USER GUIDE

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MultiSite Gateway® Three-Fragment Vector Construction Kit

Using Gateway® Technology to simultaneously clone multiple DNA fragments

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Experienced Users Guide

Introduction

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

BP Recombination Reaction

Perform a BP recombination reaction between each *att*B-flanked DNA fragment and the appropriate *att*P-containing donor vector to generate an entry clone (see page 26 for details).

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

*att*B PCR product (20–50 fmoles)	1–7 µl
pDONR™ vector (supercoiled, 150 ng/µl)	1 µl
1x TE Buffer, pH 8.0	to 8 µl

2. Vortex BP Clonase® II enzyme mix briefly. Add 2 µl to the components above and mix well by vortexing briefly twice.
3. Incubate reaction at 25°C for 1 hour.
4. Add 1 µl of 2 µg/µl Proteinase K solution and incubate at 37°C for 10 minutes.
5. Transform 1 µl of the reaction into competent *E. coli* and select for kanamycin-resistant entry clones.

MultiSite Gateway® LR Recombination Reaction

IMPORTANT: This kit contains a new enzyme (LR Clonase II Plus) Please follow the protocol below and on pages 31-35.

Perform a MultiSite Gateway® LR recombination reaction between multiple entry clones (*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 II 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, 10 fmoles each)	1–7 µl
pDEST™ R4-R3 Vector II (supercoiled, 20 fmol)	1 µl
1x TE Buffer, pH 8.0	to 8 µl

2. Vortex LR Clonase® II Plus enzyme mix briefly. Add 2 µl to the components above and mix well by vortexing briefly twice.
3. Incubate reaction at 25°C for 16 hours (or overnight).
4. Add 1 µl of 2 µg/µl Proteinase K solution and incubate at 37°C for 10 minutes.
5. Transform 2 µl of the reaction into competent *E. coli* and select for ampicillin-resistant expression clones.

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Primer Sequences

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

DONR Vector	*att* sites Flanking Insert	Primer Sequences
pDONR™ P4-P1R	*att*B4	Fwd: 5′-GGGG ACA ACT TTG TAT AGA AAA GTT GNN
	*att*B1r	Rev: 5′-GGGG AC TGC TTT TTT GTA CAA ACT TGN
pDONR™ 221	*att*B1	Fwd: 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TNN
	*att*B2	Rev: 5′-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTN
pDONR™ P2R-P3	*att*B2r	Fwd: 5′-GGGG ACA GCT TTC TTG TAC AAA GTG GNN
	*att*B3	Rev: 5′-GGGG AC AAC TTT GTA TAA TAA AGT TGN

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 (6 months) –80°C (long term)
3	LR Clonase® II Plus Enzyme Mix	–20°C (6 months) –80°C (long term)
4	One Shot® TOP10 Chemically Competent *E. coli*	–80°C

Vectors

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

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

BP Clonase® II Enzyme Mix

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

Item	Composition	Quantity
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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LR Clonase® II Plus Enzyme Mix

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

Item	Composition	Quantity
LR Clonase® II Plus 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

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	Quantity
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 *E. coli*

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

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

Product Use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

Accessory Products

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 Life Technologies. Ordering information for these reagents is provided below.

Item	Quantity	Catalog no.
BP Clonase® II Enzyme Mix	20 reactions 100 reactions	11789-020 11789-100
LR Clonase® II Plus Enzyme Mix	20 reactions 100 reactions	12538-120 12538-200
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™ 2 T1^R Chemically Competent cells	10 x 50 µl	A10460
pDONR™ 221	6 µg	12536-017
Platinum® *Pfx* DNA Polymerase	100 reactions 250 reactions	11708-013 11708-021
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions 500 reactions	11304-011 11304-029
M13 Forward (–20) Sequencing Primer	2 µg	N520-02
M13 Reverse Sequencing Primer	2 µg	N530-02
Dpn I	100 units	15242-019
PureLink® HiPure Plasmid MidiPrep Kit	25 reactions	K2100-04
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
PureLink® Gel Extraction Kit	50 reactions	K2100-12
Ampicillin	20 ml (10 mg/ml)	11593-019
Kanamycin Sulfate	100 ml (10 mg/ml)	15160-054
MultiSite Gateway® Pro 2.0 Kit	20 reactions	12537-102
MultiSite Gateway® Pro 3.0 Kit	20 reactions	12537-102
MultiSite Gateway® Pro 4.0 Kit	20 reactions	12537-104
MultiSite Gateway® Pro Plus Kit	20 reactions	12537-100

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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 Life Technologies to allow creation of entry clones using TOPO® Cloning or restriction digestion and ligation. For more information about the various entry vectors and their features go to **www.lifetechnologies.com** or contact Technical Support (see page 47).

Item	Quantity	Catalog no.
pCR™8/GW/TOPO® Cloning Kit	20 reactions	K2500-20
pENTR™/D-TOPO® Cloning Kit	20 reactions 480 reactions 500 reactions	K2400-20 K2400-480 K2400-500
pENTR™/SD/D-TOPO® Cloning Kit	20 reactions 480 reactions 500 reactions	K2420-20 K2420-480 K2420-500
pENTR™ 1A Dual Selection Vector	10 µg	A10462
pENTR™ 2B Dual Selection Vector	10 µg	A10463
pENTR™ 3C Dual Selection Vector	10 µg	A10464
pENTR™ 4 Dual Selection Vector	10 µg	A10465
pENTR™ 11 Dual Selection Vector	10 µg	A10467

Ultimate™ ORF Clones

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

Item	Quantity	Catalog no.
Ultimate™ Human ORF Clones	1 clone	HORF01
Ultimate™ Mouse ORF Clones	1 clone	MORF01

Introduction

Overview

Introduction

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

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

Important

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

Purpose of This Manual

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

1. Design three sets of forward and reverse *att*B PCR primers, and PCR-amplify your DNA sequences of interest to generate PCR products that are flanked by *att*B or *att*Br sites for BP recombination.
2. Use each PCR product in separate BP recombination reactions with the appropriate donor vectors to generate entry clones containing your DNA sequences of interest.
3. Perform a MultiSite Gateway® LR recombination reaction with your three entry clones and the provided pDEST™ R4-R3 Vector II destination vector to generate an expression clone which may then be used in the appropriate application or expression system.

Gateway® Technology

Introduction

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

Lambda Recombination Reactions

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

Recombination is conservative (*i.e.* there is no net gain or loss of nucleotides) and requires no DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector.

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

Recombination Enzymes

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

Continued on next page

Gateway® Technology, continued

*att*B, *att*P, *att*L, and *att*R

*att*B, *att*P, *att*L and *att*R are recombination sites that are utilized in the Gateway® Technology.

*att*B sites always recombine with *att*P sites in a reaction mediated by the BP Clonase® II enzyme mix:

***att*B x *att*P → *att*L x *att*R**

The BP reaction is the basis for the reaction between the donor vectors (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. The entry clone containing the PCR product is used in the LR recombination reaction.

*att*L sites always recombine with *att*R in a reaction mediated by LR Clonase® II or LR Clonase® II Plus enzyme mix:

***att*L x *att*R → *att*B x *att*P**

The LR reaction is the basis for the entry clones × destination vector reaction. Recombination between *att*L and *att*R sites yields *att*B and *att*P sites on the resulting plasmids. The expression clone containing the PCR product is used in your expression system. The by-product plasmid contains the *ccd*B gene and prevents growth if taken up by competent cells after transformation.

For More Information

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

MultiSite Gateway® Components

Introduction

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

Vector NTI Advance® Software Users

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

MultiSite Gateway® Donor Vectors

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

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

BP Clonase® II Enzyme Mix

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

LR Clonase® II Plus Enzyme Mix

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

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

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MultiSite Gateway® Components, continued

MultiSite Gateway® Destination Vector

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

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

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

pMS/GW Control Vector

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

One Shot® TOP10 Chemically Competent *E. coli*

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

MultiSite Gateway® Donor Vectors

Introduction

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

Your PCR products will be flanked by different *att*B or *att*Br sites. Three different donor vectors facilitate generation of entry clones:

Vector	Insert
pDONR™ 221 P4-P1r	*att*B4 and *att*B1r-flanked PCR products
pDONR™ 221	*att*B1 and *att*B2-flanked PCR products
pDONR™ 221 P2r-P3	*att*B2r and *att*B3-flanked PCR products

Common Features of the MultiSite Gateway® Donor Vectors

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

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

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

*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 & Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* Mach1™, TOP10, DH5α™). When recombination occurs (*i.e.* between a destination vector and an entry clone or between a donor vector and an *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.

Modifications to the *att* Sites

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

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

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

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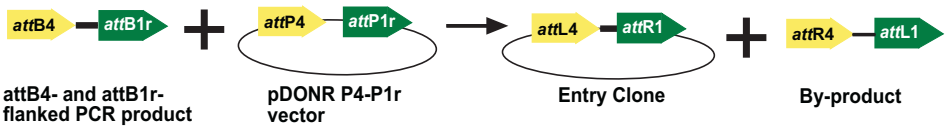
MultiSite Gateway® Donor Vectors, continued

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; for example *att*B1 sites react only with *att*P1 sites to generate *att*L1 sites
- *att*L sites react only with *att*R sites; for example *att*L1 sites react only with *att*R1 sites to generate *att*B1 sites

att sites are not palindromic and have an orientation. The direction of the arrow designates two possible orientations of the *att* sites in relation to the insert. When the arrow does not point towards the insert, the *att*P or *att*B site is designated with an "r". In the example below, the *att*B1r site flanks the PCR product and an *att*P1r site resides on the donor vector generating an *att*R1 site in the entry clone:



Performing the BP recombination reaction with an *att*Br and *att*Pr site will result in creation of an *att*R site instead of an *att*L site in the entry clone.

In the BP recombination reactions:

- *att*B1r sites react with *att*P1r sites to generate *att*R1 sites in the entry clone
- *att*B2r sites react with *att*P2r sites to generate *att*R2 sites in the entry clone

Example

In this example, an *att*B4 and *att*B1r-flanked PCR product is used in a BP recombination reaction with pDONR™ P4-P1r:

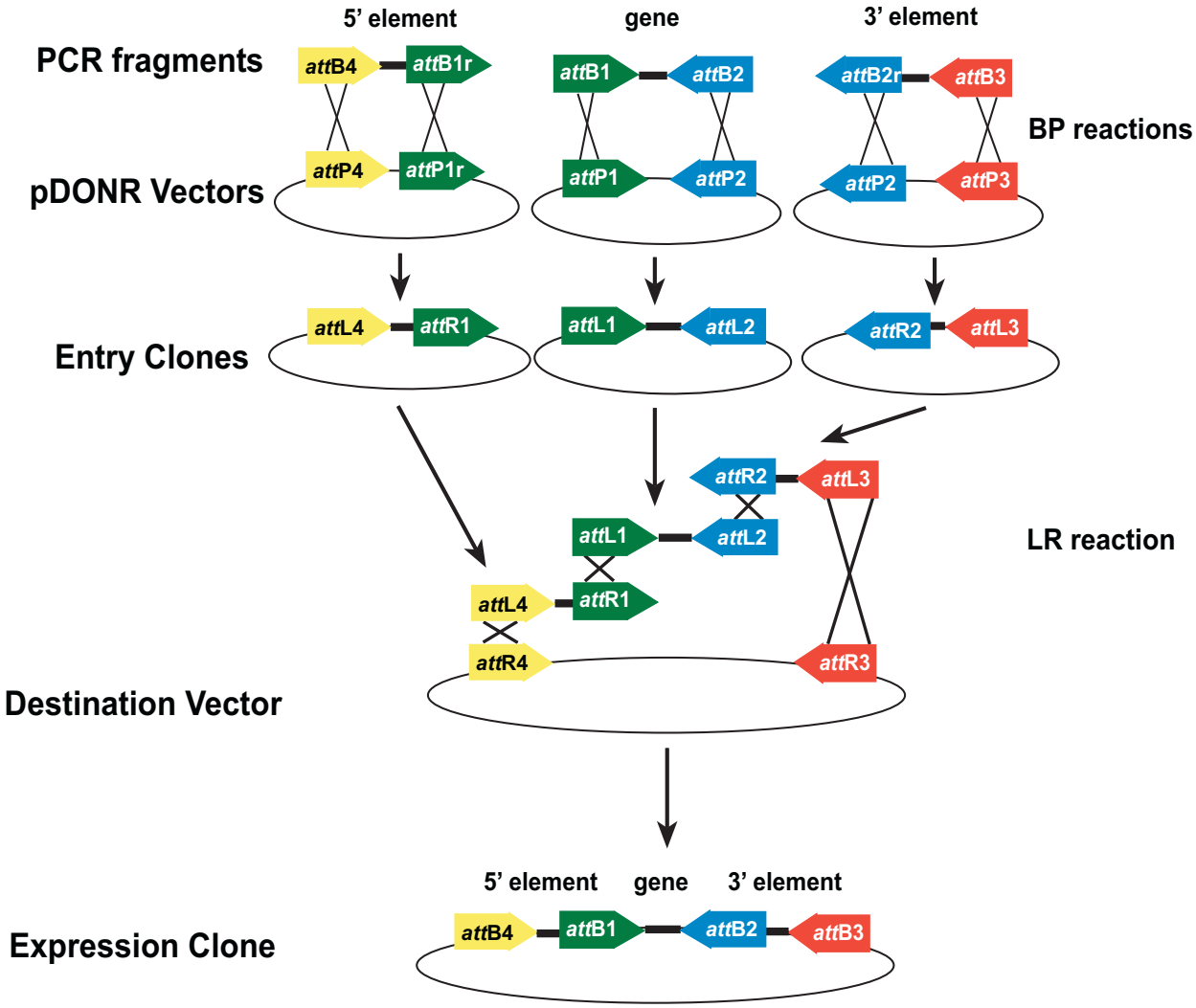
_att_B4-PCR product-_att_B1r × pDONR™ P4-P1r→ pENTR™ _att_L4-PCR product-_att_R1

Because of the orientation and position of the *att*B1r site in the PCR product and the *att*P1r site in the donor vector, 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.

Experimental Overview

Overview

In the MultiSite Gateway® 3-Fragment recombination reaction, three PCR products (5′ element, gene of interest, and 3′ element) flanked by specific *att*B or *att*Br sites and three MultiSite Gateway® Donor vectors are used in separate BP recombination reactions to generate three entry clones. The three entry clones and the destination vector, pDEST™ R4-R3 Vector II are used together in a MultiSite Gateway® LR recombination reaction to create your expression clone.



Methods

Propagating the MultiSite Gateway® Vectors

Introduction

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

See the guidelines below to propagate and maintain these vectors.

Propagating Donor and Destination Vectors

If you wish to propagate and maintain the MultiSite Gateway® donor and destination vectors prior to recombination, we recommend using One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells for transformation. These cells are available separately from Life Technologies (page ix).

The *ccd*B Survival 2 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 pDEST™ R4-R3 Vector II, use LB plates containing 100 µg/ml ampicillin and 15–30 µg/ml chloramphenicol.

Important

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

Propagating the pMS/GW Vector

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

General Information for Entry Clones

Introduction

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

- An *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 pages 12-14.

Important

If you construct an expression clone containing the elements described above (*i.e.* promoter of choice + gene of interest + termination or polyadenylation sequence of choice), remember that this expression clone will be expressed **transiently** in mammalian, yeast, and insect systems, but may be expressed stably in prokaryotic systems. To perform stable expression studies in mammalian, yeast, or insect systems, include a resistance marker in one of the entry clones (generally the *att*R2 and *att*L3-flanked entry clone).

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Designing PCR Primers

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

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

Each PCR product must be flanked by a different combination of *att*B or *att*Br sites:

DNA Element	Flanking *att* sites
5′ element	*att*B4, *att*B1r
Gene of interest	*att*B1, *att*B2
3′ element	*att*B2r, *att*B3

For more information on designing *att*B and *att*Br-flanked primers, see the following sections on page 16-17.

The MultiSite Gateway® 3 Fragment Vector Construction Kit is compatible with Vector NTI Advance® software version 10 and above. Go to **www.lifetechnologies.com** for detailed instructions to use the Vector NTI Advance® software to design *att*B and *att*Br primers for your DNA elements of choice.

Primer Concentration

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

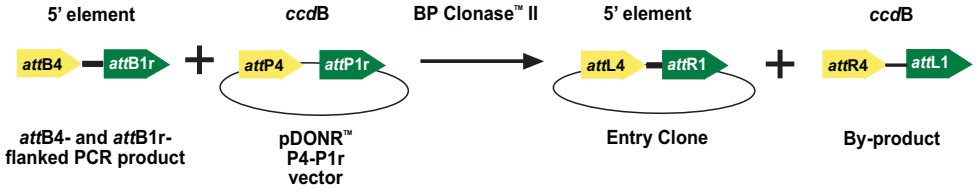
For your convenience, Life Technologies offers a custom primer synthesis service. Go to **www.lifetechnologies.com** for more information.

Making Entry Clones

Generating Entry Clone for 5' Element

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

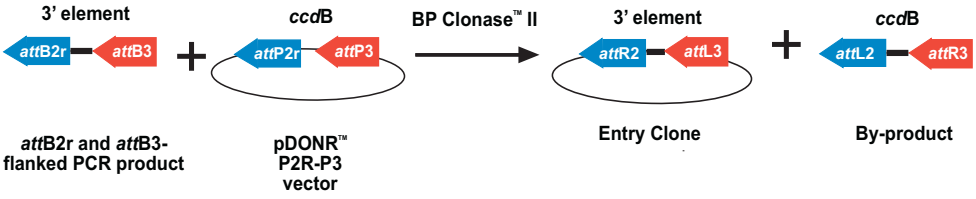
1. Design appropriate PCR primers and produce your *att*B4 and *att*B1r-flanked PCR product (see pages 16-17).
2. Perform a BP recombination reaction between the *att*B4 and *att*B1r-flanked PCR product and pDONR™ P4-P1R to generate the entry clone (see figure below).



Generating Entry Clone for 3' Element

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*B2r and *att*B3-flanked PCR product (see pages 16-17).
2. Perform a BP recombination reaction between the *att*B2r and *att*B3-flanked PCR product and pDONR™ P2R-P3 to generate the entry clone (see figure below).



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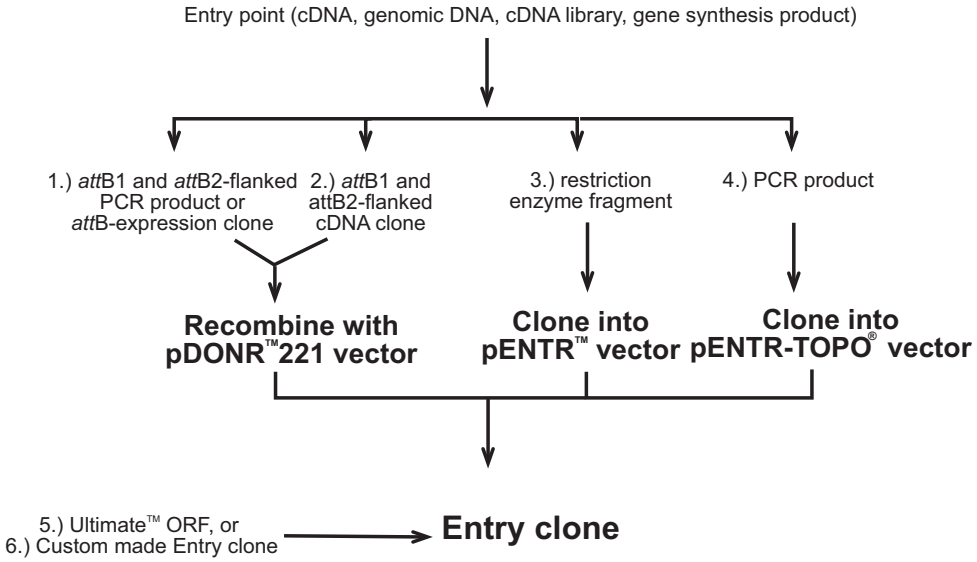
Generating *att*L1 and *att*L2-Flanked Entry Clones

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.

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, provided with the kit. To use this method, refer to the guidelines and instructions provided on the next page.
2. Generate or obtain a cDNA library cloned into a Gateway®-compatible vector (*i.e. 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).
3. Clone a restriction enzyme fragment into a pENTR™ vector (see page 14 for more information).
4. TOPO® Clone a PCR product into a pENTR™-TOPO® vector (see page 14 for more information).
5. Purchase a clone from the Life Technologies Ultimate™ ORF collection (see page x).
6. Use Life Technologies's Custom Cloning Service to make a custom vector. Go to **www.lifetechnologies.com** for more information.



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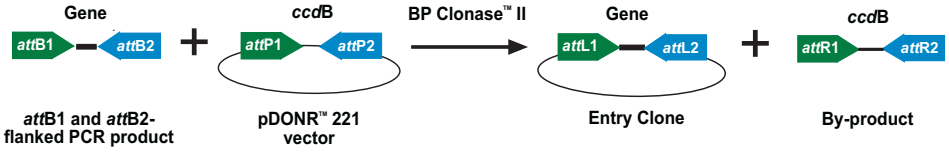
Making Entry Clones, continued

Generating Entry Clone for the Gene of Interest

To generate an *att*L1 and *att*L2-flanked entry clone containing your gene of interest:

1. Design appropriate PCR primers and produce your *att*B1 and *att*B2-flanked PCR product (see pages 16-17).
2. Perform a BP recombination reaction between the *att*B1 and *att*B2-flanked PCR product and pDONR™ 221 to generate the entry clone (see figure below).



Entry Vectors

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

- *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 & 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*.

Entry Vector	Kozak	Shine-Dalgarno	Catalog no.
pENTR™/D-TOPO®	•		K2400-20
pENTR™/SD/D-TOPO®	•	•	K2420-20
pENTR™ 1A Dual Selection Vector	•	•	A10462
pENTR™ 2B Dual Selection Vector	•		A10463
pENTR™ 3C Dual Selection Vector	•	•	A10464
pENTR™ 4 Dual Selection Vector	•		A10465
pENTR™ 11 Dual Selection Vector	•	•	A10467

To construct an entry clone usinf 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 form **www.lifetechnologies.com** or by contacting Technical Support (see page 47).

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. We strongly suggest Vector NTI Advance® software to design *att*B and *att*Br primers. Guidelines are provided below to help you design appropriate PCR primers.

Designing Your PCR Primers

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

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

Continued on next page

Designing *att*B PCR Primers, continued

Guidelines to Design the Forward PCR Primers

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

- To enable efficient MultiSite Gateway® cloning, the forward primer **MUST** contain the following structure:
  1. Four guanine (G) residues at the 5′ end followed by
  2. The 22 or 25 bp *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 & Dalgarno, 1975) or Kozak consensus sequence (Kozak, 1987; Kozak, 1990; Kozak, 1991), respectively, in the *att*B1 forward PCR primer.

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

  ***att*B2r** 5′-GGGG-ACA-GCT-TTC-TTG-**TAC-AAA**-GTG-GNN--(template-specific sequence)-3′
  *att*B2r

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

- The *att*B4 and *att*B2r 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

Guidelines to Design the Reverse PCR Primers

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

- To enable efficient MultiSite Gateway® cloning, the reverse primer **MUST** contain the following structure:
  1. Four guanine (G) residues at the 5′ end followed by
  2. The 22 or 25 bp *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*B1r 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.

***att*B1r** 5′-GGGG-AC-TGC-**TTT-TTT**-GTA-CAA-ACT-TGN--(template-specific sequence)-3′
*att*B1r

***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

- 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.

Producing *att*B PCR Products

DNA Templates

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

- Genomic DNA
- cDNA from reverse transcription reaction
- cDNA libraries
- Plasmids containing cloned DNA sequences
- *De novo* gene synthesis

Recommended Polymerases

We recommend using the following DNA polymerases available from Life Technologies to produce your *att*B PCR products. See page ix for ordering information.

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

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: *att*B sequences do not affect PCR product yield or specificity.

Checking the PCR Product

Remove 1–5 µl from each PCR reaction and use agarose gel electrophoresis to verify the quality and yield of your PCR product. If the PCR product is of the appropriate quality and quantity, proceed to **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:

- *Dpn* I
- 10x REact® Buffer, included with the enzyme

Protocol:

1. To your 50 µl PCR reaction mixture, add 5 µl of 10X REact® 4 Buffer (included with enzyme) and ≥ 5 units of *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)
- 1x 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)
- 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 1x 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 20. 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 PureLink® Gel Extraction Kit available from Life Technologies (see page ix).

Creating Entry Clones Using the BP Recombination Reaction

Introduction

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

Experimental Outline

To generate an entry clone, you will:

1. Perform a BP recombination reaction using the appropriate linear *att*B PCR product and a supercoiled, *att*P-containing donor vector (see page 26).
2. Transform the reaction mixture into a suitable *E. coli* host (see page 27).
3. Select for entry clones (see page 30).

Important

For optimal results, perform the BP recombination reaction using:

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

Recombination Regions

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

Continued on next page

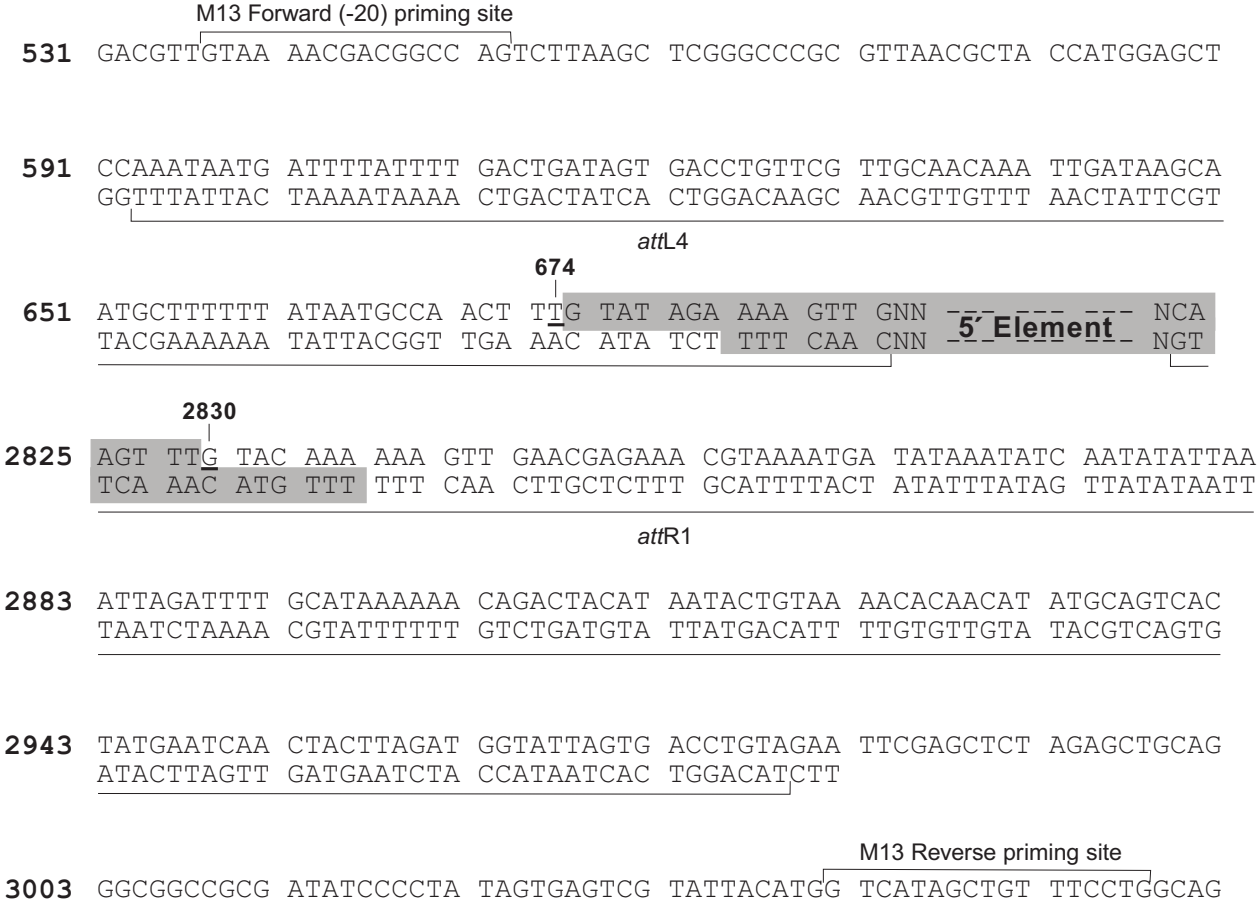
Creating Entry Clones Using the BP Recombination Reaction,
continued

Recombination Region of the *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*B1r 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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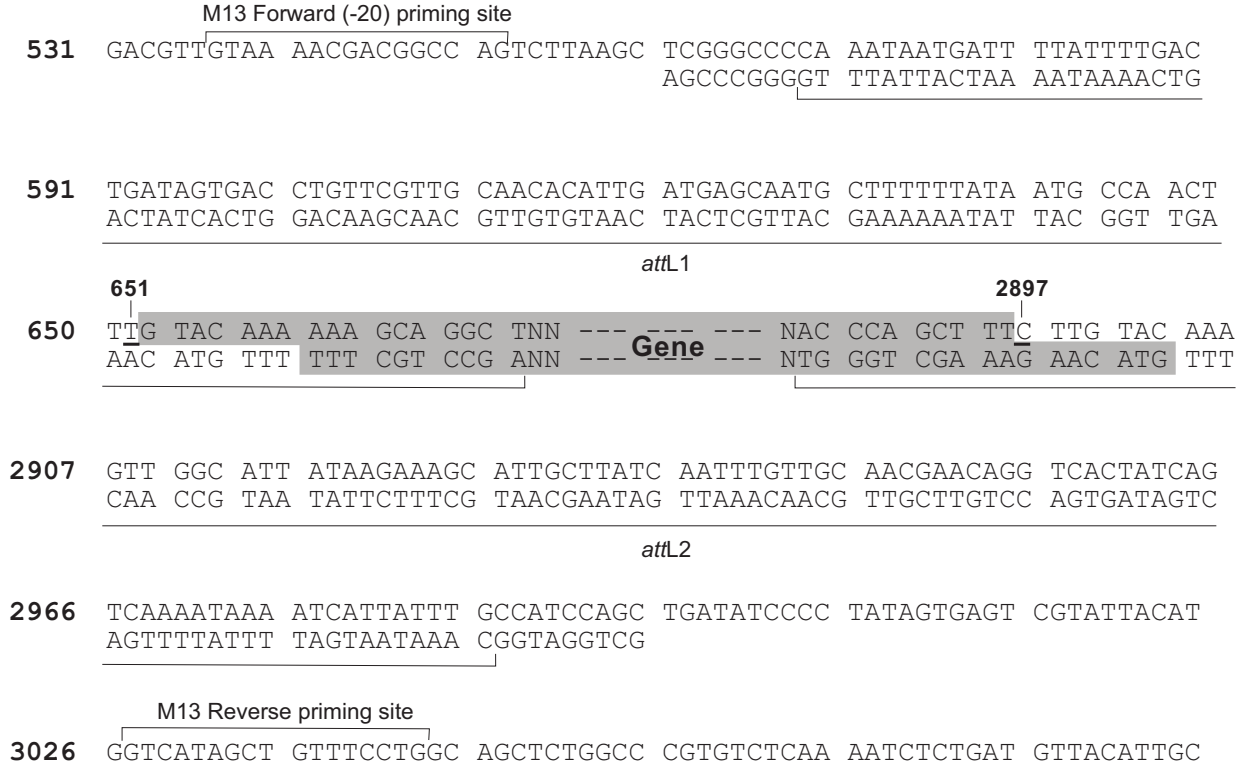
Recombination Region of the *att*L1 and *att*L2-Flanked Entry Clone

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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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*B2r-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.

Donor Vectors

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

Positive Control

pMS/GW is included with the MultiSite Gateway® Three-Fragment Vector Construction Kit for use as a positive control for each BP reaction, and contains multiple DNA fragments that have been joined using MultiSite Gateway® Technology (see page 46 for a map and more information). For an alternate positive control when creating an *att*L1 and *att*L2-flanked entry clone, see below.

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

Linearizing the Positive Control

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

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

Alternate Positive Control

When creating *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).

Continued on next page

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 below.

- Do not use more than 250 ng of donor vector in a 10 µl BP reaction, as this will affect the efficiency of the reaction.
- Do not exceed more than 500 ng of total DNA (donor vector plus *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:

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

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:

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

Continued on next page

Performing the BP Recombination Reaction, continued

Materials Needed

You should have the following materials on hand before beginning.

Supplied with the kit:

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

Supplied by the user:

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

Setting Up the BP Reaction

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

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

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

2. Remove the BP Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. To each sample above **except the negative control**, add 2 µl of BP Clonase® II enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return BP Clonase® II enzyme mix to –20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: A 1-hour incubation generally yields a sufficient number of entry clones. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. An overnight incubation typically yields 5–10 times more colonies than a 1-hour incubation. For large PCR products (≥5 kb), longer incubations (*i.e.* overnight incubation) will increase the yield of colonies and are recommended.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® TOP10 Competent Cells**, next page.

   Note: You may store the BP reaction at –20°C for up to 1 week before transformation.

Transforming One Shot® TOP10 Competent Cells

Introduction

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

Note

You may use any *rec*A, *end*A *E. coli* strain including One Shot® TOP10 chemically competent *E. coli* (supplied with the kit), DH5α™, DH10B™ or equivalent for transformation. Other strains are suitable.

Do not use *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′) for transformation. These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Materials Needed

You should have the following materials on hand before beginning.

Supplied with the kit:

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

Supplied by the user:

- BP recombination reaction (from **Setting Up the BP Reaction**, Step 7, page 26)

  OR

  MultiSite Gateway® LR recombination reaction (from **Setting Up the MultiSite Gateway® LR Reaction**, Step 7, page 35)
- 2 LB prewarmed plates containing 50 µg/ml kanamycin (for BP reaction)

  OR

  2 LB prewarmed plates containing 50–100 µg/ml ampicillin (for LR reaction).
- 42°C water bath (for chemical transformation)
- 37°C shaking and non-shaking incubators

Continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

One Shot® TOP10 Chemical Transformation Protocol

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

   OR

   - Add 2 µl of the MultiSite Gateway® LR recombination reaction (from **Setting Up the MultiSite Gateway® LR Reaction**, Step 7, page 35)

   Reminder: If you are including the transformation control, add 1 µl (10 pg) of pUC19.
2. Incubate on ice for 5 to 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread the following amount from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We generally plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies.
   - BP recombination reaction: spread 20 µl and 100 µl
   - MultiSite Gateway® LR recombination reaction: spread 50 µl and 100 µl

What You Should See

- **BP reaction:** An efficient BP recombination reaction may produce hundreds of colonies (greater than 1,500 colonies if the entire reaction is transformed and plated).
- **MultiSite Gateway® LR reaction:** An efficient MultiSite Gateway® LR recombination reaction may produce approximately 100 colonies (approximately 1,000 to 5,000 if 10 µl is transformed and plated).

Continued on next page

Transforming One Shot® TOP10 Competent Cells, continued

Transformation by Electroporation

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

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

   OR

   - 2 µl of the MultiSite Gateway® LR recombination reaction (from **Setting Up the MultiSite Gateway® LR Reaction**, Step 7, page 35).
2. Electroporate your samples using an electroporator and the manufacturer's suggested protocol.

   Note: If you have problems with arcing, see below.
3. Immediately add 450 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance marker.
5. Spread 50–100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating 2 different volumes to ensure that at least 1 plate has well-spaced colonies.
6. An efficient recombination reaction may produce several hundred colonies.

To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing during transformation, try one of the following:

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

Sequencing Entry Clones

Introduction

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

Sequencing Primers

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

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

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

MultiSite Gateway® LR Recombination Reaction

Introduction

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

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

Experimental Outline

To generate an expression clone, you will:

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

Substrates for the MultiSite Gateway® LR Recombination Reaction

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

- *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 Vector II destination vector (see the next page for more information)

Keep in mind the following:

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

Important

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

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

Continued on next page

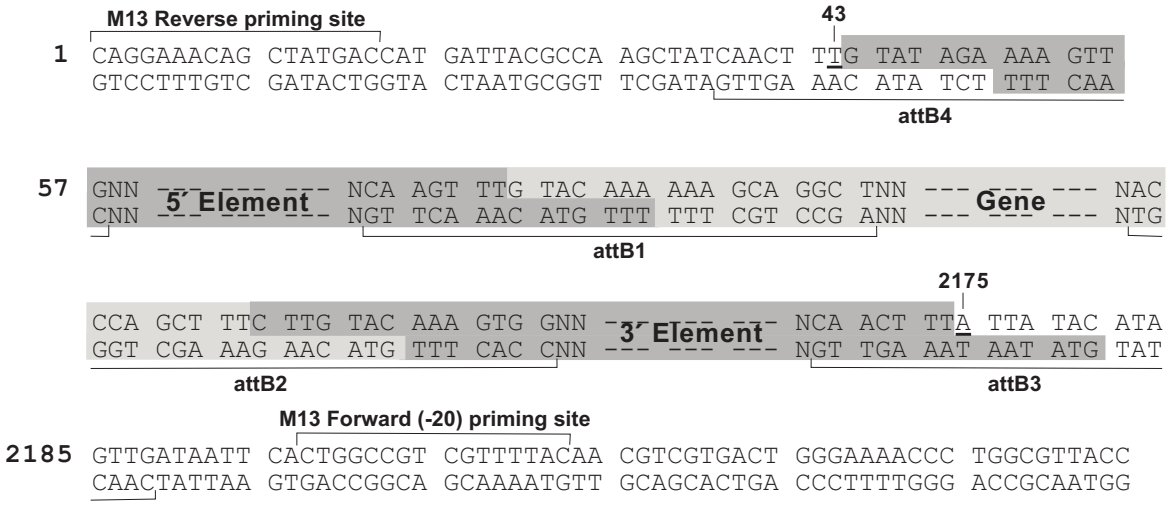
MultiSite Gateway® LR Recombination Reaction, continued

Recombination Region of the Expression Clone

The recombination region of the expression clone resulting from pDEST™ R4-R3 Vector II × *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 II 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 II vector.
- Bases 43 and 2175 of the pDEST™ R4-R3 Vector II sequence are indicated.



Performing the LR Recombination Reaction

Important

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

Introduction

Guidelines and instructions are provided in this section to:

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

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

pDEST™ R4-R3 Vector II

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

Determining How Much DNA to Use in the Reaction

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

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

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

Continued on next page

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 Vector II.

Preparing Purified Plasmid DNA

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

Materials Needed

You should have the following materials on hand before beginning.

Supplied with the kit:

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

Supplied by the user:

- Purified plasmid DNA of your *att*L4 and *att*R1-flanked entry clone (supercoiled, 10 fmoles)
- Purified plasmid DNA of your *att*L1 and *att*L2-flanked entry clone (supercoiled, 10 fmoles)
- Purified plasmid DNA of your *att*R2 and *att*L3-flanked entry clone (supercoiled, 10 fmoles)

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

Continued on next page

Performing the LR Recombination Reaction, continued

Setting Up the MultiSite Gateway® LR Reaction

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

Component	Sample	Negative Control
*att*L4 and *att*R1 entry clone (10 fmoles) *att*L1 and *att*L2 entry clone (10 fmoles) *att*R2 and *att*L3 entry clone (10 fmoles)	1–7 µl	--
pDEST™ R4-R3 Vector II (20 fmoles)	0.4 µl	0.4 µl
TE Buffer, pH 8.0	to 8 µl	7 µl

2. Remove the LR Clonase® II Plus enzyme mix from –20ºC or –80°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase® II Plus enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 2 µl of LR Clonase® II Plus enzyme mix. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return LR Clonase® II Plus enzyme mix to –20ºC or –80°C immediately after use.
5. Incubate reactions at 25°C for 16 hours or overnight.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. If you are transforming One Shot® TOP10 chemically competent *E. coli*, follow the protocol on page 28.

   Note: You may store the MultiSite Gateway® LR reaction at –20°C for up to 1 week before transformation, if desired.

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1 x 10^9 cfu/µg, the MultiSite Gateway® LR reaction should give approximately 1,000 to 5,000 colonies if the entire reaction is transformed and plated.

Next Steps

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

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

Troubleshooting

MultiSite Gateway® LR & BP Reactions

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

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.
	Used incorrect *att* sites for the reaction	• Use the appropriate entry clones and pDEST™ R4-R3 Vector II for the MultiSite Gateway® LR reaction (see page 8 for details about the types of entry clones required). • Use the correct *att*B PCR product and donor vector (*att*P) for the BP reaction (see page 12 for details).
	BP Clonase® Plus or LR Clonase® II Plus enzyme mix is inactive; or didn't use suggested amount of BP Clonase® Plus or LR Clonase® II Plus enzyme mix	• Test another aliquot of the BP Clonase® Plus or LR Clonase® II Plus enzyme mix. • Store the LR Clonase® II Plus at –20º or –80°C for long-term storage, and the BP Clonase® II at –20°C. • Do not freeze/thaw the BP Clonase® Plus or LR Clonase® II Plus enzyme mix >10 times. • Use the recommended amount of BP Clonase® Plus or LR Clonase® II Plus (see page 26 or 35).
	Used incorrect BP Clonase® Plus or LR Clonase® II Plus enzyme mix	• Use the LR Clonase® II Plus enzyme mix for the LR reaction. • 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 60 fmoles total DNA in the reaction.

Continued on next page

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* when plating cells.

Continued on next page

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* (Life Technologies, 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 26).

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.

Continued on next page

*att*B PCR Cloning, continued

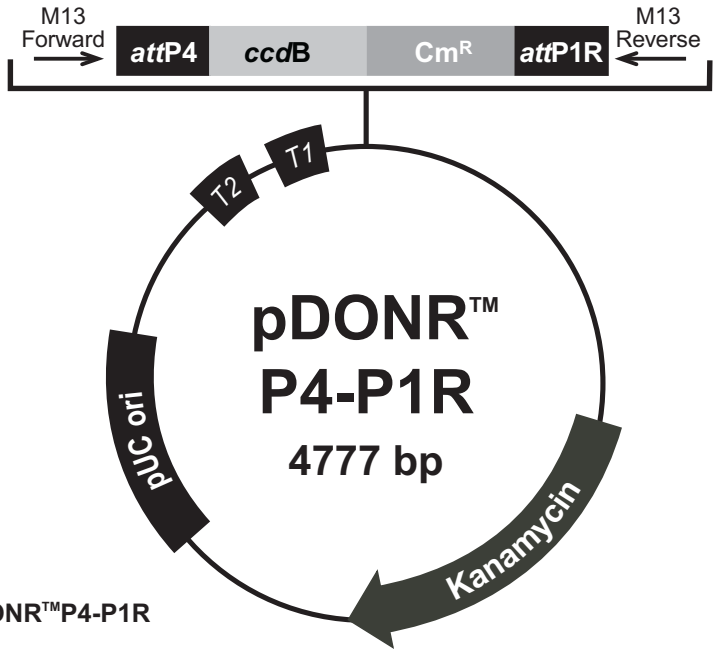
Problem	Reason	Solution
Few or no colonies obtained from a BP reaction with *att*B PCR product **and** both *att*B positive control and transformation control gave expected number of colonies	*att*B PCR primers incorrectly designed	Make sure that each *att*B PCR primer includes four 5′ terminal Gs and the 22 or 25 bp *att*B site as specified on page 11.
	*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 19 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 www.lifetechnologies.com or by contacting Technical Support (see page 47).**



Comments for pDONR™P4-P1R
4777 nucleotides

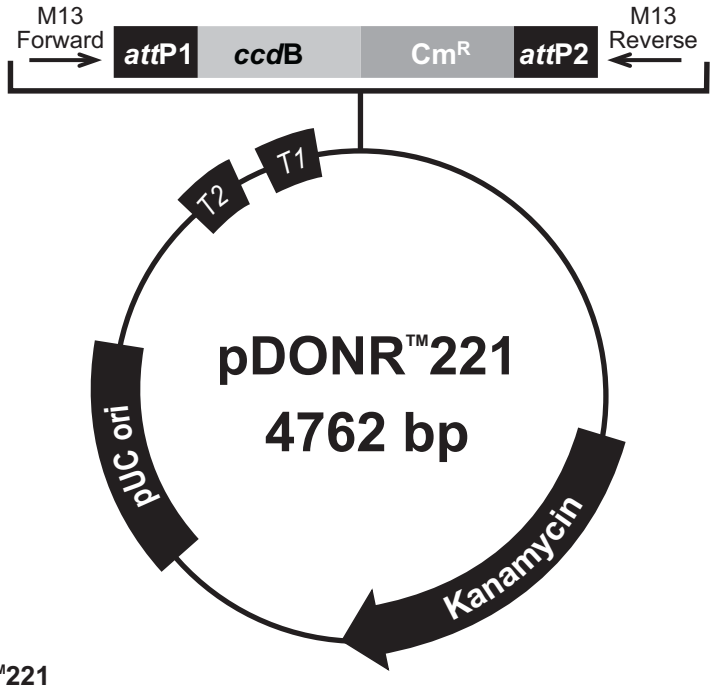
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P4 recombination site: bases 593-824 (c)
*ccd*B gene: bases 1181-1486 (c)
Chloramphenicol resistance gene: bases 1828-2487 (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 www.lifetechnologies.com or by contacting Technical Support (see page 47).**



Comments for pDONR™221
4762 nucleotides

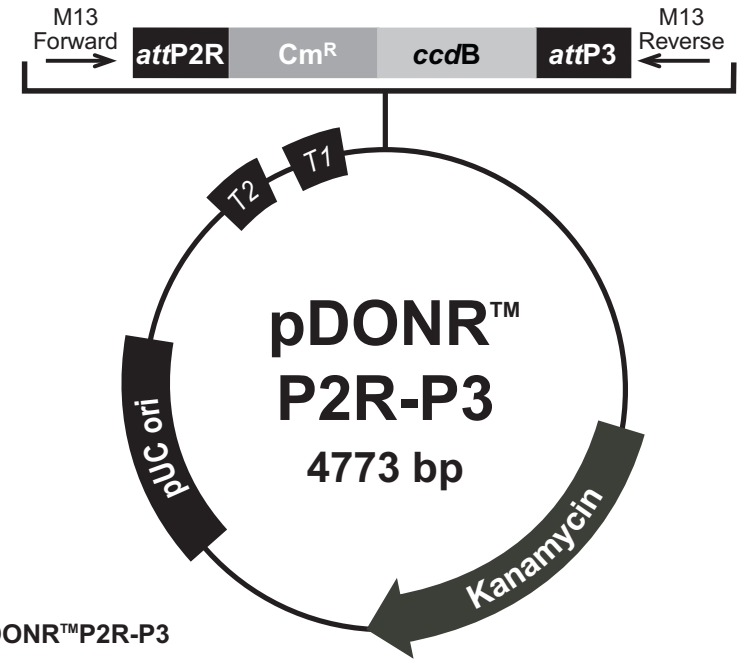
*rrn*B T2 transcription termination sequence (c): bases 268-295
rrnB T1 transcription termination sequence (c): bases 427-470
M13 Forward (-20) priming site: bases 537-552
*att*P1: bases 570-801
*ccd*B gene (c): bases 1197-1502
Chloramphenicol resistance gene (c): bases 1847-2506
*att*P2 (c): bases 2754-2985
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 www.lifetechnologies.com or by contacting Technical Support (see page 47).**



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

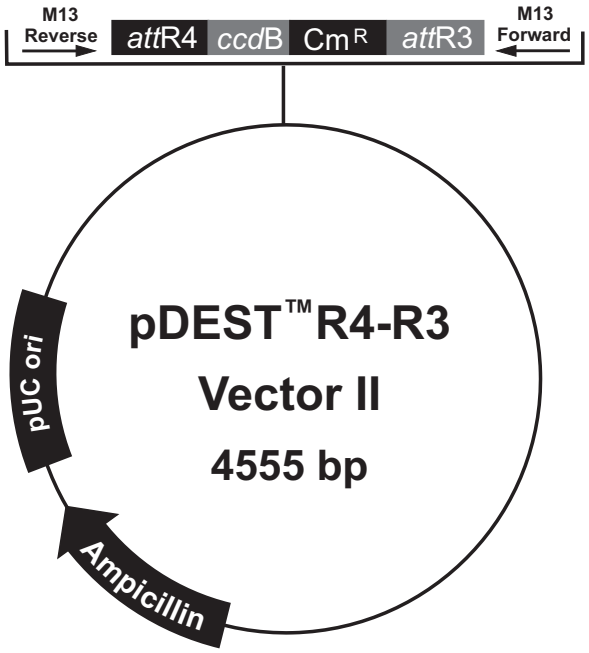
pDONR™ P4-P1R (4777 bp), pDONR™ 221 (4762 bp), and pDONR™ P2R-P3 (4773 bp) contain the following elements. Features have been functionally tested.

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 of pDEST™ R4-R3 Vector II

pDEST™ R4-R3 Vector II Map

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



Features of pDEST™R4-R3 Vector II
4555 nucleotides

M13 Reverse priming site: bases 1-17
*att*R4 recombination site: bases 37-161
*ccd*B gene: bases 508-813 (c)
Chloramphenicol resistance gene: bases 1158-1816 (c)
*att*R3 recombination site: bases 2064-2188
M13 Forward (-20) priming site: bases 2197-2212 (c)
Ampicillin resistance ORF (*bla*): bases 2791-3651
pUC origin: bases 3796-4469
(c) = complementary strand

Features of pDEST™ R4-R3 Vector II

Features of the pDEST™ R4-R3 Vector II Vector

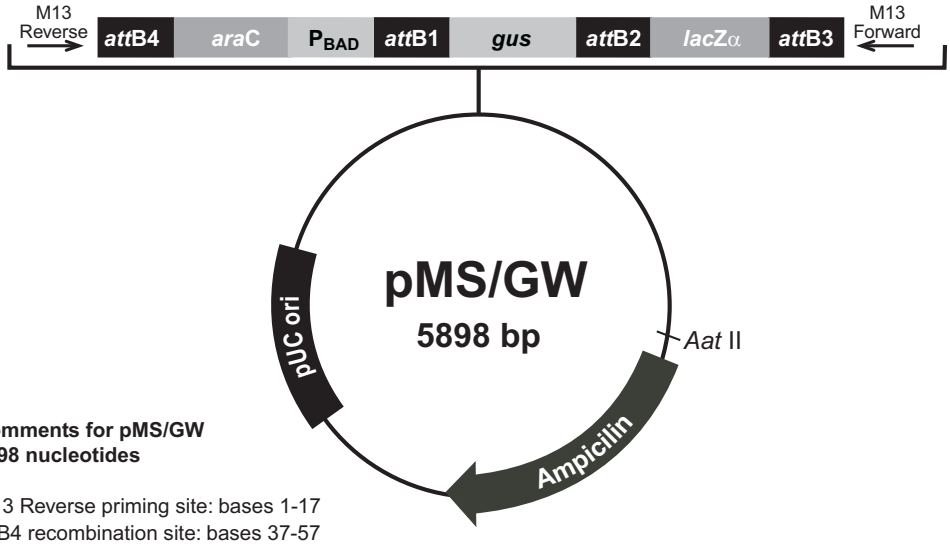
pDEST™ R4-R3 Vector II (4555 bp) contains the following elements. Features have been functionally tested.

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

pMS/GW Map

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



Comments for pMS/GW
5898 nucleotides

M13 Reverse priming site: bases 1-17
*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
*lac*Zα 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

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

Limited Use Label License: ULB ccdB Selection Technology

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

Limited Use Label License No. 48: araB Promoter

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

Gateway® Clone Distribution Policy

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

Continued on next page

Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

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Notes

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