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Instruction Manual

MultiSite Gateway® Three-Fragment Vector Construction Kit

Using Gateway® Technology to simultaneously clone multiple DNA fragments

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MultiSite Gateway® BP and LR Recombination Reaction Protocols for Experienced Users

Introduction

This quick reference sheet 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 *att*B-flanked DNA fragment and the appropriate *att*P-containing donor vector to generate an entry clone (see page 19 for details).

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

*att*B PCR product (20-50 fmoles)	1-7 µl
pDONR™ vector (supercoiled, 150 ng/µl)	1 µl
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

Perform a MultiSite Gateway® LR recombination reaction between multiple entry clones (*att*L4-5′ element-*att*R1 + *att*L1-gene of interest-*att*L2 + *att*R2-3′ element-*att*L3) and the pDEST™R4-R3 vector to generate an expression clone (*att*B4-5′ element-*att*B1-gene of interest-*att*B2-3′ element-*att*B3).

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

Entry clones (supercoiled, 20-25 fmoles each)	1-11 µl
pDEST™R4-R3 (supercoiled, 60 ng/µl)	1 µl
5X LR Clonase™ Plus reaction buffer	4 µl
TE Buffer, pH 8.0	to 16 µl

2. Vortex LR Clonase™ Plus enzyme mix briefly. Add 4 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 2 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.

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.

Box	Item	Storage
1	Vectors	**-20°C**
2	BP Clonase™ II Enzyme Mix	**-20°C**
3	LR Clonase™ Plus Enzyme Mix	**-80°C**
4	One Shot® TOP10 Chemically Competent *E. coli*	**-80°C**

Vectors

The Vectors box (Box 1) contains the following items. **Store Box 1 at -20°C.**

Item	Composition	Amount
pDONR™P4-P1R	Lyophilized in TE Buffer, pH 8.0	6 µg
pDONR™P2R-P3	Lyophilized in TE Buffer, pH 8.0	6 µg
pDONR™221	Lyophilized in TE Buffer, pH 8.0	6 µg
pDEST™R4-R3	Lyophilized in TE Buffer, pH 8.0	6 µg
pMS/GW control plasmid	Lyophilized in TE Buffer, pH 8.0	10 µg

BP Clonase™ II Enzyme Mix

The following reagents are supplied with the BP Clonase™ II enzyme mix (Box 2). **Store Box 2 at -20°C for up to 6 months.** For long-term storage, store at -80°C.

Item	Composition	Amount
BP Clonase™ II Enzyme Mix	Proprietary	40 µl
Proteinase K solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl
30% PEG/Mg solution	30% PEG 8000/30 mM $MgCl_2$	1 ml
pEXP7-tet positive control	50 ng/µl in TE Buffer, pH 8.0	20 µl

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Kit Contents and Storage, continued

LR Clonase™ Plus Enzyme Mix

The following reagents are supplied with the LR Clonase™ Plus enzyme mix (Box 3). **Store Box 3 at -80°C.**

Item	Composition	Amount
LR Clonase™ Plus Enzyme Mix	Proprietary	80 µl
5X LR Clonase™ Plus Reaction Buffer	Proprietary	100 µl
Proteinase K solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl

One Shot® TOP10 Reagents

The One Shot® TOP10 Chemically Competent *E. coli* kit (Box 4) contains the following reagents. Transformation efficiency is 1 x 10^9 cfu/µg DNA. **Store Box 4 at -80°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10 chemically competent cells	--	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of TOP10

Note that this strain cannot be used for single-strand rescue of DNA.

F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Accessory Products

Introduction

The products listed in this section may be used with the MultiSite Gateway® Three-Fragment Vector Construction Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Additional Products

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 Invitrogen. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
BP Clonase™ II Enzyme Mix	20 reactions	11789-020
	100 reactions	11789-100
LR Clonase™ Plus Enzyme Mix	20 reactions	12538-013
Library Efficiency DH5α™ Chemically Competent Cells	5 x 0.2 ml	18263-012
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µl	C4040-03
One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli*	20 x 50 µl	C7510-03
pDONR™221	6 µg	12536-017
M13 Forward (-20) Sequencing Primer	2 µg	N520-02
M13 Reverse Sequencing Primer	2 µg	N530-02
S.N.A.P.™ MiniPrep Kit	100 reactions	K1900-01
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
S.N.A.P.™ Gel Purification Kit	25 reactions	K1999-25
Ampicillin	20 ml (10 mg/ml)	11593-019
Kanamycin Sulfate	100 ml (10 mg/ml)	15160-054
Platinum® *Pfx* DNA Polymerase	100 reactions	11708-013
	250 reactions	11708-021
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions	11304-011
	500 reactions	11304-029
Dpn I	100 units	15242-019
REact® 4 Buffer	2 x 1 ml	16304-016

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Accessory Products, continued

Gateway® Entry Vectors

The MultiSite Gateway® Three-Fragment kit provides the pDONR™221 vector to facilitate creation of *att*L1 and *att*L2-flanked entry clones. Alternatively, a variety of Gateway® entry vectors are available from Invitrogen 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, see our Web site (www.invitrogen.com) or contact Technical Service (see page 48).

Item	Quantity	Catalog no.
pENTR™/D-TOPO® Cloning Kit	20 reactions	K2400-20
	480 reactions	K2400-480
	500 reactions	K2400-500
pENTR™/SD/D-TOPO® Cloning Kit	20 reactions	K2420-20
	480 reactions	K2420-480
	500 reactions	K2420-500
pENTR™1A	10 µg	11813-011
pENTR™2B	10 µg	11816-014
pENTR™3C	10 µg	11817-012
pENTR™4	10 µg	11818-010
pENTR™11	10 µg	11819-018

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.

A brief overview about the Gateway® Technology is provided in this manual. For more details about the Gateway® Technology and the recombination reactions, refer to the Gateway® Technology with Clonase™ II manual. The manual is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 48).

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 *att*B PCR primers, and amplify your three DNA sequences of interest.
2. Perform a BP recombination reaction with each *att*B PCR product and a specific donor vector to generate three types of entry clones.
3. Perform a MultiSite Gateway® LR recombination reaction with your three entry clones and the pDEST™R4-R3 destination vector to generate an expression clone which may then be used in the appropriate application or expression system.

Glossary of Terms

To help you understand the terminology used in the MultiSite Gateway® Technology, a glossary of terms is provided in the **Appendix**, page 54 for your convenience.

The Gateway® Technology

Gateway® Technology

The Gateway® Technology is a universal cloning method based on the bacteriophage lambda site-specific recombination system (Landy, 1989; Ptashne, 1992) that provides 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).

Lambda Recombination Reactions

In lambda, recombination occurs between lambda and the *E. coli* chromosome via specific recombination sequences (*att* sites), and is catalyzed by a mixture of recombination proteins (Clonase™ II enzyme mix). The reactions are described in the table below.

Pathway	Reaction	Catalyzed by…
Lysogenic	*att*B x *att*P → *att*L x *att*R	BP Clonase™ II (Int, IHF)
Lytic	*att*L x *att*R → *att*B x *att*P	LR Clonase™ II (Int, Xis, IHF)

Gateway® Recombination Reactions

The Gateway® Technology uses modified and optimized *att* sites to permit transfer of heterologous DNA sequences between vectors. Two recombination reactions constitute the basis of the Gateway® Technology:

- **BP Reaction:** Recombination of an *att*B substrate (*e.g. att*B PCR product or expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing entry clone (see diagram below). The reaction is catalyzed by BP Clonase™ II enzyme mix, a mixture of the λ Integrase (Int) and *E. coli* Integration Host Factor (IHF) proteins.

- **LR Reaction:** Recombination of an *att*L-containing entry clone with an *att*R-containing destination vector to create an *att*B-containing expression clone (see diagram below). The reaction is catalyzed by LR Clonase™ II enzyme mix, a mixture of the λ Int and Excisionase (Xis) proteins, and the *E. coli* IHF protein.

For More Information

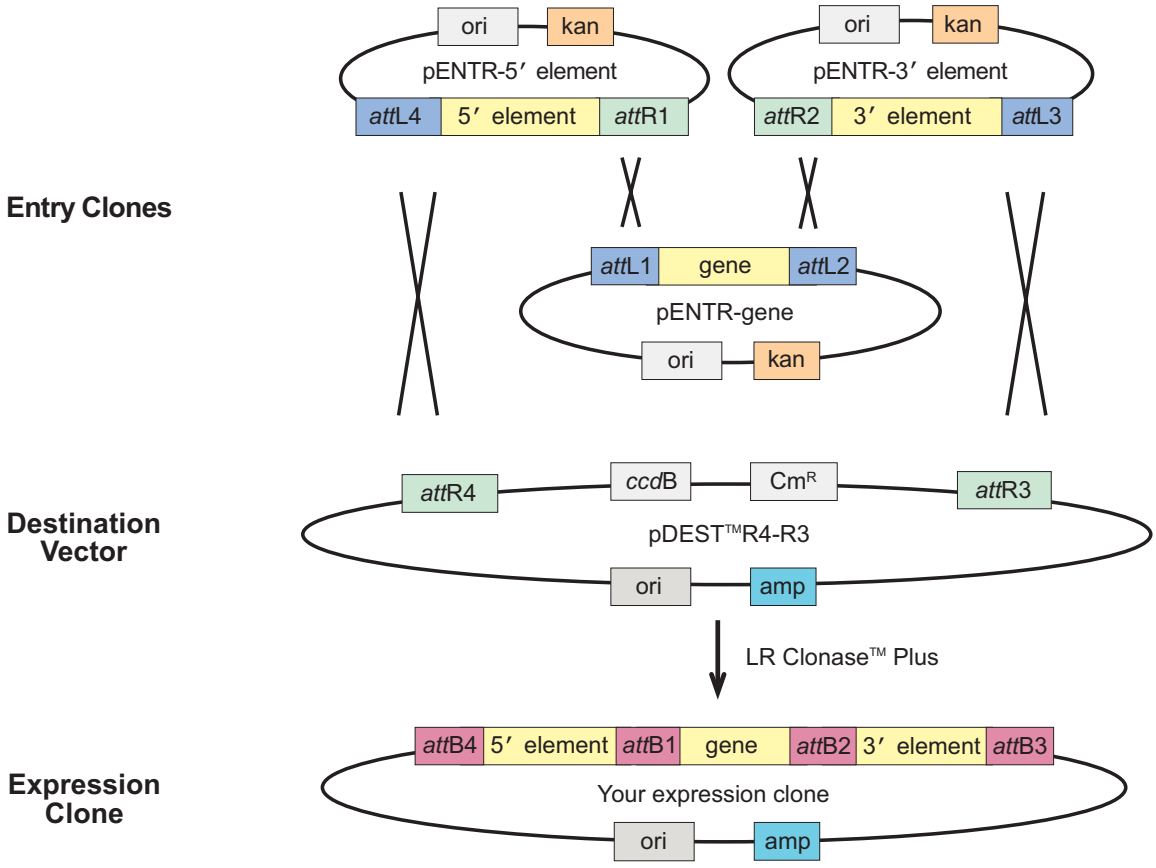
For details about the Gateway® Technology, lambda DNA recombination, *att* sites, and the BP and LR recombination reactions, refer to the Gateway® Technology with Clonase™ II manual. This manual is available for downloading from our Web site (www.invitrogen.com) or by calling Technical Service (see page 48).

MultiSite Gateway® Technology

Introduction

The MultiSite Gateway® Three-Fragment Vector Construction Kit uses modifications of the site-specific recombination reactions of the Gateway® Technology (see the next page for more information) to allow simultaneous cloning of three DNA fragments in a defined order and orientation to create your own expression clone. To generate your own expression clone, you will:

1. Amplify your three DNA sequences of interest (*i.e.* 5′ element, gene of interest, and 3′ element) using the recommended *att*B primers to generate PCR products that are flanked by *att*B sites. To ensure that your fragments are joined in a specific order, specific *att*B sites must flank each PCR product.
2. Use the PCR products in separate BP recombination reactions with three donor vectors (pDONR™P4-P1R, pDONR™221, pDONR™P2R-P3) to generate three entry clones containing your DNA sequences of interest. For more information about the donor vectors, see page 20.
3. Use the three entry clones in a single MultiSite Gateway® LR recombination reaction with a specially designed destination vector, pDEST™R4-R3, to create your expression clone of interest (see the diagram below). For more information about pDEST™R4-R3, see page 32.



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MultiSite Gateway® Technology, continued

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. In the MultiSite Gateway® Three-Fragment kit, four *att* sites are used versus two *att* sites in the standard Gateway® Technology.

For example, four *att*B sites are used in the MultiSite Gateway® Three-Fragment kit (see table below). Various combinations of these *att*B sites will flank each PCR product containing your DNA fragment of interest.

MultiSite Gateway®	Gateway®
*att*B1	*att*B1
*att*B2	*att*B2
*att*B3	
*att*B4	

Specificity of the Modified *att* Sites

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

- *att*B sites react only with *att*P sites; similarly *att*B1 sites react only with *att*P1 sites to generate *att*L1 sites
- *att*L sites react only with *att*R sites; similarly *att*L1 sites react only with *att*R1 sites to generate *att*B1 sites

However, depending on the orientation and position of the *att*B site and *att*P site in relation to the DNA fragment of interest or the donor vector, respectively, performing the BP recombination reaction can result in creation of an *att*R site instead of an *att*L site. Specifically:

- *att*B1 sites react with *att*P1R sites to generate *att*R1 sites
- *att*B2 sites react with *att*P2R sites to generate *att*R2 sites

See the next page for an example. See the next section, pages 6-8 for diagrams of these BP recombination reactions.

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MultiSite Gateway® Technology, continued

Example

In this example, an *att*B4 and *att*B1-flanked PCR product is used in a BP recombination reaction with pDONR™P4-P1R.

***att*B4-PCR product-*att*B1 x pDONR™P4-P1R → *att*L4-PCR product-*att*R1**

Because of the orientation and position of the *att*B1 and *att*P1R site in the PCR product and donor vector, respectively, the resulting entry clone contains the PCR product flanked by an *att*L4 site and an *att*R1 site rather than two *att*L sites. See page 6 for a diagram of this BP recombination reaction.

MultiSite Gateway® Donor Vectors

The MultiSite Gateway® donor vectors are used to clone *att*B-flanked PCR products to generate entry clones, and contain similar elements as other Gateway® donor vectors. However, because your PCR products will be flanked by different *att*B sites, three different donor vectors are required to facilitate generation of the three types of entry clones required for MultiSite Gateway®:

- pDONR™P4-P1R: Use to clone *att*B4 and *att*B1-flanked PCR products.
- pDONR™221: Use to clone *att*B1 and *att*B2-flanked PCR products.
- pDONR™P2R-P3: Use to clone *att*B2 and *att*B3-flanked PCR products.

For more information about the general features of the donor vectors, see page 9. For a map and a description of the features of each pDONR™ vector, see the **Appendix**, pages 41-44.

Note: While pDONR™221 may be used in standard Gateway® reactions, the pDONR™P4-P1R and pDONR™P2R-P3 vectors may **only** be used for MultiSite Gateway® applications.

MultiSite Gateway® Destination Vector

The MultiSite Gateway® destination vector, pDEST™R4-R3, is designed for use in the MultiSite Gateway® three-fragment LR recombination reaction with the three entry clones described above. The pDEST™R4-R3 vector contains *att*R4 and *att*R3 sites flanking a selection cassette and allows generation of the expression clone of interest. Note that other Gateway® destination vectors are **not** suitable for use in the MultiSite Gateway® LR reaction.

For more information about the general features of the destination vector, see page 9. For a map and a description of the features of the pDEST™R4-R3 vector, see the **Appendix**, pages 45-46.

LR Clonase™ Plus Enzyme Mix

The MultiSite Gateway® LR recombination reaction is catalyzed by an optimized LR Clonase™, LR Clonase™ Plus enzyme mix. LR Clonase™ Plus enzyme mix facilitates efficient recombinational cloning of multiple DNA fragments, but may also be used in the standard Gateway® LR recombination reaction. Note that LR Clonase™ enzyme mix is **not** suitable for use in the MultiSite Gateway® LR recombination reaction.

MultiSite Gateway® BP Recombination Reactions

Introduction

The MultiSite Gateway® BP recombination reaction facilitates production of entry clones from your three *att*B-flanked PCR products. Since each PCR product is flanked by a specific combination of *att*B sites, specific donor vectors must also be used. An illustration of each BP recombination reaction is provided in this section.

Important

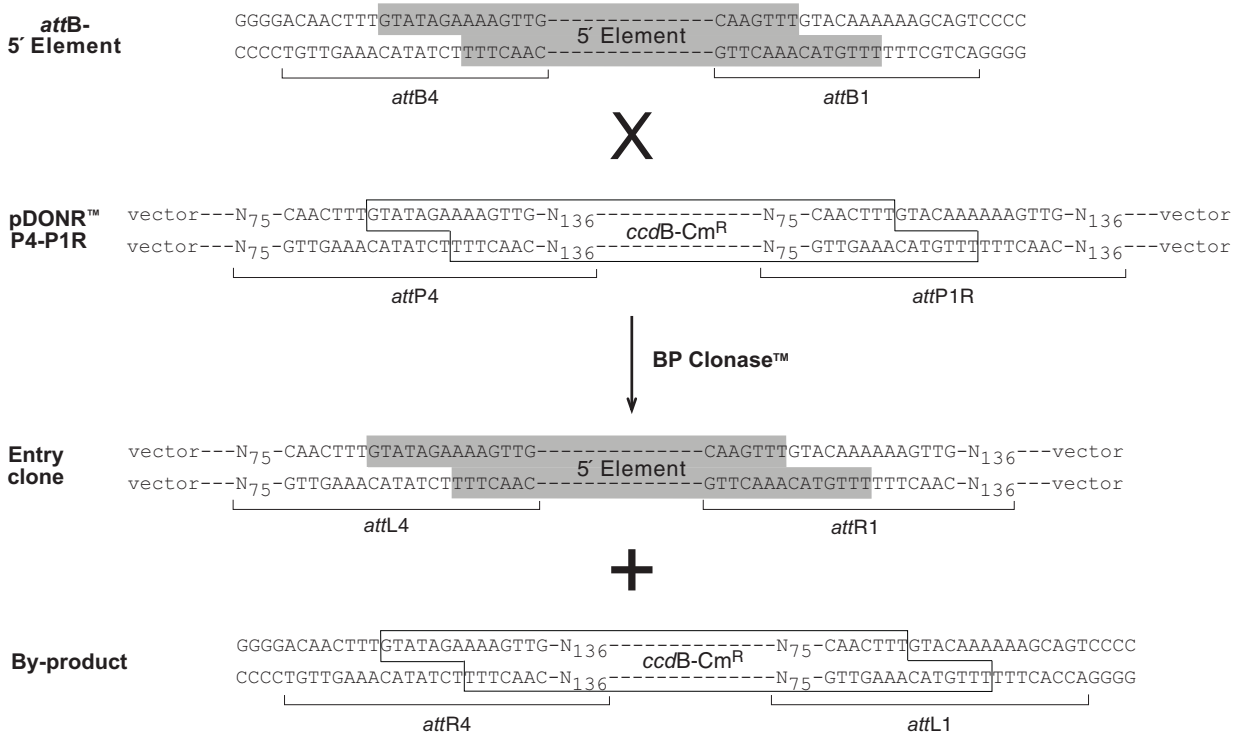
Note that the *att* sites used in MultiSite Gateway® have been optimized to improve specificity and efficiency of the MultiSite Gateway® LR recombination reaction, and may vary in size and sequence from those used in the Gateway® Technology.

*att*B 5′ Element x pDONR™P4-P1R Recombination Region

The diagram below depicts the recombination reaction between the *att*B4 and *att*B1-flanked PCR product (*i.e. att*B 5′ element) and pDONR™P4-P1R to create an entry clone and a by-product.

Features of the Recombination Region:

- Shaded regions correspond to those sequences transferred from the *att*B 5′ element into the entry clone following recombination. Note that *att*L4 and *att*R1 sites flank the 5′ element in the entry clone.
- Boxed regions correspond to those sequences transferred from the donor vector into the by-product following recombination.



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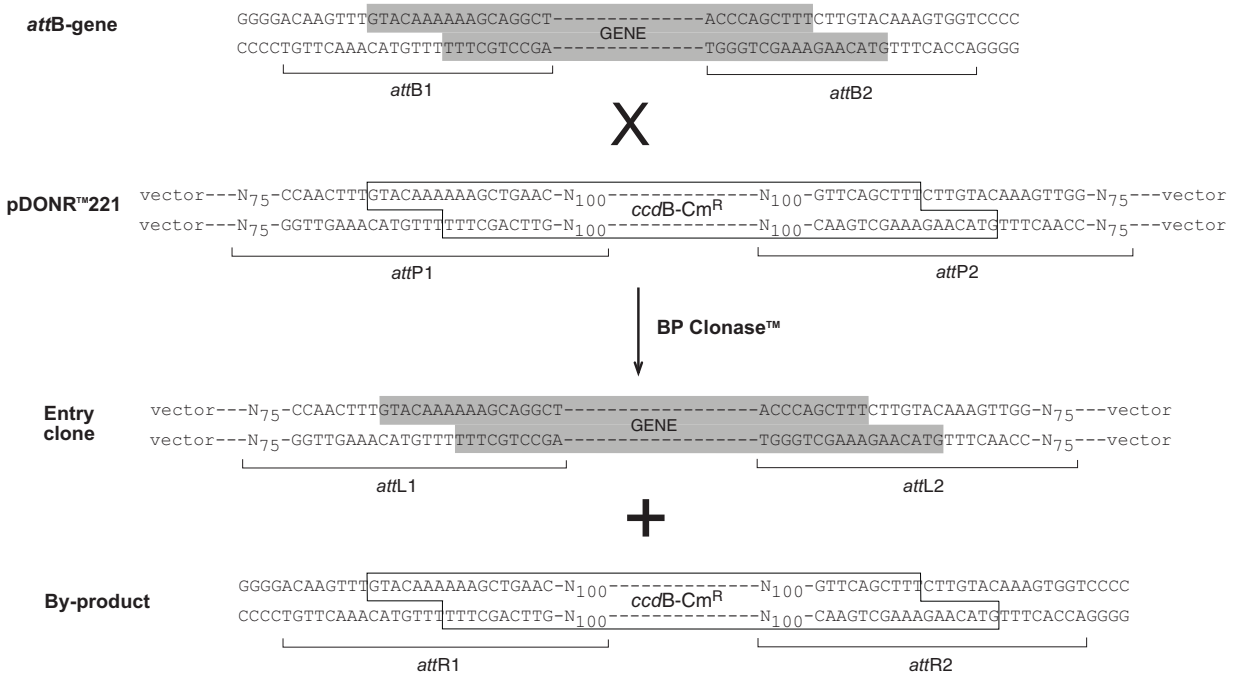
MultiSite Gateway® BP Recombination Reactions, continued

*att*B Gene x pDONR™221 Recombination Region

The diagram below depicts the recombination reaction between the *att*B1 and *att*B2-flanked PCR product (*i.e. att*B gene) and pDONR™221 to create an entry clone and a by-product.

Features of the Recombination Region:

- Shaded regions correspond to those sequences transferred from the *att*B PCR product into the entry clone following recombination. Note that the PCR product in the entry clone is flanked by *att*L1 and *att*L2 sites, and is suitable for use in all standard Gateway® applications.
- Boxed regions correspond to those sequences transferred from the donor vector into the by-product following recombination.



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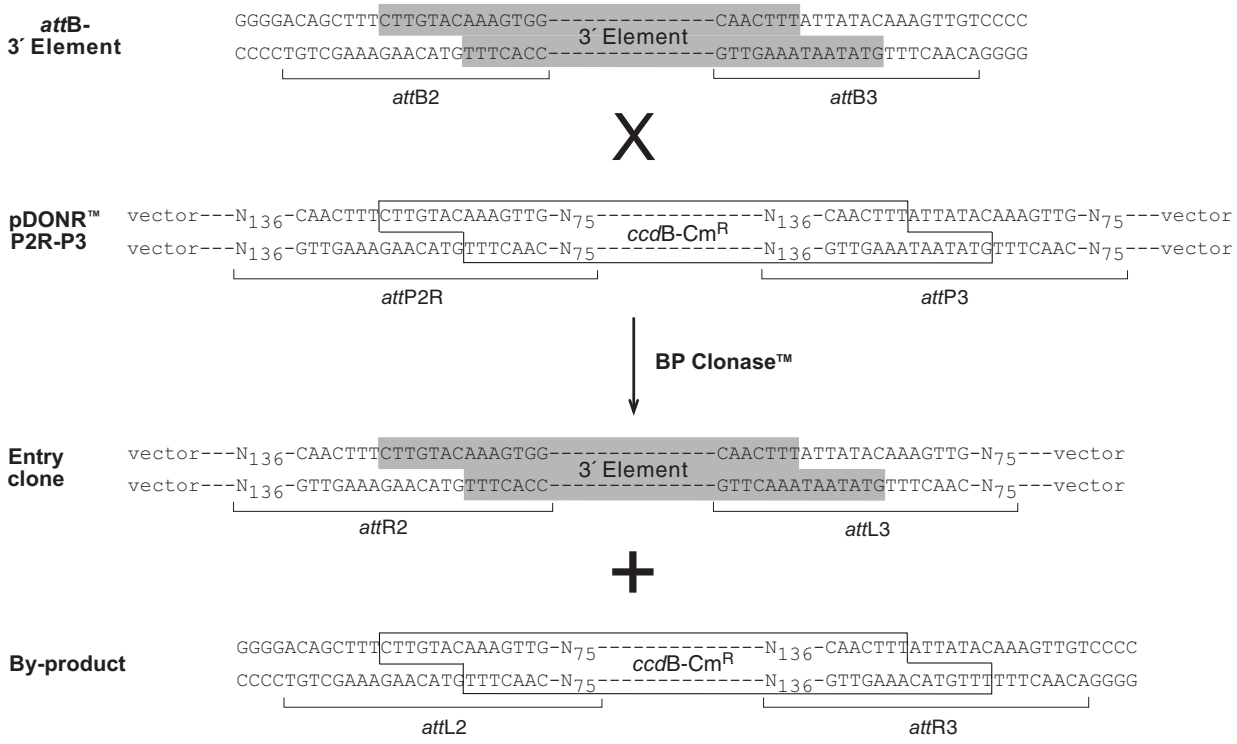
MultiSite Gateway® BP Recombination Reactions, continued

*att*B 3′ Element x pDONR™P2R-P3 Recombination Region

The diagram below depicts the recombination reaction between the *att*B2 and *att*B3-flanked PCR product (*i.e. att*B 3′ element) and pDONR™P2R-P3 to create an entry clone and a by-product.

Features of the Recombination Region:

- Shaded regions correspond to those sequences transferred from the *att*B 3′ element into the entry clone following recombination. Note that *att*R2 and *att*L3 sites flank the 3′ element in the entry clone.
- Boxed regions correspond to those sequences transferred from the donor vector into the by-product following recombination.



Features of the MultiSite Gateway® Vectors

MultiSite Gateway® Vectors

Two types of MultiSite Gateway®-adapted vectors are available from Invitrogen:

Gateway® Vector	Characteristics
Donor vector (pDONR™)	Contains *att*P sites Used to clone *att*B-flanked PCR products to generate entry clones
Destination vector	Contains *att*R sites Recombines with multiple entry clones in a MultiSite Gateway® LR reaction to generate an expression clone

Common Features of the MultiSite Gateway® Vectors

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

- The *ccd*B gene (see below) for negative selection
- Chloramphenicol resistance gene (Cm^R) for counterselection

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

*ccd*B Gene

The presence of the *ccd*B 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 and Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* TOP10, DH5α™). When recombination occurs (*i.e.* between a destination vector and an entry clone or between a donor vector and an *att*B PCR product), the gene of interest replaces the *ccd*B gene. Cells that take up unreacted vectors carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Methods

Propagating the MultiSite Gateway® Vectors

Introduction

The MultiSite Gateway® Three-Fragment Vector Construction Kit includes the following vectors. See the guidelines below to propagate and maintain these vectors.

Donor Vectors:

- pDONR™P4-P1R
- pDONR™221
- pDONR™P2R-P3

Destination Vector:

- pDEST™R4-R3

Control Vector:

- pMS/GW

Propagating Donor and Destination Vectors

If you wish to propagate and maintain the pDONR™P4-P1R, pDONR™221, pDONR™P2R-P3, and pDEST™R4-R3 vectors prior to recombination, we recommend using One Shot® *ccd*B Survival T1^R Chemically Competent *E. coli* (Catalog no. C7510-03) from Invitrogen for transformation. The *ccd*B Survival T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B 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 the pDEST™R4-R3 vector, use LB plates containing 100 µg/ml ampicillin and 15-30 µg/ml chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to ccdB effects.

pMS/GW Vector

To propagate and maintain the pMS/GW plasmid, you may use any *rec*A, *end*A *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.

Types of 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. **Note:** Depending on your needs or application of interest, other sequences are possible.

- An *att*L4 and *att*R1-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 *att*L1 and *att*L2-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 *att*R2 and *att*L3-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 below.

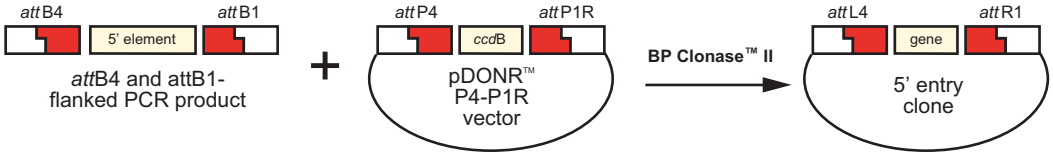
Important

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 *att*R2 and *att*L3-flanked entry clone).

Generating *att*L4 and *att*R1-Flanked Entry Clones

To generate an *att*L4 and *att*R1-flanked entry clone containing your 5′ element of interest:

1. Design appropriate PCR primers and produce your *att*B4 and *att*B1-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B4 and *att*B1-flanked PCR product and pDONR™P4-P1R to generate the entry clone (see figure below).



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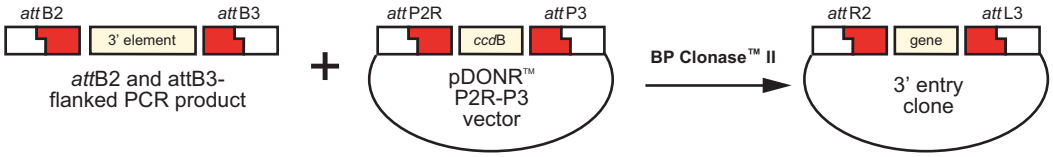
Types of Entry Clones, continued

Generating *att*R2 and *att*L3-Flanked Entry Clones

To generate an *att*R2 and *att*L3-flanked entry clone containing your 3′ element of interest:

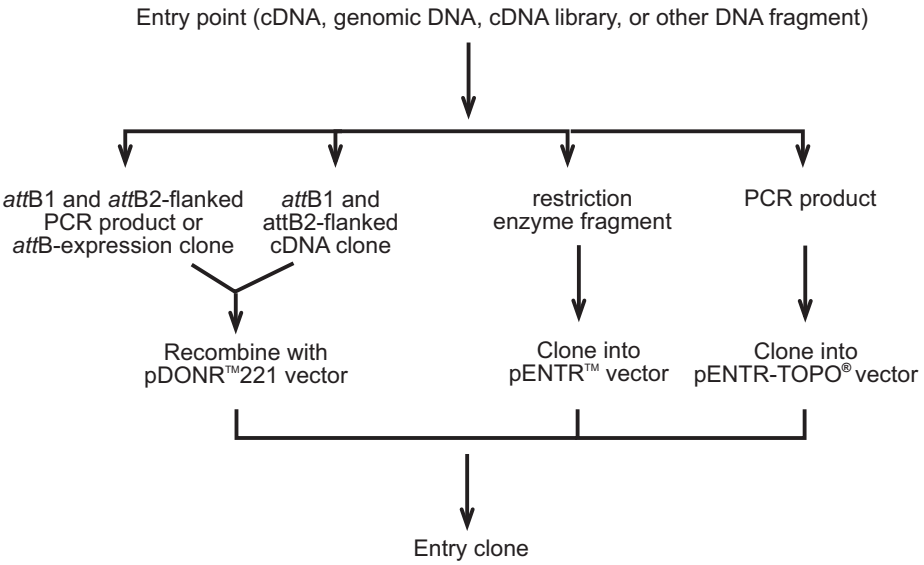
1. Design appropriate PCR primers and produce your *att*B2 and *att*B3-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B2 and *att*B3-flanked PCR product and pDONR™P2R-P3 to generate the entry clone (see figure below).



Generating *att*L1 and *att*L2-Flanked Entry Clones

The *att*L1 and *att*L2-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 (see figure below).

1. Generate a PCR product containing *att*B1 and *att*B2 sites and use this *att*B PCR product in a BP recombination reaction with the pDONR™221 vector. To use this method, refer to the guidelines and instructions provided in this manual.
2. Clone a PCR product or a restriction enzyme fragment into an entry (pENTR™) vector (see the next page for more information).
3. Generate or obtain a cDNA library cloned into a Gateway®-compatible vector (*i.e. att*B-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).



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Types of Entry Clones, continued

Entry Vectors

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

- *att*L1 and *att*L2 sites to allow recombinational cloning of the gene of interest with a destination vector to produce an expression clone.
- A Kozak consensus sequence for efficient translation initiation in eukaryotic cells. Some entry vectors include a Shine-Dalgarno sequence (Shine and Dalgarno, 1975) for initiation in *E. coli* (see table below).
- Kanamycin resistance gene for selection of plasmid in *E. coli*.
- pUC origin for high-copy replication and maintenance of the plasmid in *E coli*.

For more information about the features of each pENTR™ vector, see our Web site (www.invitrogen.com) or call Technical Service (see page 48).

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

Constructing Entry Clones

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 our Web site (www.invitrogen.com) or by calling Technical Service (see page 48).

Designing *att*B 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 *att*B sites into your PCR products. To facilitate use in MultiSite Gateway®, each PCR product must be flanked by a different combination of *att*B sites (see table below). Guidelines are provided below to help you design appropriate PCR primers.

DNA Sequence of Interest	Forward PCR Primer	Reverse PCR Primer
5′ element	*att*B4	*att*B1
Gene of interest	*att*B1	*att*B2
3′ element	*att*B2	*att*B3

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.

Guidelines to Design the Forward PCR Primer

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 *att*B 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 and Dalgarno, 1975) or Kozak consensus sequence (Kozak, 1987; Kozak, 1991; Kozak, 1990), respectively, in the *att*B1 forward PCR primer.
- The *att*B4 and *att*B2 sites end with a guanine (G), and the *att*B1 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 *att*B1 primer **cannot** be AA, AG, or GA because these additions will create a translation termination codon.

continued on next page

Designing *att*B PCR Primers, continued

*att*B Forward Primers

Design each *att*B forward primer to contain the following recommended sequence as listed below:

***att*B1** 5′-GGGG-ACA-AGT-TTG-TAC-**AAA-AAA**-GCA-GGC-TNN--(template-specific sequence)-3′
*att*B1

***att*B2** 5′-GGGG-ACA-GCT-TTC-TTG-**TAC-AAA**-GTG-GNN--(template-specific sequence)-3′
*att*B2

***att*B4** 5′-GGGG-ACA-ACT-TTG-TAT-**AGA-AAA**-GTT-GNN--(template-specific sequence)-3′
*att*B4

Guidelines to Design the Reverse PCR Primer

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 *att*B 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 *att*B1 and *att*B2 reverse primers must include one additional nucleotide to maintain the proper reading frame (see diagram below).
  2. Any in-frame stop codons between the *att*B sites and your gene of interest must be removed.
- If you do not wish to fuse your PCR product in frame with a C-terminal tag, your gene of interest or the *att*B2 primer must include a stop codon.

*att*B Reverse Primers

Design each *att*B reverse primer to contain the following recommended sequence as listed below:

***att*B1** 5′-GGGG-AC-TGC-**TTT-TTT**-GTA-CAA-ACT-TGN--(template-specific sequence)-3′
*att*B1

***att*B2** 5′-GGGG-AC-CAC-**TTT-GTA**-CAA-GAA-AGC-TGG-GTN--(template-specific sequence)-3′
*att*B2

***att*B3** 5′-GGGG-AC-AAC-**TTT-GTA**-TAA-TAA-AGT-TGN--(template-specific sequence)-3′
*att*B3

continued on next page

Designing *att*B PCR Primers, continued

Important

- 50 nmoles of standard purity, desalted oligonucleotides is sufficient for most applications.
- Dissolve oligonucleotides to 20-50 mM in water or TE Buffer and verify the concentration before use.
- For more efficient cloning of large PCR products (greater than 5 kb), we recommend using HPLC or PAGE-purified oligonucleotides.

Producing *att*B PCR Products

DNA Templates

The following DNA templates can be used for amplification with *att*B-containing PCR primers:

- Genomic DNA
- mRNA
- cDNA libraries
- Plasmids containing cloned DNA sequences

Recommended Polymerases

We recommend using the following DNA polymerases available from Invitrogen to produce your *att*B PCR products. Other DNA polymerases are suitable.

- To generate PCR products less than 5-6 kb for use in protein expression, use Platinum® *Pfx* DNA Polymerase (Catalog no. 11708-013).
- To generate PCR products for use in other applications (*e.g.* functional analysis), use Platinum® *Taq* DNA Polymerase High Fidelity (Catalog no. 11304-011).

Producing PCR Products

Standard PCR conditions can be used to prepare *att*B PCR products. Follow the manufacturer's instructions for the DNA polymerase you are using, and use the cycling parameters suitable for your primers and template. **Note:** In general, *att*B sequences do not affect PCR product yield or specificity.

Checking the PCR Product

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

Note

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

Materials Needed:

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

Protocol:

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer and ≥ 5 units of *Dpn* I.
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the *Dpn* I at 65°C for 15 minutes.
4. Proceed to **Purifying *att*B PCR Products**, next page.

Purifying *att*B PCR Products

Introduction

After you have generated your *att*B PCR products, we recommend purifying each PCR product to remove *att*B primers and any *att*B 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 *att*B 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 *att*B PCR product (in a 50 µl volume)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 30% PEG 8000/30 mM $MgCl_2$ (supplied with the kit, Box 2)
- Agarose gel of the appropriate percentage to resolve your *att*B PCR products

PEG Purification Protocol

Use the protocol below to purify *att*B 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 *att*B PCR product.
2. Add 100 µl of 30% PEG 8000/30 mM $MgCl_2$. 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 TE, pH 8.0 (to concentration > 10 ng/µl).
5. Check the quality and quantity of the recovered *att*B 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 19. If the PCR product is not suitably purified (*e.g. att*B primer-dimers are still detectable), see below.

Additional Purification

If you use the procedure above and your *att*B PCR product is not suitably purified, you may gel purify your *att*B PCR product. We recommend using the S.N.A.P.™ Gel Purification Kit available from Invitrogen (Catalog no. K1999-25).

Creating Entry Clones Using the BP Recombination Reaction

Introduction

Once you have generated your *att*B PCR products, you will perform a BP reaction to transfer the DNA sequence of interest into an *att*P-containing donor vector to create an entry clone. To ensure that you obtain the best possible results, we suggest that you read this section and the ones entitled **Performing the BP Recombination Reaction** (pages 24-26) and **Transforming One Shot® TOP10 Competent Cells** (pages 27-29) before beginning.

Choosing a Donor Vector

Since different *att*B sites flank each *att*B PCR product, a specific donor vector is required for each BP recombination reaction. Refer to the table below to determine which donor vector to use in the BP recombination reaction. See the diagrams on pages 21-23 for an illustration of the recombination region of each entry clone after the BP reaction.

If your PCR product contains…	Then use…
*att*B4-PCR product-*att*B1	pDONR™P4-P1R
*att*B1-PCR product-*att*B2	pDONR™221
*att*B2-PCR product-*att*B3	pDONR™P2R-P3

Experimental Outline

To generate an entry clone, you will:

1. Perform a BP recombination reaction using the appropriate linear *att*B PCR product and a supercoiled, *att*P-containing donor vector (see above).
2. Transform the reaction mixture into a suitable *E. coli* host (see page 27).
3. Select for entry clones.

Important

For optimal results, perform the BP recombination reaction using:

- **Linear** *att*B PCR products
- **Supercoiled** donor vector

continued on next page

Creating Entry Clones Using the BP Recombination Reaction, continued

Donor Vectors

The pDONR™P4-P1R, pDONR™221, and pDONR™P2R-P3 vectors are supplied with the kit to facilitate generation of entry clones using the BP recombination reaction. The donor vectors contain the following elements:

- Two *att*P sites for recombinational cloning of *att*B-containing PCR products
- The *ccd*B gene located between the *att*P sites for negative selection
- The chloramphenicol resistance gene (Cm^R) located between the two *att*P sites for counterselection
- M13 forward (-20) and M13 reverse primer binding sites to facilitate sequencing of the entry clone, if desired
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*
- Kanamycin resistance gene for selection of the plasmid in *E. coli*

For a map and a description of the features of each donor vector, see the **Appendix**, pages 41-44.

Resuspending the Donor Vectors

All donor vectors are supplied as 6 µg of supercoiled plasmid, lyophilized in TE Buffer, pH 8.0. To use, resuspend the pDONR™ plasmid DNA in 40 µl of sterile water to a final concentration of 150 ng/µl. To propagate donor vectors, see page 10.

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 previously supplied as separate components in BP Clonase™ enzyme mix (Catalog no. 11789-019) into an optimized single-tube format to allow easier 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.

Note: You may perform the BP recombination reaction using BP Clonase™ enzyme mix (not supplied), if desired. To use BP Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for BP Clonase™ II enzyme mix provided on page 26 as reaction conditions differ.

continued on next page

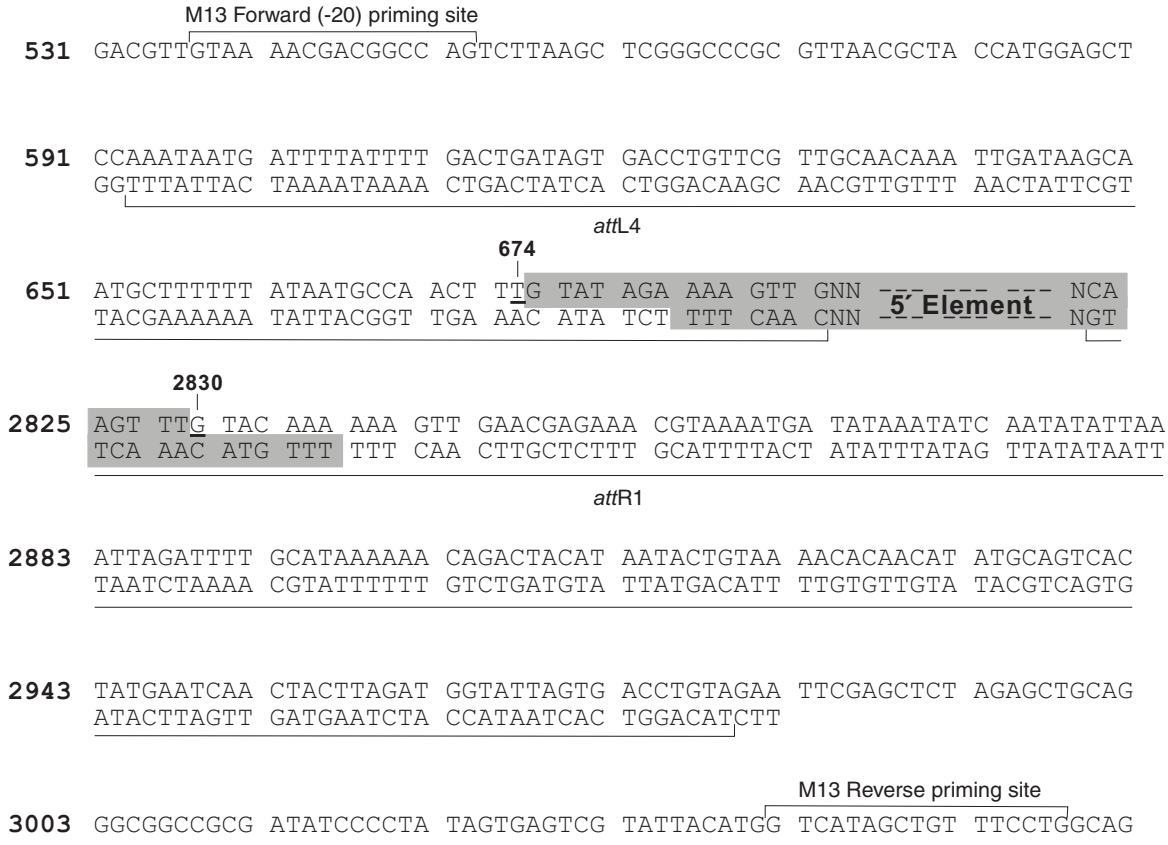
Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of the *att*L4 and *att*R1-Flanked Entry Clone

The recombination region of the entry clone resulting from pDONR™P4-P1R × *att*B4-5′ element-*att*B1 is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the *att*B 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.



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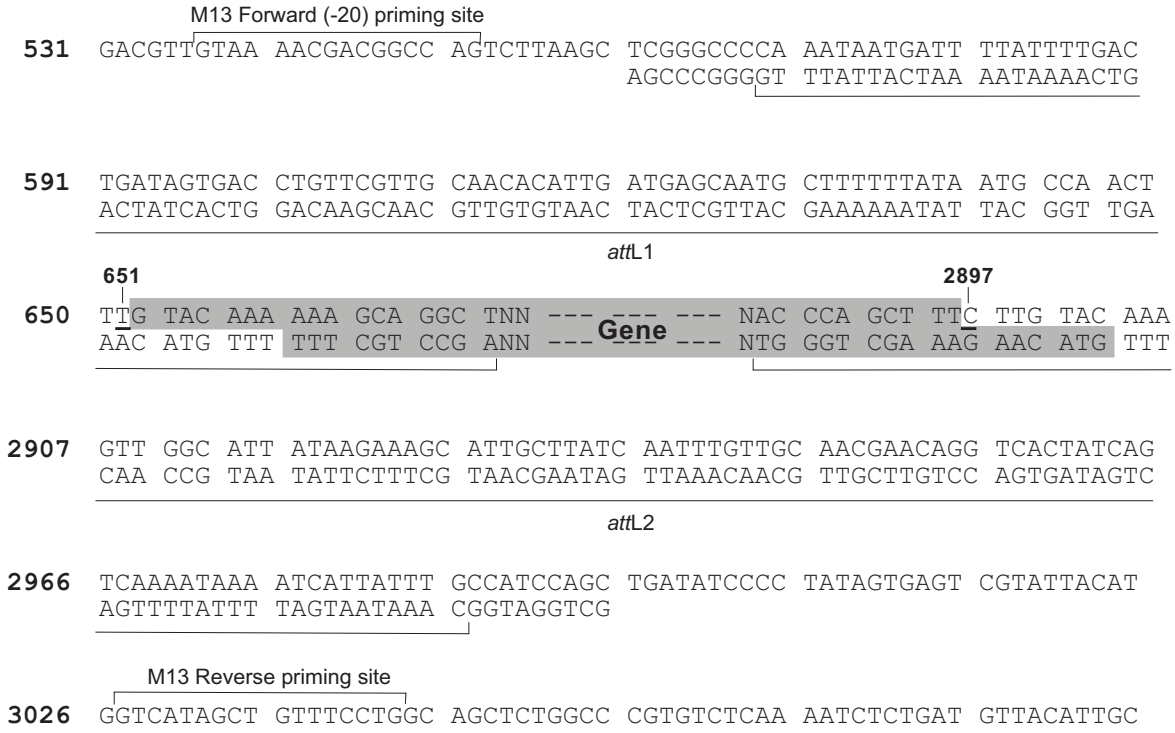
Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of the *att*L1 and *att*L2-Flanked Entry Clone

The recombination region of the entry clone resulting from pDONR™221 × *att*B1-gene of interest-*att*B2 is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the *att*B 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.



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Creating Entry Clones Using the BP Recombination Reaction, continued

Recombination Region of the *att*R2 and *att*L3-Flanked Entry Clone

The recombination region of the entry clone resulting from pDONR™P2R-P3 × *att*B2-3′ element-*att*B3 is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the *att*B 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 TCGGGCCCTG CAGCTCTAGA GCTCGAATTC

591  TACAGGTCAC TAATACCATC TAAGTAGTTG ATTCATAGTG ACTGCATATG TTGTGTTTTA
     ATGTCCAGTG ATTATGGTAG ATTCATCAAC TAAGTATCAC TGACGTATAC AACACAAAAT
```

*att*R2

```
651  CAGTATTATG TAGTCTGTTT TTTATGCAAA ATCTAATTTA ATATATTGAT ATTTATATCA
     GTCATAATAC ATCAGACAAA AAATACGTTT TAGATTAAAT TATATAACTA TAAATATAGT
```

733

```
711  TTTTACGTTT CTCGTTCA ACT TTC TTG TAC AAA GTG GNN ___ ___ ___ NCA
     AAAATGCAAA GAGCAAGT TGA AAG AAC ATG TTT CAC CNN _3´Element_ NGT
```

2889

```
2884 ACT TTA TTA TAC AAA GTT GGCATTATA AAAAAGCATT GCTTATCAAT TTGTTGCAAC
     TGA AAT AAT ATG TTT CAA CCGTAATAT TTTTTCGTAA CGAATAGTTA AACAACGTTG
```

*att*L3

```
2941 GAACAGGTCA CTATCAGTCA AAATAAAATC ATTATTTGGA GCTCCATGGT AGCGTTAACG
     CTTGTCCAGT GATAGTCAGT TTTATTTTAG TAATAAACCT
```

M13 Reverse priming site

```
3001 CGGCCGCGAT ATCCCCTATA GTGAGTCGTA TTACATGGTC ATAGCTGTTT CCTGGCAGCT
```

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 *att*B 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.

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 the **Appendix**, page 47 for a map and more information). For an alternate positive control to use when creating an *att*L1 and *att*L2-flanked entry clone, see below.

The pMS/GW plasmid is supplied as 10 µg of supercoiled plasmid, lyophilized in TE Buffer, pH 8.0. To use, resuspend the pMS/GW DNA in 10 µl of sterile water to a final concentration of 1 µg/µl. To propagate the plasmid, see page 10.

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 (New England Biolabs, Catalog no. R0117S).

1. Digest 5 µg of pMS/GW plasmid in a 50 µl reaction using *Aat* II. Follow 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 *att*L1 and *att*L2-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 *att*B1 and *att*B2 sites flanking the tetracycline resistance gene and its promoter (Tc^r).

Determining How Much *att*B PCR Product and Donor Vector to Use in the Reaction

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

- An equimolar amount of *att*B PCR product and the donor vector
- 50 femtomoles (fmoles) **each** of *att*B PCR product and donor vector is preferred, but the amount of *att*B 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 *att*B PCR product, but no more than 250 ng

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

continued on next page

Performing the BP Recombination Reaction, continued

- 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 *att*B 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:

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

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 *att*B PCR product in the BP reaction. The *att*B PCR product is 2.5 kb in size. Calculate the amount of *att*B PCR product required for the reaction (in ng) by using the equation above:

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

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:

- *att*B PCR products (*i.e. att*B4-PCR product-*att*B1, *att*B1-PCR product-*att*B2, or *att*B2-PCR product-*att*B3; see the previous page and above to determine the amount of DNA to use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

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Performing the BP Recombination Reaction, continued

Setting Up the BP Reaction

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

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

Components	Sample	Negative Control	Positive Control
*att*B PCR product (20-50 fmoles)	1-7 µl	1-7 µl	--
pDONR™ vector (150 ng/µl)	1 µl	1 µl	1 µl
pMS/GW control plasmid (100 ng/µl)	--	--	2 µl
TE Buffer, pH 8.0	to 8 µl	to 10 µl	5 µl

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, 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, if desired.

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 *rec*A, *end*A *E. coli* strain including TOP10 (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 *ccd*A gene and will prevent negative selection with the *ccd*B gene.

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

Item	Quantity	Catalog No.
Library Efficiency® DH5α™	5 x 200 µl	18263-012
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µl	C4040-03
One Shot® Max Efficiency® DH10B™ T1 Phage Resistant Chemically Competent *E. coli*	20 x 50 µl	12331-013
One Shot® TOP10 Electrocomp *E. coli*	20 x 50 µl	C4040-52
ElectroMax™ DH10B™	5 x 100 µl	18290-015

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, previous page) or MultiSite Gateway® LR recombination reaction (from **Setting Up the MultiSite Gateway® LR Reaction**, Step 7, page 36)
- LB plates containing 50 µg/ml kanamycin (for the BP reaction) or 50-100 µg/ml ampicillin (for the MultiSite Gateway® LR reaction). Prepare two plates for each transformation; warm at 37°C for 30 minutes.
- 42°C water bath (for chemical transformation)
- 37°C shaking and non-shaking incubator

continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

1. Into a vial of One Shot® TOP10 chemically competent *E. coli*, add the following 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 36). **Note:** You may transform up to 5 µl of the reaction, if desired.

   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 2,000 to 8,000 if the entire reaction 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 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 36).
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, then transform 1 µl into cells

Sequencing Entry Clones

Introduction

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

Sequencing Primers

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:

Forward primer	M13 Forward (-20): 5′-GTAAAACGACGGCCAG-3′
Reverse primer	M13 Reverse: 5′-CAGGAAACAGCTATGAC-3′

See the diagrams on page 21-23 for the location of the M13 forward (-20) and M13 reverse primer binding sites in each entry clone. The M13 Forward (-20) and M13 Reverse Primers (Catalog nos. N520-02 and N530-02, respectively) are available separately from Invitrogen. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 48).

Sequencing Using BigDye™ Chemistry

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

- Use at least 500 ng of DNA
- Use 5-50 pmoles of primers
- Use 1/4 reaction and the PCR conditions listed below

PCR Conditions

Use the following PCR conditions for sequencing using BigDye™ chemistry. These conditions are suitable for most inserts, including small inserts.

Step	Time	Temperature	Cycles
Initial Denaturation	5 minutes	95°C	1X
Denaturation	10-30 seconds	96°C	30X
Annealing	5-15 seconds	50°C	
Extension	4 minutes	60°C	

Creating Expression Clones Using the 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 destination vector to create an *att*B-containing expression clone with the following structure:

***att*B4-5′ element-*att*B1-gene of interest-*att*B2-3′ element-*att*B3**

To ensure that you obtain the best results, we suggest reading this section and the next section entitled **Performing the MultiSite Gateway® LR Recombination Reaction** (pages 34-36) 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 (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 33 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.

- *att*L4 and *att*R1-containing entry clone
- *att*L1 and *att*L2-containing entry clone
- *att*R2 and *att*L3-containing entry clone
- pDEST™R4-R3 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 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

continued on next page

Creating Expression Clones Using the MultiSite Gateway® LR Recombination Reaction, continued

pDEST™R4-R3 Vector

The pDEST™R4-R3 vector is supplied with the kit for use in the MultiSite Gateway® LR recombination reaction to generate an expression clone containing your three DNA fragments of choice. The pDEST™R4-R3 plasmid contains the following elements:

- *att*R4 and *att*R3 sites for recombinational cloning of three DNA fragments from the appropriate Gateway® entry clones
- M13 forward (-20) and M13 reverse primer binding sites to facilitate sequencing of the expression clone, if desired

  Note: When sequencing your expression clone, you will use the M13 reverse primer to sequence the sense strand and the M13 forward (-20) primer to sequence the anti-sense strand. Refer to the diagram on page 33 for the location of the priming sites.
- pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*
- Ampicillin resistance gene for selection of the plasmid in *E. coli*

Important: Note that all other elements required to express your gene of interest in the system of choice must be supplied by the entry clones.

Resuspending the pDEST™R4-R3 Vector

pDEST™R4-R3 is supplied as 6 µg of plasmid, lyophilized in TE, pH 8.0. To use, resuspend the destination plasmid in 100 µl of sterile water to a final concentration of 60 ng/µl. To propagate the vector, see page 10.

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 20 µl MultiSite Gateway® LR recombination reaction:

- An equimolar amount of each plasmid
- 20-25 fmoles of **each** entry clone and pDEST™R4-R3 is recommended. Do not use more than 30 fmoles of each plasmid.

  Note: 20 fmoles of pDEST™R4-R3 is approximately 60 ng

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

- Do not use more than 120 fmoles of total plasmid DNA in a 20 µl MultiSite Gateway® LR reaction as this will affect the efficiency of the reaction.
- Do not exceed more than 1 µg of total DNA (*i.e.* 250 ng of each entry clone plus destination vector) in a 20 µl MultiSite Gateway® LR reaction as excess DNA will inhibit the reaction. If you need to use more than 1 µg of total DNA, scale up the volume of the MultiSite Gateway® LR reaction.

continued on next page

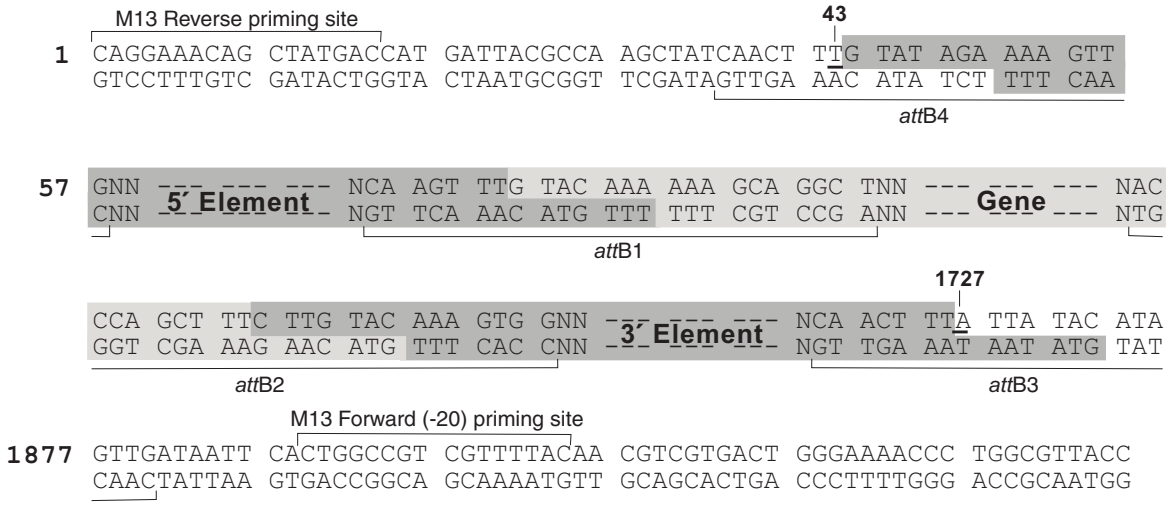
Creating Expression Clones Using the MultiSite Gateway® LR Recombination Reaction, continued

Recombination Region of the Expression Clone

The recombination region of the expression clone resulting from pDEST™R4-R3 × *att*L4-5′ entry clone-*att*R1 × *att*L1-entry clone-*att*L2 × *att*R2-3′ entry clone-*att*L3 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 by recombination. Note that the sequences comprising the *att*B1 and *att*B2 sites are entirely supplied by the entry clones. Non-shaded regions are derived from the pDEST™R4-R3 vector.
- Bases 43 and 1867 of the pDEST™R4-R3 sequence are indicated.



Performing the MultiSite Gateway® LR Recombination Reaction

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 using LR Clonase™ 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™ Plus) in your experiment to help you evaluate your results.

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 *rec*A, *end*A *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 *ccd*A gene and will prevent negative selection with the *ccd*B 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.

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 S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K2100-01) available from Invitrogen.

Important

You **must** use LR Clonase™ Plus enzyme mix to catalyze the MultiSite Gateway® LR recombination reaction. Note that the LR Clonase™ II enzyme mix (Catalog no. 11791-020) used for standard Gateway® LR recombination reactions **cannot** be used for MultiSite Gateway® LR recombination reactions.

LR Clonase™ Plus enzyme mix is supplied with the kit, but is also available separately from Invitrogen (see page ix for ordering information).

continued on next page

Performing the MultiSite Gateway® LR Recombination Reaction, continued

Materials Needed

You should have the following materials on hand before beginning.

Supplied with the kit:

- pDEST™R4-R3 (60 ng/µl in TE, pH 8.0)
- LR Clonase™ Plus enzyme mix (Box 3, keep at -80°C until immediately before use)
- 5X LR Clonase™ Plus Reaction Buffer (thaw and keep on ice before use)
- 2 µg/µl Proteinase K solution

Supplied by the user:

- Purified plasmid DNA of your *att*L4 and *att*R1-flanked entry clone (supercoiled, 20-25 fmoles)
- Purified plasmid DNA of your *att*L1 and *att*L2-flanked entry clone (supercoiled, 20-25 fmoles)
- Purified plasmid DNA of your *att*R2 and *att*L3-flanked entry clone (supercoiled, 20-25 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 a 20 µl MultiSite Gateway® LR reaction does not exceed 11 µl.
- 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) and growth media for expression
- S.O.C. Medium
- LB agar plates containing 50-100 µg/ml ampicillin

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Performing the MultiSite Gateway® 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.

Component	Sample	Negative Control
*att*L4 and *att*R1 entry clone (20-25 fmoles)	3-11 µl	--
*att*L1 and *att*L2 entry clone (20-25 fmoles)		
*att*R2 and *att*L3 entry clone (20-25 fmoles)		
pDEST™R4-R3 vector (60 ng/reaction)	1 µl	1 µl
5X LR Clonase™ Plus Reaction Buffer	4 µl	4 µl
TE Buffer, pH 8.0	to 16 µl	11 µl

2. Remove the LR Clonase™ Plus enzyme mix from -80°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ Plus enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 4 µl of LR Clonase™ Plus enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return LR Clonase™ Plus enzyme mix to -80°C immediately after use.
5. Incubate reactions at 25°C for 16 hours or overnight.
6. Add 2 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. If you are transforming 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 10^9 cfu/µg, the MultiSite Gateway® LR reaction should give approximately 2,000 to 8,000 colonies if the entire reaction is transformed and plated.

Once you have obtained an expression clone, proceed to express your recombinant protein in the appropriate system.

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.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Check the antibiotic resistance marker and use the correct antibiotic to select for entry clones or expression clones.
	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
	Used incorrect *att* sites for the reaction	• Use the appropriate entry clones and pDEST™R4-R3 for the MultiSite Gateway® LR reaction (see page 11 for details about the types of entry clones required). • Use the correct *att*B PCR product and donor vector (*att*P) for the BP reaction (see page 19 for details).
	Clonase™ (Plus) enzyme mix is inactive or didn't use suggested amount of Clonase™ (Plus) enzyme mix	• Test another aliquot of the Clonase™ (Plus) enzyme mix. • Store the LR Clonase™ Plus at -80°C and the BP Clonase™ II at -20°C. • Do not freeze/thaw the Clonase™ (Plus) enzyme mix more than 10 times. • Use the recommended amount of Clonase™ (Plus) enzyme mix (see page 26 or 36 as appropriate).
	Used incorrect Clonase™ enzyme mix	• Use the LR Clonase™ Plus enzyme mix for the MultiSite Gateway® LR reaction. **Do not** use the LR Clonase™ enzyme mix. • Use the BP Clonase™ II enzyme mix for the BP reaction.
	Too much *att*B PCR product was used in a BP reaction	Reduce the amount of *att*B PCR product used. Use an equimolar ratio of *att*B PCR product and donor vector (*i.e.* ~50 fmoles each).
	Long *att*B PCR product or linear *att*B expression clone (≥5 kb)	Incubate the BP reaction overnight.
	Too much DNA was used in a MultiSite Gateway® LR reaction	Use an equimolar amount of each entry clone and destination vector. Do not exceed 120 fmoles or 1 µg of total DNA in the reaction.

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Troubleshooting

MultiSite Gateway® LR and BP Reactions, continued

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies, continued	MultiSite Gateway® LR reaction not incubated for sufficient time	Incubate the MultiSite Gateway® LR reaction at 25°C for 16 hours or overnight.
	Insufficient amount of *E. coli* transformed or plated	**MultiSite Gateway® LR reaction:** Transform 2 to 5 µl of the reaction; plate 50 µl or 100 µl. **BP reaction:** Transform 1 µl of the reaction; plate 20 µl and 100 µl.
MultiSite Gateway® LR Reaction: High background in the absence of the entry clones	MultiSite Gateway® LR reaction transformed into an *E. coli* strain containing the F′ episome and the *ccd*A gene	Use an *E. coli* strain that does not contain the F′ episome for transformation (*e.g.* TOP10, DH5α™).
	Deletions (full or partial) of the *ccd*B 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* plated.

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Troubleshooting

MultiSite Gateway® LR and BP Reactions, continued

Problem	Reason	Solution
Two distinct types of colonies (large and small) appear	**BP reaction:** The pDONR™ vector contains deletions or point mutations in the *ccd*B gene **Note:** The negative control will give a similar number of colonies	Obtain a new pDONR™ vector.
	Loss of plasmid during culture (generally those containing large genes or toxic genes)	• Incubate selective plates at 30°C instead of 37°C. • Confirm whether a deletion has occurred by analyzing the DNA derived from the colonies. • Use Stbl2™ *E. coli* (Invitrogen, Catalog no. 10268-019) to help stabilize plasmids containing large genes during propagation (Trinh *et al.*, 1994).

*att*B PCR Cloning

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

Problem	Reason	Solution
Low yield of *att*B PCR product obtained after PEG purification	*att*B PCR product not diluted with TE	Dilute with 150 µl of 1X TE, pH 8.0 before adding the PEG/$MgCl_2$ solution.
	Centrifugation step too short or centrifugation speed too low	Increase time and speed of the centrifugation step to 30 minutes and 15,000 x g.
	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.

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*att*B PCR Cloning, continued

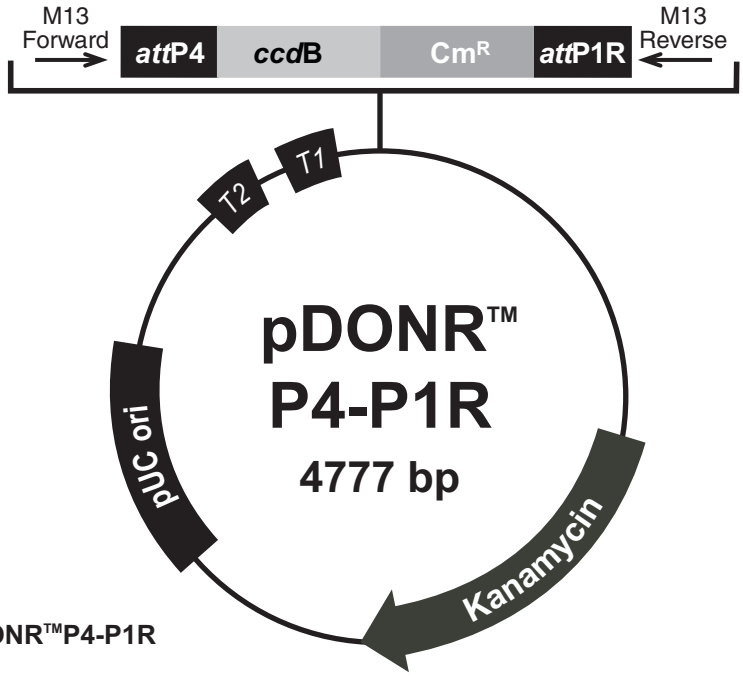
Problem	Reason	Solution
Few or no colonies obtained from a BP reaction with *att*B PCR product **and** both *att*B positive control and transformation control gave expected number of colonies	*att*B PCR primers incorrectly designed	Make sure that each *att*B PCR primer includes four 5′ terminal Gs and the 22 or 25 bp *att*B site as specified on page 15.
	*att*B PCR primers contaminated with incomplete sequences	Use HPLC or PAGE-purified oligonucleotides to generate your *att*B PCR product.
	*att*B PCR product not purified sufficiently	Gel purify your *att*B PCR product to remove *att*B primers and *att*B primer-dimers.
	For large PCR products (>5 kb), too few *att*B PCR molecules added to the BP reaction	• Increase the amount of *att*B PCR product to 20-50 fmoles per 10 µl reaction. **Note:** Do not exceed 250 ng DNA per 10 µl reaction. • Incubate the BP reaction overnight.
	Insufficient incubation time	Increase the incubation time of the BP reaction up to 18 hours.
Entry clones migrate as 2.2 kb supercoiled plasmids	BP reaction may have cloned *att*B primer-dimers	• Purify *att*B PCR product using the PEG/$MgCl_2$ purification protocol on page 18 or gel-purify the *att*B PCR product. • Use a Platinum® DNA polymerase with automatic hot-start capability for higher specificity amplification. • Redesign *att*B PCR primers to minimize potential mutual priming sites leading to primer-dimers.

Appendix

Map of pDONR™P4-P1R

pDONR™P4-P1R Map

The map below shows the elements of pDONR™P4-P1R. **The complete sequence of pDONR™P4-P1R is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pDONR™P4-P1R
4777 nucleotides

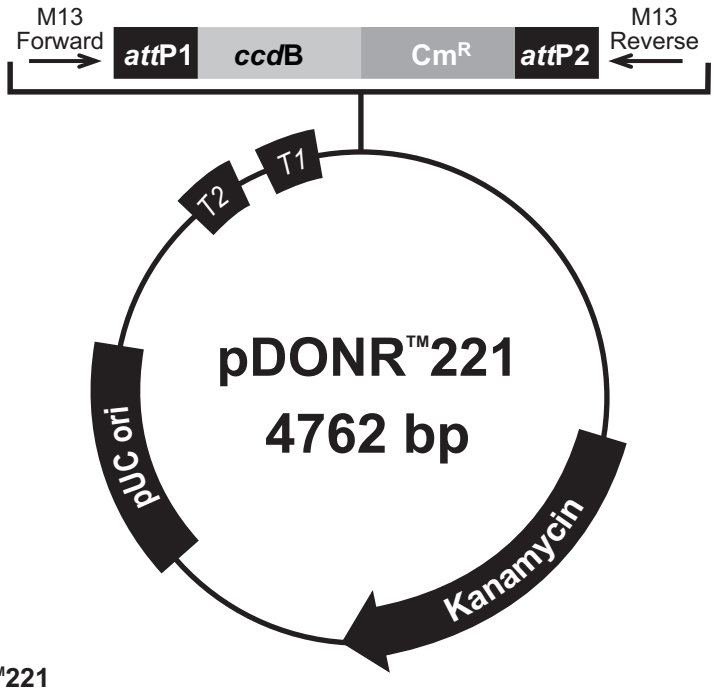
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P4 recombination site: bases 593-824 (c)
*ccd*B gene: bases 1181-1486 (c)
Chloramphenicol resistance gene: bases 1828-2487 (c)
*att*P1R 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

pDONR™221 Map

The map below shows the elements of pDONR™221. **The complete sequence of pDONR™221 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pDONR™221
4762 nucleotides

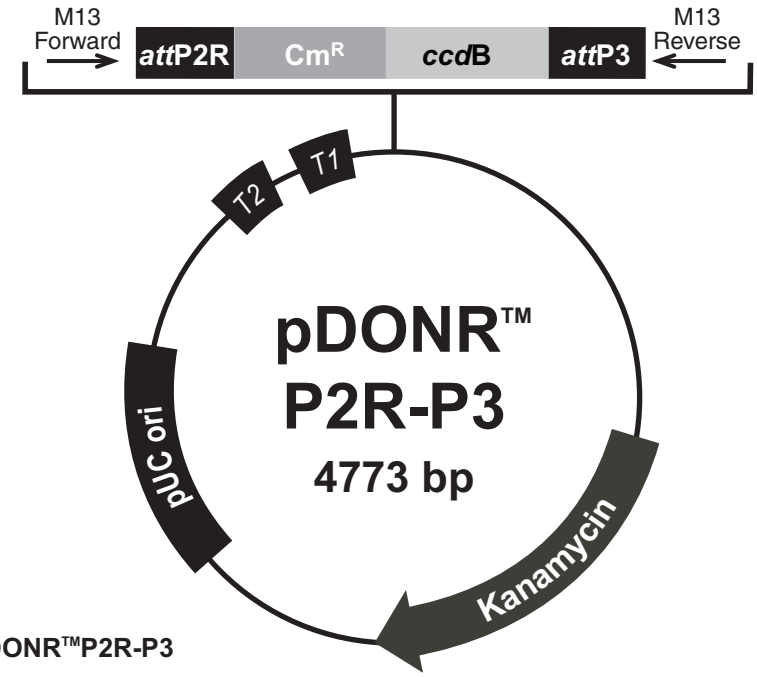
*rrn*B T2 transcription termination sequence (c): bases 268-295
rrnB T1 transcription termination sequence (c): bases 427-470
M13 Forward (-20) priming site: bases 537-552
*att*P1: bases 570-801
*ccd*B gene (c): bases 1197-1502
Chloramphenicol resistance gene (c): bases 1847-2506
*att*P2 (c): bases 2754-2985
M13 Reverse priming site: bases 3027-3043
Kanamycin resistance gene: bases 3156-3965
pUC origin: bases 4086-4759

(c) = complementary strand

Map of pDONR™P2R-P3

pDONR™P2R-P3 Map

The map below shows the elements of pDONR™P2R-P3. **The complete sequence of pDONR™P2R-P3 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pDONR™P2R-P3
4773 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P2R recombination site: bases 591-822
Chloramphenicol resistance gene: bases 1083-1742
*ccd*B gene: bases 2084-2389
*att*P3 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

Features of the pDONR™ Vectors

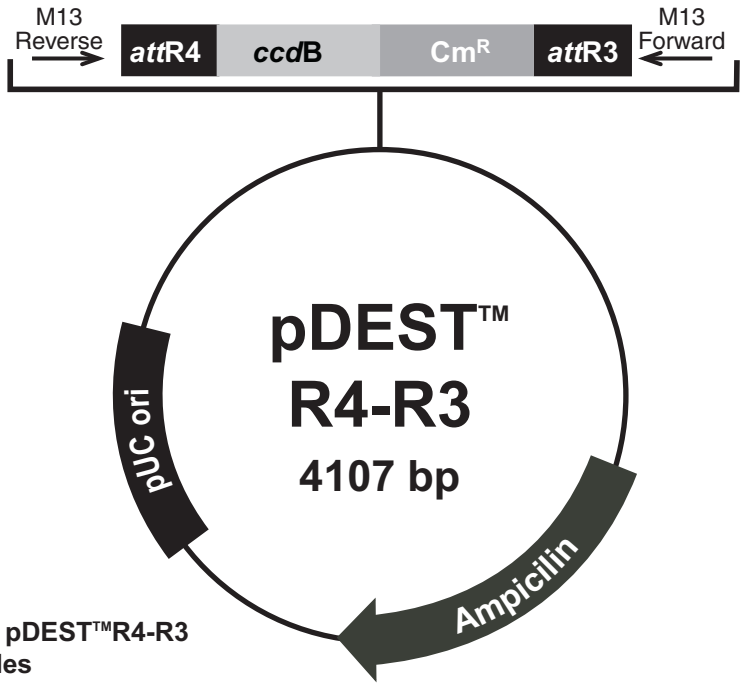
pDONR™P4-P1R (4777 bp), pDONR™221 (4762 bp), and pDONR™P2R-P3 (4773 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz *et al.*, 1991).
M13 forward (-20) priming site	Allows sequencing in the sense orientation.
*att*P4 and *att*P1R site (pDONR™P4-P1R) *att*P1 and *att*P2 sites (pDONR™221) *att*P2R and *att*P3 sites (pDONR™P2R-P3)	Bacteriophage λ-derived DNA recombination sequences that have been optimized to permit recombinational cloning of DNA fragments from specific *att*B PCR products (Landy, 1989).
*ccd*B gene	Permits negative selection of the plasmid.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
M13 reverse priming site	Permits sequencing in the anti-sense orientation.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin and replisome assembly site	Permits high-copy replication and maintenance of the plasmid in *E. coli*.

Map and Features of pDEST™R4-R3

pDEST™R4-R3 Map

The map below shows the elements of pDEST™R4-R3. **The complete sequence of pDEST™R4-R3 is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pDEST™R4-R3
4107 nucleotides

M13 Reverse priming site: bases 1-17
*att*R4 recombination site: bases 37-161
*ccd*B gene: bases 201-506 (c)
Chloramphenicol resistance gene: bases 848-1507 (c)
*att*R3 recombination site: bases 1616-1740 (c)
M13 Forward (-20) priming site: bases 1749-1764 (c)
bla promoter: bases 2244-2342
Ampicillin (*bla*) resistance gene: bases 2343-3203
pUC origin: bases 3348-4021
(c) = complementary strand

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Map and Features of pDEST™R4-R3

Features of the pDEST™R4-R3 Vector

pDEST™R4-R3 (4107 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
M13 reverse priming site	Permits sequencing in the sense orientation.
*att*R4 and *att*R3 sites	Bacteriophage λ-derived DNA recombination sequences that have been optimized to permit recombinational cloning of DNA fragments from specific *att*L-flanked entry clones (Landy, 1989).
*ccd*B gene	Permits negative selection of the plasmid.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
M13 forward (-20) priming site	Allows sequencing in the anti-sense orientation.
bla promoter	Permits expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allow selection of the plasmid in *E. coli*.
pUC origin and replisome assembly site	Permits high-copy replication and maintenance of the plasmid in *E. coli*.

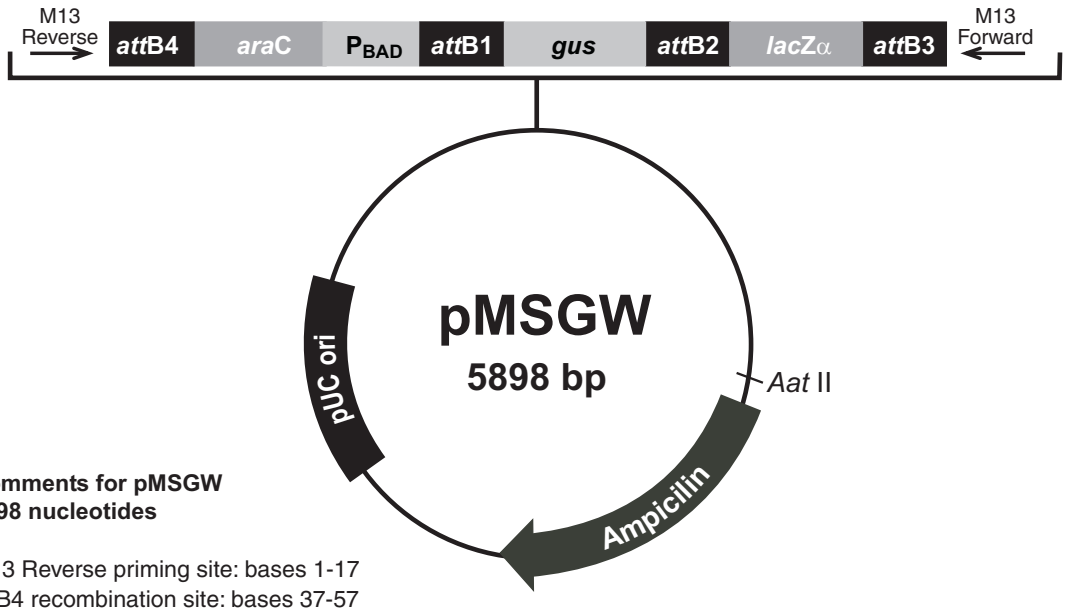
Map of pMS/GW

Description

pMS/GW is a 5898 bp control vector, and was generated using the MultiSite Gateway® LR recombination reaction between pDEST™R4-R3 and three entry clones containing the *ara*C gene and *ara*BAD promoter, *gus* gene, and *lacZ*α fragment, respectively. This expression clone is designed for use as a control for each BP recombination reaction (see page 24 for details).

Map of pMS/GW

The map below shows the elements of pMS/GW. **The complete sequence of pMS/GW is available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 48).**



Comments for pMSGW
5898 nucleotides

M13 Reverse priming site: bases 1-17
*att*B4 recombination site: bases 37-57
AraC ORF: bases 58-936 (c)
Arabinose O_2 operator region: bases 966-981
Arabinose O_1 operator region: bases 1123-1144
CAP binding site: bases 1165-1178
Arabinose I_1 and I_2 region: bases 1175-1213
Arabinose minimal promoter: bases 1175-1213
Ribosome binding site: bases 1267-1270
*att*B1 recombination site: bases 1285-1308
gus gene: bases 1306-3149
*att*B2 recombination site: bases 3154-3174
*lacZ*α gene: bases 3175-3509
*att*B3 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 Service

World Wide Web

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

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

Once connected to the Internet, launch your web browser (Netscape 3.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

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

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MSDS Requests

To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

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Technical Service, continued

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Purchaser Notification

Introduction

Use of the MultiSite Gateway® Three-Fragment Vector Construction Kit is covered under the licenses detailed below.

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 52.

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Purchaser Notification, continued

Limited Use Label License No. 23: GUS Control Vector	The GUS positive control vector in these products is claimed in patents and patent applications (See U.S. Patent No. 5,599,670 and Great Britain Patent No. 2,197,653) licensed to Invitrogen by Cambia Biosystems, L.L.C. ("CBL"). Use of the GUS gene is restricted to use as a positive control. Any other use may require a license from CBL.
Limited Use Label License No. 28: CMV Promoter	The use of the CMV promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned and licensed by the University of Iowa Research Foundation and is sold for research use only. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation (UIRF), 214 Technology Innovation Center, Iowa City, Iowa 52242. For further information, please contact the Associate Director of UIRF, at 319-335-4546.
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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

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

Product Qualification

Introduction

This section describes the criteria used to qualify the components of the MultiSite Gateway® Three-Fragment Vector Construction Kit.

Vectors

The vectors are qualified as described below.

pDONR™ vectors (pDONR™P4-P1R, pDONR™P2R-P3, pDONR™221)

- Structure of the vector is verified by restriction enzyme digestion.
- Functionality is verified in a 1 hour recombination assay with Gateway® BP Clonase™ II enzyme mix.
- The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

pDEST™R4-R3

- Structure of the vector is verified by restriction enzyme digestion.
- Functionality is verified in a 16 hour recombination assay with Gateway® LR Clonase™ Plus enzyme mix.
- The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.

pMS/GW

- Structure of the vector is verified by restriction enzyme digestion.

BP Clonase™ II Enzyme Mix

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

LR Clonase™ Plus Enzyme Mix

Gateway® LR Clonase™ Plus enzyme mix is functionally tested in a 16 hour MultiSite Gateway® LR recombination reaction followed by a transformation assay.

One Shot® TOP10 Chemically Competent *E. coli*

1. One Shot® TOP10 chemically competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^9 cfu/µg plasmid DNA.
2. To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
3. Untransformed cells are plated on LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Glossary of Terms

***att*L, *att*R, *att*B, and *att*P**	The recombination sites from bacteriophage lambda that are utilized in the Gateway® Technology. • *att*L always recombines with *att*R in a reaction mediated by the LR Clonase™ II enzyme mix (for standard Gateway® reactions) or LR Clonase™ Plus enzyme mix (for MultiSite Gateway® reactions). The LR reaction is the basis for the entry clone(s) × destination vector reaction. Recombination between *att*L and *att*R sites yields *att*B and *att*P sites on the resulting plasmids. • *att*B sites always recombine with *att*P sites in a reaction mediated by the BP Clonase™ II enzyme mix. The BP reaction is the basis for the reaction between the donor vector (pDONR™) and PCR products or other clones containing *att*B sites. Recombination between *att*B and *att*P sites yields *att*L and *att*R sites on the resulting plasmids.
BP Clonase™ II Enzyme Mix	A proprietary mix of lambda recombination proteins that mediates the *att*B × *att*P recombination reaction.
***ccd*B Gene**	A gene that encodes a protein that interferes with *E. coli* DNA gyrase, thereby inhibiting the growth of standard *E. coli* hosts. This gene is present on Gateway® destination, donor, and supercoiled entry vectors. When recombination occurs between a destination vector and an entry clone, the gene of interest replaces the *ccd*B gene. Cells that take up unreacted vectors carrying the *ccd*B gene, or by-product molecules that retain the *ccd*B gene, will fail to grow. This allows high-efficiency recovery of only the desired clones.
Destination Vector	Gateway®-adapted expression vectors which contain *att*R sites and allow recombination with entry clones.
Donor Vector (pDONR™)	A Gateway® vector containing *att*P sites. This vector is used for cloning PCR products and DNA sequences of interest flanked by *att*B sites (expression clones) to generate entry clones. When PCR fragments modified with *att*B sites are recombined with the pDONR™ vector in a BP reaction, they yield an entry clone. **PCR fragment (*att*B sites) + pDONR™ vector (*att*P sites) → entry clone**
Entry Clone	The result of cloning a DNA segment into an entry vector or donor vector. For MultiSite Gateway® applications, the entry clone contains the DNA sequence of interest flanked by *att*L sites or a combination of *att*L and *att*R sites. The entry clone can be used for subsequent transfers into destination vectors.
Entry Vector (pENTR™)	A Gateway® vector containing *att*L1 and *att*L2 sites used for cloning DNA fragments using either TOPO® Cloning or conventional restriction enzymes and ligase.

continued on next page

Glossary of Terms, continued

Expression Clone	The result of subcloning the DNA of interest from an entry clone into a destination vector of choice by LR recombination. For MultiSite Gateway® applications, the expression clone contains DNA fragments transferred from multiple entry clones into a single destination vector. Each DNA fragment of interest in the expression clone is flanked by *att*B sites. **Entry clone(s) + destination vector → expression clone**
Gateway® Technology	A universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989) that allows highly efficient transfer of a DNA sequence of interest into multiple vector systems.
LR Clonase™ Plus Enzyme Mix	A proprietary mix of lambda and *E. coli* recombination proteins that mediates the *att*L × *att*R recombination reaction. This enzyme has been optimized for demanding applications including MultiSite Gateway®, but is also suitable for use in standard Gateway® applications.
MultiSite Gateway® Technology	An extension of the Gateway® Technology that facilitates simultaneous cloning of multiple DNA fragments in a defined order and orientation.

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