USER GUIDE

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Gateway® Technology with Clonase® II

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

Catalog numbers 12535-029 and 12535-037

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For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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Contents

BP and LR Recombination Reaction Protocols for Experienced Users

Introduction

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

BP Recombination Reaction

Perform a BP recombination reaction between an *att*B-flanked DNA fragment and an *att*P-containing donor vector to generate an entry clone.

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

*att*B-PCR product or linearized *att*B expression clone (≥ 10 ng/µL; final amount ~15–150 ng)	1–7 µL
pDONR™ vector (supercoiled, 150 ng/µL)	1 µL
TE Buffer, pH 8.0	to 8 µL

2. Vortex Gateway® BP Clonase® II enzyme mix briefly. Add 2 µL to the components above and mix well by vortexing briefly twice.
3. Incubate the 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 competent *E. coli* and select for the appropriate antibiotic-resistant entry clones.

LR Recombination Reaction

Perform an LR recombination reaction between an *att*L-containing entry clone and an *att*R-containing destination vector to generate an expression clone.

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

Entry clone (supercoiled, 50–150 ng)	1–7 µL
Destination vector (supercoiled, 150 ng/µL)	1 µL
TE Buffer, pH 8.0	to 8 µL

2. Vortex Gateway® LR 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 competent *E. coli* and select for the appropriate antibiotic-resistant expression clones.

Kit Contents and Storage

Types of Products

This manual is supplied with the following products.

Product	Quantity	Catalog no.
PCR Cloning System with Gateway® Technology		
with pDONR™221 and One Shot® OmniMAX™ 2-T1^R Chemically Competent E. coli	20 reactions	12535-029
with pDONR™/Zeo and One Shot® OmniMAX™ 2-T1^R Chemically Competent E. coli	20 reactions	12535-037

Shipping/Storage

The PCR Cloning System with Gateway® Technology is shipped on dry ice as described in the following table. Upon receipt, store each box as detailed in the following table.

Box	Item	Storage
1	Donor Vector (pDONR™221 or pDONR™/Zeo)	Vector: **–30°C to –10°C** Zeocin™ Selection Reagent (supplied with pDONR™/Zeo): **–30°C to –10°C, protected from light**
2	Gateway® BP Clonase® II Enzyme Mix and Reagents	Gateway® BP Clonase® II Enzyme Mix: **–30°C to –10°C** ***or*** **–85°C to –68°C** All other reagents: **–30°C to –10°C**
3–4	M13 Sequencing Primers	**–30°C to –10°C**
5	One Shot® OmniMAX™ 2-T1^R Chemically Competent *E. coli*	**–85°C to –68°C**

Product Use

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

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Kit Contents and Storage, Continued

Contents

The Donor Vector box, the Gateway® BP Clonase® II Enzyme Mix and Reagents box, and the M13 Sequencing Primers box (Boxes 1–4) contain the following items. Store the Gateway® BP Clonase® II enzyme mix at –30°C to –10°C for up to 6 months. For long-term storage, store at –85°C to –68°C. Store all other components at –30°C to –10°C. Store Zeocin™ Selection Reagent at –30°C to –10°C, protected from light.

Item	Composition	Amount
pDONR™ Vector (pDONR™221 or pDONR™/Zeo)	150 ng/µL in TE buffer, pH 8.0	40 µL
Zeocin™ Selection Reagent (supplied with pDONR™/Zeo)	100 mg/mL in deionized, sterile water	1.25 mL
Gateway® BP Clonase® II Enzyme Mix	Proprietary	40 µL
Proteinase K solution	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µL
30% PEG/Mg solution	30% PEG 8000/30 mM $MgCl_2$	1 mL
pEXP7-tet positive control	50 ng/µL in TE Buffer, pH 8.0	20 µL
M13 Forward (–20) Primer	Lyophilized in TE Buffer, pH 8.0	2 µg
M13 Reverse Primer	Lyophilized in TE Buffer, pH 8.0	2 µg

Sequence of Primers

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

Primer	Sequence	pMoles Supplied
M13 Forward (–20)	5´-GTAAAACGACGGCCAG-3´	407
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385

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One Shot® OmniMAX™ 2-T1^R Reagents

The One Shot® OmniMAX™ 2-T1^R Chemically Competent *E. coli* box (Box 5) includes the following items. The transformation efficiency is ≥ 5×10^9 cfu/µg DNA. Store Box 5 at −85°C to −68°C.

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature at 15°C to 30°C, or in a cold room at 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
OmniMAX™ 2-T1^R 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 OmniMAX™ 2-T1^R

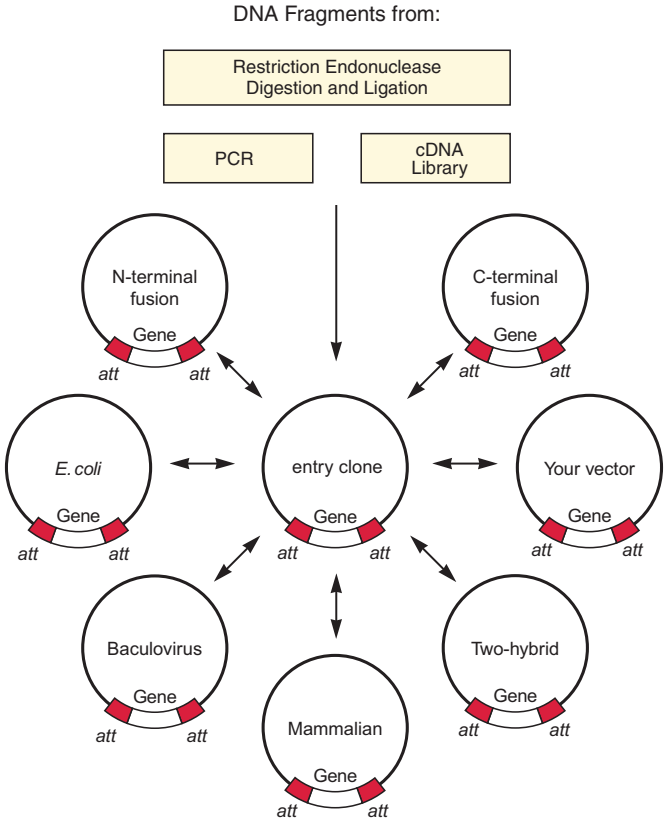
F′ [*pro*AB$^+$ *lac*I^q *lac*ZΔM15 *Tn*10(Tet^R) Δ(*ccd*AB)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80(*lac*Z)ΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D

Introduction

Overview

Introduction

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



Advantages of the Gateway® Technology

Using the Gateway® Technology provides the following advantages:

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

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Purpose of This Manual

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

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

For details about a particular Life Technologies destination vector or expression system, refer to the manual for the specific destination vector or system. All Gateway® product manuals are available from **www.lifetechnologies.com/mauals** or by contacting Technical Support (see page 57).

Glossary of Terms

To help you understand the terminology used in the Gateway® Technology, a glossary of terms is provided in the **Appendix**, page 61.

The Gateway® Technology

The Basis of Gateway®

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

Recombination Components

Lambda-based recombination involves two major components:

- The DNA recombination sequences (*att* sites)

  and

- The proteins that mediate the recombination reaction (i.e. Clonase® II enzyme mix)

Characteristics of the Recombination Reactions

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

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

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

att Sites

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

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Recombination Proteins

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

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

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

Gateway® Recombination Reactions

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

- **BP Reaction:** Facilitates recombination of an *att*B substrate (*att*B-PCR product or a linearized *att*B expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing entry clone (see the following diagram). This reaction is catalyzed by the Gateway® BP Clonase® II enzyme mix.

- **LR Reaction:** Facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone (see the following diagram). This reaction is catalyzed by the Gateway® LR Clonase® II enzyme mix.

Gateway® BP and LR Recombination Reactions

Introduction

The wild-type λ *att* recombination sites have been modified to improve the efficiency and specificity of the Gateway® BP and LR recombination reactions. This section describes the modifications and provides examples of the Gateway® recombination reactions between the *att*B x *att*P and *att*L x *att*R sites.

Modifications to the *att* Sites

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

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

Important

In addition to the modifications described in the preceding section, site-specific point mutations have been made to some *att* sites to increase recombination efficiency. As a result, sequence variations may exist among the *att* sites. These sequence variations do not affect the specificity of the recombination reactions or the functionality of the vectors.

Characteristics of the Modified *att* Sites

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

Site	Length	Found in…
*att*B	25 bp	Expression vector
		Expression clone
*att*P	200 bp	Donor vector
*att*L	100 bp	Entry vector
		Entry clone
*att*R	125 bp	Destination vector

Specificity:

- *att*B1 sites react only with *att*P1 sites
- *att*B2 sites react only with *att*P2 sites
- *att*L1 sites react only with *att*R1 sites
- *att*L2 sites react only with *att*R2 sites

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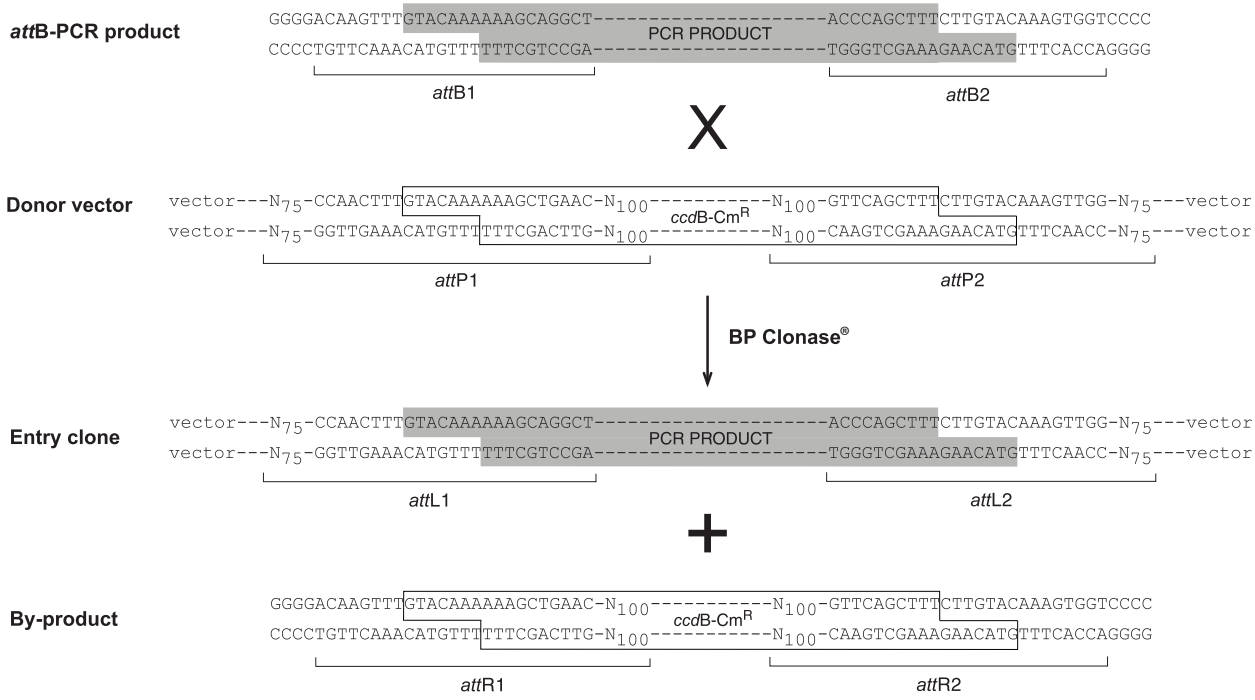
Example of an *att*B x *att*P Recombination Reaction

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

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

Features of the Recombination Region:

- Shaded regions correspond to those sequences transferred from the *att*B-PCR product into the entry clone following recombination. Note that the *att*L sites are composed of sequences from *att*B and *att*P.
- Boxed regions correspond to those sequences transferred from pDONR™221 or pDONR™/Zeo into the by-product following recombination.



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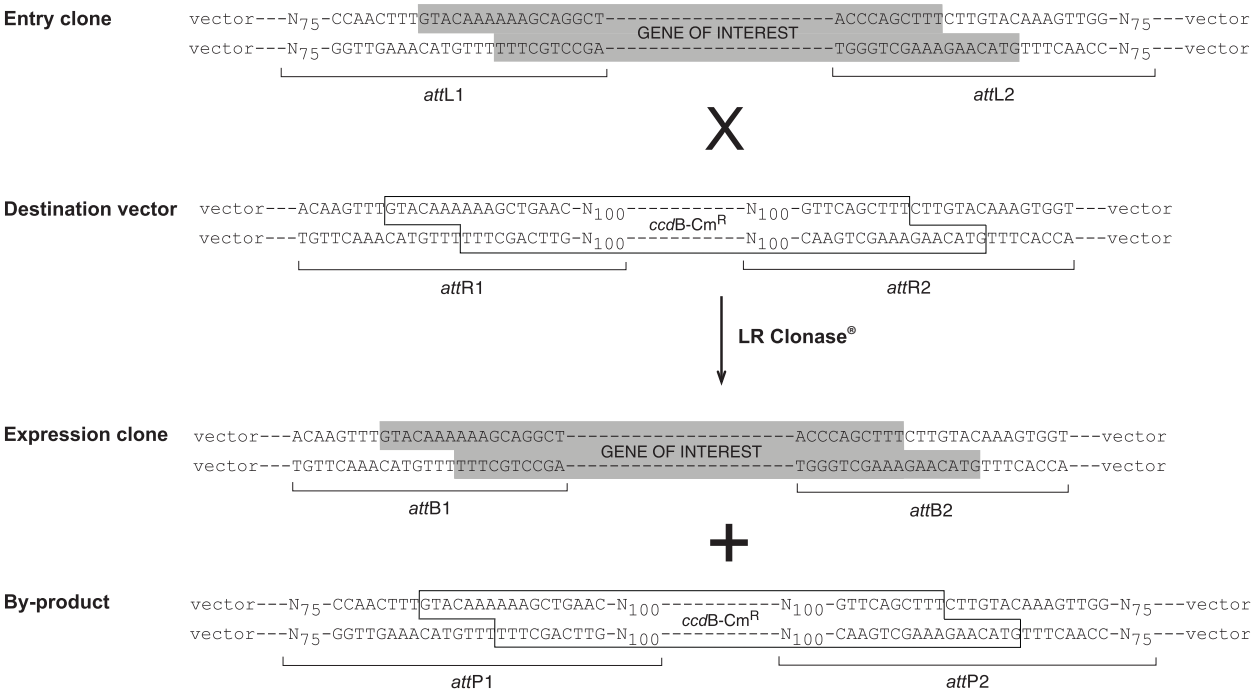
Example of an *att*L x *att*R Recombination Reaction

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

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

Features of the Recombination Region:

- Shaded regions correspond to those sequences transferred from the pENTR™/D-TOPO® entry clone into the expression clone following recombination. Note that the *att*B sites are composed of sequences from *att*L and *att*R sites.
- Boxed regions correspond to those sequences transferred from pcDNA™6.2/V5-DEST into the by-product following recombination.



Features of the Gateway® Vectors

Gateway® Vectors

Four different types of Gateway®-adapted vectors are available:

Gateway® Vector	Characteristics
Donor vector (pDONR™)	• Contains *att*P sites • Used to clone *att*B-flanked PCR products and genes of interest to generate entry clones
Entry vector (pENTR™)	• Contains *att*L sites • Used to clone PCR products or restriction fragments that do not contain *att* sites to generate entry clones
Destination vector	• Contains *att*R sites • Recombines with the entry clone in an LR reaction to generate an expression clone • Contains elements necessary to express the gene of interest in the appropriate system (*i.e. E. coli*, mammalian, yeast, insect)
Expression vector	• Contains a multiple cloning site (MCS) or pre-made cDNA libraries flanked by attB sites • Designed for directional cloning of cDNA or genomic DNA to construct libraries, or contain pre-made cDNA libraries • Recombines with donor vectors containing attP sites in a BP reaction to generate entry clones

Common Features of the Gateway® Vectors

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

- The *ccd*B gene (see the following section) for negative selection (present in donor, destination, and supercoiled entry vectors)
- Chloramphenicol resistance gene (Cm^R) for counterselection (present in donor and destination vectors)

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

*ccd*B Gene

The presence of the *ccd*B gene allows negative selection of the donor and destination (and some entry) vectors in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase Bernard & Couturier, 1992, thereby inhibiting growth of most *E. coli* strains (e.g. OmniMAX™ 2-T1^R, DH5α™, TOP10). 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 *ccd*B gene is replaced by the gene of interest. 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.

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Propagating Gateway® Vectors

If you intend to propagate and maintain the pccdB Vector, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (see page 55 for ordering information). 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.

Gateway® Nomenclature

Suggested Naming Convention

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

Plasmid Type	Description	Individual Vector or Clone Names
*att*L Vector	Entry Vector	pENTR™1, 2,…
*att*L Subclone	Entry Clone	pENTR™3-gus,..; pENTR™221-gus • The number 3 refers to the entry vector • 221 refers to the donor vector used to make the entry clone • gus is the subcloned gene
*att*R Vector	Destination Vector	pDEST1, 2, 3..; p…-DEST
*att*B Vector	Expression Vector	pEXP501, 502,… • This vector is used to prepare expression cDNA libraries
*att*B Subclone	Expression Clone	pEXP14-cat,…; pcDNA/GW-47/cat • 14 and 47 refers to the destination vector (i.e. pDEST™14 and pcDNA-DEST47™, respectively) used to make the expression clone • cat is the subcloned gene
*att*P Vector	Donor Vector	pDONR221,...

Example: LR Reaction

pENTR™221-cat × pcDNA-DEST47 → pcDNA/GW-47/cat

Examples: BP Reaction

*att*B-p53 PCR product × pDONR221 → pENTR™221-p53

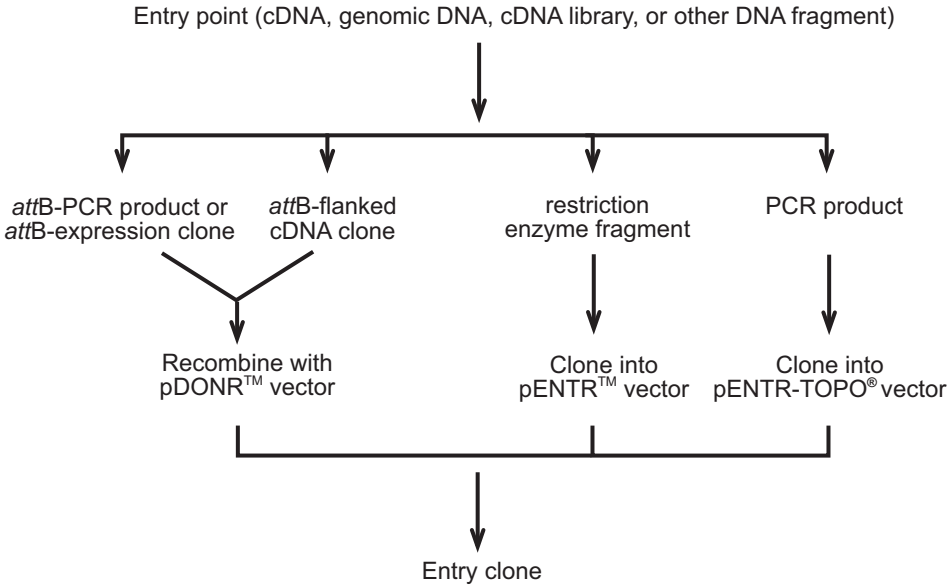
Methods

Options to Create Entry Clones

Introduction

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

1. Clone a PCR product or a restriction enzyme fragment into an entry (pENTR™) vector (see page 12 for more information). For an alternative, see below.
2. Generate a PCR product containing *att*B sites and use this *att*B-PCR product in a BP recombination reaction with a donor (pDONR™) vector. To use this method, refer to the guidelines and instructions provided in this manual.
3. Generate or obtain a cDNA library cloned into a Gateway®-compatible vector (*i.e. att*B-containing pCMV SPORT6 or pEXP-AD502 vectors), and use the cDNA clones in a BP recombination reaction with a donor vector (see the **Appendix**, page 50 for more information).



Note

If you intend to express a particular human or murine gene, we recommend using an Ultimate™ ORF Human or Mouse Clone. Each Ultimate™ hORF or mORF Clone is a fully sequenced clone provided in a Gateway® entry vector that is ready-to-use in an LR recombination reaction with a Gateway® destination vector. For more information about the Ultimate™ ORF Clones available, refer to **www.lifetechnologies.com** or contact Technical Support (see page 57).

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Options to Create Entry Clones, Continued

Entry Vectors

Many entry vectors are available to facilitate generation of entry clones. The pCR™8/GW/TOPO® and pENTR™ D-TOPO® vectors allow rapid TOPO® TA Cloning and directional TOPO® Cloning of *Taq*-amplified and blunt-end PCR products, respectively while the pENTR™ vectors allow ligase-mediated cloning of restriction enzyme fragments. All entry vectors include:

- *att*L1 and *att*L2 sites to allow recombinational cloning of the gene of interest with a destination vector to produce an expression clone.
- Antibiotic resistance gene for selection of plasmid in *E. coli*.
- pUC origin for high-copy replication and maintenance of the plasmid in *E coli*.

Some entry vectors include:

- A Kozak consensus sequence for efficient translation initiation in eukaryotic cells.
- A Shine-Dalgarno sequence Shine & Dalgarno, 1975 for initiation in *E. coli.*
- Primer binding sites within the *att*L sites to facilitate sequencing.

For more information about the features of each pENTR™ vector, refer to **www.lifetechnologies.com** or contact Technical Support (see page 57).

Entry Vector	Kozak	Shine-Dalgarno	Sequencing within *att*L Sites	Catalog no.
pCR™8/GW/TOPO®			•	K2500-20, K2520-20
pENTR™/D-TOPO®	•			K2400-20, K2435-20
pENTR™/SD/D-TOPO®	•	•		K2420-20, K2635-20
pENTR™/TEV/D-TOPO®	•			K2525-20, K2535-20
pENTR™1A	•	•		11813-011
pENTR™2B	•			11816-014
pENTR™3C	•	•		11817-012
pENTR™4	•			11818-010
pENTR™11	•	•		11819-018

Constructing Entry Clones

To construct an entry clone, refer to the manual for the specific entry vector you are using. All entry vector manuals are available from **www.lifetechnologies.com/manuals** or by contacting Technical Support (see page 57).

Designing *att*B PCR Primers

Introduction

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

Designing Your PCR Primers

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

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

Guidelines to Design the Forward PCR Primer

When designing your forward PCR primer, consider the points below. Refer to the following diagram and **Examples 1 and 2**, page 14, for more help.

- To enable efficient Gateway® cloning, it is **required** that the forward primer contain the following structure:
  1. Four guanine (G) residues at the 5′ end, followed by:
  2. The 25-bp *att*B1 site, followed by:
  3. At least 18–25 bp of template- or gene-specific sequences.

  Note: If you plan to express native protein in *E. coli* or mammalian cells, you may want to include a Shine-Dalgarno Shine & Dalgarno, 1975 or Kozak consensus sequence Kozak, 1987; Kozak, 1990; Kozak, 1991, respectively, in your PCR primer (see **Example 1**, next page).
- The *att*B1 site ends with a thymidine (T). If you wish to fuse your PCR product in frame with an N-terminal tag, the primer must include two additional nucleotides to maintain the proper reading frame with the *att*B1 region (see the following diagram and **Example 2**, page 14). These two nucleotides **cannot** be AA, AG, or GA, because these additions will create a translation termination codon.

*att*B1 Forward Primer:

```
5′-GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TNN--(template-specific sequence)-3′
                         attB1
```

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Designing *att*B PCR Primers, Continued

Example 1: Forward Primer Design for Native Expression

In the following example, we design the following forward *att*B PCR primer to allow expression of native protein of interest. The *att*B1 site is indicated in bold and the ATG initiation codon for the protein of interest is underlined. Inclusion of the Shine-Dalgarno and Kozak consensus sequence allows protein expression in both *E. coli* and mammalian cells.

Note: The ATG initiation codon in this example is in frame with the *att*B1 sequence, so the PCR product can also be expressed from an N-terminal fusion destination vector.

Shine-Dalgarno Kozak

5′-GGGG**ACAAGTTTGTACAAAAAAGCAGGCT**TCGAAGGAGATAGAACCATGG(18-25 gene-specific nucleotides)-3′

Example 2: Forward Primer Design for N-terminal Fusions

In the following example, we design the following forward *att*B PCR primer to allow expression of an N-terminal fusion protein of interest. The *att*B1 site is indicated in bold. Remember that the gene-specific nucleotides need to be in frame with the *att*B1 sequence and that no stop codons should be introduced.

Tip: Keep the -AAA-AAA- triplets in the *att*R1 site in frame with the translation reading frame of the fusion protein.

Lys Lys

5′-GGGG **ACA AGT TTG TAC AAA AAA GCA GGC T**TC(18-25 gene-specific nucleotides)-3′

Guidelines to Design the Reverse PCR Primer

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

- To enable efficient Gateway® cloning, it is **required** that the reverse primer contain the following structure:
  1. Four guanine (G) residues at the 5′ end, followed by:
  2. The 25-bp *att*B2 site, followed by:
  3. 18–25 bp of template- or gene-specific sequences.
- If you intend to fuse your PCR product in frame with a C-terminal tag:
  - The primer must include one additional nucleotide to maintain the proper reading frame with the *att*B2 region (see the following diagram and **Example 2**, page 15)
  - Any in-frame stop codons between the *att*B2 site and your gene of interest must be removed
- If you do not intend to fuse your PCR product in frame with a C-terminal tag, your gene of interest or the primer must include a stop codon (see **Example 1**, page 15)

*att*B2 Reverse Primer:

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

*att*B2

Continued on next page

Designing *att*B PCR Primers, Continued

Example 1: Reverse Primer Design

In the following example, we design the following reverse *att*B PCR primer to allow expression of a protein of interest with no C-terminal fusion tag. The *att*B2 site is indicated in bold and the stop codon for the protein of interest is underlined. Remember that the gene-specific nucleotides need to be in frame with the stop codon.

5′-GGGG**ACCACTTTGTACAAGAAAGCTGGGT**C<u>CTA</u>(18-25 gene-specific nucleotides)-3′

Example 2: Reverse Primer Design for C-terminal Fusions

In the following example, we design the following reverse *att*B PCR primer to allow expression of a C-terminal fusion protein of interest. The *att*B2 site is indicated in bold. Remember that the gene-specific nucleotides need to be in-frame with the *att*B2 sequence and that stop codons should be removed.

Tip: Keep the -TTT-GTA (TAC-AAA on the complementary strand) triplets in the *att*R2 site in frame with the translation reading frame of the fusion protein.

```
              Lys Tyr
5′-GGG G AC CAC TTT GTA CAA GAA AGC TGG GTC(18-25 gene-specific nucleotides)-3′
```

Note

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

Important

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

The Next Step

Proceed to the next section for guidelines to produce your *att*B-PCR products.

If you are performing high throughput applications or are using long PCR primers (greater than 70 nucleotides) to generate your PCR products, we recommend using the *att*B adapter protocol provided in the **Appendix**, pages 47–48.

Producing *att*B-PCR Products

DNA Templates

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

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

Recommended Polymerases

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

- To generate PCR products less than 5–6 kb for use in protein expression, use Platinum® *Pfx* DNA Polymerase (see page 55 for ordering information)
- To generate PCR products for use in other applications (e.g. functional analysis), use Platinum® *Taq* DNA Polymerase High Fidelity (see page 55 for ordering information)

Producing PCR Products

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

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

Checking the PCR Product

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

Note

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

Materials Needed:

- *Dpn* I (see page 55 for ordering information)
- 10X Buffer T (supplied with *Dpn* I)

Protocol:

1. To your 50 µL PCR reaction mixture, add 5 µL of Buffer T and ≥5 units of *Dpn* I.
2. Incubate at 37°C for 15 minutes.
3. Heat-inactivate the *Dpn* I at 65°C for 15 minutes.
4. Proceed to **Purifying *att*B-PCR Products**, page 17.

Purifying *att*B-PCR Products

Introduction

After generating your *att*B-PCR product, we recommend purifying the PCR product to remove *att*B primers and any *att*B primer-dimers. Primers and primer-dimers can recombine efficiently with the donor vector in the BP reaction and may increase background after transformation into *E. coli*. A protocol is provided below to purify your PCR product.

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.

Required Materials

Components required but not supplied:

- *att*B-PCR product (in a 50 µL volume)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Agarose gel of the appropriate percentage to resolve your *att*B-PCR product

Components supplied with the kit:

- 30% PEG 8000/30 mM $MgCl_2$ Solution

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 150 µL of TE, pH 8.0 to a 50 µL amplification reaction containing your *att*B-PCR product.
2. Add 100 µL of 30% PEG 8000/30 mM $MgCl_2$. Vortex to mix thoroughly and centrifuge immediately at 10,000 × 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 50 µL of TE, pH 8.0 (to concentration > 10 ng/µL).
5. Check the quality and quantity of the recovered *att*B-PCR product on an agarose gel.
6. If the PCR product is suitably purified, proceed to **Creating Entry Clones Using the BP Recombination Reaction**, page 18. If the PCR product is not suitably purified (e.g. *att*B primer-dimers are still detectable), see the next section.

Additional Purification

If you use the preceding procedure and your *att*B-PCR product is not suitably purified, you may gel purify your *att*B-PCR product. We recommend using the PureLink® Quick Gel Extraction Kit available from Life Technologies (see page 55 for ordering information).

Creating Entry Clones Using the BP Recombination Reaction

Introduction

The BP recombination reaction facilitates transfer of a gene of interest in an *att*B expression clone or *att*B-PCR product to an *att*P-containing donor vector to create an entry clone. Once you have created an entry clone, your gene of interest may then be easily shuttled into a large selection of destination vectors using the LR recombination reaction. To ensure that you obtain the best possible results, we suggest that you read this section and the ones entitled **Performing the BP Recombination Reaction** (pages 21–23) and **Transforming Competent Cells** (pages 24–26) before beginning.

Note: If you intend to go directly from an *att*B-PCR product or *att*B expression clone into a destination vector, see the **Appendix**, page 46 for a 1-tube protocol.

Experimental Outline

To generate an entry clone, you will:

1. Perform a BP recombination reaction using the appropriate *att*B and *att*P-containing substrates (see the next section)
2. Transform the reaction mixture into a suitable *E. coli* host (see page 24)
3. Select for entry clones

Substrates for the BP Recombination Reaction

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

- *att*B-flanked PCR products **or** *att*B-containing expression clones
- *att*P-containing donor (pDONR™) vector (see the next sections)

Important

For optimal efficiency, perform the BP recombination reaction using:

- **Linear** *att*B substrates (see the next page for guidelines to linearize *att*B expression clones)
- **Supercoiled** *att*P-containing donor vector

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

Donor Vectors

The PCR Cloning System with Gateway® Technology includes a choice of donor (pDONR™221 or pDONR™/Zeo) vectors. Other donor vectors are available (see page 55 for ordering information). For a map and a description of the features of pDONR™221 and pDONR™/Zeo, see the **Appendix**, pages 53–54.

Continued on next page

Propagating Donor Vectors

If you intend to propagate and maintain the pDONR™ vectors, we recommend using One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli* (see page 55 for ordering information) for transformation. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene.

To maintain the integrity of the vector, select for transformants in media containing the appropriate antibiotic and 15 µg/mL chloramphenicol. If you are using pDONR™/Zeo, you will need to select transformants in Low Salt LB medium containing Zeocin™ Selection Reagent and 15 µg/mL chloramphenicol (see page 24 for more information).

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

Linearizing Expression Clones

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

1. Linearize 1–2 µg of the expression clone with a unique restriction enzyme that does not digest within the gene of interest and is located outside the *att*B region.
2. Ethanol precipitate the DNA after digestion by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Pellet the DNA by centrifugation. Wash the pellet twice with 70% ethanol.
4. Dissolve the DNA in TE Buffer, pH 8.0 to a final concentration of 50–150 ng/µL.

Continued on next page

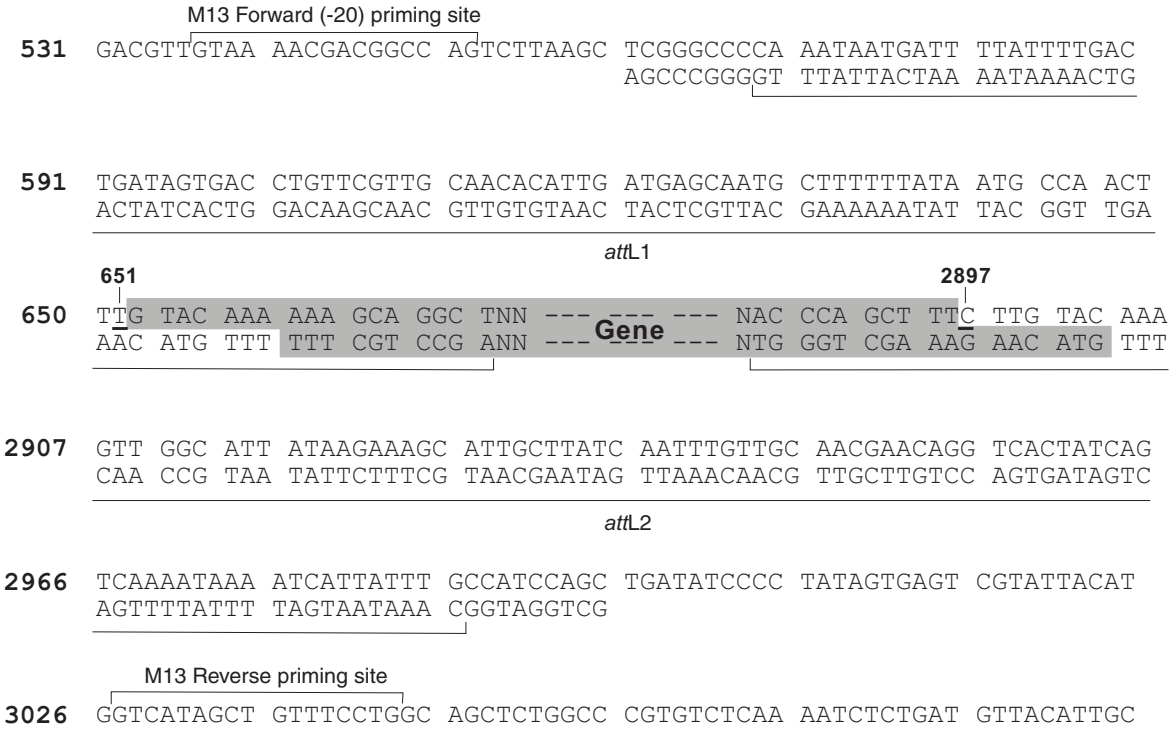
Creating Entry Clones Using the BP Recombination Reaction, Continued

Recombination Region of pDONR™221 and pDONR™/Zeo

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

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the *att*B substrate into pDONR™221 or pDONR™/Zeo by recombination. Non-shaded regions are derived from the pDONR™221 or pDONR™/Zeo vector.
- Bases 651 and 2897 of the pDONR™221 or pDONR™/Zeo vector sequence are marked.



Performing the BP Recombination Reaction

Introduction

General guidelines and instructions are provided in the following sections to perform a BP recombination reaction using an appropriate *att*B substrate and a donor vector, and to transform the reaction mixture into a suitable *E. coli* host to select for entry clones. We recommend that you include a positive control (see the next section) and a negative control (no Gateway® BP Clonase® II) in your experiment to help you evaluate your results.

Positive Control

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

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

For optimal efficiency, we recommend using the following amounts of *att*B-PCR product (or linearized *att*B expression clone) and donor vector in a 20 µL BP recombination reaction:

- An equimolar amount of *att*B-PCR product (or linearized *att*B expression clone) and the donor vector
- 50 femtomoles (fmol) **each** of *att*B-PCR product (or linearized *att*B expression clone) and donor vector is preferred, but the amount of *att*B-PCR product used may range from 20–50 fmol

  Note: 50 fmol of donor vector (pDONR™221 or pDONR™/Zeo) is approximately 150 ng
- For large PCR products (>4 kb), use at least 50 fmol of *att*B-PCR product, but no more than 250 ng

For a formula to convert fmol of DNA to nanograms (ng), see the following section. For an example, see the page 22.

- Do not use more than 250 ng of donor vector in a 10 µL BP reaction as this will affect the efficiency of the reaction
- Do not exceed more than 0.5 µg of total DNA (donor vector plus *att*B-PCR product) in a 10 µL BP reaction as excess DNA will inhibit the reaction

Converting Femtomoles (fmol) to Nanograms (ng)

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

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

where N is the size of the DNA in bp. For an example, see page 22.

Continued on next page

Example of fmol to ng Conversion

In this example, you need to use 50 fmol 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 on page 21:

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

Gateway® BP Clonase® II Enzyme Mix

The Gateway® BP Clonase® II enzyme mix is supplied with the PCR Cloning System with Gateway® Technology to catalyze the BP recombination reaction. The Gateway® BP Clonase® II enzyme mix combines the proprietary enzyme formulation and 5X BP Clonase® Reaction Buffer previously supplied as separate components in Gateway® BP Clonase® enzyme mix into an optimized single tube format to allow easier set-up of the BP recombination reaction. Use the protocol provided on page 23 to perform the BP recombination reaction using Gateway® BP Clonase® II enzyme mix.

Note: You may perform the BP recombination reaction using Gateway® BP Clonase® enzyme mix, if desired. To use Gateway® BP Clonase® enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for Gateway® BP Clonase® II enzyme mix provided on page 23.

Materials Needed

You should have the following materials on hand before beginning:

- *att*B-PCR product or linearized *att*B expression clone (see the page 21to determine the amount of DNA to use)
- pDONR™ vector (150 ng/µL, supplied with the kit)
- Gateway® BP Clonase® II enzyme mix (supplied with the PCR Cloning System; keep at −20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µL Proteinase K solution (supplied with the Gateway® BP Clonase® II enzyme mix; thaw and keep on ice until use)
- pEXP7-tet positive control (50 ng/µL; supplied with the Gateway® BP Clonase® II enzyme mix)

Continued on next page

Setting Up the BP Recombination Reaction

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

   Note: To include a negative control, set up a second sample reaction and omit the Gateway® BP Clonase® enzyme mix (see Step 4).

Components	Sample	Positive Control	Negative Control
*att*B-PCR product or linearized *att*B expression clone (20–50 fmol)	1–7 µL	—	1–7 µL
pDONR™ vector (150 ng/µL)	1 µL	1 µL	1 µL
pEXP7-tet positive control (50 ng/µL)	—	2 µL	—
TE Buffer, pH 8.0	to 8 µL	5 µL	to 10 µL

2. Remove the Gateway® BP Clonase® II enzyme mix from −20°C and thaw on ice (~ 2 minutes).
3. Vortex the Gateway® BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. Add 2 µL of Gateway® BP Clonase® II enzyme mix to the sample and positive control vials. Do not add Gateway® BP Clonase® II to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).

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

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

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

Transforming Competent Cells

Introduction

After performing the BP recombination reaction, you will transform competent *E. coli* and select for entry clones. If you are using the PCR Cloning System with Gateway® Technology, One Shot® OmniMAX™ 2-T1^R chemically competent *E. coli* are included with the kit for use in transformation. You may transform electrocompetent cells, if desired. Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

E. coli Host Strain

You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α™, DH10B™or equivalent for transformation. Other strains are suitable. **Do not** use *E. coli* strains that contain the F′ episome (e.g. TOP10F′) for transformation. These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Note: For the pEXP7-tet control reaction, we recommend that you transform TOP10 or DH5 ™ competent cells. **Do not** use the OmniMAX™ 2-T1^R cells because these cells have resistance to tetracycline. Use the transformation protocol on the following page, and assess the efficiency of the BP reaction by streaking entry clones onto LB agar plates containing 20 µg/mL tetracycline. True entry clones should be tetracycline-resistant.

For your convenience, OmniMAX™ 2-T1^R, TOP10, DH5α™, and DH10B™ *E. coli* are available as chemically competent or electrocompetent cells (see the following table).

Item	Quantity	Catalog No.
One Shot® OmniMAX™ 2-T1^R Chemically Competent *E. coli*	20 × 50 µL	C8540-03
Library Efficiency® DH5α™ *E. coli*	5 × 200 µL	18263-012
One Shot® TOP10 Chemically Competent *E. coli*	20 × 50 µL	C4040-03
One Shot® Max Efficiency® DH10B™ T1 Phage Resistant Chemically Competent *E. coli*	20 × 50 µL	12331-013
One Shot® TOP10 Electrocomp *E. coli*	20 × 50 µL	C4040-52
ElectroMax™ DH10B™ *E. coli*	5 × 100 µL	18290-015

Selection Media

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

Important: If you are using pDONR™/Zeo, use Low Salt LB agar for selection (see the **Note** on page 25).

Donor Vector	Selection Media
pDONR™221	LB + 50 µg/ml kanamycin
pDONR™/Zeo	**Low Salt LB** + 50 µg/ml Zeocin™

Continued on next page

Transforming Competent Cells, Continued

Note

The Zeocin™ resistance gene in pDONR™/Zeo allows selection of *E. coli* transformants using Zeocin™ Selection Reagent. For selection, use Low Salt LB agar plates containing 50 µg/mL Zeocin™ Selection Reagent (see page 52 for a recipe). Note that for Zeocin™ Selection Reagent to be active, the salt concentration of the bacterial medium must remain low (< 90mM) and the pH must be 7.5. For information about storing and handling Zeocin™ Selection Reagent, see page 51.

Materials Needed

- BP recombination reaction (from Step 7, page 23)
- One Shot® OmniMAX™ 2-T1^R chemically competent *E. coli* (supplied with the PCR Cloning System; 1 vial per transformation; thaw on ice before use) or another suitable *E. coli* strain
- TOP10 or DH5 ™ competent cells for transforming the pEXP7-tet control reaction
- S.O.C. Medium (supplied with the PCR Cloning System; warm to room temperature)
- Positive control (e.g. pUC19 supplied with the PCR Cloning System; use as a control for transformation if desired)
- LB Medium
- LB plates containing the appropriate antibiotic, refer to table on the page 24 (2 for each transformation; warm at 37°C for 30 minutes)
- 42°C water bath (for chemical transformation)
- 37°C shaking and non-shaking incubator

Continued on next page

One Shot® Chemical Transformation Protocol

Use this procedure to transform the BP recombination reaction into One Shot® OmniMAX™ 2-T1^R chemically competent *E. coli*. If you are using another *E. coli* strain, follow the manufacturer's instructions. **Do not** use the OmniMAX™ 2-T1^R to transform the pEXP7-tet control reaction (see page 24).

1. Thaw, on ice, 1 vial of One Shot® OmniMAX™ 2-T1^R chemically competent cells for each transformation.
2. Add 1 µL of the BP recombination reaction (from **Performing the BP Recombination Reaction**, Step 7, page 23) into a vial of One Shot® OmniMAX™ 2-T1^R cells and mix gently. **Do not mix by pipetting up and down.** For the pUC19 control, add 10 pg (1 µL) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C bath and place them on ice for 2 minutes.
6. Add 250 µL of room temperature S.O.C. medium to each vial.
7. Cap the vial(s) tightly and shake horizontally (225 rpm) at 37°C for 1 hour.
8. Before plating, dilute the transformation mix 1:10 into LB Medium (e.g. remove 20 µL of the transformation mix and add to 180 µL of LB Medium).
9. Spread 20 µL and 100 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

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

Transformation by Electroporation

Use **only** electrocompetent cells for electroporation to avoid arcing. **Do not** use the One Shot® OmniMAX™ 2-T1^R chemically competent cells for electroporation.

1. Add 1 µL of the BP recombination reaction (from **Performing the BP Recombination Reaction**, Step 7, page 23) into a 0.1-cuvette containing 50 µL of electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down. Avoid formation of bubbles.**
2. Electroporate your samples using an electroporator and the manufacturer's suggested protocol.

   Note: If you have problems with arcing, see page 27.
3. Immediately add 450 µL of room temperature S.O.C. medium.
4. Transfer the solution to a 15-mL snap-cap tube (i.e., BD Falcon®) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance marker.
5. Spread 50–100 µL from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

   An efficient BP recombination reaction may produce hundreds of colonies.

Continued on next page

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

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

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

Sequencing Entry Clones

Introduction

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

Sequencing Primers

You may use the M13 Sequencing Primers included with the PCR Cloning System with Gateway® Technology kits to sequence entry clones derived from BP recombination with pDONR™221 or pDONR™/Zeo. Refer to the diagram on page 20 for the location of the primer binding sites.

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

Sequencing Using BigDye® Chemistry

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

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

PCR Conditions

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

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

Creating Expression Clones Using the LR Recombination Reaction

Introduction

After generating an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into an *att*R-containing destination vector to create an *att*B-containing expression clone. To ensure that you obtain the best possible results, we suggest that you read this section and the next section entitled **Performing the LR Recombination Reaction** (pages 31–32) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the appropriate *att*L and *att*R-containing substrates (see the following sections)
2. Transform the reaction mixture into a suitable *E. coli* host (see page 24)
3. Select for expression clones

Substrates for the LR Recombination Reaction

To perform an LR recombination reaction, you need to have the following substrates:

- *att*L-containing entry clone
- A Life Technologies destination vector **or** your converted destination vector (see the page 30)

Important

For most applications, we recommend performing the LR recombination reaction using a:

- Supercoiled *att*L-containing entry clone
- Supercoiled *att*R-containing destination vector

Exception: If your destination vector or entry clone is large (>10 kb), you may do the following to increase recombinational efficiency by up to 2-fold:

- Linearize either the destination vector or the entry clone. To linearize the destination vector, choose a unique restriction site that cuts within the *att*R cassette but does not disrupt the *att*R sites or the *ccd*B gene. To linearize the entry clone, choose a unique restriction site that does not cut within the *att*L sites or the gene of interest.
- Relax the destination vector using topoisomerase I if suitable restriction sites are unavailable. Refer to the **Appendix**, page 49 for a protocol to perform a modified LR reaction using a relaxed destination vector.

Note

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

Continued on next page

Destination Vectors

A large selection of destination vectors is available to allow expression of your gene of interest in virtually any protein expression system. For more information about the options available, refer to **www.lifetechnologies.com** or call Technical Support (see page 57).

Converting Your Vector to a Destination Vector

You may convert any vector to a destination vector using the Gateway® Vector Conversion System. For guidelines and instructions, see **Constructing a Gateway® Destination Vector**, pages 33–39.

Performing the LR Recombination Reaction

Introduction

After obtaining an entry clone containing the gene of interest, you may perform an LR recombination reaction between the entry clone and a destination vector of choice, and transform the reaction mixture into a suitable *E. coli* host to select for an expression clone. We recommend including the pENTR™-gus positive control in your experiments to help evaluate your results.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α™, DH10B™or equivalent for transformation (see page 24 for ordering information). **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (e.g. TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Note: If you are transforming One Shot® OmniMAX™ 2-T1^R cells, see pages 24–26.

Positive Control

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

Gateway® LR Clonase® II Enzyme Mix

Gateway® LR Clonase® II enzyme mix is available separately (see page 55) to catalyze the LR recombination reaction. The Gateway® LR Clonase® II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase® Reaction Buffer previously supplied as separate components in Gateway® LR Clonase® enzyme mix (see page 55) into an optimized single tube format to allow easier set-up of the LR recombination reaction. Use the protocol provided on the next page to perform the LR recombination reaction using Gateway® LR Clonase® II enzyme mix.

Note: You may perform the LR recombination reaction using Gateway® LR Clonase® enzyme mix, if desired. To use Gateway® LR Clonase® enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for Gateway® LR Clonase® II enzyme mix provided on page 32.

Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/µL in TE, pH 8.0)
- Destination vector of choice (150 ng/µL in TE, pH 8.0)
- Gateway® LR Clonase® II enzyme mix (keep at −20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µL Proteinase K solution (supplied with the Gateway® LR Clonase® II enzyme mix; thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µL; supplied with the Gateway® LR Clonase® II enzyme mix)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates with the appropriate antibiotic to select for expression clones

Continued on next page

Setting Up the LR Recombination Reaction

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

   Note: To include a negative control, set up a second sample reaction and omit the Gateway® LR Clonase® II enzyme mix (see Step 4).

Component	Sample	Negative Control	Positive Control
Entry clone (50–150 ng/reaction)	1–7 µL	1–7 µL	—
Destination vector (150 ng/µL)	1 µL	1 µL	1 µL
pENTR™-gus (50 ng/µL)	—	—	2 µL
TE Buffer, pH 8.0	to 8 µL	to 10 µL	5 µL

2. Remove the Gateway® LR Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the Gateway® LR Clonase® II enzyme mix briefly twice (2 seconds each time).
4. Add 2 µL of Gateway® LR Clonase® II enzyme mix to the sample and positive control vials. Do not add Gateway® LR Clonase® II enzyme mix to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).

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

   Note: For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation times (i.e. overnight incubation) will yield more colonies and are recommended.
6. Add 1 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. To transform One Shot® OmniMAX™ 2-T1^R *E. coli*, follow the protocol on page 26.

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

What You Should See

If you use *E. coli* cells with a transformation efficiency of ≥1 × 10^8 cfu/µg, the LR reaction should give >5000 colonies if the entire LR reaction is transformed and plated. **Note:** The transformation efficiency of One Shot® OmniMAX™ 2-T1^R chemically competent cells is ≥5 × 10^9 cfu/µg.

Expressing Your Recombinant Protein

After obtaining an expression clone, you may express your recombinant protein. Refer to the manual for the destination vector you are using for guidelines and instructions to express your recombinant protein in the appropriate system. Manuals for all Gateway® destination vectors are available from **www.lifetechnologies.com/manuals** or by contacting Technical Support (see page 57).

Constructing a Gateway® Destination Vector

Introduction

You may easily convert any vector of choice to a Gateway® destination vector by ligating a blunt-ended cassette containing *att*R sites flanking the *ccd*B gene and the chloramphenicol resistance gene into the multiple cloning site (MCS) of the vector. The Gateway® Vector Conversion System is available from Life Technologies (see page 55 for ordering information) to facilitate conversion of your vector into a destination vector for expression of native, N-, or C-terminally-tagged proteins.

Important

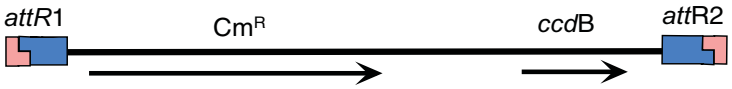
Most entry vectors contain the kanamycin resistance gene for selection. For maximal compatibility within the Gateway® Technology, we recommend that your vector **not** contain a kanamycin resistance marker. If this is unavoidable, you will need to perform the LR recombination reaction with an entry clone that carries a selection marker other than the kanamycin resistance gene (e.g. use pDONR™/Zeo and your *att*B-flanked gene of interest in a BP recombination reaction to generate an entry clone that confers Zeocin™ resistance, or TOPO® Clone a *Taq*-amplified PCR product into pCR®8/GW/TOPO® to generate an entry clone that confers spectinomycin resistance).

Gateway® Vector Conversion System

The Gateway® Vector Conversion System includes three conversion cassettes:

- Reading Frame (Rf) Cassette A (RfA)
- Reading Frame (Rf) Cassette B (RfB)
- Reading Frame (Rf) Cassette C.1 (RfC.1)

Each reading frame cassette contains the chloramphenicol resistance gene (Cm^R) and the *ccd*B gene flanked by *att*R1 and *att*R2 sites (see below). Each reading frame cassette also differs by one nucleotide to allow generation of *att*R sites in all three reading frames.



Note

Each reading frame cassette contains a unique restriction site to allow you to distinguish between them (see the following table).

Cassette	Restriction Site	Location (bp)
RfA	*Mlu* I	898
RfB	*Bgl* II	899
RfC.1	*Xba* I	899

Ligating the Reading Frame Cassette to Your Vector

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

Continued on next page

Constructing a Gateway® Destination Vector, Continued

Experimental Outline

To convert your vector to a destination vector:

1. Choose an appropriate reading frame cassette depending on your needs.
2. Linearize the vector with a restriction enzyme of choice. If you use a restriction enzyme that generates an overhang, you will need to blunt the ends.
3. Remove the 5′ phosphates using calf intestinal alkaline phosphatase.
4. Ligate the reading frame cassette into your vector using T4 DNA ligase.
5. Transform the ligation reaction into *ccd*B Survival T1^{R} *E. coli* and select for transformants.
6. Analyze transformants.

Factors to Consider

To determine which Gateway® reading frame cassette to use when converting your vector, you should consider the following:

- If you plan to express a fusion protein from the destination vector, use a reading frame cassette with the correct translation reading frame
- If you plan to linearize your vector using a restriction enzyme that generates an overhang, choose the correct reading frame cassette based on what the sequence of the ends will be after the vector has been made blunt (*i.e.* after filling in a protruding 5′ end or polishing a protruding 3′ end)

N-terminal Fusions

If you wish to create a destination vector to express N-terminal fusion proteins, use the following table and the diagram on page 35 to help you determine which reading frame cassette to use.

Tip: Keep the -AAA-AAA- triplets in the *att*R1 site in frame with the translation reading frame of the fusion protein.

If the coding sequence of the blunt end…	Then use…
Terminates after a complete codon triplet	RfA
Encodes two bases of a complete codon triplet	RfB
Encodes one base of a complete codon triplet	RfC.1

C-terminal Fusions

If you wish to create a destination vector to express C-terminal fusion proteins, use the following table and the diagram on page 35 to help you determine which reading frame cassette to use.

Tip: Keep the -TAC-AAA- triplets in the *att*R2 site in frame with the translation reading frame of the fusion protein.

If the coding sequence of the blunt end…	Then use…
Terminates after a complete codon triplet	RfB
Encodes two bases of a complete codon triplet	RfC.1
Encodes one base of a complete codon triplet	RfA

Continued on next page

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

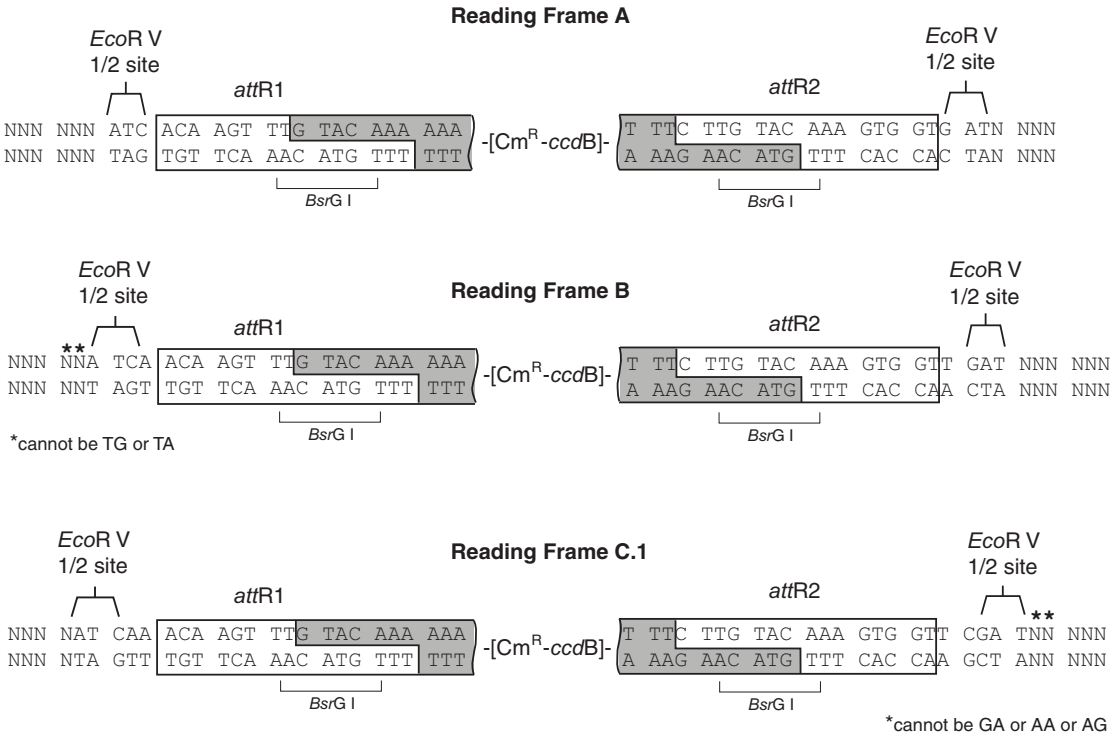
Sequences of the Reading Frame Cassettes

The sequences of the ends of each reading frame cassette are shown in the following diagram. The complete sequence of each reading frame cassette is available from **www.lifetechnologies.com** or by calling Technical Support (see page 57).

Features of the reading frame cassettes:

- Non-shaded regions correspond to those DNA sequences that are transferred into the *att*B expression clone following the LR recombination reaction.
- The *Eco*R V half-site present on the 5′ and 3′ ends of each cassette is labeled.
- Sequences contributed by your vector are denoted by Ns.

  Note: If you are using RfB to create an N-terminal fusion vector, the two nucleotides next to the 5′ *Eco*R V half-site cannot be TG or TA otherwise this will generate a stop codon. Similarly, if you are using RfC.1 to create a C-terminal fusion vector, the two nucleotides next to the 3′ *Eco*R V half-site cannot be GA, AA, or AG.
- The *Bsr*G I restriction site common to all *att*1 and *att*2 sites is indicated.



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Constructing a Gateway® Destination Vector, Continued

E. coli Host

To propagate and maintain your destination vector, you must use *ccd*B Survival T1^R *E. coli*. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli* are provided with the Gateway® Vector Conversion System and are also available separately (see page 55 for ordering information).

Note: Do not use general *E. coli* cloning strains including OmniMAX™ 2-T1^R, TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects.

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

Materials Needed

- Your vector of choice
- Appropriate restriction enzymes to linearize your vector at the position where you wish your gene (flanked by *att* sites) to be after recombination (see the preceding **Recommendation**)
- T4 DNA polymerase or Klenow fragment (if necessary to create blunt ends in your vector)
- Calf intestinal alkaline phosphatase (see page 55 for ordering information)
- 10X CIAP Buffer (supplied with Calf intestinal alkaline phosphatase)
- Sterile water (autoclaved, distilled)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA)
- T4 DNA ligase (see page 55 for ordering information)
- 5X T4 DNA ligase buffer (supplied with T4 DNA ligase)
- Appropriate Gateway® reading frame cassette (5 ng/µL)
- One Shot® *ccd*B Survival™ 2 T1^R chemically competent *E. coli* (supplied with the Gateway® Vector Conversion System)
- S.O.C. Medium (supplied with the Gateway® Vector Conversion System)
- LB agar plates containing the appropriate antibiotic to select for your vector **and** 30 µg/mL chloramphenicol

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Constructing a Gateway® Destination Vector, Continued

Conversion Procedure

1. Digest 1–5 µg of your plasmid vector with the appropriate restriction enzyme(s).
2. If necessary, convert the ends of the vector to blunt double-stranded DNA using T4 DNA polymerase or Klenow fragment according to the manufacturer's recommendations.
3. Remove the 5′ phosphates with calf intestinal alkaline phosphatase (CIAP) to decrease the background associated with self-ligation of the vector.
   a. Determine the mass of DNA required for 1 pmol of the DNA 5′ end.
   b. Add the following reagents to a 1.5-mL microcentrifuge tube:

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

   c. Dilute the CIAP in dilution buffer such that 1 µL contains the amount of enzyme required to dephosphorylate the appropriate 5′ end (i.e. 1 unit for blunt ends). Add 1 µl of CIAP and incubate for 1 hour at 50°C.
   d. Heat-inactivate CIAP for 15 minutes at 65°C.
4. Adjust the DNA to a final concentration of 20–50 ng/µl in TE Buffer, pH 8.0. Run 20–100 ng of DNA on an agarose gel to verify digestion and recovery.
5. To set up the ligation reaction, add the following reagents to a 1.5-mL microcentrifuge tube:

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

6. Incubate at room temperature for 1 hour.

   Note: Overnight incubation at 16°C is also suitable.
7. Transform the ligation reaction into competent One Shot® *ccd*B Survival T1^R *E. coli*. Follow the instructions provided with the cells.
8. After expression in S.O.C. medium, spread 20 µL and 100 µL from each transformation on a prewarmed selective plate containing the appropriate antibiotic to select for your vector **and** 30 µg/mL chloramphenicol. Incubate plates overnight at 37°C.

Important

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

Continued on next page

Analyzing Transformants

1. Pick 10 colonies and culture them overnight in 3–5 mL of LB medium containing 30 µg/mL chloramphenicol.
2. Isolate plasmid DNA using your method of choice Ausubel *et al.*, 1994; Sambrook *et al.*, 1989. We recommend the PureLink® HQ Mini Plasmid Purification Kit (see page 55 for ordering information).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the cassette. You can use the *Bsr*G I restriction enzyme to identify clones containing the reading frame cassette (see page 35).

Verifying the Function of the *ccd*B Gene in the Destination Vector

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

Materials Needed:

- *ccd*B Survival™ 2 T1^R competent *E. coli*
- OmniMAX™ 2-T1^R or TOP10 competent *E. coli* (or any other strain sensitive to CcdB effects)
- Positive control plasmid (e.g. pUC 19) to verify success of transformation
- Selective plates (e.g. LB + ampicillin)

Procedure:

1. Transform equal amounts (10–50 pg) of your destination vector into competent OmniMAX™ 2-T1^R and *ccd*B Survival T1^R cells using the protocol provided with the cells. Also transform each strain with 50 pg of the positive control plasmid.
2. Plate onto selective plates and incubate overnight at 37°C.
3. Use the pUC19 control DNA to verify that the transformation efficiency is as expected for each strain.
4. Determine the number of colonies obtained in both strains transformed with the destination vector.

What You Should See:

The destination vector should give 10,000 times **more** colonies in *ccd*B Survival T1^R cells than in OmniMAX™ 2-T1^R cells. Any ratio less than 10,000:1 indicates either an inactive *ccd*B gene or contamination of the plasmid prep with another antibiotic-resistant plasmid.

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Preparing the Destination Vector

Once purified, you may use your supercoiled destination vector directly in the LR recombination reaction. If your destination vector is large (>10 kb), you may increase the efficiency of the LR reaction by linearizing the destination vector with a restriction enzyme or relaxing the DNA with topoisomerase I (see the protocol on page 49), if desired.

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

- *Alw*N I
- *Bss*H II
- *Eco*R I
- *Nco* I
- *Not* I
- *Pvu* II
- *Sal* I
- *Sca* I
- *Sfc* I

Troubleshooting

LR and BP Reactions

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

Observation	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Check the antibiotic resistance marker and use the correct antibiotic to select for entry clones or expression clones.
	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
	Used incorrect *att* sites for the reaction	• Use an entry clone (*att*L) and a destination vector (*att*R) for the LR reaction. • Use an expression clone (or *att*B-PCR product) and a donor vector (*att*P) for the BP reaction.
	Clonase® II enzyme mix is inactive or didn't use suggested amount of Clonase® II enzyme mix	• Test another aliquot of the Gateway® Clonase® II enzyme mix. • Make sure that you store the Gateway® Clonase® II enzyme mix at −20°C or −80°. • Do not freeze/thaw the Gateway® Clonase® II enzyme mix more than 10 times. • Use the recommended amount of Gateway® Clonase® II enzyme mix (see page 23 or 32 as appropriate).
	Used incorrect Gateway® Clonase® II enzyme mix	Use the Gateway® LR Clonase® II enzyme mix for the LR reaction and the Gateway® BP Clonase® II enzyme mix for the BP reaction.
	Too much *att*B-PCR product was used in a BP reaction	Reduce the amount of *att*B-PCR product used. Remember to use an equimolar ratio of *att*B-PCR product and donor vector (i.e. ~50 fmol each).
	Long *att*B-PCR product or linear *att*B expression clone (≥5 kb)	Incubate the BP reaction overnight.
	Too much entry clone was used in an LR reaction	Use equal fmol of destination vector and entry clone.
	Large destination vector or entry clone (>10 kb)	• Incubate the LR reaction overnight. • Linearize the destination vector or the entry clone. • Relax the destination vector with topoisomerase I.

Continued on next page

LR and BP Reactions, Continued

Observation	Reason	Solution
Two distinct types of colonies (large and small) appear	**LR reaction:** Small colonies can be unreacted entry clone that cotransforms with expression clone **Note:** When small colonies are restreaked onto selective plates to select for unreacted entry clones (e.g. LB + kanamycin) and expression clones (e.g. LB + ampicillin), small colonies often only grow on the selective plates used to select for unreacted entry clones.	• Reduce the amount of entry clone to 50 ng per 10 µL reaction. • Reduce the volume of sample used for transformation to 1 µL. • If you are using a destination vector that contains the ampicillin resistance gene for selection, increase the ampicillin concentration to 300 µg/mL.
	BP reaction: The pDONR™ vector contains deletions or point mutations in the *ccd*B gene **Note:** The negative control will give a similar number of colonies.	Obtain a new pDONR™ vector.
	Loss of plasmid during culture (generally those containing large genes or toxic genes)	• Incubate selective plates at 30°C instead of 37°C. • Confirm whether a deletion has occurred by analyzing the DNA derived from the colonies. • Use Stbl2™ *E. coli* (see page 55 for ordering information) to help stabilize plasmids containing large genes during propagation Trinh *et al.*, 1994.
High background of Zeocin™-resistant transformants that do not contain the entry clone	Selection of entry clones derived from pDONR™/Zeo not performed on Low Salt LB agar plates	Use Low Salt LB agar plates with 50 µg/mL Zeocin™ to select entry clones derived from pDONR™/Zeo. See page 25 for more information and page 52 for a recipe.

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LR and BP Reactions, Continued

Observation	Reason	Solution
LR Reaction: High background in the absence of the entry clone	LR reaction transformed into an *E. coli* strain containing the F′ episome and the *ccd*A gene	Use an *E. coli* strain that does not contain the F′ episome for transformation (e.g. OmniMAX™ 2-T1^R, TOP10).
	Deletions (full or partial) of the *ccd*B gene from the destination vector	• To maintain the integrity of the vector, propagate in media containing the appropriate antibiotic (e.g. ampicillin) and 15–30 µg/mL chloramphenicol. • Prepare plasmid DNA from one or more colonies and verify the integrity of the vector before use. • If you have converted your own vector to a destination vector, try using a different vector backbone to reduce instability of the plasmid.
	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 LR reaction. • Test for bacterial contamination by plating an aliquot of each solution directly onto LB plates containing ampicillin.
Few or no colonies obtained from the transformation control	Competent cells stored incorrectly	Store competent cells at −80°C.
	Transformation performed incorrectly	If you are using One Shot® OmniMAX™ 2-T1^R, follow the protocol on page 26 to transform cells. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.

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Troubleshooting

*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 (see page 40).

Observation	Reason	Solution
Low yield of *att*B-PCR product obtained after PEG purification	*att*B-PCR product not diluted with TE	Dilute with 150 µL of 1X TE, pH 8.0 before adding the PEG/$MgCl_2$ solution.
	Centrifugation step too short or centrifugation speed too low	Increase time and speed of the centrifugation step to 30 minutes and 15,000 × 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.
Few or no colonies obtained from a BP reaction with *att*B- PCR product **and** both *att*B positive control and transformation control gave expected number of colonies	*att*B PCR primers incorrectly designed	• Make sure that the *att*B PCR primers include four 5′ terminal Gs and the 25 bp *att*B1 or *att*B2 site (see page 13).
	*att*B PCR primers contaminated with incomplete sequences	• Use HPLC or PAGE-purified oligonucleotides to generate your *att*B-PCR product. • Use the *att*B adapter PCR protocol to generate your *att*B-PCR product.
	*att*B-PCR product not purified sufficiently	Gel purify your *att*B-PCR product to remove *att*B primers and *att*B primer-dimers.
	For large PCR products (>5 kb), too few *att*B-PCR molecules added to the BP reaction	• Increase the amount of *att*B-PCR product to 40–100 fmol per 20 µL reaction. **Note:** Do not exceed 500 ng DNA per 20 µL reaction. • Incubate the BP reaction overnight.
	Insufficient incubation time	Increase the incubation time of the BP reaction up to 18 hours.

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***att*B PCR Cloning,** Continued

Observation	Reason	Solution
Entry clones migrate as 2.2 kb supercoiled plasmids	BP reaction may have cloned *att*B primer-dimers	• Purify *att*B-PCR product using the PEG/$MgCl_2$ purification protocol on page 17 or gel-purify the *att*B-PCR product. • Use a Platinum® DNA polymerase with automatic hot-start capability for higher specificity amplification. • Redesign *att*B PCR primers to minimize potential mutual priming sites leading to primer-dimers.

Appendix

"One-Tube" Protocol for Cloning *att*B-PCR Products Directly into Destination Vectors

Introduction

Use this one-tube protocol to:

- Move *att*B-PCR products into a destination vector in 2 steps; (1) a BP reaction followed by (2) an LR reaction without purification of the intermediate entry clone. See page 16 for guidelines to generate *att*B-PCR products.
- Transfer a gene from one expression clone into another destination vector.

Note: Using this protocol allows you to generate expression clones more rapidly than the protocols provided on pages 21–32; however, fewer expression clones will be obtained (at least 10–20% of the total number of expression clones). If you wish to maximize the number of expression clones generated, **do not** use this protocol. Use the protocols on pages 21–32 instead.

Expression Clones Containing PCR Products

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

Note

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

Materials Needed

You should have the following materials on hand before beginning:

- *att*B-PCR product (50–100 ng)
- *att*P DNA (i.e. pDONR™ vector; 150 ng/µL in 1X TE Buffer, pH 8.0)
- Gateway® BP Clonase® II enzyme mix (keep at –20°C until immediately before use)
- TE Buffer, pH 8.0
- Proteinase K solution (supplied with the Gateway® BP and LR Clonase® II enzyme mixes)
- Destination vector (supercoiled; 150 ng/µL in TE Buffer, pH 8.0)
- Gateway® LR Clonase® II enzyme mix (keep at –20°C until immediately before use)
- Competent *E. coli* cells (see page 24 to choose an appropriate host strain)
- LB agar plates containing the appropriate antibiotic to select for entry clones (e.g. kanamycin or Zeocin™)
- LB agar plates containing the appropriate antibiotic to select for expression clones (e.g. ampicillin)

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"One-Tube" Protocol for Cloning *att*B-PCR Products Directly into Destination Vectors, Continued

"One-Tube" Protocol

1. In a 1.5-mL microcentrifuge tube, prepare the following 15 µL BP reaction:

*att*B DNA (50–100 ng)	1.0–5.0 µL
*att*P DNA (pDONR™ vector, 150 ng/µL)	1.3 µL
Gateway® BP Clonase® II enzyme mix	3.0 µL
TE Buffer, pH 8.0	add to a final volume of 15 µL

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

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

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

4. To the remaining 10 µL reaction, add:

Destination vector (150 ng/µL)	2.0 µL
Gateway® LR Clonase® II enzyme mix	3.0 µL
Final volume	15 µL

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

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

6. Add 2 µL of proteinase K solution. Incubate at 37°C for 10 minutes.

7. Transform 50 µL of the appropriate competent *E. coli* with 1 µL of the reaction. Plate on LB plates containing the appropriate antibiotic to select for expression clones.

Assessing the Efficiency of the BP Reaction

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

2. Transform 50 µL of the appropriate competent *E. coli* with 1 µL of the reaction. Plate on LB plates containing the appropriate antibiotic to select for entry clones.

Preparing *att*B-PCR Products Using *att*B Adapter PCR

Introduction

We recommend using this protocol to produce *att*B-PCR products if your PCR primers are greater than 70 bp. To use this protocol, you will need to have 2 sets of PCR primers, one set for the gene-specific amplification and a second set to install the complete *att*B sequences (adapter-primers *att*B1 and *att*B2).

Template-Specific Primers

Design the following template-specific primers. Include 12 bases of the *att*B1 or *att*B2 site on the 5′ end of each primer, as appropriate.

- *att*B1 forward: 5′-AA AAA GCA GGC TNN - template-specific sequences-3′
- *att*B2 reverse: 5′-A GAA AGC TGG GTN - template-specific sequences-3′

Adapter Primers

Design the following adapter primers that are required to install the complete *att*B sequences.

- *att*B1 adapter: 5′-G GGG ACA AGT TTG TAC AAA AAA GCA GGC T -3′
- *att*B2 adapter: 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT -3′

*att*B Adapter PCR Protocol

1. Set up a 50 µL PCR reaction containing 10 pmoles of each template-specific primer and the appropriate amount of template DNA.

   Note: Do not use more than 10 pmoles of each template-specific primer as this can lead to reduced yield of clonable full-length *att*B-PCR product.
2. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minutes	95°C	1X
Denaturation	15 seconds	94°C	10X
Annealing	30 seconds	50 to 60°C	
Extension	1 minute/kb	68°C	

3. Transfer 10 µL of the PCR reaction to a 40 µL PCR mixture containing 40 pmoles each of the *att*B1 and *att*B2 adapter primers.
4. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	1 minutes	95°C	1X
Denaturation	15 seconds	94°C	5X
Annealing	30 seconds	45°C	
Extension	1 minute/kb	68°C	

Continued on next page

Preparing *att*B-PCR Products Using *att*B Adapter PCR, Continued

*att*B Adapter PCR Protocol, Continued

5. Adjust cycling parameters and amplify for 15–20 cycles using the following parameters:

Step	Time	Temperature	Cycles
Denaturation	15 seconds	94°C	15–20X
Annealing	30 seconds	55°C	
Extension	1 minute/kb	68°C	

6. Use agarose gel electrophoresis to check quality and yield of the *att*B-PCR product.
7. Proceed to page 17 to purify the *att*B-PCR product.

Relaxing Destination Vectors Using Topoisomerase I

Introduction

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

Materials Needed

- Destination vector (supercoiled; 300 ng per reaction)
- Entry clone (supercoiled, 100–300 ng per reaction)
- Topoisomerase I (see page 55 for ordering information; use 15 units/µg of total DNA)
- TE Buffer, pH 8.0
- Gateway® LR Clonase® II enzyme mix (see page 55 for ordering information; keep at −20°C until immediately before use)
- Proteinase K solution (supplied with the Gateway® LR Clonase® II; thaw and keep on ice until use)

Protocol

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

Entry clone (50–150 ng)	1–6 µL
Destination vector (150 ng/µl)	1 µL
Topoisomerase I (15 units/µg total DNA)	0.6–2 µL
TE Buffer, pH 8.0	add to a final volume of 8 µL

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

   Reminder: Return Gateway® LR Clonase® II enzyme mix to −20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 µL of the Proteinase K solution to the reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. If you are transforming One Shot® OmniMAX™ 2-T1^R competent *E. coli*, follow the protocol on page 26.

Transferring Clones from cDNA Libraries Made in Gateway® Vectors

Introduction

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

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

Expressing Full-Length vs. Other cDNAs

Most cDNA libraries typically contain a mixture of:

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

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

Expressing Fusion Proteins

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

- For full-length cDNAs containing 5′ untranslated sequence, the 5′ UTR will be translated as part of the fusion protein. This may present problems as the additional codons may interfere with expression or function of the protein, or may include stop codons.
- **N-terminal fusions:** To express any cDNA as an N-terminal fusion protein, the reading frame of the gene must be in frame with the reading frame of the *att*B1 site. If the identity of the cDNAs is unknown, there is a one in three chance that the cDNA will be in frame with the N-terminal tag. You may construct three destination vectors, each allowing expression of the fusion protein in a different reading frame or alternatively, you may amplify the cDNA using *att*B primers designed to be in frame with the ORF.
- **C-terminal fusions:** Stop codons present in full-length cDNAs must be removed to permit expression of a C-terminal fusion protein. This may be done by amplifying the gene using *att*B PCR primers in which the stop codon has been eliminated from the gene-specific sequence. Alternatively, the gene may be subcloned into any entry vector in such a way that no stop codon is present.

Zeocin™ Selective Antibiotic

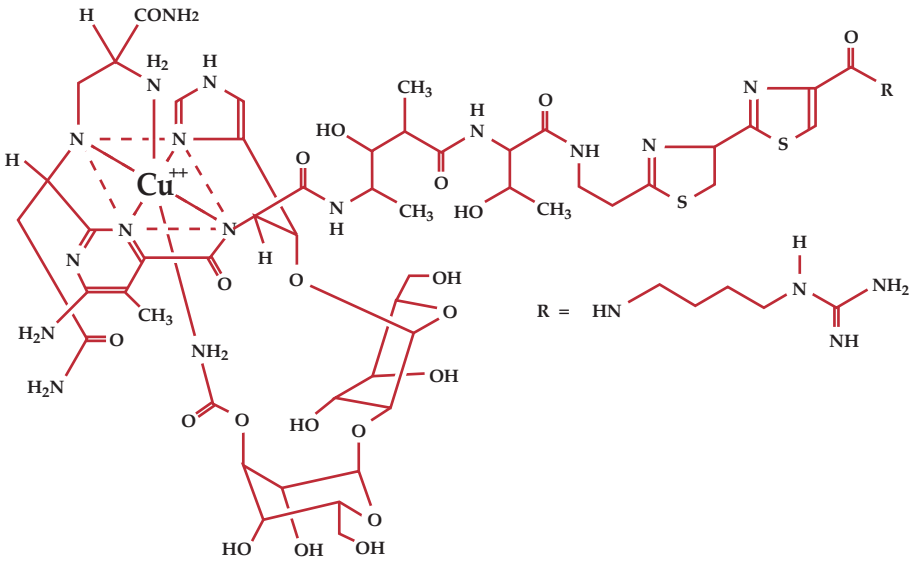
Introduction

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

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

Molecular Weight, Formula, and Structure

The formula for Zeocin™ selective antibiotic is $C_{55}H_{86}O_{21}N_{20}S_2Cu$-HCl and the molecular weight is 1527.5. The following diagram shows the structure of Zeocin™ selective antibiotic.



Handling Zeocin™ Selective Antibiotic

- High ionic strength and acidity or basicity inhibit the activity of Zeocin™ selective antibiotic. Therefore, we recommend that you reduce the salt in bacterial medium and adjust the pH to 7.5 to keep the drug active (see page 52 for a recipe).
- Store Zeocin™ selective antibiotic at –20°C and thaw on ice before use.
- Zeocin™ selective antibiotic is light sensitive. Store the drug and plates or medium containing the drug in the dark.
- Wear gloves, a laboratory coat, and safety glasses when handling Zeocin™-containing solutions.
- Do not ingest or inhale solutions containing the drug.
- Be sure to bandage any cuts on your fingers to avoid exposure to the drug.

Recipes

Low Salt LB Medium with Zeocin™ Selective Antibiotic

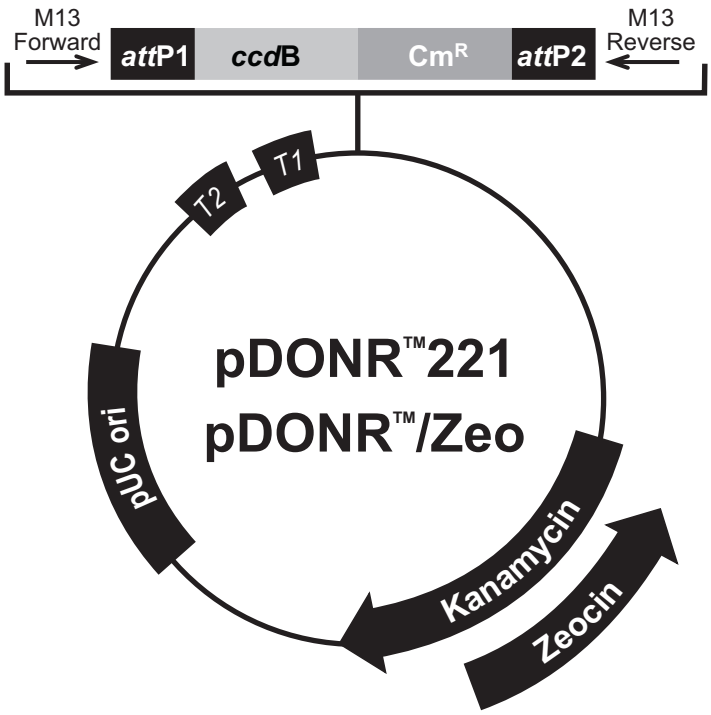
10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust the pH to 7.5 with 5 M NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 lbs/sq. in. and 121°C for 20 minutes.
3. Thaw Zeocin™ selective antibiotic on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ selective antibiotic to 50 µg/mL final concentration.
5. Store plates at 4°C in the dark. Plates containing Zeocin™ selective antibiotic are stable for 1–2 weeks.

Map and Features of pDONR™221 and pDONR™/Zeo

pDONR™221 and pDONR™/Zeo Map

The following map shows the elements of pDONR™221 and pDONR™/Zeo. The complete sequences of pDONR™221 and pDONR™/Zeo are available from www.lifetechnologies.com or by contacting Technical Support (see page 57).



Comments for:	pDONR™221 4761 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
*att*P1:	570-801	570-801
*ccd*B gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1825-2505	1847-2506
*att*P2 (c):	2753-2984	2754-2985
M13 Reverse priming site:	3026-3042	3027-3043
Kanamycin resistance gene:	3155-3964	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4085-4758	3615-4288

(c) = complementary strand

Continued on next page

Map and Features of pDONR™221 and pDONR™/Zeo, Continued

Features of the Vectors

pDONR™221 (4761 bp) and pDONR™/Zeo (4291 bp) contain the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity Orosz *et al.*, 1991.
M13 forward (–20) priming site	Allows sequencing in the sense orientation.
*att*P1 and *att*P2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from an *att*B-containing expression clone or *att*B-PCR product Landy, 1989.
*ccd*B gene	Allows negative selection of the plasmid.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
M13 reverse priming site	Allows sequencing in the anti-sense orientation.
Kanamycin resistance gene **(pDONR™221 only)**	Allows selection of the plasmid in *E. coli*.
EM7 promoter **(pDONR™/Zeo only)**	Allows expression of the Zeocin™ resistance gene in *E. coli*.
Zeocin™ resistance gene **(pDONR™/Zeo only)**	Allows selection of the plasmid in *E. coli*.
pUC origin and replisome assembly site	Allows high-copy replication and maintenance of the plasmid in *E. coli*.

Accessory Products

Introduction

The products listed in this section may be used with the PCR Cloning System with Gateway® Technology. For more information, refer to **www.lifetechnologies.com** or call Technical Support (see page 57).

Additional Products

Many of the reagents supplied in the PCR Cloning System with Gateway® Technology as well as other products suitable for use with the kit are available separately. Ordering information is provided in the following table.

Product	Quantity	Catalog no.
Gateway® BP Clonase® II Enzyme Mix	20 reactions	11789-020
	100 reactions	11789-100
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-100
Gateway® LR Clonase® enzyme mix	80 µL	11791-019
One Shot® OmniMAX™ 2-T1^R Chemically Competent *E. coli*	20 × 50 µL	C8540-03
One Shot® TOP10 Chemically Competent *E. coli*	20 × 50 µL	C4040-03
One Shot® *ccd*B Survival™ T1^R Chemically Competent *E. coli*	10 × 50 µL	C7510-03
pDONR™221	6 µg	12536-017
pDONR™/Zeo	6 µg	12535-035
Gateway® Vector Conversion System with One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent *E. coli*	20 reactions	11828-029
PureLink® Quick Gel Extraction Kit	50 reactions	K210012
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
Ampicillin	20 mL (10 mg/mL)	11593-019
Kanamycin Sulfate	100 mL (10 mg/mL)	15160-054
Zeocin™	1 g	R250-01
	5 g	R250-05
Platinum® *Pfx* DNA Polymerase	100 reactions	11708-013
	250 reactions	11708-021
Platinum® *Taq* DNA Polymerase High Fidelity	100 reactions	11304-011
	500 reactions	11304-029
Dpn I	100 units	15242-019
Calf intestinal alkaline phosphatase	1,000 units	18009-019
T4 DNA ligase	100 units	15224-017
MAX Efficiency® Stbl2™ Competent Cells	1 mL	10268-019
Topoisomerase I	500 units	38042-024

Continued on next page

Gateway® Entry Vectors

A variety of Gateway® entry vectors are available to facilitate creation of entry clones.

- For rapid TOPO® Cloning of *Taq* polymerase-amplified PCR products, use one of the pCR®8/GW/TOPO® TA Cloning® Kits.
- For directional TOPO® Cloning of PCR products, use one of the pENTR™ D-TOPO® vectors.
- For traditional restriction enzyme digestion and ligase-mediated cloning, use one of the other pENTR™ vectors.

For more information about the features of the entry vectors, see **www.lifetechnologies.com** or contact Technical Support (see page 57).

Product	Quantity	Catalog no.
pCR®8/GW/TOPO® TA Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2500-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2520-20
pENTR™/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2400-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2435-20
pENTR™/SD/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2420-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2635-20
pENTR™/TEV/D-TOPO® Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2525-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2535-20
pENTR™1A Dual Selection	10 µg	A10462
pENTR™2B Dual Selection	10 µg	A10463
pENTR™3C Dual Selection	10 µg	A10464
pENTR™4 Dual Selection	10 µg	A10465

Gateway® Destination Vectors

A large selection of Gateway® destination vectors is available to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available and their features, see **www.lifetechnologies.com/support** or contact Technical Support (see page 57).

Technical Support

Obtaining support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

Certificate of Analysis

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

Limited warranty

Life Technologies 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**.

Purchaser Notification

Introduction

Use of the PCR Cloning System with Gateway® Technology is covered under the licenses detailed below.

Information for European Customers

The OmniMAX™ 2-T1^R *E. coli* strain is genetically modified and carries the F′ episome containing *pro*AB *lac*I^q *lac*ZΔM15 *Tn*10 Δ(*ccd*AB). As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

Limited Use Label License No. 19: Gateway® Cloning Products

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Life Technologies Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase® purchased from Life Technologies Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made by the employment of this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the employment of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Notwithstanding the preceding, any buyer who is employed in an academic or government institution may transfer materials made with this product to a third party who has a license from Life Technologies under the patents identified above to distribute such materials. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (i) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Life Technologies Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. If the purchaser is not willing to accept the limitations of this limited use statement, Life Technologies is willing to accept return of the product with a full refund. For information on purchasing a license to use this product for purposes other than those permitted above, contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Continued on next page

Purchaser Notification, Continued

Gateway® Clone Distribution Policy

For additional information about Life Technologies' policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 60.

Limited Use Label License: Research Use Only

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

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.

Glossary of Terms

***att*L, *att*R, *att*B, and *att*P**	The recombination sites from bacteriophage lambda that are utilized in the Gateway® Technology. • *att*L always recombines with *att*R in a reaction mediated by the LR Gateway® Clonase® II enzyme mix. The LR reaction is the basis for the entry clone x destination vector reaction. Recombination between *att*L and *att*R sites yields *att*B and *att*P sites on the resulting plasmids. • *att*B sites always recombine with *att*P sites in a reaction mediated by the Gateway® BP Clonase® II enzyme mix. The BP reaction is the basis for the reaction between the PCR cloning vector (pDONR™) and PCR products, source clones, or cDNA library clones containing *att*B sites. Recombination between *att*B and *att*P sites yields *att*L and *att*R sites on the resulting plasmids.
Gateway® BP Clonase® II Enzyme Mix	A proprietary mix of lambda recombination proteins that mediates the *att*B x *att*P recombination reaction.
***ccd*B Gene**	A gene which encodes a protein that interferes with *E. coli* DNA gyrase, thereby inhibiting the growth of standard *E. coli* hosts. This gene is present on Gateway® destination, donor, and supercoiled entry vectors. When recombination occurs between a destination vector and an entry clone, the *ccd*B gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the *ccd*B gene, or by-product molecules that retain the *ccd*B gene, will fail to grow. This allows high-efficiency recovery of only the desired clones.
***ccd*B Survival™ 2 T1^R Competent Cells**	These cells are resistant to the effects of the *ccd*B gene product and are used to propagate vectors that contain the *ccd*B gene (i.e. donor, supercoiled entry, and destination vectors).
Destination Vector	Gateway®-adapted expression vectors which contain *att*R sites and allow recombination with entry clones.
Donor Vector (pDONR™)	A Gateway® vector containing *att*P sites. This vector is used for cloning PCR products and genes of interest flanked by *att*B sites (expression clones) to generate entry clones. When PCR fragments modified with *att*B sites are recombined with the pDONR™ vector in a BP reaction, they yield an entry clone. **PCR fragment (*att*B sites) + pDONR™ vector (*att*P sites) → entry clone**
Entry Clone	The result of cloning a DNA segment into an entry vector or donor vector. The entry clone contains the gene of interest flanked by *att*L sites. It can be used for subsequent transfers into destination vectors.

Continued on next page

Glossary of Terms, Continued

Entry Vector (pENTR™)	A Gateway® vector containing *att*L sites used for cloning DNA fragments using either TOPO® Cloning or conventional restriction enzymes and ligase.
Expression Clone	The result of subcloning the DNA of interest from an entry clone into a destination vector of choice by LR recombination. The gene or DNA of interest in the expression clone is flanked by *att*B sites. Expression clones can be either fusion or native proteins. **Entry clone + destination vector → expression clone**
Gateway® LR Clonase® II Enzyme Mix	A proprietary mix of lambda recombination proteins that mediates the *att*L x *att*R recombination reaction.

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