB2r and attB3-flanked PCR product (see pages 16-17).

2. Perform a BP recombination reaction between the attB2r and attB3-flanked PCR product and pDONR™ P2R-P3 to generate the entry clone (see figure below).

Continued on next page
Making Entry Clones, continued

Generating attL1 and attL2-Flanked Entry Clones

The attL1 and attL2-flanked entry clone contains your gene of interest and can be used with both MultiSite Gateway® and traditional Gateway® applications. This entry clone may be generated using a variety of methods.

1. Generate a PCR product containing attB1 and attB2 sites and use this attB PCR product in a BP recombination reaction with the pDONR™ 221 vector, provided with the kit. To use this method, refer to the guidelines and instructions provided on the next page.

2. 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 the pDONR™ 221 vector (see the Gateway® Technology with Clonase® II manual for more information).

3. Clone a restriction enzyme fragment into a pENTR™ vector (see page 14 for more information).

4. TOPO® Clone a PCR product into a pENTR™-TOPO® vector (see page 14 for more information).

5. Purchase a clone from the Life Technologies Ultimate™ ORF collection (see page x).

6. Use Life Technologies’s Custom Cloning Service to make a custom vector. Go to www.lifetechnologies.com for more information.

Entry point (cDNA, genomic DNA, cDNA library, gene synthesis product)

1.) attB1 and attB2-flanked PCR product or attB-expression clone

2.) attB1 and attB2-flanked cDNA clone

3.) restriction enzyme fragment

4.) PCR product

Recombine with pDONR™221 vector

Clone into pENTR™ vector

Clone into pENTR-TOPO® vector

5.) Ultimate™ ORF, or

6.) Custom made Entry clone

Entry clone

Continued on next page
Making Entry Clones, continued

Generating Entry Clone for the Gene of Interest

To generate an attL1 and attL2-flanked entry clone containing your gene of interest:

1. Design appropriate PCR primers and produce your attB1 and attB2-flanked PCR product (see pages 16-17).
2. Perform a BP recombination reaction between the attB1 and attB2-flanked PCR product and pDONR™ 221 to generate the entry clone (see figure below).

Entry Vectors

Many entry vectors are available from Life Technologies 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 & 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.

<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 Dual Selection Vector</td>
<td>•</td>
<td>•</td>
<td>A10462</td>
</tr>
<tr>
<td>pENTR™ 2B Dual Selection Vector</td>
<td>•</td>
<td>•</td>
<td>A10463</td>
</tr>
<tr>
<td>pENTR™ 3C Dual Selection Vector</td>
<td>•</td>
<td>•</td>
<td>A10464</td>
</tr>
<tr>
<td>pENTR™ 4 Dual Selection Vector</td>
<td>•</td>
<td>•</td>
<td>A10465</td>
</tr>
<tr>
<td>pENTR™ 11 Dual Selection Vector</td>
<td>•</td>
<td>•</td>
<td>A10467</td>
</tr>
</tbody>
</table>

To construct an entry clone using one of the pENTR™ vectors, refer to the manual for the specific entry vector you are using. All entry vector manuals are available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 47).
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. To facilitate use in MultiSite Gateway®, each PCR product must be flanked by a different combination of *attB* sites. We strongly suggest Vector NTI Advance® software to design *attB* and *attBr* primers. Guidelines are provided below to help you design appropriate PCR primers.

**Designing Your PCR Primers**

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

- Sequences required to facilitate MultiSite Gateway® cloning.
- Sequences required for efficient expression of the protein of interest *(i.e. promoter sequences, termination or polyadenylation sequences, Shine-Dalgarno or Kozak consensus sequences).*
- Whether or not you wish your PCR product(s) to be fused in frame with any N- or C-terminal fusion tags. Note that sequences encoding the tag are generally incorporated into your PCR product as part of the 5' or 3' element.

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

Guidelines to Design the Forward PCR Primers

When designing the appropriate forward PCR primer, consider the points below. Refer to the diagram on the next page for more help.

- To enable efficient MultiSite Gateway® cloning, the forward primer **MUST** contain the following structure:
  1. Four guanine (G) residues at the 5′ end followed by
  2. The 22 or 25 bp $attB$ site followed by
  3. 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 & Dalgarno, 1975) or Kozak consensus sequence (Kozak, 1987; Kozak, 1990; Kozak, 1991), respectively, in the $attB1$ forward PCR primer.

\[
attB1 \quad 5′-\text{GGGG-ACG-AGT-TTG-TAC-AAA-AAA-GCA-GUC-TNN}-(\text{template-specific sequence})-3′
\]

\[
attB2r \quad 5′-\text{GGGG-ACG-GCT-TTC-TTG-TAC-AAA-GTN}-(\text{template-specific sequence})-3′
\]

\[
attB4 \quad 5′-\text{GGGG-ACG-ACT-TTG-TAC-AGA-AAA-GTT-GNN}-(\text{template-specific sequence})-3′
\]

- The $attB4$ and $attB2r$ sites end with a guanine (G), and the $attB1$ site with a thymine (T). If you wish to fuse your PCR product in frame with an N- or C-terminal tag (as appropriate), the primer must include two additional nucleotides to maintain the proper reading frame (see diagram on the next page). Note that the two additional nucleotides in the $attB1$ primer **cannot** be AA, AG, or GA because these additions will create a translation termination codon.

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

**Guidelines to Design the Reverse PCR Primers**

When designing your reverse PCR primer, consider the points below. Refer to the diagram below for more help.

- To enable efficient MultiSite Gateway® cloning, the reverse primer **MUST** contain the following structure:
  1. Four guanine (G) residues at the 5′ end followed by
  2. The 22 or 25 bp *attB* site followed by
  3. 18–25 bp of template- or gene-specific sequences

- If you wish to fuse your PCR product in frame with an N- or C-terminal tag:
  1. The *attB*1r and *attB*2 reverse primers must include one additional nucleotide to maintain the proper reading frame (see diagram below).
  2. Any in-frame stop codons between the *attB* sites and your gene of interest must be removed.

  - *attB*1r 5′-GGGG-AC-TGC-TTT-TTT-GTA-CAA-ACT-TGN--(template-specific sequence)-3′
  - *attB*2 5′-GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTN--(template-specific sequence)-3′
  - *attB*3 5′-GGGG-AC-AAC-TTT-GTA-TAA-TAA-AGT-TGN--(template-specific sequence)-3′

- If you do not wish to fuse your PCR product in frame with a C-terminal tag, your gene of interest or the *attB*2 primer must include a stop codon.
Producing \textit{attB} PCR Products

**DNA Templates**

The following DNA templates can be used for amplification with \textit{attB}-containing PCR primers:

- Genomic DNA
- cDNA from reverse transcription reaction
- cDNA libraries
- Plasmids containing cloned DNA sequences
- \textit{De novo} gene synthesis

**Recommended Polymerases**

We recommend using the following DNA polymerases available from Life Technologies to produce your \textit{attB} PCR products. See page ix for ordering information.

- To generate PCR products less than 5–6 kb for use in protein expression, use Platinum® \textit{Pfx} DNA Polymerase.
- To generate PCR products for use in other applications (e.g. functional analysis), use Platinum® \textit{Taq} DNA Polymerase High Fidelity.

**Producing PCR Products**

Standard PCR conditions can be used to prepare \textit{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.

\textbf{Note:} \textit{attB} sequences do not affect PCR product yield or specificity.

**Checking the PCR Product**

Remove 1–5 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 \textbf{Purifying \textit{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 \textit{Dpn I} before purifying the \textit{attB} PCR product. This treatment degrades the plasmid \textit{(i.e. Dpn I recognizes methylated GATC sites)} and helps to reduce background in the BP-recombination reaction associated with template contamination.

**Materials Needed:**

- \textit{Dpn I}
- 10X REact® Buffer, included with the enzyme

**Protocol:**

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer (included with enzyme) and ≥ 5 units of \textit{Dpn I}.
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the \textit{Dpn I} at 65°C for 15 minutes.
4. Proceed to \textbf{Purifying \textit{attB} PCR Products}, next page.
Purifying *attB* PCR Products

**Introduction**

After you have generated your *attB* PCR products, we recommend purifying each 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 products.

**Important**

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.

**Materials Needed**

You should have the following materials on hand before beginning:

- Each *attB* PCR product (in a 50 μl volume)
- 1x TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 30% PEG 8000/30 mM MgCl₂ (supplied with the kit)
- Agarose gel of the appropriate percentage to resolve your *attB* PCR products

**PEG Purification Protocol**

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

1. Add 150 μl of TE, pH 8.0 to a 50 μl amplification reaction containing your *attB* PCR product.
2. 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.
3. Carefully remove the supernatant. The pellet will be clear and nearly invisible.
4. Dissolve the pellet in 50 μl of 1x TE, pH 8.0 (to concentration > 10 ng/μl).
5. Check the quality and quantity of the recovered *attB* PCR product on an agarose gel.
6. If the PCR product is suitably purified, proceed to *Creating Entry Clones Using the BP Recombination Reaction*, page 20. If the PCR product is not suitably purified (*e.g. attB* primer-dimers are still detectable), see below.

**Additional Purification**

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 PureLink® Gel Extraction Kit available from Life Technologies (see page ix).
Creating Entry Clones Using the BP Recombination Reaction

**Introduction**

Once you have generated your attB PCR products, you will perform BP reactions to transfer the DNA sequence of interest into an attP-containing donor vector to create entry clones. To ensure that you obtain the best possible results, we suggest that you read this section (pages 20-26) and Transforming One Shot® TOP10 Competent Cells (pages 27-29) before beginning.

**Experimental Outline**

To generate an entry clone, you will:

1. Perform a BP recombination reaction using the appropriate linear attB PCR product and a supercoiled, attP-containing donor vector (see page 26).

2. Transform the reaction mixture into a suitable E. coli host (see page 27).

3. Select for entry clones (see page 30).

**Important**

For optimal results, perform the BP recombination reaction using:

- **Linear** attB PCR products
- **Supercoiled** donor vector

**Recombination Regions**

The MultiSite Gateway® BP recombination reactions involve a specific combination of attB- and attBr-flanked PCR products and specific corresponding donor vectors. An illustration of the BP recombination regions of each pDONR™ vectors are provided on the following pages.

*Continued on next page*
Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of the attL4 and attR1-Flanked Entry Clone

The recombination region of the entry clone resulting from pDONR™ P4-P1R × attB4-5' element-attB1r is shown below.

Features of the Recombination Region:
- Shaded regions correspond to those DNA sequences transferred from the attB PCR product into the pDONR™ P4-P1R vector by recombination. Non-shaded regions are derived from the pDONR™ P4-P1R vector.
- Bases 674 and 2830 of the pDONR™ P4-P1R sequence are marked.

Continued on next page
Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of the attL1 and attL2-Flanked Entry Clone

The recombination region of the entry clone resulting from pDONR™ 221 × attB1-gene of interest-attB2 is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the attB PCR product into the pDONR™ 221 vector by recombination. Non-shaded regions are derived from the pDONR™ 221 vector.
- Bases 651 and 2897 of the pDONR™ 221 sequence are marked.

Continued on next page
Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of the
attR2 and attL3-Flanked Entry Clone

The recombination region of the entry clone resulting from pDONR™ P2R-P3 × attB2r-3' element-attB3 is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the attB PCR product into the pDONR™ P2R-P3 vector by recombination. Non-shaded regions are derived from the pDONR™ P2R-P3 vector.
- Bases 733 and 2889 of the pDONR™ P2R-P3 sequence are marked.

M13 Forward (-20) priming site

531 GACGTTGTAA AACGACGGCC AGTCTTAAGC TCGGCCCCCTG CAGCTCTAGA GCTCGAATTCT

591 TACAGGTCAC TAATACACAC TAAGTATTTG ATTCCATAGT ACTGCATATG TTGTGTATTA
ATGTCCAGTG ATTATGCTAG ATTCATCAAAC TAAGTATAC ACAGCTATAC AACACAAAT

attR2

651 CAGTATATATG TAATCCTGTGT TTTATGCAAAT ATCTAATTTA ATATATTGAT ATTTATATAC
GTCATAATAC ATCAGACAAGAA AAATACGTTT TAGATTAATAT TATATAACTA TAAAATATAGT

733

711 TTTTACGTTT CTGCCTCA ACT TTA TTA TAC AAA GTG GNN PPP PPP PPP PPP PPP PPP PPP PPP PPP PPP 3' Element NCA
AAATGCAAAA GACGAAGT TGA AAG AAC ATG TTT CAC CNN

2889

2884 ACT TTA TTA TAC AAA GTT GGCATTA AAAAAAGCAT TGGTATCAAT TGTATGGCAAC
TGA AAT AAT ATG TTT CAA CCGTAATAT TTTTCCGTAA CGAATAGTTA AACAACGTTG

attL3

2941 GAACAGGTCA CTATCAGTCA AAATAAATAT CTATATTGGA GCTCCATGGT AGCGTAAACG
CTGTCCAGT GATAGTCAGT TTTATTTTAG TAAATAACCT

3001 CGGCCGGCAT ATCC CCTATA GTGAGTCGTA TTACATGGTC ATAGCTTTT CCTGCAGCT

M13 Reverse priming site
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 the appropriate attB PCR product and donor vector, and to transform the reaction mixture into a suitable E. coli host to select for entry clones. We recommend including a positive control and a negative control (no BP Clonase® II) to help you evaluate your results.

Donor Vectors

All donor vectors are supplied as 6 μg of supercoiled plasmid, resuspended in 40 μl of TE Buffer, pH 8.0 at a final concentration of 150 ng/μl.

Positive Control

pMS/GW is included with the MultiSite Gateway® Three-Fragment Vector Construction Kit for use as a positive control for each BP reaction, and contains multiple DNA fragments that have been joined using MultiSite Gateway® Technology (see page 46 for a map and more information). For an alternate positive control when creating an attL1 and attL2-flanked entry clone, see below.

The pMS/GW plasmid is supplied as 10 μg of supercoiled plasmid, resuspended in 20 μl TE Buffer, pH 8.0 at a final concentration of 0.5 μg/μl. To propagate the plasmid, see page 9.

Linearizing the Positive Control

You will need to linearize the pMS/GW plasmid before it may be used as a control for each BP reaction. We recommend linearizing the vector by restriction digest using Aat II.

1. Digest 5 μg of pMS/GW plasmid with Aat II in a 50 μl reaction using the manufacturer’s instructions.
2. Incubate the reaction at 70°C for 1 hour to inactivate the Aat II.
3. Proceed to Setting Up the BP Reaction, page 26. Note that the concentration of the digested DNA is 100 ng/μl.

Alternate Positive Control

When creating attL1 and attL2-flanked entry clones, you may use the pEXP7-tet supplied with the kit as a positive control in a BP reaction with pDONR™ 221. pEXP7-tet is an approximately 1.4 kb linear fragment and contains attB1 and attB2 sites flanking the tetracycline resistance gene and its promoter (Tc').

Continued on next page
Performing the BP Recombination Reaction, continued

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

For optimal efficiency, we recommend using the following amounts of attB PCR product and donor vector in a 10 μl BP recombination reaction:

- An equimolar amount of attB PCR product and the donor vector
- 50 femtomoles (fmoles) each of attB PCR product and donor vector is preferred, but the amount of attB PCR product used may range from 20–50 fmoles

Note: 50 fmoles of donor vector (pDONR™ P4-P1R, pDONR™ 221, or pDONR™ P2R-P3) is approximately 150 ng

- For large PCR products (>4 kb), use at least 50 fmoles of attB PCR product, but no more than 250 ng

For a formula to convert fmoles of DNA to nanograms (ng) and an example, see below.

- Do not use more than 250 ng of donor vector in a 10 μl BP reaction, as this will affect the efficiency of the reaction.
- Do not exceed more than 500 ng of total DNA (donor vector plus attB PCR product) in a 10 μl BP reaction, as excess DNA will inhibit the reaction.

Converting Femtomoles (fmoles) to Nanograms (ng)

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

\[
ng = (x \text{ fmoles})(N) \left(\frac{660 \text{ fg}}{\text{fmoles}}\right) \left(\frac{1 \text{ ng}}{10^6 \text{ fg}}\right)
\]

where x is the number of fmoles and N is the size of the DNA in bp. For an example, see below.

Example of fmoles to ng Conversion

In this example, you need to use 50 fmoles 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 above:

\[
(50 \text{ fmoles})(2500 \text{ bp}) \left(\frac{660 \text{ fg}}{\text{fmoles}}\right) \left(\frac{1 \text{ ng}}{10^6 \text{ fg}}\right) = 82.5 \text{ ng of PCR product required}
\]

Continued on next page
Performing the BP Recombination Reaction, continued

Materials Needed

You should have the following materials on hand before beginning.

**Supplied with the kit:**
- pDONR™ vectors (i.e. pDONR™ P4-P1R, pDONR™ 221, and pDONR™ P2R-P3; resuspend each vector to 150 ng/μl with water)
- BP Clonase® II enzyme mix (keep at –20°C until immediately before use)
- 2 μg/μl Proteinase K solution (thaw and keep on ice until use)
- pMS/GW control plasmid (linearize before use; 100 ng/μl)
- pEXP7-tet positive control (50 ng/μl; optional)

**Supplied by the user:**
- attB PCR products (i.e. attB4-PCR product-attB1, attB1-PCR product-attB2, or attB2-PCR product-attB3; see the previous page and above to determine the amount of DNA to use)
- 1x TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

Setting Up the BP Reaction

1. For each BP recombination reaction between an appropriate attB PCR product and donor vector, add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.

<table>
<thead>
<tr>
<th>Components</th>
<th>Sample</th>
<th>Negative Control</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB PCR product (20–50 fmoles)</td>
<td>1–7 μl</td>
<td>1–7 μl</td>
<td>--</td>
</tr>
<tr>
<td>pDONR™ vector (150 ng/μl)</td>
<td>1 μl</td>
<td>1 μl</td>
<td>1 μl</td>
</tr>
<tr>
<td>pMS/GW control plasmid (100 ng/μl)</td>
<td>--</td>
<td>--</td>
<td>2 μl</td>
</tr>
<tr>
<td>TE Buffer, pH 8.0</td>
<td>to 8 μl</td>
<td>to 10 μl</td>
<td>5 μl</td>
</tr>
</tbody>
</table>

**Note:** If you are using pEXP7-tet as a positive control, use 100 ng (2 μl) in place of the pMS/GW DNA.

2. Remove the BP Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).

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

4. To each sample above except the negative control, add 2 μl of BP Clonase® II enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

**Reminder:** Return BP Clonase® II enzyme mix to –20°C immediately after use.

5. Incubate reactions at 25°C for 1 hour.

**Note:** A 1-hour incubation generally yields 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 1 μl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.

7. Proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

**Note:** You may store the BP reaction at –20°C for up to 1 week before transformation.
Transforming One Shot® TOP10 Competent Cells

Introduction

Use the guidelines and procedures provided in this section to transform competent E. coli with the BP recombination reaction or the MultiSite Gateway® LR recombination reaction to select for entry clones or expression clones, respectively. One Shot® TOP10 chemically competent E. coli (Box 4) are included with the kit for use in transformation. However, you may also transform electrocompetent cells. Instructions to transform chemically competent or electrocompetent E. coli are provided in this section.

Note

You may use any recA, endA E. coli strain including One Shot® TOP10 chemically competent E. coli (supplied with the kit), 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.

Materials Needed

You should have the following materials on hand before beginning.

Supplied with the kit:

- One Shot® TOP10 chemically competent E. coli (thaw on ice 1 vial of One Shot® TOP10 cells for each transformation)
- S.O.C. medium (warm to room temperature)
- Positive control (e.g. pUC19; use as a control for transformation if desired)

Supplied by the user:

- BP recombination reaction (from Setting Up the BP Reaction, Step 7, page 26)
  OR
  MultiSite Gateway® LR recombination reaction (from Setting Up the MultiSite Gateway® LR Reaction, Step 7, page 35)
- 2 LB prewarmed plates containing 50 μg/ml kanamycin (for BP reaction)
  OR
  2 LB prewarmed plates containing 50–100 μg/ml ampicillin (for LR reaction).
- 42°C water bath (for chemical transformation)
- 37°C shaking and non-shaking incubators

Continued on next page
Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Add ONE of the following into a vial of One Shot® TOP10 chemically competent E. coli and mix gently. Do not mix by pipetting up and down.
   - Add 1 μl of the BP recombination reaction (from Setting Up the BP Reaction, Step 7, page 26)
   - OR
   - Add 2 μl of the MultiSite Gateway® LR recombination reaction (from Setting Up the MultiSite Gateway® LR Reaction, Step 7, page 35)

   Reminder: If you are including the transformation control, add 1 μl (10 pg) of pUC19.

2. Incubate on ice for 5 to 30 minutes.

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

4. Immediately transfer the tubes to ice.

5. Add 250 μl of room temperature S.O.C. medium.

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

7. Spread the following amount from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We generally plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies.
   - BP recombination reaction: spread 20 μl and 100 μl
   - MultiSite Gateway® LR recombination reaction: spread 50 μl and 100 μl

What You Should See

- BP reaction: An efficient BP recombination reaction may produce hundreds of colonies (greater than 1,500 colonies if the entire reaction is transformed and plated).

- MultiSite Gateway® LR reaction: An efficient MultiSite Gateway® LR recombination reaction may produce approximately 100 colonies (approximately 1,000 to 5,000 if 10 μl is transformed and plated).

Continued on next page
Transforming One Shot® TOP10 Competent Cells, continued

Transformation by Electroporation

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

1. Into a 0.1 cuvette containing 50 μl of electrocompetent E. coli, add ONE of the following and mix gently. Do not mix by pipetting up and down. Avoid formation of bubbles.
   • 1 μl of the BP recombination reaction (from Setting Up the BP Reaction, Step 7, page 26)
   OR
   • 2 μl of the MultiSite Gateway® LR recombination reaction (from Setting Up the MultiSite Gateway® LR Reaction, Step 7, page 35).

2. Electroporate your samples using an electroporator and the manufacturer’s suggested protocol.
   Note: If you have problems with arcing, see below.

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

4. Transfer the solution to a 15 ml snap-cap tube (i.e. Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance marker.

5. 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.

6. An efficient recombination reaction may produce several hundred 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, transform 1 μl into cells
Sequencing Entry Clones

**Introduction**

After BP recombination, we strongly recommend sequencing the entry clones to ensure that the inserts do not contain errors introduced during PCR. Sequencing can be performed using any method of choice using the M13 Forward (–20) and M13 Reverse primers (available separately from Life Technologies, see page ix).

**Sequencing Primers**

To sequence entry clones derived from BP recombination with pDONR™ P4-P1R, pDONR™ 221, and pDONR™ P2R-P3, we recommend using the following sequencing primers:

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>M13 Forward (–20): 5’-GTAAAACGACGGCCAG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse primer</td>
<td>M13 Reverse: 5’-CAGGAAACAGCTATGAC-3’</td>
</tr>
</tbody>
</table>

See the diagrams on pages 21-23 for the location of the M13 forward (–20) and M13 reverse primer binding sites in each entry clone.
MultiSite Gateway® LR Recombination Reaction

Introduction

After you have generated entry clones containing your 5′ element, gene of interest, and 3′ element, you will perform the MultiSite Gateway® LR recombination reaction to simultaneously transfer the three DNA fragments into the pDEST™ R4-R3 Vector II destination vector to create an attB-containing expression clone.

To ensure that you obtain the best results, we suggest reading this section and the next section entitled Performing the LR Recombination Reaction (pages 33-35) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform a MultiSite Gateway® LR recombination reaction using the appropriate entry clones and pDEST™ R4-R3 Vector II (see below).
2. Transform the reaction mixture into a suitable E. coli host (see page 27).
3. Select for MultiSite Gateway® expression clones (see page 32 for a diagram of the recombination region).

Substrates for the MultiSite Gateway® LR Recombination Reaction

To perform a three-fragment MultiSite Gateway® LR recombination reaction, you must have the substrates listed below.

- attL4 and attR1-containing entry clone
- attL1 and attL2-containing entry clone
- attR2 and attL3-containing entry clone
- pDEST™ R4-R3 Vector II destination vector (see the next page for more information)

Keep in mind the following:

- You cannot successfully create a three-fragment expression clone using the MultiSite Gateway® LR recombination reaction if you have any combination of att-flanked entry clones other than the ones listed above.
- You must use the pDEST™ R4-R3 Vector II destination vector for the three-fragment MultiSite Gateway® LR recombination reaction. Other Gateway® destination vectors cannot be used.

Important

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

- Supercoiled entry clones
- Supercoiled pDEST™ R4-R3 Vector II

Continued on next page
MultiSite Gateway® LR Recombination Reaction, continued

Recombination Region of the Expression Clone

The recombination region of the expression clone resulting from pDEST™ R4-R3 Vector II × attL4-5' entry clone-attR1 × attL1-entry clone-attL2 × attR2-3' entry clone-attL3 is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the three entry clones into the pDEST™ R4-R3 Vector II vector by recombination. Note that the sequences comprising the attB1 and attB2 sites are entirely supplied by the entry clones. Non-shaded regions are derived from the pDEST™ R4-R3 Vector II vector.
- Bases 43 and 2175 of the pDEST™ R4-R3 Vector II sequence are indicated.
Performing the LR Recombination Reaction

Important

A new enzyme (LR Clonase® II Plus) is supplied in this kit, and the MultiSite Gateway® LR recombination reaction protocol has been changed. Follow the protocol below carefully.

Introduction

Guidelines and instructions are provided in this section to:

• Perform a MultiSite Gateway® LR recombination reaction between suitable entry clones and pDEST™ R4-R3 Vector II using LR Clonase® II Plus enzyme mix.
• Transform the reaction mixture into a suitable E. coli host (see below)
• Select for an expression clone

We recommend including a positive control (see below) and a negative control (no LR Clonase® II Plus) in your experiment to help you evaluate your results.

pDEST™ R4-R3 Vector II

pDEST™ R4-R3 Vector II is supplied as 6 μg of plasmid, resuspended in 40 μl of TE Buffer, pH 8.0 at a final concentration of 150 ng/μl. To propagate the vector, see page 9.

Determining How Much DNA to Use in the Reaction

For optimal efficiency, we recommend using the following amounts of plasmid DNA (i.e. entry clones and destination vector) in a 10 μl MultiSite Gateway® LR recombination reaction:

• An equimolar amount of each plasmid
• 10 fmoles of each entry clone and 20 fmoles of pDEST™ R4-R3 Vector II is recommended
• Do not use more than 60 fmoles of total plasmid DNA in a 10 μl MultiSite Gateway® LR reaction as this will affect the efficiency of the reaction

For a formula to convert fmoles of DNA to nanograms (ng) and an example, see page 25.

Continued on next page
Performing the LR Recombination Reaction, continued

**E. coli Host**

We recommend using the One Shot® TOP10 Chemically Competent E. coli supplied with the kit for transformation. If you wish to use another E. coli strain, note that any recA, endA E. coli strain is suitable.

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: To use the One Shot® TOP10 chemically competent cells for transformation, see the section entitled *Transforming One Shot® TOP10 Competent Cells*, pages 27-29.

**Positive Control**

If you used the pMS/GW plasmid as a control for each BP recombination reaction, you may use the resulting three entry clones as controls in a MultiSite Gateway® LR recombination reaction with pDEST™ R4-R3 Vector II.

**Preparing Purified Plasmid DNA**

You will need to have purified plasmid DNA of each entry clone to perform the MultiSite Gateway® LR recombination reaction. You may use any method of choice to isolate purified plasmid DNA. We recommend using the PureLink® HiPure Plasmid MidiPrep Kit or the PureLink® HQ Mini Plasmid Purification Kit available from Life Technologies (see page ix).

**Materials Needed**

You should have the following materials on hand before beginning.

Supplied with the kit:
- pDEST™ R4-R3 Vector II (150 ng/μl in TE, pH 8.0)
- LR Clonase® II Plus enzyme mix
- 2 μg/μl Proteinase K solution

Supplied by the user:
- Purified plasmid DNA of your attL4 and attR1-flanked entry clone (supercoiled, 10 fmoles)
- Purified plasmid DNA of your attL1 and attL2-flanked entry clone (supercoiled, 10 fmoles)
- Purified plasmid DNA of your attR2 and attL3-flanked entry clone (supercoiled, 10 fmoles)

Important: Remember that you will need to add plasmid DNA from three entry clones to the MultiSite Gateway® LR reaction. Make sure that the plasmid DNA for each entry clone is sufficiently concentrated such that the total amount of entry clone plasmid DNA added to the MultiSite Gateway® LR reaction does not exceed 7 μl.
- 1x TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Appropriate competent E. coli host (e.g. One Shot® TOP10, included with the kit) and growth media for expression
- LB agar plates containing 50–100 μg/ml ampicillin

Continued on next page
Performing the LR Recombination Reaction, continued

Setting Up the MultiSite Gateway® LR Reaction

1. Add the following components to 1.5 ml microcentrifuge tubes at room temperature and mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>attL4 and attR1 entry clone (10 fmoles)</td>
<td>1–7 μl</td>
<td>--</td>
</tr>
<tr>
<td>attL1 and attL2 entry clone (10 fmoles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>attR2 and attL3 entry clone (10 fmoles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDEST™ R4-R3 Vector II (20 fmoles)</td>
<td>0.4 μl</td>
<td>0.4 μl</td>
</tr>
<tr>
<td>TE Buffer, pH 8.0</td>
<td>to 8 μl</td>
<td>7 μl</td>
</tr>
</tbody>
</table>

2. Remove the LR Clonase® II Plus enzyme mix from –20°C or –80°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase® II Plus enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 2 μl of LR Clonase® II Plus enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).
   
   **Reminder:** Return LR Clonase® II Plus enzyme mix to –20°C or –80°C immediately after use.
5. Incubate reactions at 25°C for 16 hours or overnight.
6. Add 1 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 One Shot® TOP10 chemically competent *E. coli*, follow the protocol on page 28.
   
   **Note:** You may store the MultiSite Gateway® 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 10⁹ cfu/μg, the MultiSite Gateway® LR reaction should give approximately 1,000 to 5,000 colonies if the entire reaction is transformed and plated.

Next Steps

If your recombination reaction was successful (*i.e.* provided the expected number of colonies) proceed to express your recombinant protein in the appropriate system.

If your recombination reaction was not satisfactory (*i.e.* resulted in fewer than expected or no colonies) use the troubleshooting guide on the following pages to troubleshoot your experiment.
# Troubleshooting

## MultiSite Gateway® LR & BP Reactions

The table below lists some potential problems and possible solutions that may help you troubleshoot the BP or MultiSite Gateway® 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>Used incorrect att sites for the reaction</td>
<td>Use the appropriate entry clones and pDEST™ R4-R3 Vector II for the MultiSite Gateway® LR reaction (see page 8 for details about the types of entry clones required).</td>
<td>Use the correct attB PCR product and donor vector (attP) for the BP reaction (see page 12 for details).</td>
</tr>
<tr>
<td>BP Clonase® Plus or LR Clonase® II Plus enzyme mix is inactive; or didn’t use suggested amount of BP Clonase® Plus or LR Clonase® II Plus enzyme mix</td>
<td>Test another aliquot of the BP Clonase® Plus or LR Clonase® II Plus enzyme mix. Store the LR Clonase® II Plus at −20° or −80°C for long-term storage, and the BP Clonase® II at −20°C. Do not freeze/thaw the BP Clonase® Plus or LR Clonase® II Plus enzyme mix &gt;10 times. Use the recommended amount of BP Clonase® Plus or LR Clonase® II Plus (see page 26 or 35).</td>
<td></td>
</tr>
<tr>
<td>Used incorrect BP Clonase® Plus or LR Clonase® II Plus enzyme mix</td>
<td>Use the LR Clonase® II Plus enzyme mix for the LR reaction. Use the BP Clonase® II enzyme mix for the BP reaction.</td>
<td></td>
</tr>
<tr>
<td>Too much attB PCR product used in a BP reaction</td>
<td>Reduce the amount of attB PCR product used. Use an equimolar ratio of attB PCR product and donor vector (i.e. ~50 fmoles 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 DNA was used in a MultiSite Gateway® LR reaction</td>
<td>Use an equimolar amount of each entry clone and destination vector. Do not exceed 60 fmoles total DNA in the reaction.</td>
<td></td>
</tr>
</tbody>
</table>

*Continued on next page*
### Troubleshooting, continued

#### MultiSite Gateway® LR and BP Reactions, continued

<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 <strong>and</strong> the transformation control gave colonies, continued</td>
<td>MultiSite Gateway® LR reaction not incubated for sufficient time</td>
<td>Incubate the MultiSite Gateway® LR reaction at 25°C for 16 hours or overnight.</td>
</tr>
<tr>
<td>Insufficient amount of <em>E. coli</em> transformed or plated</td>
<td>MultiSite Gateway® LR reaction: Transform 2 to 5 μl of the reaction; plate 50 μl or 100 μl. <strong>BP reaction</strong>: Transform 1 μl of the reaction; plate 20 μl and 100 μl.</td>
<td></td>
</tr>
<tr>
<td><strong>MultiSite Gateway® LR Reaction</strong>: High background in the absence of the entry clones</td>
<td>MultiSite Gateway® 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 (<em>e.g.</em> TOP10, DH5α™).</td>
</tr>
</tbody>
</table>
| Deletions (full or partial) of the *ccdB* gene from the destination vector | • To maintain the integrity of the vector, propagate in media containing 50–100 μg/ml ampicillin and 15–30 μg/ml chloramphenicol.  
• Prepare plasmid DNA from one or more colonies and verify the integrity of the vector before use. |
| Contamination of solution(s) with another plasmid carrying the same antibiotic resistance, or by bacteria carrying a resistance plasmid | • Test for plasmid contamination by transforming *E. coli* with aliquots of each of the separate solutions used in the MultiSite Gateway® LR reaction.  
• Test for bacterial contamination by plating an aliquot of each solution directly onto LB plates containing ampicillin. |
| Few or no colonies obtained from the transformation control | Competent cells stored incorrectly | Store competent cells at –80°C. |
| Transformation performed incorrectly | If you are using One Shot® TOP10 *E. coli*, follow the protocol on page 28 to transform cells.  
If you are using another *E. coli* strain, follow the manufacturer’s instructions. |
| Insufficient amount of *E. coli* plated | Increase the amount of *E. coli* when plating cells. |

*Continued on next page*
### Troubleshooting, continued

#### MultiSite Gateway® 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 | **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. |

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
</table>
| 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* (Life Technologies, Catalog no. 10268-019) to help stabilize plasmids containing large genes during propagation (Trinh et al., 1994). |

### 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 26).

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low yield of <em>attB</em> PCR product obtained after PEG purification</td>
<td><em>attB</em> 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>
</tbody>
</table>
| Lost PEG pellet | | • 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. |

Continued on next page
## Troubleshooting, continued

### attB PCR Cloning, continued

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<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 each attB PCR primer includes four 5' terminal Gs and the 22 or 25 bp attB site as specified on page 11.</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.</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>For large PCR products (&gt;5 kb), too few attB PCR molecules added to the BP reaction</td>
<td></td>
<td>• Increase the amount of attB PCR product to 20–50 fmole per 10 μl reaction.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Note: Do not exceed 250 ng DNA per 10 μl reaction.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Incubate the BP reaction overnight.</td>
</tr>
<tr>
<td>Insufficient incubation time</td>
<td></td>
<td>Increase the incubation time of the BP reaction up to 18 hours.</td>
</tr>
<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 19 or gel-purify the attB PCR product.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Use a Platinum® DNA polymerase with automatic hot-start capability for higher specificity amplification.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Redesign attB PCR primers to minimize potential mutual priming sites leading to primer-dimers.</td>
</tr>
</tbody>
</table>
Appendix

Map of pDONR™ P4-P1R

The map below shows the elements of pDONR™ P4-P1R. The complete sequence of pDONR™ P4-P1R is available from www.lifetechnologies.com or by contacting Technical Support (see page 47).

Comments for pDONR™ P4-P1R
4777 nucleotides

- *rrnB* T2 transcription termination sequence: bases 268-295 (c)
- *rrnB* T1 transcription termination sequence: bases 427-470 (c)
- M13 Forward (-20) priming site: bases 537-552
- *attP4* recombination site: bases 593-824 (c)
- *ccdB* gene: bases 1181-1486 (c)
- Chloramphenicol resistance gene: bases 1828-2487 (c)
- *attP1R* recombination site: bases 2748-2979 (c)
- M13 Reverse priming site: bases 3042-3058
- Kanamycin resistance gene: bases 3171-3980
- pUC origin: bases 4101-4774

(c) = complementary strand
Map of pDONR™ 221

The map below shows the elements of pDONR™ 221. The complete sequence of pDONR™ 221 is available from www.lifetechnologies.com or by contacting Technical Support (see page 47).

Comments for pDONR™ 221
4762 nucleotides

*rm*B T2 transcription termination sequence (c): bases 268-295
*rm*B T1 transcription termination sequence (c): bases 427-470
M13 Forward (-20) priming site: bases 537-552
*att*P1: bases 570-801
*ccdB* gene (c): bases 1197-1502
Chloramphenicol resistance gene (c): bases 1847-2506
*att*P2 (c): bases 2754-2985
M13 Reverse priming site: bases 3027-3043
Kanamycin resistance gene: bases 3156-3965
pUC origin: bases 4086-4759
(c) = complementary strand
The map below shows the elements of pDONR™ P2R-P3. The complete sequence of pDONR™ P2R-P3 is available from www.lifetecnologies.com or by contacting Technical Support (see page 47).

**Comments for pDONR™ P2R-P3**

- rrnB T2 transcription termination sequence: bases 268-295 (c)
- rrnB T1 transcription termination sequence: bases 427-470 (c)
- M13 Forward (−20) priming site: bases 537-552
- attP2R recombination site: bases 591-822
- Chloramphenicol resistance gene: bases 1083-1742
- ccdB gene: bases 2084-2389
- attP3 recombination site: bases 2746-2977
- M13 Reverse priming site: bases 3038-3054
- Kanamycin resistance gene: bases 3167-3976
- pUC origin: bases 4097-4770

(c) = complementary strand
## Features of pDONR™ Vectors

pDONR™ P4-P1R (4777 bp), pDONR™ 221 (4762 bp), and pDONR™ P2R-P3 (4773 bp) contain the following elements. Features have been functionally tested.

<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><strong>attP4 and attP1R site (pDONR™ P4-P1R)</strong>&lt;br&gt;<strong>attP1 and attP2 sites (pDONR™ 221)</strong>&lt;br&gt;<strong>attP2R and attP3 sites (pDONR™ P2R-P3)</strong></td>
<td>Bacteriophage λ-derived DNA recombination sequences that have been optimized to permit recombinational cloning of DNA fragments from specific attB PCR products (Landy, 1989).</td>
</tr>
<tr>
<td><strong>ccdB gene</strong></td>
<td>Permits negative selection of the plasmid.</td>
</tr>
<tr>
<td><strong>Chloramphenicol resistance gene (Cm&lt;sup&gt;®&lt;/sup&gt;)</strong></td>
<td>Allows counterselection of the plasmid.</td>
</tr>
<tr>
<td><strong>M13 reverse priming site</strong></td>
<td>Permits sequencing in the anti-sense orientation.</td>
</tr>
<tr>
<td><strong>Kanamycin resistance gene</strong></td>
<td>Allows selection of the plasmid in <em>E. coli</em>.</td>
</tr>
<tr>
<td><strong>pUC origin and replisome assembly site</strong></td>
<td>Permits high-copy replication and maintenance of the plasmid in <em>E. coli</em>.</td>
</tr>
</tbody>
</table>
Map of pDEST™ R4-R3 Vector II

The map below shows the elements of pDEST™ R4-R3 Vector II. The complete sequence of pDEST™ R4-R3 Vector II is available from www.lifetechnologies.com or by contacting Technical Support (see page 47).

Features of pDEST™R4-R3 Vector II
4555 nucleotides

- M13 Reverse priming site: bases 1-17
- attR4 recombination site: bases 37-161
- ccdB gene: bases 508-813 (c)
- Chloramphenicol resistance gene: bases 1158-1816 (c)
- attR3 recombination site: bases 2064-2188
- M13 Forward (-20) priming site: bases 2197-2212 (c)
- Ampicillin resistance ORF (bla): bases 2791-3651
- pUC origin: bases 3796-4469

(c) = complementary strand
pDEST™ R4-R3 Vector II (4555 bp) contains the following elements. Features have been functionally tested.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 reverse priming site</td>
<td>Permits sequencing in the sense orientation.</td>
</tr>
<tr>
<td><em>att</em>R4 and <em>att</em>R3 sites</td>
<td>Bacteriophage <em>λ</em>-derived DNA recombination sequences that have been optimized to permit recombinational cloning of DNA fragments from specific <em>att</em>L-flanked entry clones (Landy, 1989).</td>
</tr>
<tr>
<td><em>ccdB</em> gene</td>
<td>Permits negative selection of the plasmid.</td>
</tr>
<tr>
<td>Chloramphenicol resistance gene (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Allows counterselection of the plasmid.</td>
</tr>
<tr>
<td>M13 forward (−20) priming site</td>
<td>Allows sequencing in the anti-sense orientation.</td>
</tr>
<tr>
<td><em>bla</em> promoter</td>
<td>Permits expression of the ampicillin resistance gene.</td>
</tr>
<tr>
<td>Ampicillin resistance gene (β-lactamase)</td>
<td>Allow selection of the plasmid in <em>E. coli.</em></td>
</tr>
<tr>
<td>pUC origin and replisome assembly site</td>
<td>Permits high-copy replication and maintenance of the plasmid in <em>E. coli.</em></td>
</tr>
</tbody>
</table>
Map of pMS/GW

pMS/GW is a control vector generated using the MultiSite Gateway® LR recombination reaction between pDEST™ R4-R3 Vector II and three entry clones containing the araC gene and araBAD promoter, gus gene, and lacZα fragment, respectively. The map below shows the elements of pMS/GW. The complete sequence of pMS/GW is available from www.lifetechnologies.com or by contacting Technical Support (see page 47).

Comments for pMS/GW
5898 nucleotides

M13 Reverse priming site: bases 1-17
attB4 recombination site: bases 37-57
AraC ORF: bases 58-936 (c)
Arabinose O2 operator region: bases 966-981
Arabinose O1 operator region: bases 1123-1144
CAP binding site: bases 1165-1178
Arabinose I1 and I2 region: bases 1175-1213
Arabinose minimal promoter: bases 1175-1213
Ribosome binding site: bases 1267-1270
attB1 recombination site: bases 1285-1308
gus gene: bases 1306-3149
attB2 recombination site: bases 3154-3174
lacZα gene: bases 3175-3509
attB3 recombination site: bases 3510-3530
M13 Forward (−20) priming site: bases 3539-3554
Aat II linearization site: base 4002
bla promoter: bases 4034-4132
Ampicillin (bla) resistance gene: bases 4133-4993
pUC origin: bases 5138-5811
(c) = complementary strand
Technical Support

Obtaining Support
For the latest services and support information for all locations, go to www.lifetechnologies.com
At the website, you can:

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

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

Certificate of Analysis
The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited Warranty
Life Technologies and/or its affiliate(s) warrant their products as set forth in the Life Technologies General Terms and Conditions of Sale found on the Life Technologies website at www.lifetechnologies.com/termsandconditions. If you have any questions, please contact Life Technologies.

Life Technologies and/or its affiliate(s) disclaim all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Life Technologies and/or its affiliate(s) be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.
Purchaser Notification

Limited Use Label License No. 358: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser’s activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License: ULB ccdB Selection Technology

ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License No. 48: araB Promoter

Products containing the araB promoter are sold under patent license for research purposes only and are non-transferable. Inquiries for any commercial use, including production of material to be sold commercially or used in production or in product development efforts, which includes efforts toward regulatory approval, should be made directly to Xoma Corporation, 2910 Seventh Street, Berkeley, CA 94710, Tel: 1-510-644-1170 Fax: 1-510-649-7571.

Gateway® Clone Distribution Policy

For additional information about Life Technologies’s policy for the use and distribution of Gateway® clones, see the section entitled Gateway® Clone Distribution Policy, page 49.

Continued on next page
# Gateway® Clone Distribution Policy

## Introduction

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

## Gateway® Entry Clones

Life Technologies understands that Gateway® entry clones, containing attL1 and attL2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

## Gateway® Expression Clones

Life Technologies also understands that Gateway® expression clones, containing attB1 and attB2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Life Technologies.

## Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase® from Life Technologies is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Life Technologies’s Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Life Technologies’s licensing department at 760-603-7200.
References


© 2011 Life Technologies Corporation. All rights reserved.
The trademarks mentioned herein are the property of Life Technologies Corporation or their respective owners.
Notes