USER GUIDE

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MultiSite Gateway® Pro

Using Gateway® Technology to simultaneously clone multiple DNA fragments

Catalog Numbers 12537-102, 12537-103, 12537-104, 12537-100

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Revision A.0

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

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Experienced users guide

Introduction

This quick reference sheet is provided for experienced users of the MultiSite Gateway® Pro Technology. If you are performing the BP or MultiSite Gateway® Pro 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 pages 36–47 for details).

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

*att*B PCR product (15–150 ng)	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 2 µL of the reaction into One Shot® Mach1™ T1^R *E. coli* and select for kanamycin-resistant entry clones.

MultiSite Gateway® Pro LR recombination reaction

Perform a MultiSite Gateway® Pro LR recombination reaction between multiple entry clones and a Gateway® Destination vector using LR Clonase® II Plus to generate an expression clone (see pages 51–57 for details).

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*
Destination vector (supercoiled, 20 fmoles)	1 µL
1X TE Buffer, pH 8.0	to 8 µL

*All entry clones (2, 3, or 4, depending on the reaction) must be included. The total volume of all entry clones must not exceed 7 µL.

2. Vortex LR Clonase® II Plus Enzyme Mix briefly. Add 2 µL to the components above and mix by vortexing briefly twice.
3. Incubate reaction at 25°C for 16 hours.
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 One Shot® Mach1™ T1^R *E. coli* and select for antibiotic-resistant expression clones.

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Primer sequences for *att*B and *att*Br-flanked PCR products

Depending on what kind of recombination you are performing (*i.e.* 2-fragment, 3-fragment or 4-fragment) your PCR products will be flanked by different *att*B or *att*Br sites. The recommended primer sequences are shown in the following table for each recombination reaction. For more information about primer design, see pages 25–30.

Vector and recombination type	*att* sites flanking insert	Primer sequences
pDONR™ 221 P1-P5r 2-fragment, 4-fragment	*att*B1 *att*B5r	Fwd: 5' GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTA Rev: 5' GGGG AC AAC TTT TGT ATA CAA AGT TGT
pDONR™ 221 P5-P2 2-fragment	*att*B5 *att*B2	Fwd 5' GGGG ACA ACT TTG TAT ACA AAA GTT GTA Rev 5' GGGG AC CAC TTT GTA CAA GAA AGC TGG GTT
pDONR™ 221 P1-P4 3-fragment	*att*B1 *att*B4	Fwd 5' GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTA Rev 5' GGGG AC AAC TTT GTA TAG AAA AGT TGG GTG
pDONR™ 221 P4r-P3r 3-fragment, 4-fragment	*att*B4r *att*B3r	Fwd 5' GGGG ACA ACT TTT CTA TAC AAA GTT GTA Rev 5' GGGG AC AAC TTT ATT ATA CAA AGT TGT
pDONR™ 221 P3-P2 3-fragment, 4-fragment	*att*B3 *att*B2	Fwd 5' GGGG ACA ACT TTG TAT AAT AAA GTT GTA Rev 5' GGGG AC CAC TTT GTA CAA GAA AGC TGG GTT
pDONR™ 221 P5-P4 4-fragment	*att*B5 *att*B4	Fwd 5' GGGG ACA ACT TTG TAT ACA AAA GTT GTA Rev 5' GGGG AC AAC TTT GTA TAG AAA AGT TGG GTG

Kit contents and storage

Types of kits

This manual is supplied with the kits listed below.

Product	Catalog no.
MultiSite Gateway® Pro 2.0 Kit for 2-fragment recombination	12537-102
MultiSite Gateway® Pro 3.0 Kit for 3-fragment recombination	12537-103
MultiSite Gateway® Pro 4.0 Kit for 4-fragment recombination	12537-104
MultiSite Gateway® Pro Plus Kit for 2-, 3- or 4-fragment recombination	12537-100

Each kit supplies enough reagents for 20 recombination reactions.

Shipping/Storage

The MultiSite Gateway® Pro Kits are shipped on dry ice in four boxes. Upon receipt, store each box as detailed in the following table.

Box	Item	Storage
1	Vectors	–30°C to –10°C
2	BP Clonase® II Enzyme Mix	–30°C to –10°C (6 months) –85°C to –68°C (long term)
3	LR Clonase® II Plus Enzyme Mix	–30°C to –10°C (6 months) –85°C to –68°C (long term)
4	One Shot® Mach1™ T1^{R} Chemically Competent *E. coli*	–85°C to –68°C

Continued on next page

Kit contents and storage, continued

Vectors

Depending on the kit configuration, the vectors box (Box 1) contains the following items. All vectors except pDONR™ 221 are supplied as 60 µL of 100 ng/µL supercoiled DNA. pDONR™ 221 is supplied as 6 µg of plasmid (150 ng/µL) in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. M13 forward (–20) and M13 reverse primers are 20 µL at 0.1 µg/µL. **Store Box 1 at –20°C.**

Item	Catalog Number			
	12537-102	12537-103	12537-104	12537-100
pDONR™ 221 P1-P5r	✓		✓	✓
pDONR™ 221 P5-P2	✓			✓
pDONR™ 221 P1-P4		✓		✓
pDONR™ 221 P4r-P3r		✓	✓	✓
pDONR™ 221 P3-P2		✓	✓	✓
pDONR™ 221 P5-P4			✓	✓
pENTR™ L1-pLac-lacZalpha-R5	✓		✓	✓
pENTR™ L5-pLac-Spect-L2	✓			✓
pENTR™ L1-pLac-lacZalpha-L4		✓		✓
pENTR™ R4-pLac-Spect-R3		✓	✓	✓
pENTR™ L3-pLac-Tet-L2		✓	✓	✓
pENTR™ L5-LacI-L4			✓	✓
M13 (–20) Forward primer	✓	✓	✓	✓
M13 Reverse primer	✓	✓	✓	✓
pDONR™ 221	✓	✓	✓	✓

BP Clonase® II Enzyme Mix

The following reagents are supplied with 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	50 ng/µL in TE Buffer, pH 8.0	20 µL

Continued on next page

LR Clonase® II Plus Enzyme Mix

The following reagents are supplied with LR Clonase® II Plus Enzyme Mix (Box 3). **Store Box 3 at –30°C to –10°C for up to 6 months.** For long-term storage, store at **–85°C to –68°C.**

Item	Composition	Amount
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® Mach1™ T1^R Chemically Competent Cells

The following reagents are supplied with One Shot® Mach1™ T1^R Chemically Competent *E. coli* kit (Box 4). **Store Box 4 at –85°C to –68°C.**

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature, 15°C to 30°C, or in a cold room, 2°C to 8°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
Mach1™ T1^R chemically competent cells	—	21 × 50 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µL

Genotype of Mach1™ T1^R

F- ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R(r_K-m_K+) Δ*rec*A1398 *end*A1 *ton*A

Introduction

Product overview

Introduction

The MultiSite Gateway® Pro Kits facilitate rapid and highly efficient construction of an expression clone containing your choice of two, three or four separate DNA elements. Based on the Gateway® Technology (Hartley *et al.*, 2000; Sasaki *et al.*, 2005; Sasaki *et al.*, 2004), the MultiSite Gateway® Technology uses site-specific recombinational cloning to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation.

Important

The MultiSite Gateway® Pro Kits are designed to help you create a multiple-fragment 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 these products are geared towards 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 from **www.lifetechnologies.com/manuals** or by contacting Technical Support (see page 81).

Overview

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

1. Design 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. Use the entry clones in a single MultiSite Gateway® Pro LR recombination reaction with any destination vector of choice that contains *att*R1 and *att*R2 sites to create your expression clone of interest, 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

*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.

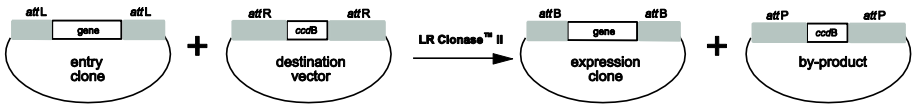
*att*B sites always recombine with *att*P sites in a reaction mediated by the BP Clonase® II Enzyme Mix:

$$attB \times attP \rightarrow attL \times attR$$

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. 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 II Plus Enzyme Mix:

$$attL \times attR \rightarrow attB \times attP$$



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. 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 Mach1™ T1^R competent cells after transformation.

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 from **www.lifetechnologies.com/manuals** or by contacting Technical Support (see page 81).

MultiSite Gateway® Pro components

Introduction

MultiSite Gateway® Pro Kits contain enzymes that catalyze the Gateway® recombination reactions (BP Clonase® II and LR Clonase® II Plus), donor vectors, and a set of control entry clones. More details about each component can be found below.

Note: You will need a Gateway® Destination vector to create an expression clone using the MultiSite Gateway® Pro kits. See the next page for further information about suitable destination vectors.

Vector NTI Advance® software users

The MultiSite Gateway® Pro kits are compatible with Vector NTI Advance® sequence analysis software version 10.2 and higher. To begin using Vector NTI Advance® software to design your MultiSite Gateway® Pro experiments, go to **www.lifetechnologies.com/vectornti** for detailed instructions.

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 47 to perform the BP recombination reaction using BP Clonase® II Enzyme Mix.

MultiSite Gateway® Pro Donor vectors

The MultiSite Gateway® Pro 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 following section for detailed information.

For more information about the general features of the donor vectors, see page 72. For a map and a description of the features of each MultiSite Gateway® Pro donor vector, see the **Appendix**, pages 66–71.

Note: pDONR™ 221 is provided as a positive control for the BP recombination reaction, but should not be used to generate multi-fragment entry clones.

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® Pro components, continued

Control entry clones

Depending on the MultiSite Gateway® Pro kit configuration, 2, 3, 4 or 6 control entry clones are provided as a positive control for the LR recombination reaction and to troubleshoot the LR recombination reaction in 2-, 3- and 4-fragment reactions.

For more information about performing control reactions with the entry clones, see page 58. For a map and a description of the features of each MultiSite Gateway® Pro Control Entry clone, see the **Appendix**, pages 73–78.

Destination vector

To create the expression clone containing your 2, 3, or 4 DNA elements of choice, you will need to provide an appropriate Gateway® destination vector for the LR recombination reaction. You may use any destination vector of choice that contains *att*R1 and *att*R2 sites.

A large variety of destination vectors are available (see **www.lifetechnologies.com**). If one or more of the fragments you wish to recombine is a promoter, you may want to use one of the promoterless DEST vectors such as pcDNA6.2/V5-pL-DEST vector available separately (page 79).

Important

Do not use pDEST R4-R3 Vector from the MultiSite Gateway® Three-Fragment Vector Construction Kit, because the *att*R3 and *att*R4 sites are incompatible with recombination with *att*L1 and *att*L2- containing entry clones.

MultiSite Gateway® Pro Donor vectors

Common features of the MultiSite Gateway® Pro Donor vectors

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

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

After a BP recombination reaction, this cassette is replaced by the gene 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.

Modify 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® Pro kits, up to five *att* sites are used versus two *att* sites in the standard Gateway® Technology.

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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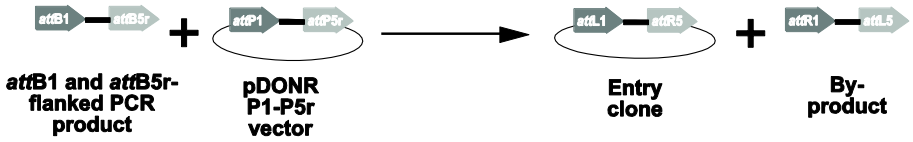
MultiSite Gateway® Pro Donor vectors, continued

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*B5r site flanks the PCR product and an *att*P5r site resides on the donor vector generating an *att*R5 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 reaction:

- *att*B5r sites react with *att*P5r sites to generate *att*R5 sites in the entry clone
- *att*B4r sites react with *att*P4r sites to generate *att*R4 sites in the entry clone
- *att*B3r sites react with *att*P3r sites to generate *att*R3 sites in the entry clone

Example

In this example, an *att*B1 and *att*B5r-flanked PCR product is used in a BP recombination reaction with pDONR™ P1-P5r:

_att_B1-PCR product-_att_B5r × pDONR™ P1-P5r→ _att_L1-PCR product-_att_R5

Because of the orientation and position of the *att*B5r and *att*P5r site in the PCR product and donor vector, respectively, the resulting entry clone contains the PCR product flanked by an *att*L1 site and an *att*R5 site rather than two *att*L sites.

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MultiSite Gateway® Pro Donor vectors, continued

MultiSite Gateway® Pro Donor vectors

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

Depending on what kind of recombination you are performing (*i.e.* 2-fragment, 3-fragment or 4-fragment) your PCR products will be flanked by different *att*B or *att*Br sites. Six different donor vectors facilitate generation of entry clones:

Vector	Insert	Recombination Type
pDONR™ 221 P1-P5r	*att*B1 and *att*B5r-flanked PCR products	2-fragment, 4-fragment
pDONR™ 221 P5-P2	*att*B5 and *att*B2-flanked PCR products	2-fragment
pDONR™ 221 P1-P4	*att*B1 and *att*B4-flanked PCR products	3-fragment
pDONR™ 221 P4r-P3r	*att*B4r and *att*B3r-flanked PCR products	3-fragment, 4-fragment
pDONR™ 221 P3-P2	*att*B3 and *att*B2-flanked PCR products	3-fragment, 4-fragment
pDONR™ 221 P5-P4	*att*B5 and *att*B4-flanked PCR products	4-fragment

Note: pDONR™ 221 is also supplied for the positive control for the BP reaction only (page 47). **DO NOT** use pDONR™ 221 to generate multi-fragment entry clones.

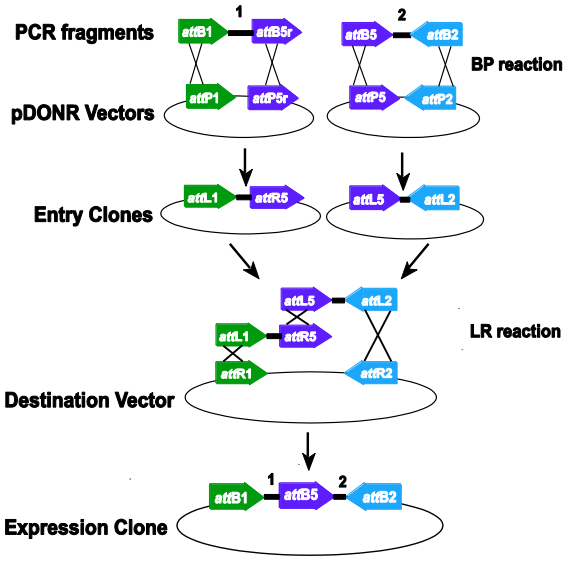
The MultiSite Gateway® Pro kits are compatible with Vector NTI Advance® software version 10 and above.

Go to **www.lifetechnologies.com/vectornti** for detailed instructions to use the Vector NTI Advance® software to design *att*B and *att*Br primers for your DNA elements of choice.

Experimental outline

MultiSite Gateway® Pro 2-fragment recombination

Two PCR products flanked by specific *att*B or *att*Br sites and two MultiSite Gateway® Pro Donor vectors are used in separate BP recombination reactions to generate two entry clones. The two entry clones and a destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your expression clone containing two DNA elements.



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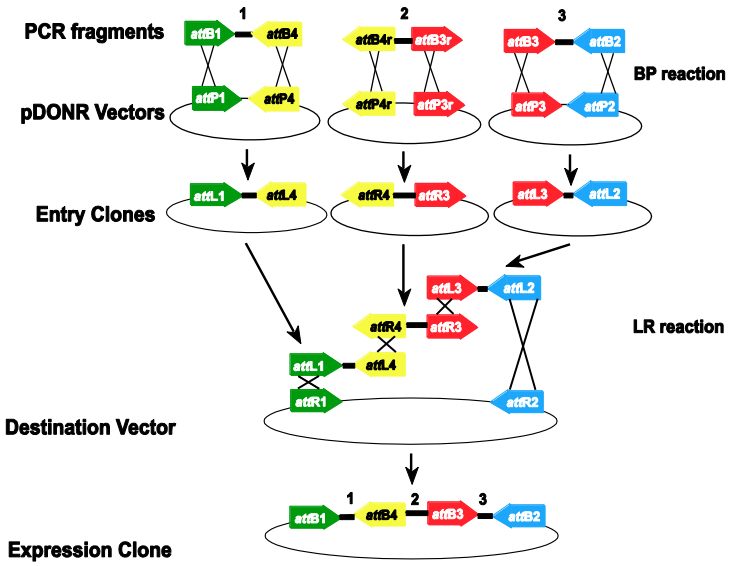
MultiSite Gateway® Pro 3-fragment recombination

Three PCR products flanked by specific *att*B or *att*Br sites and three MultiSite Gateway® Pro Donor vectors are used in separate BP recombination reactions to generate three entry clones. The three entry clones and a destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your expression clone containing three DNA elements.



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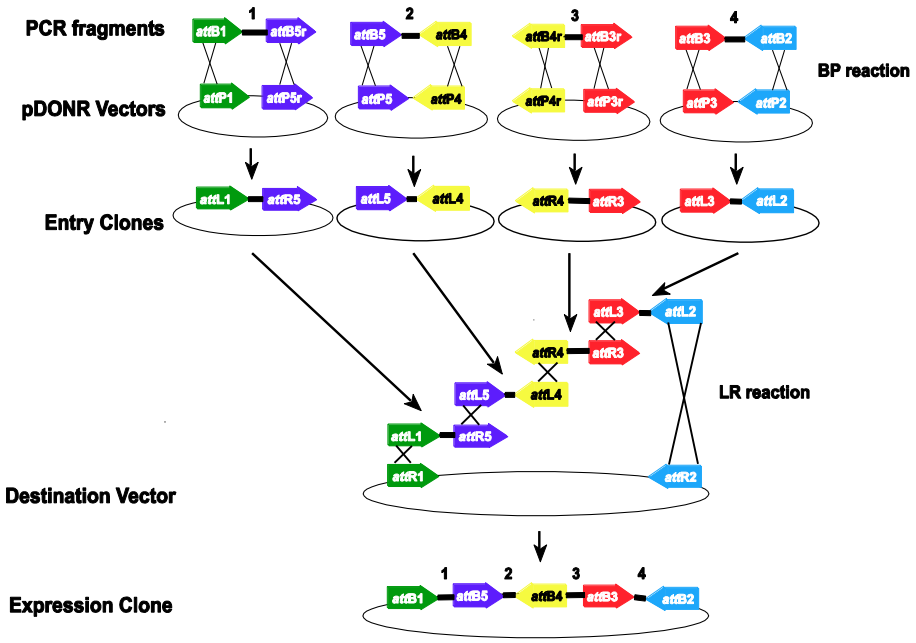
MultiSite Gateway® Pro 4-fragment recombination

Four PCR products flanked by specific *att*B or *att*Br sites and four MultiSite Gateway® Pro Donor vectors are used in separate BP recombination reactions to generate four entry clones. The four entry clones and a destination vector are used together in a MultiSite Gateway® Pro LR recombination reaction to create your expression clone containing four DNA elements.



Methods

Propagate the MultiSite Gateway® Pro Donor vectors

Introduction

The MultiSite Gateway® Pro Kits (MultiSite Gateway® Pro 2.0, MultiSite Gateway® Pro 3.0, MultiSite Gateway® Pro 4.0, and MultiSite Gateway® Pro Plus Kit) contain 2, 3, 4 or 6 pDONR™ vectors, respectively. See page 10 for a description of the vectors included in each kit. See the guidelines in the following section to propagate and maintain these vectors.

Propagate donor vectors

If you wish to propagate and maintain the MultiSite Gateway® Pro pDONR™ vectors prior to recombination, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells. These cells are available separately (see page 79). The *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

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

General information for making entry clones

Introduction

Using the MultiSite Gateway® Pro Kit, you will create 2, 3, or 4 entry clones, depending on how many DNA elements you wish to recombine into your expression clone. To create entry clones, you will PCR amplify a single DNA element flanked by specific *att*B sites, which is recombined using BP Clonase® II with the corresponding MultiSite Gateway® Pro Donor vector.

The following sections provide instructions for designing and producing entry clones.

DNA elements

Depending on the kit configuration, MultiSite Gateway® Pro allows flexibility in combining up to four specific DNA elements into a single expression clone. These elements may include, but are not limited to:

- 5′ element to control expression of your gene of interest, such as an IRES or promoter
- N- or C-terminal fusion tag that will be in frame with your gene of interest
- ORFs of interest, including sequences necessary for efficient translation initiation (*i.e.* Shine-Dalgarno, Kozak consensus sequences, yeast consensus sequences)
- Double-stranded DNA oligos for miRNA
- Reporter gene such as GFP or LacZ
- Resistance gene for selection in various systems
- 3′ element of interest, such as transcription termination sequences or

polyadenylation sequences required for efficient transcription termination and polyadenylation of mRNA

Depending on your application, these elements will be recombined in a specific orientation and order (see **Examples**, in the following section).

Examples

If you are, for example, performing 2-fragment recombination using MultiSite Gateway® Pro 2.0, you could produce the following entry clone(s):

Example	Element 1	Element 2
Gene under control of a specific promoter	Promoter	ORF
N-terminal tagged gene of choice	N-term tag	ORF
C-terminal tagged gene of choice	ORF*	C-term tag

*ORF should not have a stop codon.

In the preceding example, if you wish to express a gene of interest under control of a specific promoter element, we recommend recombining these entry clones into a promoterless DEST eukaryotic expression vector (such as pcDNA6.2/V5 pL-DEST), available separately (page 79).

Important

If you want to perform stable expression studies in mammalian, yeast, or insect systems, make sure your DEST vector contains a resistance marker such as G418 or blasticidin.

Continued on next page

Design 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® Pro Technology. Your primer design must incorporate:

- Sequences required to facilitate MultiSite Gateway® Pro 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), respectively, in the *att*B1 forward PCR primer, if they are not provided by your chosen DEST vector.

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

MultiSite Gateway® Pro	DNA Element	Flanking *att* sites
2-fragment recombination	Element 1	*att*B1, *att*B5r
	Element 2	*att*B5, *att*B2
3-fragment recombination	Element 1	*att*B1, *att*B4
	Element 2	*att*B4r, *att*B3r
	Element 3	*att*B3, *att*B2
4-fragment recombination	Element 1	*att*B1, *att*B5r
	Element 2	*att*B5, *att*B4
	Element 3	*att*B4r, *att*B3r
	Element 4	*att*B3, *att*B2

For more information to design *att*B and *att*Br-flanked primers, see the following sections.

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.

Make entry clones for 2-fragment recombination

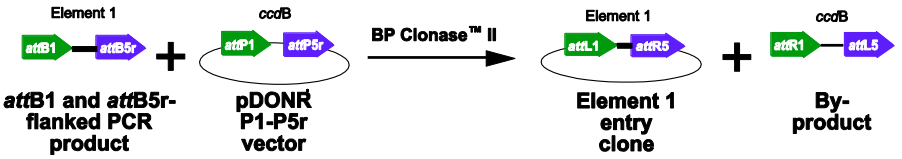
Introduction

Guidelines are provided in this section to create entry clones for 2 fragment recombination using the MultiSite Gateway® Pro 2.0 kit (Cat. no. 12537-102) or MultiSite Gateway® Pro Plus kit (Cat. no. 12537-100).

Generate the entry clone for Element 1

To generate an entry clone containing Element 1:

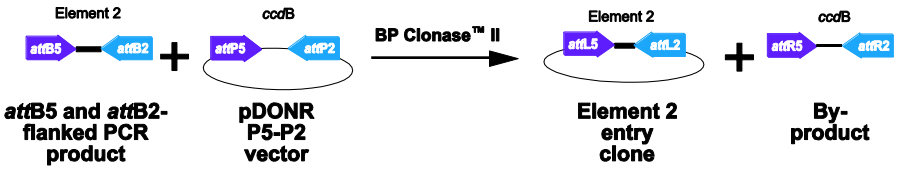
- Design appropriate PCR primers and produce your *att*B1 and *att*B5r-flanked PCR product.
- Perform a BP recombination reaction between the *att*B1 and *att*B5r-flanked PCR product and pDONR™ P1-P5r to generate the entry clone for Element 1 (see the following figure).



Generate the entry clone for Element 2

To generate an entry clone containing Element 2:

- Design appropriate PCR primers and produce your *att*B5 and *att*B2-flanked PCR product.
- Perform a BP recombination reaction between the *att*B5 and *att*B2 -flanked PCR product and pDONR™ P5-P2 to generate the entry clone for Element 2 (see the following figure).



Design 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. To facilitate use in MultiSite Gateway®, each PCR product must be flanked by a different combination of *att*B sites (see the following table). Guidelines are provided on the next page to help you design appropriate PCR primers.

MultiSite Gateway® Pro reaction	DNA element	Flanking *att* sites	PCR primers
Generate entry clones for 2-fragment recombination	Element 1	*att*B1, *att*B5r	*att*B1 forward, *att*B5r reverse
	Element 2	*att*B5, *att*B2	*att*B5 forward, *att*B2 reverse

Continued on next page

Guidelines to design the forward PCR primers

Consider the following when designing forward PCR primers:

- The forward primer **MUST** contain the following structure:
  - 4 guanine (G) residues at the 5′ end followed by
  - The 22- or 25-bp *att*B site followed by
  - At least 18–25 bp of template- or gene-specific sequences
- Two additional nucleotides are included in each diagram below between the *attB* site and the template-specific sequence to maintain the proper reading frame. In this example, the nucleotides TA are used. Note that the nucleotides cannot be AA, AG, or GA, as these will generate a stop codon.

attB1 5′-GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TTA--(template-specific sequence)-3′
*att*B1

attB5 5′-GGGG-ACA-ACT-TTG-TAT-ACA-AAA-GTT-GTA--(template-specific sequence)-3′
*att*B5

Guidelines to design the reverse PCR primers

Consider the following when designing reverse PCR primers:

- The reverse primer **MUST** contain the following structure:
  - 4 guanine (G) residues at the 5′ end followed by
  - The 22- or 25-bp *att*B or *att*Br site followed by
  - 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:
  - An additional nucleotide (T) is included in each following diagram (between the *attB* site and the template-specific sequence) to maintain the proper reading frame.
  - Any in-frame stop codons between the *att*B sites and your gene of interest must be removed.

attB2 5′-GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTT--(template-specific sequence)-3′
*att*B2

attB5r 5′-GGGG AC AAC TTT TGT ATA CAA AGT TGT--(template-specific sequence)-3′
*att*B5r

- If a C-terminal tag is present in your destination vector of choice and you do not wish to fuse it with your PCR product, your gene of interest or primer must include a stop codon.

Next step

Proceed to **Produce *att*B PCR products**, page 34.

Make entry clones for 3-fragment recombination

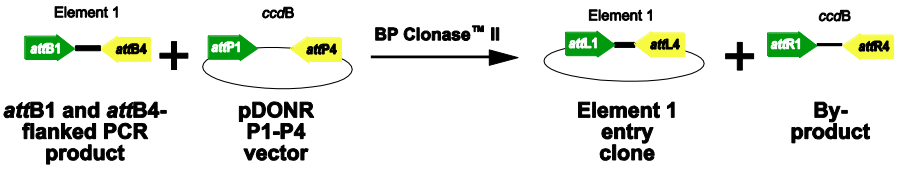
Introduction

Guidelines are provided in this section to create entry clones for 3-fragment recombination using the MultiSite Gateway® Pro 3.0 kit (Cat. no. 12537-103) or MultiSite Gateway® Pro Plus kit (Cat. no. 12537-100).

Generate the entry clone for Element 1

To generate an entry clone containing Element 1:

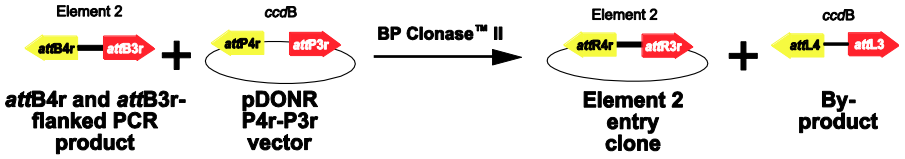
1. Design appropriate PCR primers and produce your *att*B1 and *att*B4-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B1 and *att*B4-flanked PCR product and pDONR™ P1-P4 to generate the entry clone for Element 1 (see the following figure).



Generate the entry clone for Element 2

To generate an entry clone containing Element 2:

1. Design appropriate PCR primers and produce your *att*B4r and *att*B3r-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B4r and *att*B3r-flanked PCR product and pDONR™ P4r-P3r to generate the entry clone for Element 2 (see the following figure).



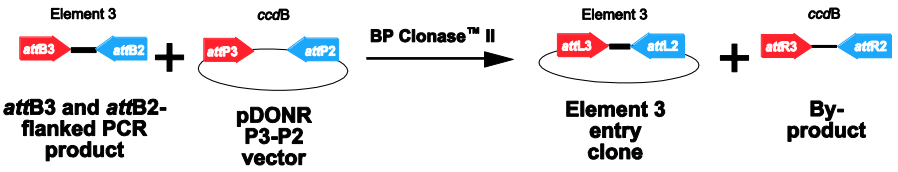
Continued on next page

Make entry clones for 3-fragment recombination, continued

Generate the entry clone for Element 3

To generate an entry clone containing Element 3:

1. Design appropriate PCR primers and produce your *att*B3 and *att*B2-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B3 and *att*B2-flanked PCR product and pDONR™ P3-P2 to generate the entry clone for Element 3 (see the following figure).



Design 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. To facilitate use in MultiSite Gateway®, each PCR product must be flanked by a different combination of *att*B or *att*Br sites (see table below). Guidelines are provided on the next page to help you design appropriate PCR primers.

MultiSite Gateway® Pro reaction	DNA element	Flanking *att* sites	PCR primers
Generate entry clones for 3-fragment recombination	Element 1	*att*B1, *att*B4	*att*B1 forward, *att*B4 reverse
	Element 2	*att*B4r, *att*B3r	*att*B4r forward, *att*B3r reverse
	Element 3	*att*B3, *att*B2	*att*B3 forward, *att*B2 reverse

Continued on next page

Guidelines to design the forward PCR primers

Consider the following when designing forward PCR primers:

- The forward primer **MUST** contain the following structure:
  - 4 guanine (G) residues at the 5′ end followed by
  - The 22- or 25-bp *att*B or *att*Br site followed by
  - At least 18–25 bp of template- or gene-specific sequences
- If you want to fuse your PCR product in frame with an N- or C-terminal tag, the primer must include two additional nucleotides between the *att* site and the gene of interest to maintain the proper reading frame (shown as TA in the following diagram). Note that the nucleotides cannot be AA, AG, or GA, as these will generate a stop codon.

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

***att*B4r** 5′-GGGG ACA ACT TTT CTA TAC AAA GTT GTA--(template-specific sequence)-3′
*att*B4r

***att*B3** 5′-GGGG ACA ACT TTG TAT AAT AAA GTT GTA--(template-specific sequence)-3′
*att*B3

Guidelines to design the reverse PCR primers

Consider the following when designing reverse PCR primers:

- The reverse primer **MUST** contain the following structure:
  - 4 guanine (G) residues at the 5′ end followed by
  - The 22- or 25-bp *att*B or *att*Br site followed by
  - 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:
  - The reverse primers must include one additional nucleotide to maintain the proper reading frame (shown as G or T in the following diagram).
  - Any in-frame stop codons between the *att*B or *att*Br sites and your gene of interest must be removed.

***att*B4** 5′-GGGG AC AAC TTT GTA TAG AAA AGT TGG GTG--(template-specific sequence)-3′
*att*B4

***att*B3r** 5′-GGGG AC AAC TTT ATT ATA CAA AGT TGT--(template-specific sequence)-3′
*att*B3r

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

- If a C-terminal tag is present in your destination vector of choice and you do not wish to fuse it with your PCR product, your gene of interest or primer must include a stop codon.

Next step

Proceed to **Produce *att*B PCR products**, page 34.

Make entry clones for 4-fragment recombination

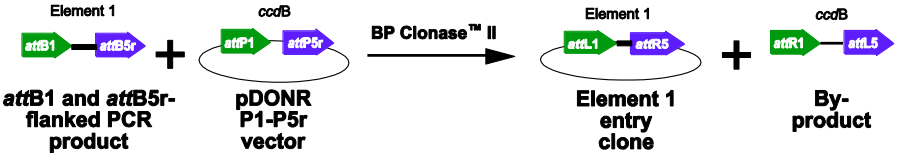
Introduction

Guidelines are provided in this section to create entry clones for 4-fragment recombination using the MultiSite Gateway® Pro 4.0 kit (Cat. no. 12537-104) or MultiSite Gateway® Pro Plus kit (Cat. no. 12537-100).

Generate the entry clone for Element 1

To generate an entry clone containing Element 1:

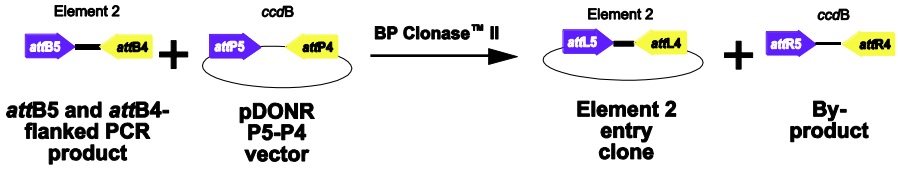
1. Design appropriate PCR primers and produce your *att*B1 and *att*B5r-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B1 and *att*B5r-flanked PCR product and pDONR™ P1-P5r to generate the entry clone for Element 1 (see the following figure).



Generate the entry clone for Element 2

To generate an entry clone containing Element 2:

1. Design appropriate PCR primers and produce your *att*B5 and *att*B4-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B5 and *att*B4-flanked PCR product and pDONR™ P5-P4 to generate the entry clone for Element 2 (see the following figure).



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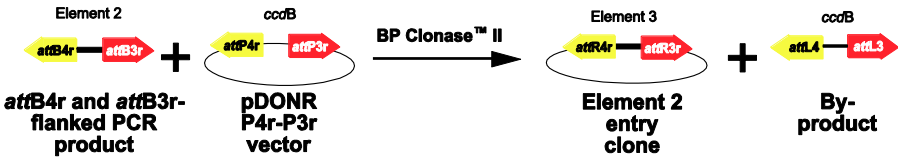
Make entry clones for 4-fragment recombination, continued

Generate the entry clone for Element 3

To generate an entry clone containing Element 3:

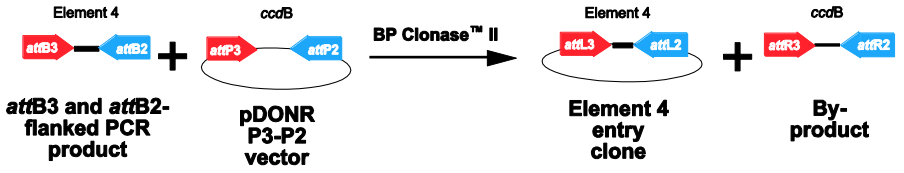
1. Design appropriate PCR primers and produce your *att*B4r and *att*B3r-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B4r and *att*B3r-flanked PCR product and pDONR™ P4r-P3r to generate the entry clone for Element 3 (see the following figure).



Generate the entry clone for Element 4

To generate an entry clone containing Element 4:

1. Design appropriate PCR primers and produce your *att*B3 and *att*B2-flanked PCR product.
2. Perform a BP recombination reaction between the *att*B3 and *att*B2-flanked PCR product and pDONR™ P3-P2 to generate the entry clone for Element 4 (see the following figure).



Design 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. To facilitate use in MultiSite Gateway®, each PCR product must be flanked by a different combination of *att*B or *att*Br sites (see the following table). Guidelines are provided on the next page to help you design appropriate PCR primers.

MultiSite Gateway® Pro reaction	DNA Element	Flanking *att* sites	PCR primers
Generate entry clones for 4-fragment recombination	Element 1	*att*B1, *att*B5r	*att*B1 forward, *att*B5r reverse
	Element 2	*att*B5, *att*B4	*att*B5 forward, *att*B4 reverse
	Element 3	*att*B4r, *att*B3r	*att*B4r forward, *att*B3r reverse
	Element 4	*att*B3, *att*B2	*att*B3 forward, *att*B2 reverse

Continued on next page

Guidelines to design the forward PCR primers

Consider the following when designing forward PCR primers

- The forward primer **MUST** contain the following structure:
  - 4 guanine (G) residues at the 5′ end followed by
  - The 22- or 25-bp *att*B or *att*Br site followed by
  - At least 18–25 bp of template- or gene-specific sequences
- If you want to fuse your PCR product in frame with an N- or C-terminal tag, the primer must include two additional nucleotides between the *att* site and the gene of interest to maintain the proper reading frame (shown as TA in the following diagram). Note that the nucleotides cannot be AA, AG, or GA, as these will generate a stop codon.

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

***att*B5** 5′-GGGG ACA ACT TTG TAT ACA AAA GTT GTA--(template-specific sequence)-3′
***att*B5**

***att*B4r** 5′-GGGG ACA ACT TTT CTA TAC AAA GTT GTA--(template-specific sequence)-3′
***att*B4r**

***att*B3** 5′-GGGG ACA ACT TTG TAT AAT AAA GTT GTA--(template-specific sequence)-3′
***att*B3**

Guidelines to design the reverse PCR primers

Consider the following when designing reverse PCR primers:

- The reverse primer **MUST** contain the following structure:
  - 4 guanine (G) residues at the 5′ end followed by
  - The 22- or 25-bp *att*B or *att*Br site followed by
  - 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:
  - The reverse primers must include one additional nucleotide to maintain the proper reading frame.
  - Any in-frame stop codons between the *att*B sites and your gene of interest must be removed.

***att*B5r** 5′-GGGG AC AAC TTT TGT ATA CAA AGT TGT--(template-specific sequence)-3′
***att*B5r**

***att*B4** 5′-GGGG AC AAC TTT GTA TAG AAA AGT TGG GTG--(template-specific sequence)-3′
***att*B4**

***att*B3r** 5′-GGGG AC AAC TTT ATT ATA CAA AGT TGT--(template-specific sequence)-3′
***att*B3r**

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

- If a C-terminal tag is present in your destination vector of choice and you do not wish to fuse it with your PCR product, your gene of interest or primer must include a stop codon.

Next step

Proceed to **Produce *att*B PCR products**, next page.

Produce *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 separately from Life Technologies to produce your *att*B PCR products. Other DNA polymerases are also suitable. See page 79 for ordering information.

- To generate PCR products less than 5–6 kb for use in protein expression, use *Pfx* 50 DNA Polymerase
- To generate PCR products for use in other applications (*e.g.* functional analysis), use Platinum® *Taq* DNA Polymerase High Fidelity

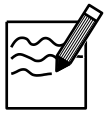
Producing PCR products

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

Check 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 **Purify *att*B PCR products**.



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 (supplied with enzyme)
- *Dpn* I (see page 79)

Protocol:

1. To your 25 µL PCR reaction mixture, add 5 µL of 10X REact® 4 Buffer and ≥ 5 units of *Dpn* I. Add water to a final volume of 50 µL.
2. Incubate at 37°C for 1 hour.
3. Heat-inactivate the *Dpn* I at 65°C for 15 minutes.
4. Proceed to **Purify *att*B PCR products**, page 35.

Purify *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 in the following sections 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 will need the following materials before beginning:

- Each *att*B PCR product (in a 25 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, Box 3)
- Agarose gel of the appropriate percentage to resolve your *att*B PCR products

PEG purification protocol

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

1. Add 75 µL of 1X TE, pH 8.0 to a 25 µL amplification reaction containing your *att*B PCR product.
2. Add 50 µL of 30% PEG 8000/30 mM $MgCl_2$. Vortex to mix thoroughly and centrifuge immediately at 10,000 × *g* for 15 minutes at room temperature.

   Note: In most cases, centrifugation at 10,000 × *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 25 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 by running an aliquot on an agarose gel.
6. If the PCR product is suitably purified, proceed to **Create entry clones using the BP recombination reaction**, page 36. If the PCR product is not suitably purified (*e.g. att*B primer-dimers are still detectable), see the following section.

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 separately (see page 79).

Create entry clones using the BP recombination reaction

Introduction

Once you have generated your *att*B or *att*Br-flanked PCR products, you will perform a BP reaction to transfer the DNA sequence of interest into the appropriate *att*P-containing MultiSite Gateway® Pro Donor vector to create entry clones.

Depending on your MultiSite Gateway® Pro kit configuration, you will perform 2, 3 or 4 BP recombination reactions. The following sections provide recombination regions for each of these reactions in detail.

To ensure that you obtain the best possible results, we suggest that you read this section and **Transforming One Shot® Mach1™ T1R Competent Cells** (pages 48–49) entirely before beginning.

Experimental outline

To generate entry clones, you will:

1. Perform 2, 3, or 4 BP recombination reactions using the appropriate linear *att*B- or *att*Br-flanked PCR products and the appropriate supercoiled MultiSite Gateway® Pro donor vectors
2. Transform each reaction mixture separately into One Shot® Mach1™ T1R Competent Cells
3. Select for entry clones on LB plates containing kanamycin
4. Sequence entry clones using M13 Forward (–20) and Reverse Primers

Recombination regions

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

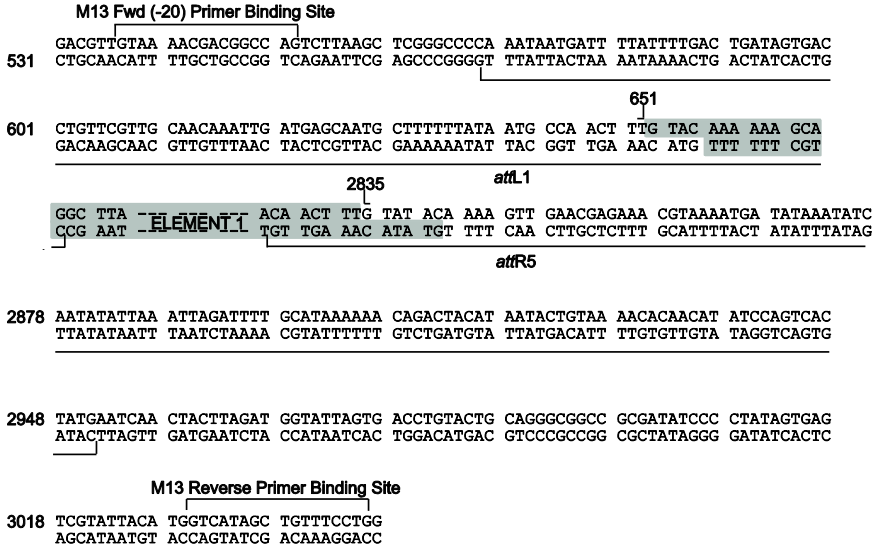
2-Fragment BP recombination regions

Recombination Region of pDONR™ 221 P1-P5r + Element 1

The recombination region of the entry clone resulting from pDONR™ 221 P1-P5r × *att*B1-Element1-*att*B5r is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P1-P5r vector by recombination. Non-shaded regions are derived from pDONR™ 221 P1-P5r vector.
- Bases 651 and 2835 of the pDONR™ 221 P1-P5r vector sequence are marked.



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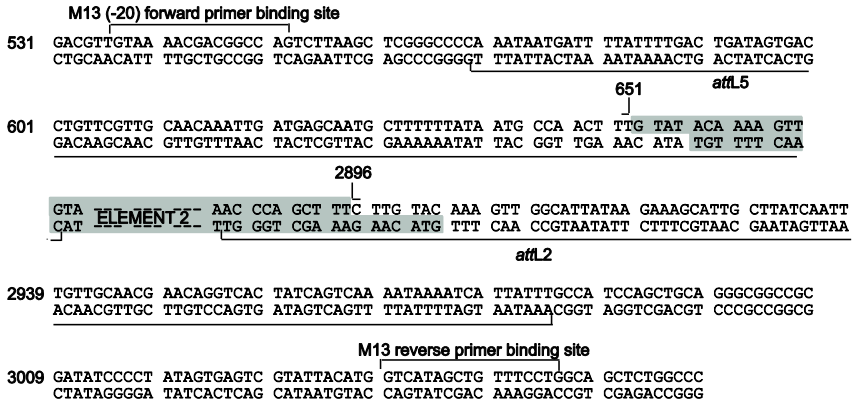
Recombination region of pDONR™ 221 P5-P2 + Element 2

The recombination region of the entry clone resulting from pDONR™ 221 P5-P2 × *att*B5-Element 2-*att*B2 is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into pDONR™ 221 P5-P2 by recombination. Non-shaded regions are derived from the pDONR™ 221 P5-P2 vector.
- Bases 651 and 2896 of the pDONR™ 221 P5-P2 sequence are marked.



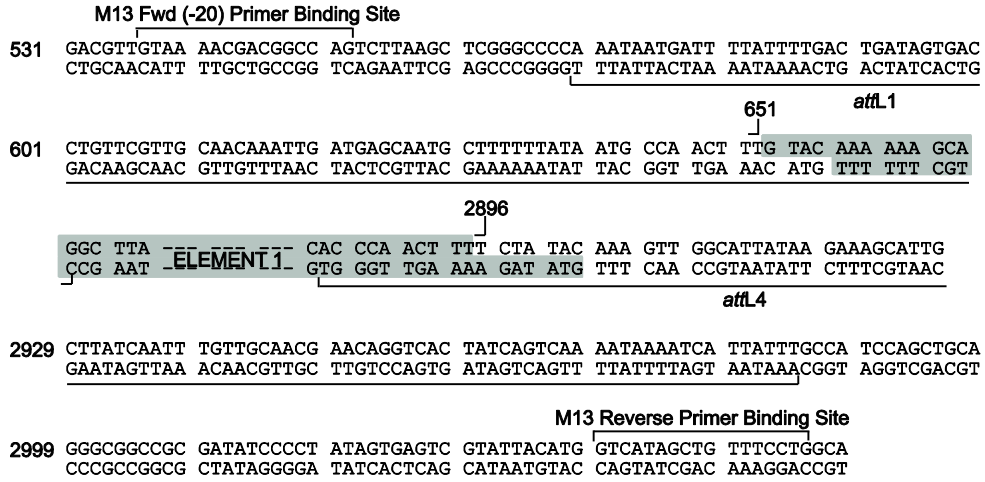
3-Fragment BP recombination regions

Recombination region of pDONR™ 221 P1-P4 + Element 1

The recombination region of the entry clone resulting from pDONR™ 221 P1-P4 × *att*B1-Element 1–*att*B4 is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P1-P4 vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P1-P4 vector.
- Bases 651 and 2896 of the pDONR™ 221 P1-P4 sequence are marked.



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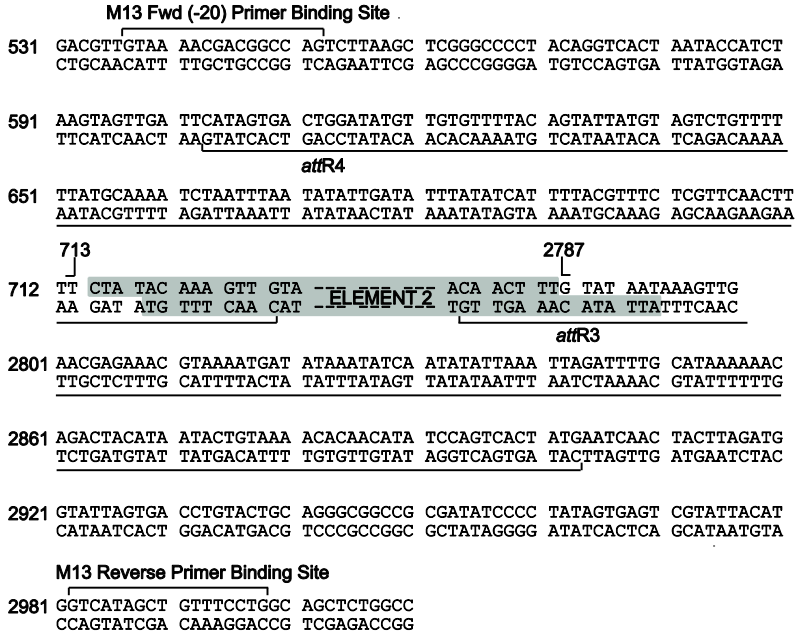
3-Fragment BP recombination regions, continued

Recombination region of pDONR™ 221 P4r-P3r + Element 2

The recombination region of the entry clone resulting from pDONR™ 221 P4r-P3r × *att*B4r-Element 2–*att*B3r is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P4r-P3r vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P4r-P3r vector.
- Bases 713 and 2787 of the pDONR™ 221 P4r-P3r sequence are marked.



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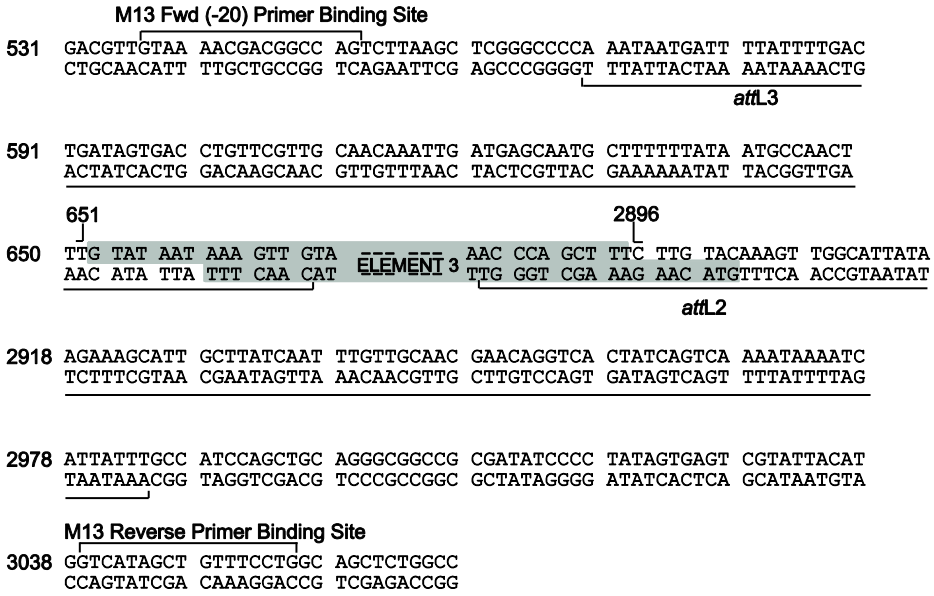
3-Fragment BP recombination regions, continued

Recombination region of pDONR™ 221 P3-P2 x Element 3

The recombination region of the entry clone resulting from pDONR™ 221 P3-P2 × *att*B3-Element 3-*att*B2 is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P3-P2 vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P3-P2 vector.
- Bases 651 and 2896 of the pDONR™ 221 P3-P2 sequence are marked.



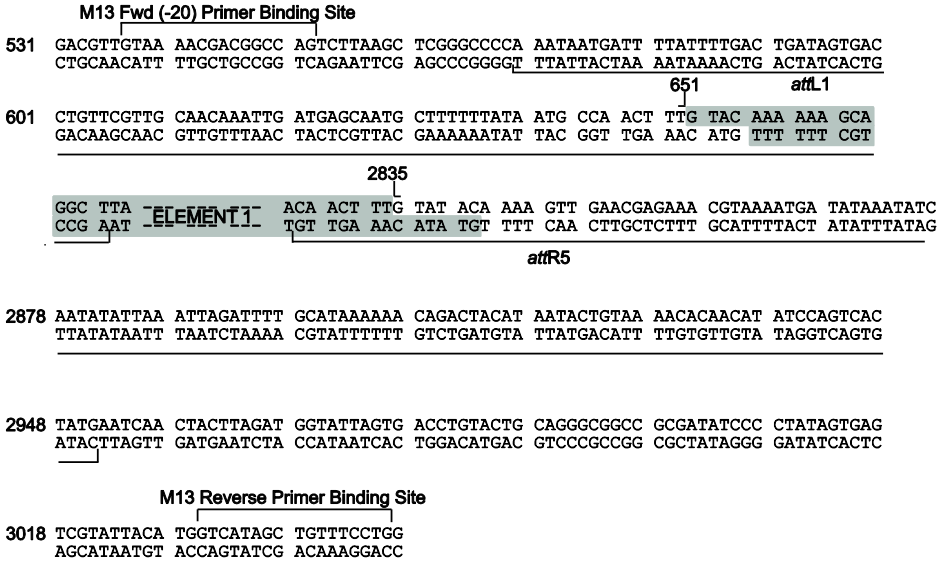
4-Fragment BP recombination regions

Recombination region of pDONR™221 P1-P5r + Element 1

The recombination region of the entry clone resulting from pDONR™ 221 P1-P5r × *att*B1-Element 1–*att*B5r is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P1-P5r vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P1-P5r vector.
- Bases 651 and 2835 of the pDONR™ 221 P1-P5r sequence are marked.



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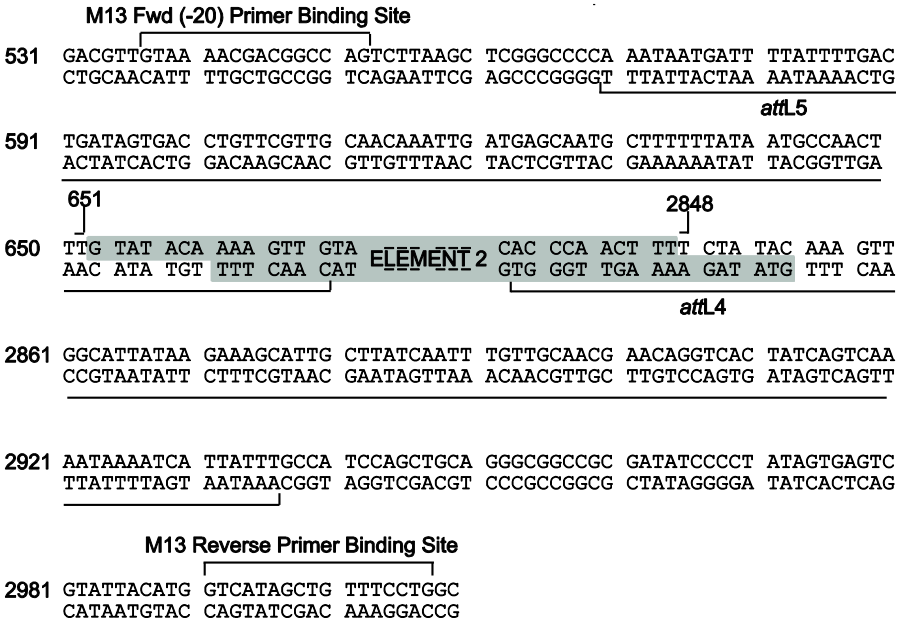
4-Fragment BP recombination regions, continued

Recombination region of pDONR™221 P5-P4 + Element 2

The recombination region of the entry clone resulting from pDONR™ 221 P5-P4 × *att*B5-Element 2-*att*B4 is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P5-P4 vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P5-P4 vector.
- Bases 651 and 2896 of the pDONR™ 221 P5-P4 sequence are marked.



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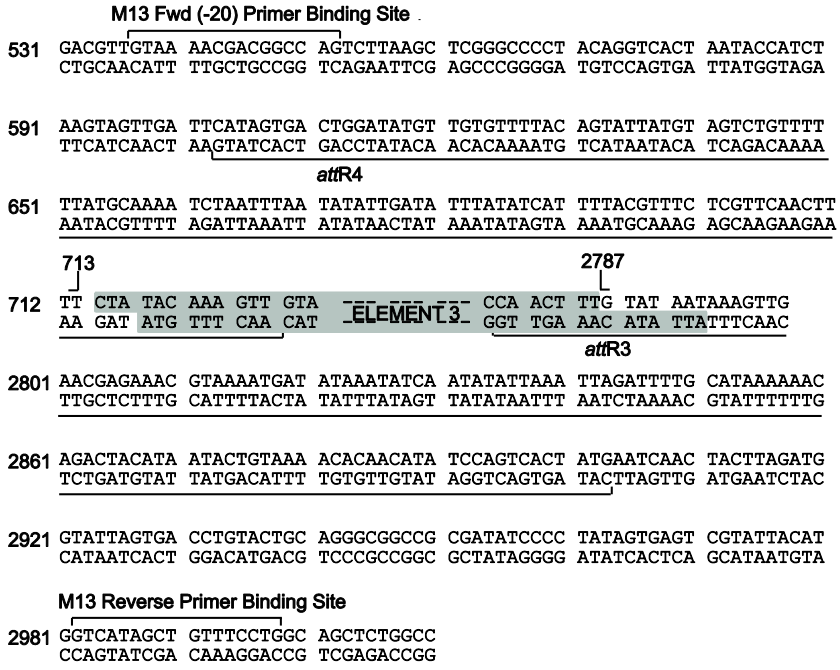
4-Fragment BP recombination regions, continued

Recombination region of pDONR™221 P4r-P3r + Element 3

The recombination region of the entry clone resulting from pDONR™ 221 P4r-P3r × *att*B4r-Element 3–*att*B3r is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P4r-P3r vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P4r-P3r vector.
- Bases 713 and 2787 of the pDONR™ 221 P4r-P3r sequence are marked.



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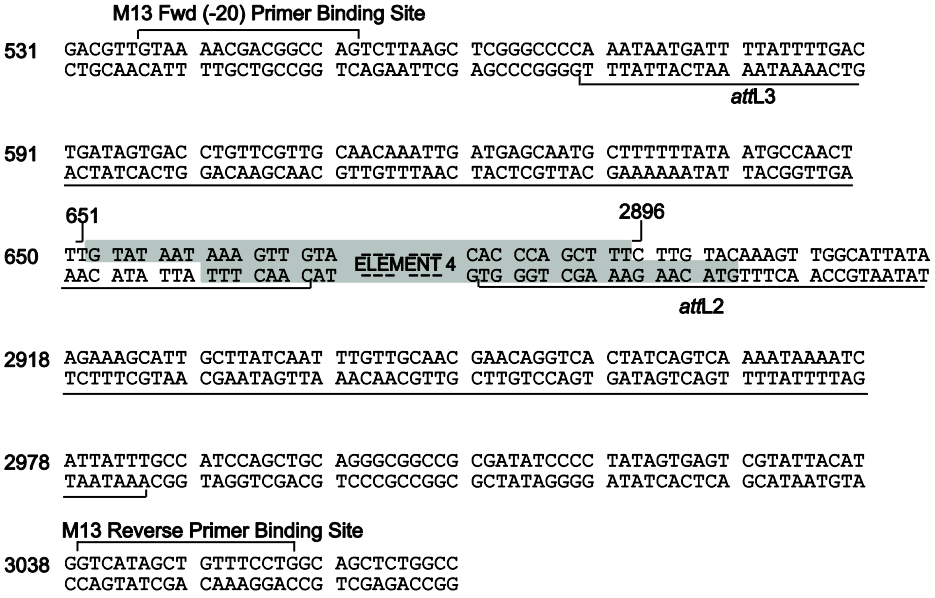
4-Fragment BP recombination regions, continued

Recombination region of pDONR™221 P3-P2 + Element 4

The recombination region of the entry clone resulting from pDONR™ 221 P3-P2 × *att*B3-Element 4-*att*B2 is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the PCR product into the pDONR™ 221 P3-P2 vector by recombination. Non-shaded regions are derived from the pDONR™ 221 P3-P2 vector.
- Bases 651 and 2896 of the pDONR™ 221 P3-P2 sequence are marked.



Perform the BP recombination reaction

Introduction

General guidelines and instructions are provided below to perform a BP recombination reaction using the appropriate *att*B PCR product and donor vector, and to transform the reaction mixture into One Shot® Mach1™ T1^R chemically competent *E. coli* and select for entry clones. We recommend including a negative control (no BP Clonase® II) and a positive control (see page 47) to help you evaluate your results.

Important

For optimal results, perform the BP recombination reaction using:

- **Linear** PCR products
- **Supercoiled** 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 in an optimized single-tube format to allow easy set-up of the BP recombination reaction.

Convert femtomoles (fmoles) to nanograms (ng)

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

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

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

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 from the preceding section:

$$(50\ fmoles)(25\ 00\ bp)\left(\frac{660\ fg}{fmoles}\right)\left(\frac{1\ ng}{10^6\ fg}\right) = 82.5\ ng\ of\ PCR\ product\ required$$

Materials needed

Supplied with the kit:

- Appropriate MultiSite Gateway® Pro pDONR™ vectors for each *att*B-or *att*Br-flanked PCR product (see page 25)
- 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)

Supplied by the user:

- *att*B-flanked PCR products
- 1X TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 37°C water bath
- Vortex

Perform the BP recombination reaction, continued

Set 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
*att*B PCR product (15–150 ng)	1–7 µL
pDONR™ vector (150 ng/µL)	1 µL
1X TE Buffer, pH 8.0	to 8 µL

2. Remove the BP Clonase® II Enzyme Mix from –20°C or –80°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 or –80°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: 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 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 **Transform One Shot® Mach1™ T1^R competent cells**, page 48.

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

BP reaction positive control

pEXP7-tet is provided as a positive control for BP Clonase® II. 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). You may perform a BP reaction with pEXP7-tet and pDONR™221 (supplied with the kit), which will result in an entry clone that expresses the tetracycline resistance gene.

To perform this reaction:

1. Add 1 µL (150 ng) pDONR™ 221, 2 µL (100 ng) of pEXP7-tet and 5 µL 1X TE, pH 8.0 to a microcentrifuge tube and mix.
2. Continue with Step 2 in **Set up the BP reaction** to perform the control BP recombination reaction.

The efficiency of the BP reaction can be easily determined after transformation into *E. coli* by streaking entry clones onto LB plates containing 20 µg/mL tetracycline.

Transform One Shot® Mach1™ T1R competent cells

Introduction

Use the protocol provided in this section to transform competent *E. coli* with the BP recombination reaction or the MultiSite Gateway® Pro LR recombination reaction to select for entry clones or expression clones, respectively. One Shot® Mach1™ T1R chemically competent *E. coli* (Box 4) are included with the kit for use in transformation.

Materials needed

You will need the following materials:

Supplied with the kit:

- One Shot® Mach1™ T1R chemically competent *E. coli* (thaw 1 vial of cells on ice for each transformation)
- S.O.C. medium (warm to room temperature)
- pUC19; use as a control for transformation (if desired)

Supplied by the user:

- **One of the following:**

  BP recombination reaction (from **Set up the BP reaction**, step 7 page 47)

  or

  MultiSite Gateway® Pro LR recombination reaction (from **Set up the MultiSite Gateway® Pro LR reaction**, Step 7, page 57)

- **One of the following:**

  2 LB plates containing 50 µg/mL kanamycin, pre-warmed to 37°C for each BP reaction

  or

  2 LB plates containing 50–100 µg/mL antibiotic of choice, pre-warmed to 37°C for each MultiSite Gateway® Pro LR reaction

  Note: Check the destination vector you are using in the LR reaction to determine what antibiotic to use.

- 42°C water bath
- 37°C shaking and non-shaking incubators

Continued on next page

Transform One Shot® Mach1™ T1R competent cells, continued

One Shot® Mach1™ T1R transformation protocol

1. Add *one* of the following into a vial of One Shot® Mach1™ T1R chemically competent *E. coli*, and mix gently. **Do not mix by pipetting up and down.**

   2 µL of the BP recombination reaction (from **Set up the BP reaction**, Step 7, page 47)

   or

   2 µL of 2- or 3-fragment MultiSite Gateway® LR recombination reaction. For 4-fragment recombination reactions, add 4 µL

   Reminders:

   - If you are including the transformation control, add 1 µL (10 pg) of pUC19 to a separate vial of One Shot® Mach1™ chemically competent *E. coli*.
   - If you are including the BP positive control reaction, add 2 µL of the recombination reaction to a separate vial of One Shot® Mach1™ chemically competent *E. coli*
2. Incubate vial on ice for 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice for 2 minutes.
5. Add 250 µL of room temperature S.O.C. medium.
6. Cap tube tightly and shake the tube horizontally (225 rpm) at 37°C for 1 hour.
7. Spread the following volumes from each transformation on pre-warmed selective plates, invert and incubate overnight at 37°C:

Gateway® recombination reaction	Volume	Selective plate
BP reaction	Plate 20 µL and 100 µL	LB plates containing 50 µg/mL kanamycin
MultiSite Gateway® Pro 2-fragment LR reaction recombination	Dilute 1:10 in S.O.C. medium and plate 50 µL and 100 µL	LB plates containing 50-100 µg/mL antibiotic of choice
MultiSite Gateway® Pro 3-fragment LR reaction recombination	Plate 50 µL and 100 µL of each transformation	LB plates containing 50-100 µg/mL antibiotic of choice
MultiSite Gateway® Pro 4-fragment LR reaction recombination	Plate the entire transformation reaction	Single LB plate containing 50-100 µg/mL antibiotic of choice

What you should see

- **BP reaction**
  An efficient BP recombination reaction may produce hundreds of colonies (greater than 1500 colonies if the entire reaction is transformed and plated).
- **MultiSite Gateway® Pro LR reaction**
  Typical numbers of colonies (per 10 µL LR reaction):

  2-fragment recombination reaction: 2000–15,000

  3-fragment recombination reaction: 1000–5000

  4-fragment recombination reaction: 50–500

Sequence entry clones

Introduction

After BP recombination, we strongly recommend sequencing the entry clones to ensure that the inserts do not contain errors that have been introduced during PCR. Sequencing can be performed using any method of choice using the M13 Forward (–20) and M13 Reverse primers provided in the kit

Sequencing primers

To sequence entry clones derived from BP recombination with MultiSite Gateway® Pro pDONR™ vectors, 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 37–45 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 are available separately (see page 79).

MultiSite Gateway® Pro LR recombination reaction

Introduction

After you have generated entry clones containing your DNA elements of choice, you will perform the MultiSite Gateway® Pro LR recombination reaction to simultaneously transfer 2, 3 or 4 DNA fragments into your 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 **Perform the MultiSite Gateway® Pro LR recombination reaction** (pages 51–57) before beginning.

Experimental outline

To generate an expression clone, you will:

1. Perform a MultiSite Gateway® Pro LR recombination reaction using the appropriate entry clones and an *att*R1, *att*R2-containing destination vector of choice.
2. Transform the reaction mixture into One Shot® Mach1™ T1^{R} *E. coli*.
3. Select for expression clones (see pages 52–54 for representative diagrams of the recombination regions).

Substrates for the MultiSite Gateway® Pro LR recombination reaction

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

Configuration	Entry clones containing...	DEST vector
MultiSite Gateway® Pro 2.0	*att*L1-Element 1-*att*R5 *att*L5-Element 2-*att*L2	Any containing *att*R1 and *att*R2 sites
MultiSite Gateway® Pro 3.0-	*att*L1-Element 1-*att*L4 *att*R4-Element 2-*att*R3 *att*L2-Element 3-*att*L2	
MultiSite Gateway® Pro 4.0	*att*L1-Element 1-*att*R5 *att*L5-Element 2-*att*L4 *att*R4-Element 3-*att*R3 *att*L3-Element 4-*att*L2	

You **cannot** successfully create an expression clone using the MultiSite Gateway® Pro LR recombination reaction if you have any combination of *att*-flanked entry clones other than the ones listed in the preceding table.

See the following pages for representative recombination regions of the expression clones.

Recombination region of the 2-fragment expression clone

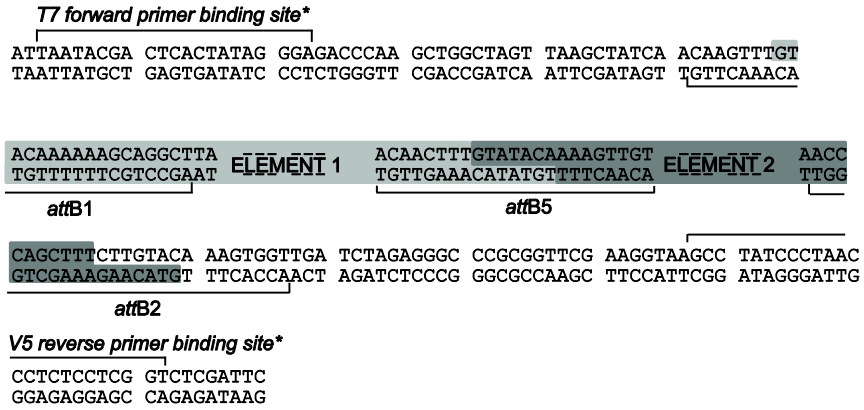
Recombination region of the 2-fragment expression clone

As an example, the recombination region of the expression clone resulting from **pcDNA6.2/V5-pL-DEST × *att*L1-Element 1-*att*R5 × *att*L5-Element 2-*att*L2** is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the two entry clones into the pcDNA6.2/V5-pL-DEST vector by recombination. Note that the sequences comprising the *att*B5 site are entirely supplied by the entry clones. Non-shaded regions are derived from the pcDNA6.2/V5-pL-DEST vector.

*Note that *T7 forward primer binding site* and *V5 reverse primer binding site* are features of the pcDNA6.2/V5-pL-DEST vector and are not conferred by the LR reaction. Your destination vector may have different primer binding sites.



Recombination region of the 3-fragment expression clone

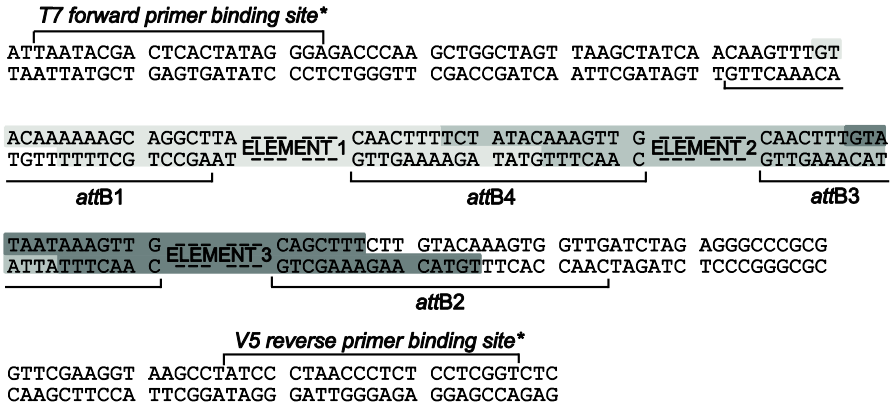
Recombination region of the 3-fragment expression clone

As an example, the recombination region of the expression clone resulting from **pcDNA6.2/V5-pL-DEST × *att*L1-Element 1-*att*L4 × *att*R4-Element 2-*att*R3 × *att*L3-Element 3-*att*L2** is shown in the following figure.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the three entry clones into the pcDNA6.2/V5-pL-DEST vector by recombination. Note that the sequences comprising the *att*B4 and *att*B3 sites are entirely supplied by the entry clones. Non-shaded regions are derived from the pcDNA6.2/V5-pL-DEST vector.

*Note that *T7 forward primer binding site* and *V5 reverse primer binding sites* are features of the pcDNA6.2/V5-pL-DEST vector and are not conferred by the LR reaction. Your destination vector may have different primer binding sites.



Recombination region of the 4-fragment expression clone

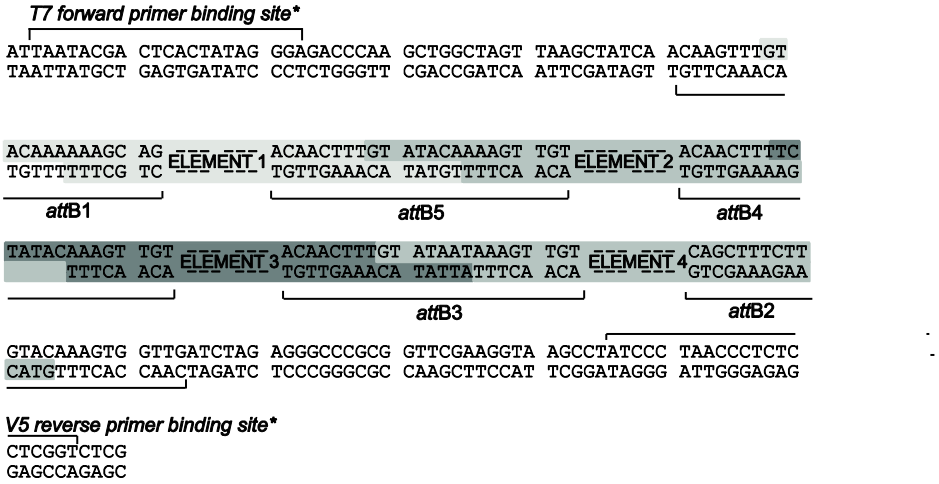
Recombination region of the 4-fragment expression clone

As an example, the recombination region of the expression clone resulting from **pcDNA6.2/V5-pL-DEST × *att*L1-Element 1-*att*R5 × *att*L5-Element 2-*att*L4 × *att*R4-Element 3-*att*R3 × *att*L3-Element 4-*att*L2** is shown in the following figure.

Features of the recombination region:

- Shaded regions correspond to those DNA sequences transferred from the four entry clones into the pcDNA6.2/V5-pL-DEST vector by recombination. Note that the sequences comprising the *att*B5, *att*B4 and *att*B3 sites are entirely supplied by the entry clones. Non-shaded regions are derived from the pcDNA6.2/V5-pL-DEST vector.

*Note that *T7 forward primer binding site* and *V5 reverse primer binding sites* are features of the pcDNA6.2/V5-pL-DEST vector and are not conferred by the LR reaction. Your destination vector may have different primer binding sites.



Perform the MultiSite Gateway® Pro LR recombination reaction

Introduction

Guidelines and instructions are provided in this section to perform a MultiSite Gateway® Pro LR recombination reaction between suitable supercoiled entry clones and a supercoiled destination vector using LR Clonase® II Plus Enzyme Mix. We recommend including and negative control (no LR Clonase® II Plus) and one or more positive control reactions (see page 58) in your experiment to help you evaluate your results.

You must use LR Clonase® II Plus Enzyme Mix to catalyze the MultiSite Gateway® Pro LR recombination reaction. LR Clonase® II Plus Enzyme Mix is supplied with the kit, but is also available separately (see page 79 for ordering information).

Note: LR Clonase® II Enzyme Mix used for standard Gateway® LR recombination reactions is not suitable for MultiSite Gateway® Pro LR recombination reactions.

***E. coli* host**

Use One Shot® Mach1™ T1^{R} Chemically Competent *E. coli* supplied with the kit for transformation.

Do not transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Destination vector

Depending on your downstream applications, you will need to provide an appropriate Gateway® destination vector for the LR recombination reaction. You may use any destination vector of choice that contains *att*R1 and *att*R2 sites.

You cannot use pDEST R4-R3 from the MultiSite Gateway® Three-Fragment Vector Construction Kit, because the *att*R3 and *att*R4 sites are incompatible with recombination with *att*L1- and *att*L2-containing entry clones.

Positive control

To perform the control LR reactions using the MultiSite Gateway® Pro control entry clones included with the kit, see page 58.

Prepare purified plasmid DNA

You will need to have purified midiprep plasmid DNA of each entry clone to perform the MultiSite Gateway® LR recombination reaction. We recommend using the PureLink® HiPure Plasmid MidiPrep Kit available separately (see page 79).

Continued on next page

Perform the MultiSite Gateway® Pro LR recombination reaction, continued

Materials needed

You will need the following materials before beginning.

Supplied with the kit:

- LR Clonase® II Plus Enzyme Mix (Box 3, keep at –20°C or –80°C until immediately before use)
- 2 µg/µL Proteinase K solution
- One Shot® Mach1™ T1^R chemically competent *E. coli*
- S.O.C. Medium

Supplied by the user:

- Midiprep-purified plasmid DNA of your entry clones (supercoiled, 10 fmoles)

  Important: You will need to add plasmid DNA from two, three, or four entry clones to the MultiSite Gateway® Pro 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 8 µL MultiSite Gateway® Pro LR reaction does not exceed 7 µL total.
- Purified plasmid DNA of your destination vector (supercoiled, 20 fmoles)
- 1X TE Buffer, pH 8.0
- Bacterial growth media for expression
- Selective LB agar plates containing 50–100 µg/mL antibiotic, depending on the resistance gene present in your destination vector

Continued on next page

Perform the MultiSite Gateway® Pro LR recombination reaction, continued

Set up the MultiSite Gateway® Pro LR reaction

To convert fmoles to ng, see page 46.

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

Component	Sample
Entry clones (10 fmoles each)	1–7 µL
Destination vector (20 fmoles)	1 µL
1X TE Buffer, pH 8.0	to 8 µL

2. Remove the LR Clonase® II Plus Enzyme Mix from– 20°C or –80°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase® II Plus Enzyme Mix briefly twice (2 seconds each time).
4. To each sample above, add 2 µL of LR Clonase® Plus Enzyme Mix. Mix well by vortexing briefly twice (2 seconds each time).
5. Return LR Clonase® II Plus Enzyme Mix to –20 °C or –80°C immediately after use. The Enzyme Mix can be stored at –20°C for up to 6 months or at –80°C for long-term storage.
6. Incubate reactions at 25°C for 16 hours.
7. Add 1 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.

Proceed to **Transform One Shot® Mach1™ T1^R competent cells** (page 48).

Next steps

If your recombination reaction was successful (*i.e.* provided the expected number of colonies) you may express your clone in the system appropriate for your destination vector. Depending on the length of the inserts in your clone and the presence of specific primer binding sites on your destination vector, you may sequence your expression clone.

If your recombination reaction was not satisfactory (*i.e.* resulted in fewer than expected or no colonies) you should perform the control reactions described in the following section to troubleshoot your MultiSite Gateway® Pro LR recombination reaction.

Perform control LR reactions

Introduction

Depending on the MultiSite Gateway® Pro kit configuration, 2, 3, 4 or 6 Control Entry clones are provided as a positive control for the LR recombination reaction and to troubleshoot the LR recombination reaction in 2-, 3- and 4-fragment reactions.

MultiSite Gateway® Pro control entry clones

The following control entry clones are provided with MultiSite Gateway® Pro kits. For plasmid maps and features, see pages 73–78.

Control Entry Clone	MultiSite Gateway® Pro 2.0	MultiSite Gateway® Pro 3.0	MultiSite Gateway® Pro 4.0	MultiSite Gateway® Pro Plus
pENTR L1-pLac-lacZalpha-R5	✓		✓	✓
pENTR L5-pLac-Spec-L2	✓			✓
pENTR L1-pLac-lacZalpha-L4		✓		✓
pENTR R4-pLac-Spec-R3		✓	✓	✓
pENTR L3-pLac-TetL2		✓	✓	✓
pENTR L5-LacI-L4			✓	✓

Experimental outline

If your LR recombination reaction does not yield the expected number of colonies, you should:

1. Perform the positive control reaction using all Control Entry Clones supplied with your kit to determine the activity of the LR Clonase® II Plus Enzyme Mix and to test if your DEST vector is intact.

   If the LR Clonase® II Plus positive control is successful, then:

2. Troubleshoot your LR recombination reaction by substituting one of the Control Entry Clones with your entry clone.

LR Clonase® II Plus positive control

To set up a positive control for LR Clonase® II Plus enzyme activity, you can substitute all Entry clones with the supplied Control Entry clones in the LR recombination reaction. You will analyze of the number of colonies expected and/or the phenotype of the resulting clones to determine the efficiency of the LR recombination reaction.

Note: In the unlikely event that the *att*R sites in the destination vector are incorrect, then the LR reaction will result in zero clones.

Troubleshooting the LR recombination reaction

To troubleshoot your LR recombination reaction, **you can substitute one of the supplied Control Entry Clones at a time with one of your entry clones.** You will analyze the number of colonies produced in the control reaction compared to the expected number in a given LR reaction type (see page 49). By performing vector substitution reactions, you can identify which entry clone may be flawed.

2-Fragment LR Clonase® II Plus positive control

To perform a positive control for LR Clonase® II Plus activity in a 2-fragment MultiSite Gateway® reaction, perform the following reaction using the following entry clones and the LR reaction conditions on page 57:

- pENTR L1-pLac-LacZα-R5
- pENTR L5-pLac-Spect-L2

You should see at least 2000 colonies on a selective antibiotic plate after transformation into Mach1™ T1^R *E. coli* if you transformed the entire 10 µL LR reaction.
The resulting expression clone should contain:

--*att*B1-pLac-LacZα-*att*B5-pLac-Spec-*att*B2—

Additionally, you can use the phenotypic reporter genes present in the Control Entry Clones to determine the cloning efficiency:

Selection	Expected*
Chloramphenicol	S
Kanamycin**	S
X-gal	Blue
Spectinomycin	R
Tetracycline	S

*S= sensitive; R= resistant
**Clones should be sensitive unless your DEST vector confers resistance to kanamycin

Troubleshooting the 2-fragment LR recombination reaction

To troubleshoot your LR recombination reaction, you can **substitute one of the supplied Control Entry Clones at a time with one of your entry clones:**

Reaction 1: Your entry clone #1 + pENTR L5-pLac-Spec-L2 + DEST vector

Reaction 2: pENTR L1-pLac-LacZα-R5 + Your entry clone #2 + DEST vector

You will analyze the number of colonies produced compared to the expected number in a given LR reaction type (see page 49).

By performing these 2 substitution reactions, you can identify which entry clone may be flawed. In the preceding example, if Reaction 1 resulted in <50 colonies and Reaction 2 resulted in >5000 colonies, then the problem is likely to be in your entry clone #1.

Continued on next page

Perform control LR reactions, continued

3-Fragment LR Clonase® II Plus Positive Control

To perform a positive control for LR Clonase® II Plus activity in a 3-fragment MultiSite Gateway® reaction, perform the following reaction using the following entry clones and the LR recombination reaction conditions on page 57:

- pENTR L1-pLac-LacZα-L4
- pENTR R4-pLac-Spec-R3
- pENTR L3-pLac-Tet-L2

You should see at least 1,000 colonies on a selective antibiotic plate after transformation into Mach1™ T1^R *E. coli* if you transformed the entire 10 µL LR reaction.
The resulting expression clone should contain:

--*att*B1-pLac-LacZα-*att*B4-pLac-Spec-*att*B3-pLac-Tet-*att*B2—

Additionally, you can use the phenotypic reporter genes present in the Control Entry Clones to determine the cloning efficiency:

Selection	Expected*
Chloramphenicol	S
Kanamycin**	S
X-gal	Blue
Spectinomycin	R
Tetracycline	R

*S= sensitive; R= resistant

**Clones should be sensitive unless your DEST vector confers resistance to kanamycin

Troubleshooting the 3-fragment LR recombination reaction

To troubleshoot your LR recombination reaction, you can **substitute one of the supplied Control Entry Clones at a time with one of your entry clones:**

Reaction 1: Your Entry Clone #1 + pENTR R4-pLac-Spec-R3 + pENTR L3-pLac-Tet-L2 + DEST vector

Reaction 2: pENTR L1-pLac-LacZα-L4 + Your Entry Clone #2 + pENTR L3-pLac-Tet-L2 + DEST vector

Reaction 3: pENTR L1-pLac-LacZα-L4 + pENTR R4-pLac-Spec-R3 + Your Entry Clone #3 + DEST vector

You will analyze the number of colonies produced compared to the expected number in a given LR reaction type (see page 49).

By performing these 3 substitution reactions, you can identify which entry clone may be flawed. In the preceding example, if Reaction 1 resulted in >2500 colonies, Reaction 2 resulted in >2300 colonies, and Reaction 3 resulted in <25 colonies, then the problem is likely to be in your entry clone #3.

4-Fragment LR Clonase® II Plus positive control

To perform a positive control for LR Clonase® II Plus activity in a 4-fragment MultiSite Gateway® reaction, perform the following reaction using the following entry clones and the LR recombination reaction conditions on page 57:

- pENTR L1-pLac-LacZα-R5
- pENTR L5-LacI-L4
- pENTR R4-pLac-Spec-R3
- pENTR L3-pLac-Tet-L2

You should see at least 50 colonies on a selective antibiotic plate after transformation into Mach1™ T1^R *E. coli* if you transformed the entire 10 µL LR reaction. The resulting expression clone should contain:

--*att*B1-pLac-LacZα-*att*B5-LacI-*att*B4-pLac-Spec-*att*B3-pLac-Tet-*att*B2—

Additionally, you can use the phenotypic reporter genes present in the Control Entry Clones to determine the cloning efficiency.

Selection	Expected*
Chloramphenicol	S
Kanamycin**	S
X-gal	White
X-gal + IPTG	Blue
Spectinomycin	S
Spectinomycin + IPTG	R
Tetracycline	S
Tetracycline +IPTG	R

*S= sensitive; R= resistant

**Clones should be sensitive unless your DEST vector confers resistance to kanamycin

Troubleshooting the 4-fragment LR recombination reaction

To troubleshoot your LR recombination reaction, you can **substitute one of the supplied Control Entry Clones at a time with one of your entry clones:**

Reaction 1: Your Entry Clone #1 + pENTR L5-LacI-L4 + pENTR R4-pLac-Spec-R3 + pENTR L3-pLac-Tet-L2 + DEST vector

Reaction 2: pENTR L1-pLac-LacZα-R5 + Your Entry Clone #2 + pENTR R4-pLac-Spec-R3 + pENTR L3-pLac-Tet-L2 + DEST vector

Reaction 3: pENTR L1-pLac-LacZα-R5 + pENTR L5-LacI-L4 + Your Entry Clone #3 + pENTR L3-pLac-Tet-L2 + DEST vector

Reaction 4: pENTR L1-pLac-LacZα-R5 + pENTR L5-LacI-L4 + pENTR R4-pLac-Spec-R3 + Your Entry Clone #4 + DEST vector

You will analyze the number of colonies produced compared to the expected number in a given reaction type (see page 49).

By performing these 4 substitution reactions, you can identify which entry clone may be flawed. In the example above, if Reaction 1 resulted in >70 colonies, Reaction 2 resulted in <10 colonies, and Reaction 3 resulted in >250 colonies, and Reaction 4 resulted in >90 colonies, then the problem is likely Entry Clone #2.

Troubleshooting

MultiSite Gateway® BP reactions

The following table lists some potential problems and possible solutions that may help you troubleshoot the BP reaction.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Used incorrect combination of *att*B or *att*Br flanked PCR products and pDONR™ vectors	Use the correct *att*B or *att*Br PCR product and donor vector for the BP reaction (see page 25 for details).
	BP Clonase® Plus Enzyme Mix is inactive or didn't use suggested amount	• Perform positive control as described on page 47 to verify activity of BP Clonase® Plus. • Store BP Clonase® Plus at –20°C for up to 6 months; or at –80°C for long-term storage. Do not freeze/thaw BP Clonase® Plus Enzyme Mix more than 10 times. • Use the recommended amount of BP Clonase® Plus Enzyme Mix (see page 47).
	Used incorrect Clonase® enzyme mix	Use the BP Clonase® II Enzyme Mix for the BP reaction. Do not use the LR Clonase® Plus II Enzyme Mix for the BP reaction.
	Too much PCR product was used in a BP reaction	Reduce the amount of *att*B or *att*Br PCR product in the reaction. Use an equimolar ratio of *att*B PCR product and donor vector.
	Long *att*B PCR product or linear *att*B expression clone (≥5 kb)	Incubate the BP reaction overnight at 25°C.
	Insufficient amount of *E. coli* transformed or plated	Transform 2 µL of the BP reaction; plate 20 µL and 100 µL.
	Incorrect antibiotic used to select for transformants	MultiSite Gateway® Pro pDONR™ plasmids are all kanamycin-resistant; use 50 µg/mL kanamycin to select for entry clones.

Continued on next page

MultiSite Gateway® BP reactions, continued

Problem	Reason	Solution
Two distinct types of colonies (large and small) appear	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 MAX Efficiency® Stbl2™ *E. coli* (see page 79) to help stabilize plasmids containing large genes during propagation (Trinh *et al.*, 1994).

*att*B PCR cloning

The following table 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.

Problem	Reason	Solution
Few or no colonies obtained from a BP reaction with PCR product **and** both *att*B positive control and transformation control gave expected number of colonies	*att*B or *att*Br PCR primers incorrectly designed	Make sure that each *att*B or *att*Br PCR primer includes four 5′ terminal Gs and the 22- or 25-bp *att*B or *att*Br site as specified on pages 26–33. Use Vector NTI Advance® software to help design primers for appropriate *att*B sites. See page 15 for details.
	*att*B or *att*Br PCR primers contaminated with incomplete sequences	Use HPLC or PAGE-purified oligonucleotides to generate your *att*B or *att*Br PCR product.
	*att*B or *att*Br PCR product not purified sufficiently	Gel purify your *att*B or *att*Br PCR product to remove primers and primer-dimers.
	For large PCR products (>5 kb), too few *att*B or *att*Br 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 at 25°C.
	Insufficient incubation time	Increase the incubation time of the BP reaction up to 18 hours.

***att*B PCR cloning, continued**

Problem	Reason	Solution
No PCR product is cloned	BP reaction may have cloned *att*B primer-dimers	• Purify *att*B PCR product using the PEG/$MgCl_2$ purification protocol on page 35 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.
Low yield of *att*B PCR product obtained after PEG purification	*att*B PCR product not diluted with TE	Dilute with 75 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 × *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.

MultiSite Gateway® Pro LR reactions

The following table lists some potential problems and possible solutions that may help you troubleshoot the MultiSite Gateway® Pro LR recombination reaction.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Used incorrect combination of entry clones for LR reaction	Use the correct entry clones for 2-, 3- or 4-fragment recombination (see page 51).
	Used incorrect Clonase® enzyme mix	Use the LR Clonase® Plus II Enzyme Mix for the LR reaction. Do not use the BP Clonase® II Enzyme Mix for the LR reaction.
	LR Clonase® II Plus enzyme inactive.	Perform LR Clonase® II Plus control reaction as described on page 58.
	Incorrect antibiotic used to select for transformants	Depending on the resistance gene present in your destination vector, use the correct antibiotic to select for expression clones.
	One or more entry clones is incorrect	Perform control reactions by substituting Control Entry Clones as described on pages 58–61.
	The *att*R sites in the destination vector are incorrect	Verify the sequence of the *att*R1 and *att*R2 sites in your destination vector and obtain a new vector if necessary.

MultiSite Gateway® Pro LR reactions, continued

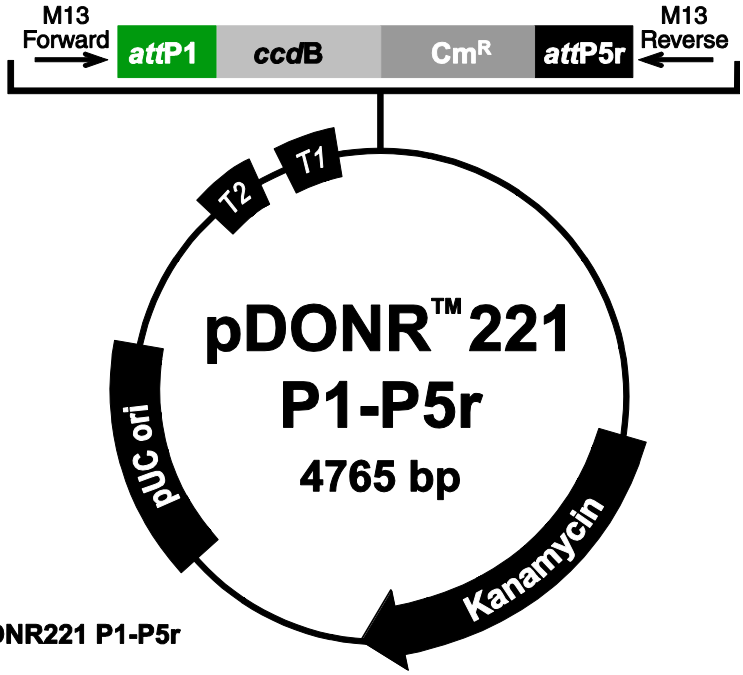
Problem	Reason	Solution
High background in the absence of the entry clone	MultiSite Gateway® Pro LR reaction transformed into an *E. coli* strain containing the F′ episome and the *ccd*A gene	Use Mach1™ T1^R Chemically Competent *E. coli* included with the kit or available separately (see page 79).
	Deletions (full or partial) of the *ccd*B gene from the destination vector	• To maintain the integrity of the destination vector, propagate in *ccd*B Survival™ 2 T1^R *E. coli* strain in media containing 50–100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol. • 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® Pro LR reaction. • Test for bacterial contamination by plating an aliquot of each solution directly onto LB plates containing ampicillin.
	Too much DNA was used in a MultiSite Gateway® Pro LR reaction	Use 10 fmoles of each entry clone and 20 fmoles of destination vector. Make sure the volume of all entry clones combined does not exceed 7 µL.
Few or no colonies obtained from the transformation control	Competent cells inactive	Make sure competent cells are stored at –80°C.
	Transformation performed incorrectly	Follow the protocol on page 49 to transform One Shot® Mach1™ T1^R cells
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.

Appendix

Map of pDONR™ P1-P5r

Map of pDONR™ P1-P5r

The following map shows the elements of pDONR™ P1-P5r. The vector sequence of pDONR™ P1-P5r is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pDONR221 P1-P5r
4765 nucleotides

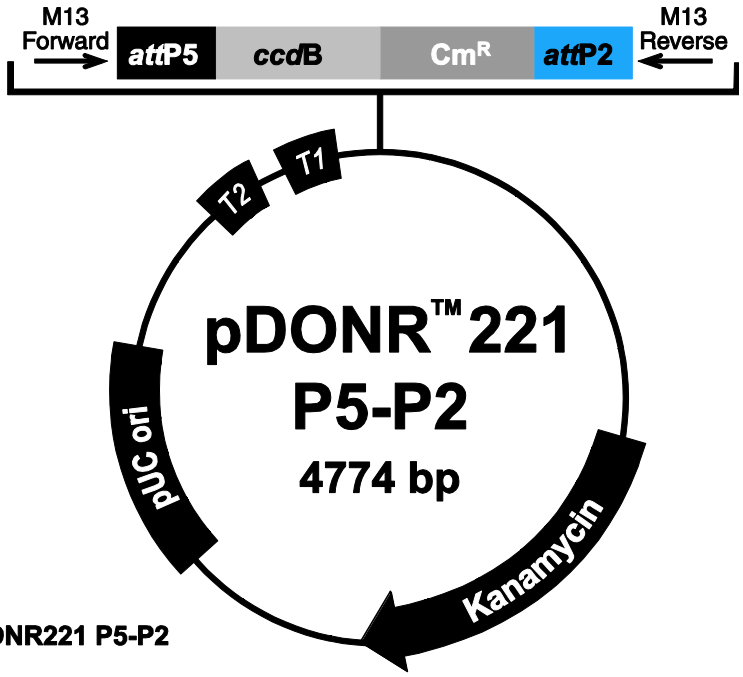
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P1 recombination site: bases 570-801
*ccd*B gene: bases 1200-1502 (c)
Chloramphenicol resistance gene: bases 1847-2506 (c)
*att*P5r recombination site: bases 2753-2984
M13 Reverse priming site: bases 3030-3046 (c)
Kanamycin resistance gene: bases 3159-3968
pUC origin: bases 4089-4762

(c) = complementary strand

Map of pDONR™ P5-P2

Map of pDONR™ P5-P2

The following map shows the elements of pDONR™ P5-P2. The vector sequence of pDONR™ P5-P2 is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pDONR221 P5-P2
4774 nucleotides

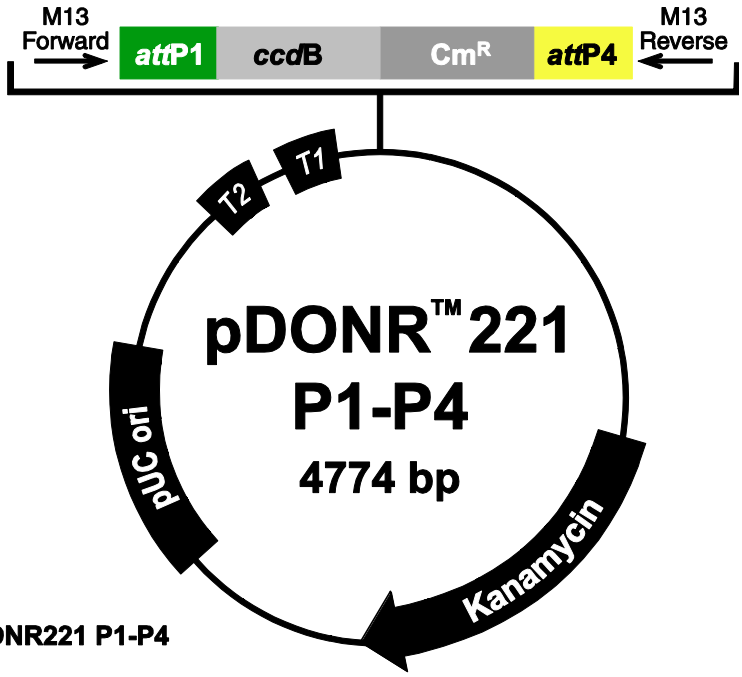
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P5 recombination site: bases 570-801
*ccd*B gene: bases 1197-1502 (c)
Chloramphenicol resistance gene: bases 1847-2506 (c)
*att*P2 recombination site: bases 2753-2984
M13 Reverse priming site: bases 3039-3055 (c)
Kanamycin resistance gene: bases 3168-3977
pUC origin: bases 4098-4771

(c) = complementary strand

Map of pDONR™ P1-P4

Map of pDONR™ P1-P4

The following map shows the elements of pDONR™ P1-P4. The vector sequence of pDONR™ P1-P4 is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pDONR221 P1-P4
4774 nucleotides

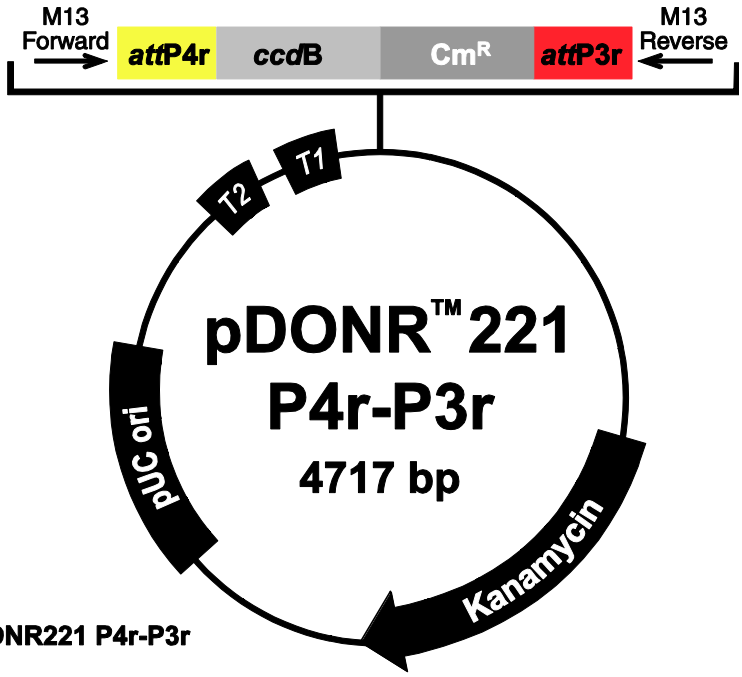
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P1 recombination site: bases 593-824 (c)
*ccd*B gene: bases 1197-1502 (c)
Chloramphenicol resistance gene: bases 1847-2506 (c)
*att*P4 recombination site: bases 2753-2984
M13 Reverse priming site: bases 3039-3055 (c)
Kanamycin resistance gene: bases 3168-3977
pUC origin: bases 4098-4771

(c) = complementary strand

Map of pDONR™ P4r-P3r

Map of pDONR™ P4r-P3r

The following map shows the elements of pDONR™ P4r-P3r. The vector sequence of pDONR™ P4r-P3r is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pDONR221 P4r-P3r
4717 nucleotides

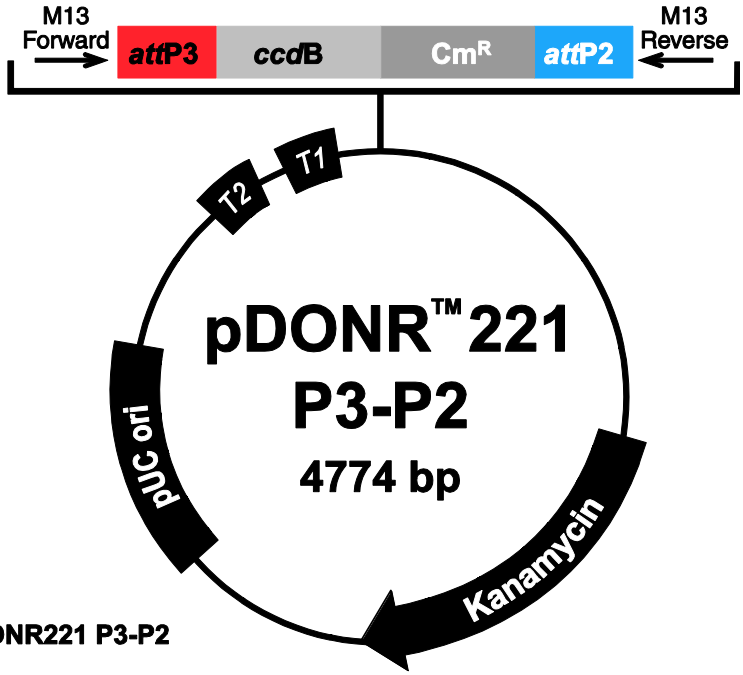
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P4r recombination site: bases 570-801
*ccd*B gene: bases 1152-1454 (c)
Chloramphenicol resistance gene: bases 1799-2458 (c)
*att*P3r recombination site: bases 2705-2936
M13 Reverse priming site: bases 2982-2998 (c)
Kanamycin resistance gene: bases 3111-3920
pUC origin: bases 4041-4714

(c) = complementary strand

Map of pDONR™ P3-P2

Map of pDONR™ P3-P2

The following map shows the elements of pDONR™ P3-P2. The vector sequence of pDONR™ P3-P2 is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pDONR221 P3-P2
4774 nucleotides

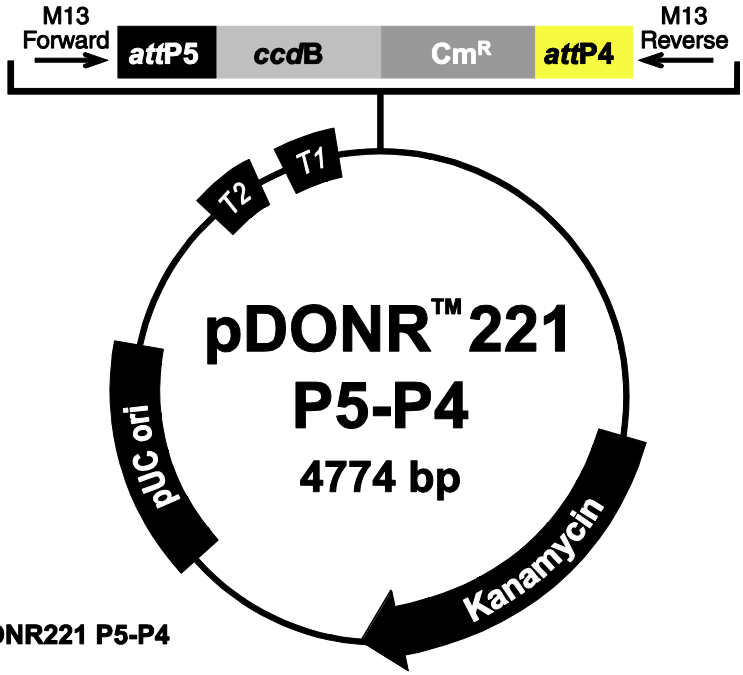
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*P3 recombination site: bases 570-801
*ccd*B gene: bases 1200-1502 (c)
Chloramphenicol resistance gene: bases 1847-2506 (c)
*att*P2 recombination site: bases 2752-2984
M13 Reverse priming site: bases 3039-3055 (c)
Kanamycin resistance gene: bases 3168-3977
pUC origin: bases 4098-4771

(c) = complementary strand

Map of pDONR™ P5-P4

Map of pDONR™ P5-P4

The following map shows the elements of pDONR™ P5-P4. The vector sequence of pDONR™ P5-P4 is available from **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pDONR221 P5-P4
4774 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*P5 recombination site: bases 570-801
*ccd*B gene: bases 1197-1502 (c)
Chloramphenicol resistance gene: bases 1847-2506 (c)
*att*P4 recombination site: bases 2753-2984
M13 Reverse priming site: bases 3039-3055 (c)
Kanamycin resistance gene: bases 3168-3977
pUC origin: bases 4098-4771

(c) = complementary strand

Features of pDONR™ vectors

Features of the MultiSite Gateway® Pro pDONR™ vectors

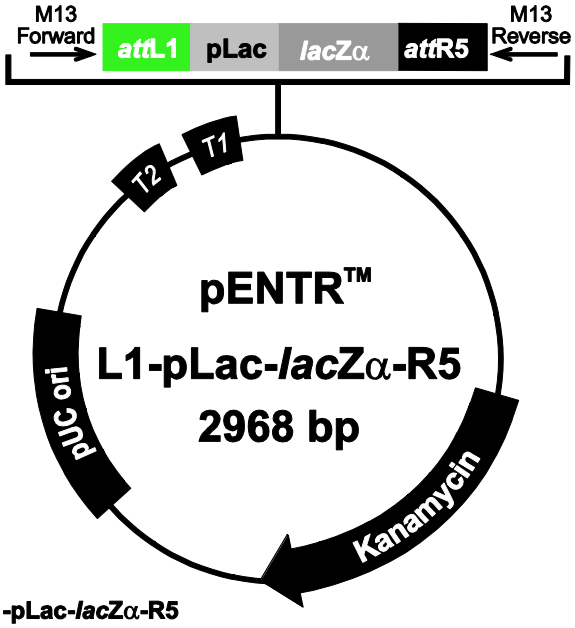
pDONR™ P1-P5r (4765 bp), pDONR™ P5-P2 (4774 bp), pDONR™ P1-P4 (4774 bp), pDONR™ P4r-P3r (4717 bp), pDONR™ P3-P2 (4774 bp), and pDONR™ P5-P4 (4774 bp) contain the following elements. Features have been functionally tested.

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*P1 and *att*P5r sites (pDONR™ P1-P5r) *att*P5 and *att*P2 sites (pDONR™ P5-P2) *att*P1 and *att*P4 sites (pDONR™ P1-P4) *att*P4r and *att*P3r sites (pDONR™ P4r-P3r) *att*P3 and *att*P2 sites (pDONR™ P3-P2) *att*P5 and *att*P4 sites (pDONR™ P5-P4)	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 counterscreening 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 pENTR™ L1-pLac-LacZalpha-R5

Map of pENTR™ L1-pLac-LacZalpha-R5

The following map shows the elements of pENTR L1-pLac-LacZalpha-R5. The vector sequence of pENTR L1-pLac-LacZalpha-R5 is available at **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pENTR L1-pLac-*lac*Zα-R5
2968 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*L1 recombination site: bases 569-668
pLac: bases 670-767
*lacZ*α: bases 768-983
*att*R5 recombination site: bases 1031-1154
M13 Reverse priming site: bases 1233-1249 (c)
Kanamycin resistance gene: bases 1362-2171
pUC origin: bases 2292-2965

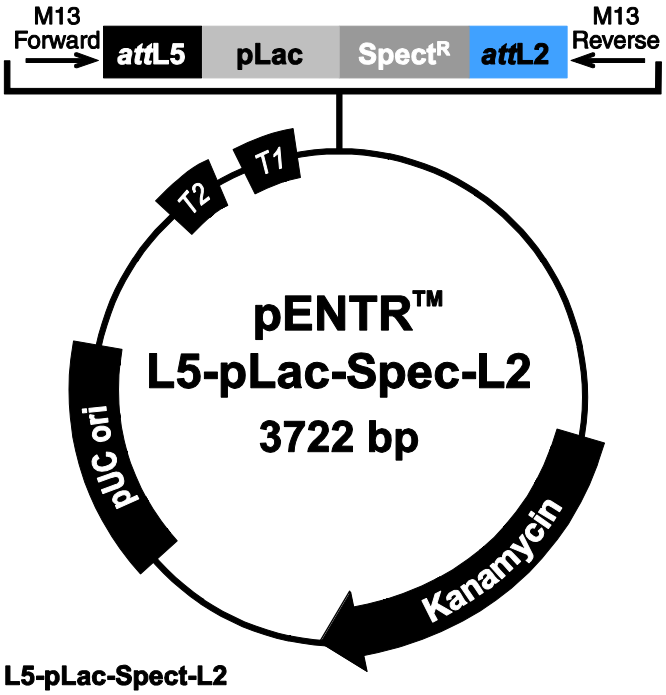
(c) = complementary strand

Continued on next page

Map and features of pENTR™ L5-pLac-Spec-L2

Map of pENTR™ L5-pLac-Spec-L2

The following map shows the elements of pENTR L5-pLac-Spec-L2. The vector sequence of pENTR L5-pLac-Spec-L2 is available at **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pENTR L5-pLac-Spect-L2
3722 nucleotides

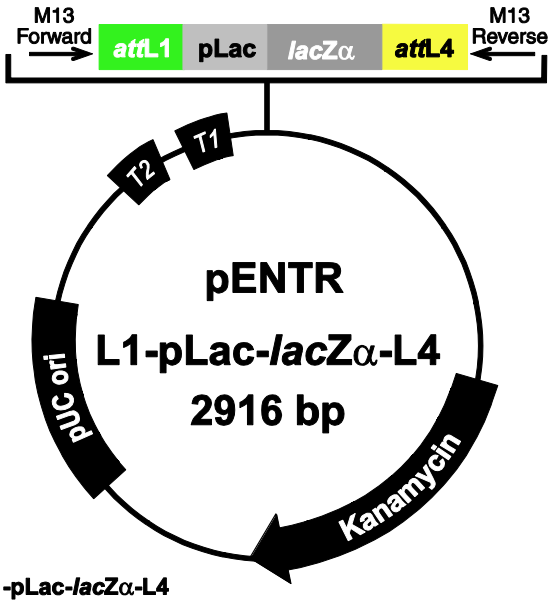
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*L5 recombination site: bases 537-552
pLac: bases 670-767
Spectinomycin resistance gene: bases 768-1781
*att*L2 recombination site: bases 1834-1933
M13 Reverse priming site: bases 1987-2003 (c)
Kanamycin resistance gene: bases 2129-2925
pUC origin: bases 3046-3719

(c) = complementary strand

Map and features of pENTR™ L1-pLac-LacZalpha-L4

Map of pENTR™ L1-pLac-LacZalpha-L4

The following map shows the elements of pENTR L1-pLac-LacZalpha-L4. The vector sequence of pENTR L1-pLac-LacZalpha-L4 is available at **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



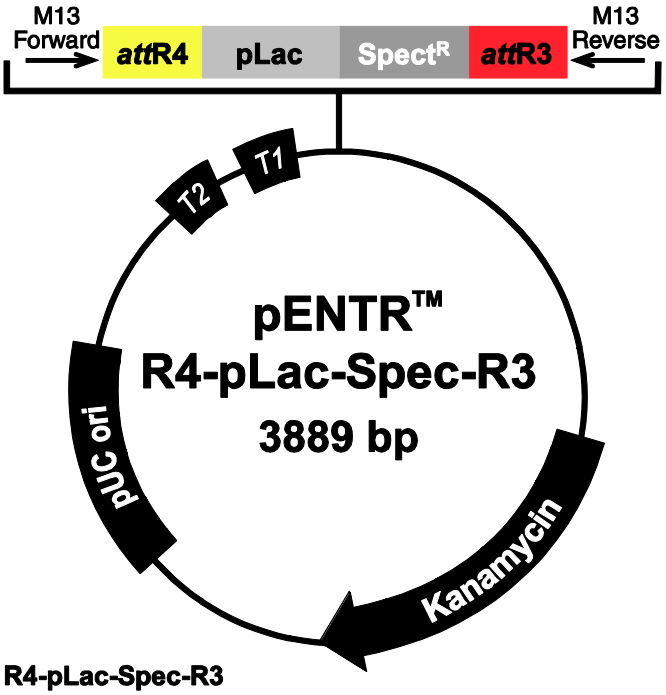
Comments for pENTR L1-pLac-*lac*Zα-L4
2916 nucleotides

*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*L1 recombination site: bases 570-665
pLac: bases 670-767
*lacZ*α: bases 768-983
*att*L4 recombination site: bases 1031-1126
M13 Reverse priming site: bases 1181-1197 (c)
Kanamycin resistance gene: bases 1310-2119
pUC origin: bases 2240-2913
(c) = complementary strand

Map and features of pENTR™ R4-pLac-Spec-R3

Map of pENTR R4-pLac-Spec-R3

The following map shows the elements of pENTR R4-pLac-Spec-R3. The vector sequence of pENTR R4-pLac-Spec-R3 is available at **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pENTR R4-pLac-Spec-R3
3889 nucleotides

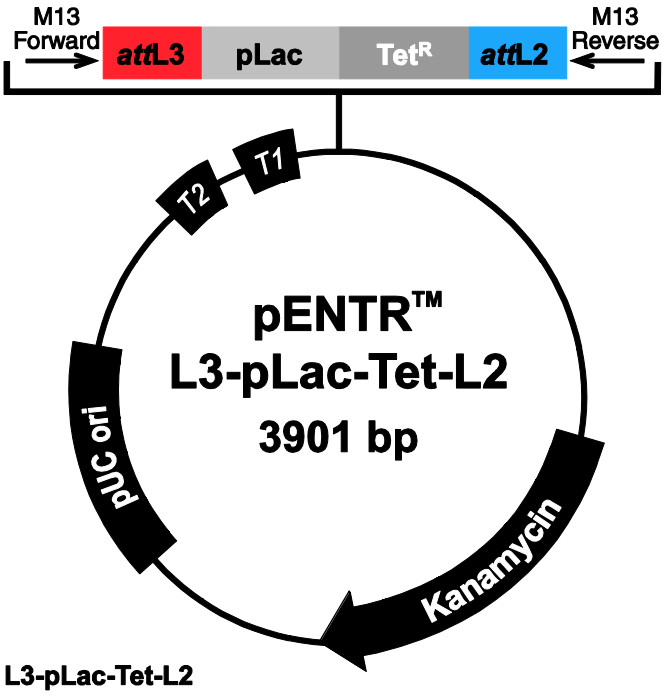
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*R4 recombination site: bases 603-727
pLac: bases 731-828
Spectinomycin resistance gene: bases 829-1839
*att*R3 recombination site: bases 1952-2075
M13 Reverse priming site: bases 2154-2170 (c)
Kanamycin resistance gene: bases 2283-3092
pUC origin: bases 3213-2886

(c) = complementary strand

Map and features of pENTR™ L3-pLac-Tet-L2

Map of pENTR L3-pLac-Tet-L2

The following map shows the elements of pENTR L3-pLac-Tet-L2. The vector sequence of pENTR L3-pLac-Tet-L2 is available at **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pENTR L3-pLac-Tet-L2
3901 nucleotides

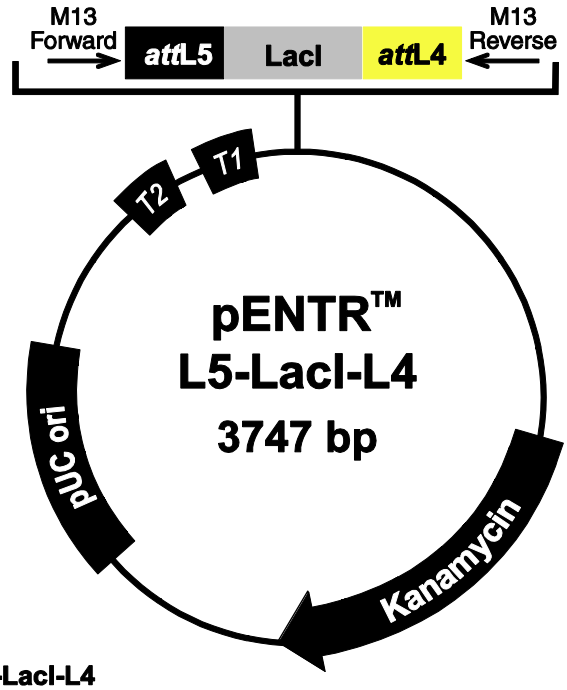
*rrn*B T2 transcription termination sequence: bases 268-295 (c)
*rrn*B T1 transcription termination sequence: bases 427-470 (c)
M13 Forward (-20) priming site: bases 537-552
*att*L3 recombination site: bases 570-665
pLac: bases 670-767
Tetracycline resistance gene: bases768-1958
*att*L2 recombination site: bases 2016-2111
M13 Reverse priming site: bases 2166-2182 (c)
Kanamycin resistance gene: bases 2295-3104
pUC origin: bases 3225-3898

(c) = complementary strand

Map and features of pENTR™ L5-LacI-L4

Map of pENTR™ L5-LacI-L4

The following map shows the elements of pENTR L5-LacI-L4. The vector sequence of pENTR L5-LacI-L4 is available at **www.lifetechnologies.com** or by contacting Technical Support (see page 81).



Comments for pENTR L5-LacI-L4
3747 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*L5 recombination site: bases 570-665
LacI: bases 765-1856
*att*L4 recombination site: bases 1862-1957
M13 Reverse priming site: bases 2012-2028 (c)
Kanamycin resistance gene: bases 2141-2950
pUC origin: bases 3071-3744
(c) = complementary strand

Accessory products

Introduction

Many of the reagents supplied in the MultiSite Gateway® Pro Kits as well as other products suitable for use with the kit are available separately. For more information, go to **www.lifetechnologies.com** or contact Technical Support (see page 81).

Item	Amount	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
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli*	11 × 50 µL	A10460
One Shot® Mach1™ T1^R Chemically Competent *E. coli*	21 × 50 µL	C8620-03
Pfx 50 DNA Polymerase	100 reactions	12355-012
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions	11304-011
M13 Forward (–20) Sequencing Primer	2 µg	N520-02
M13 Reverse Sequencing Primer	2 µg	N530-02
Dpn I	100 units	15242-019
PureLink® Gel Extraction Kit	50 reactions	K2100-12
PureLink® HiPure Plasmid Midiprep Kit	25 reactions	K2100-04
pcDNA™6.2/V5-pL-DEST	6 µg	12537-162
Ampicillin	200 mg	11593-027
Kanamycin Sulfate	100 mL (10 mg/mL)	15160-054
Gateway® Vector Conversion System	20 reactions	11828-029
MAX Efficiency® Stbl2™ *E. coli*	1 mL	10268-019

Gateway® clone distribution policy

Introduction

The information supplied in this section is intended to provide clarity concerning Life Technologies' policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Life Technologies' 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' 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 **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Technical support

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**

At the website, you can:

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

Safety Data Sheets (SDS)

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

Certificate of Analysis

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

Limited Product Warranty

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

References

Bernard, P., and Couturier, M. (1992) Cell Killing by the F Plasmid CcdB Protein Involves Poisoning of DNA-Topoisomerase II Complexes. J. Mol. Biol. *226*, 735-745

Bushman, W., Thompson, J. F., Vargas, L., and Landy, A. (1985) Control of Directionality in Lambda Site Specific Recombination. Science *230*, 906-911

Hartley, J. L., Temple, G. F., and Brasch, M. A. (2000) DNA Cloning Using *in vitro* Site-Specific Recombination. Genome Research *10*, 1788-1795

Kozak, M. (1987) An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148

Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305

Kozak, M. (1991) An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903

Landy, A. (1989) Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949

Orosz, A., Boros, I., and Venetianer, P. (1991) Analysis of the Complex Transcription Termination Region of the *Escherichia coli rrnB* Gene. Eur. J. Biochem. *201*, 653-659

Ptashne, M. (1992) *A Genetic Switch: Phage (Lambda) and Higher Organisms*, Cell Press, Cambridge, MA

Sasaki, Y., Sone, T., Yahata, K., Kishine, H., Hotta, J., Chesnut, J., Honda, T., and Imamoto, F. (2005) Multi-gene gateway clone design for expression of multiple heterologous genes in living cells: Eukaryotic clones containing two and three ORF multi-gene cassettes expressed from a single promoter. J. Biotechnol

Sasaki, Y., Sone, T., Yoshida, S., Yahata, K., Hotta, J., Chesnut, J. D., Honda, T., and Imamoto, F. (2004) Evidence for high specificity and efficiency of multiple recombination signals in mixed DNA cloning by the Multisite Gateway system. J. Biotechnol. *107*, 233-243

Shine, J., and Dalgarno, L. (1975) Terminal-Sequence Analysis of Bacterial Ribosomal RNA. Correlation Between the 3'-Terminal-Polypyrimidine Sequence of 16-S RNA and Translational Specificity of the Ribosome. Eur. J. Biochem. *57*, 221-230

Trinh, T., Jessee, J., and Bloom, F. R. (1994) STBL2: An *Escherichia Coli* Strain for the Stable Propagation of Retroviral Clones and Direct Repeat Sequences. FOCUS *16*, 78-80