Version E
22 September 2003
25-0522

Gateway® Technology

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

Catalog nos. 12535-019 and 12535-027

Table of Contents

Table of Contents iii
BP and LR Recombination Reaction Protocols for Experienced Users v
Kit Contents and Storage vii
Accessory Products x

Introduction 1

Overview 1
The Gateway® Technology 3
Gateway® BP and LR Recombination Reactions 5
Features of the Gateway® Vectors 8
Gateway® Nomenclature 9

Methods 10

Options to Create Entry Clones 10
Designing *att*B PCR Primers 12
Producing *att*B-PCR Products 15
Purifying *att*B-PCR Products 16
Creating Entry Clones Using the BP Recombination Reaction 17
Performing the BP Recombination Reaction 20
Transforming Competent Cells 23
Sequencing Entry Clones 26
Creating Expression Clones Using the LR Recombination Reaction 27
Performing the LR Recombination Reaction 29
Constructing a Gateway® Destination Vector 31
Troubleshooting 37

Appendix 42

"One-Tube" Protocol for Cloning *att*B-PCR Products Directly into Destination Vectors 42
Preparing *att*B-PCR Products Using *att*B Adapter PCR 44
Relaxing Destination Vectors Using Topoisomerase I 46
Transferring Clones from cDNA Libraries Made in Gateway® Vectors 47
Zeocin™ 48
Recipes 49
Map and Features of pDONR™221 and pDONR™/Zeo 50
Technical Service 52
Purchaser Notification 54
Product Qualification 56
Glossary of Terms 57
References 59

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 (40-100 fmol)	1-10 µl
pDONR™ vector (supercoiled, 150 ng/µl)	2 µl
5X BP Clonase™ reaction buffer	4 µl
TE Buffer, pH 8.0	to 16 µl

2. Vortex BP Clonase™ enzyme mix briefly. Add 4 µl to the components above and mix well by vortexing briefly twice.
3. Incubate reaction at 25°C for 1 hour.
4. Add 2 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, 100-300 ng)	1-10 µl
Destination vector (supercoiled, 150 ng/µl)	2 µl
5X LR Clonase™ reaction buffer	4 µl
TE Buffer, pH 8.0	to 16 µl

2. Vortex LR Clonase™ enzyme mix briefly. Add 4 µl to the components above and mix well by vortexing briefly twice.
3. Incubate reaction at 25°C for 1 hour.
4. Add 2 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 products listed below. For a description of the reagents supplied with the *E. coli*, Baculovirus, and Mammalian Expression Systems and their usage, refer to the individual Expression System manual supplied with each kit.

Product	Quantity	Catalog no.
PCR Cloning System with Gateway® Technology		
with pDONR™221	1 kit	12535-019
with pDONR™/Zeo	1 kit	12535-027
E. coli Expression System with Gateway® Technology	1 kit	11824-026
Baculovirus Expression System with Gateway® Technology	1 kit	11827-011
Mammalian Expression System with Gateway® Technology	1 kit	11826-021

Shipping/Storage

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

Box	Item	Storage
1	Donor Vector (pDONR™221 or pDONR™/Zeo)	Vector: **-20°C** Zeocin™ (supplied with pDONR™/Zeo): **-20°C, protected from light**
2	BP Clonase™ Enzyme Mix and Reagents	BP Clonase™ Enzyme Mix :**-80°C** BP Clonase™ Reaction Buffer and all other reagents: **-20°C**
3-4	M13 Sequencing Primers	**-20°C**
5	Library Efficiency® DH5α™ Chemically Competent *E. coli*	**-80°C**

continued on next page

Kit Contents and Storage, continued

Contents

The Donor Vector box, the BP Clonase™ Enzyme Mix and Reagents box, and the M13 Sequencing Primers box (Boxes 1-4) contain the following items. Store the BP Clonase™ enzyme mix at -80°C. Store all other components at -20°C. Store Zeocin™ at -20°C, protected from light.

Note: For a description of the reagents supplied with Catalog nos. 11824-026, 11827-011, and 11826-013, refer to the manuals for the *E. coli*, Baculovirus, and Mammalian Expression System with Gateway® Technology, respectively.

Item	Composition	Amount
pDONR™ Vector (pDONR™221 or pDONR™/Zeo)	Lyophilized in TE Buffer, pH 8.0	6 µg
Zeocin™ (supplied with pDONR™/Zeo)	100 mg/ml in deionized, sterile water	1.25 ml
BP Clonase™ Enzyme Mix	Proprietary	80 µl
5X BP Clonase™ Reaction Buffer	Proprietary	100 µl
Proteinase K solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl
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 table below 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

continued on next page

Kit Contents and Storage, continued

DH5α™ Competent *E. coli* Reagents

The Library Efficiency® DH5α™ Chemically Competent *E. coli* box (Box 5) includes the following items. Transformation efficiency is 1 x 10^8 cfu/µg DNA. Store Box 5 at -80°C.

Item	Composition	Amount
S.O.C. Medium (may be stored at room temperature or +4°C)	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	2 x 6 ml
Library Efficiency® Chemically Competent DH5α™	--	5 x 200 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of DH5α™

F^- *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1

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 our Web site (www.invitrogen.com) or call Technical Service (see page 52).

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 from Invitrogen. Ordering information is provided below.

Item	Quantity	Catalog no.
BP Clonase™ Enzyme Mix	20 reactions	11789-013
	100 reactions	11789-021
LR Clonase™ Enzyme Mix	20 reactions	11791-019
	100 reactions	11791-043
Library Efficiency DH5α™ Chemically Competent Cells	5 x 0.2 ml	18263-012
One Shot® TOP10 Chemically Competent *E. coli*	20 x 50 µl	C4040-03
Library Efficiency DB3.1™ Competent Cells	5 x 0.2 ml	11782-018
pDONR™201	6 µg	11798-014
pDONR™221	6 µg	12536-017
pDONR™/Zeo	6 µg	12535-035
Gateway® Vector Conversion System	20 reactions	11828-019
S.N.A.P.™ MiniPrep Kit	100 reactions	K1900-01
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
S.N.A.P.™ Gel Purification Kit	25 reactions	K1999-25
Ampicillin	20 ml (10 mg/ml)	11593-019
Kanamycin Sulfate	100 ml (10 mg/ml)	15160-054
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
REact® 4 Buffer	2 x 1 ml	16304-016

continued on next page

Accessory Products, continued

Gateway® Entry Vectors

A variety of Gateway® entry vectors are available from Invitrogen to facilitate creation of entry clones. For rapid TOPO® Cloning of PCR products, we recommend using the pENTR/D-TOPO® or pENTR/SD/D-TOPO® Cloning Kits. 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 our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

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

Gateway® Destination Vectors

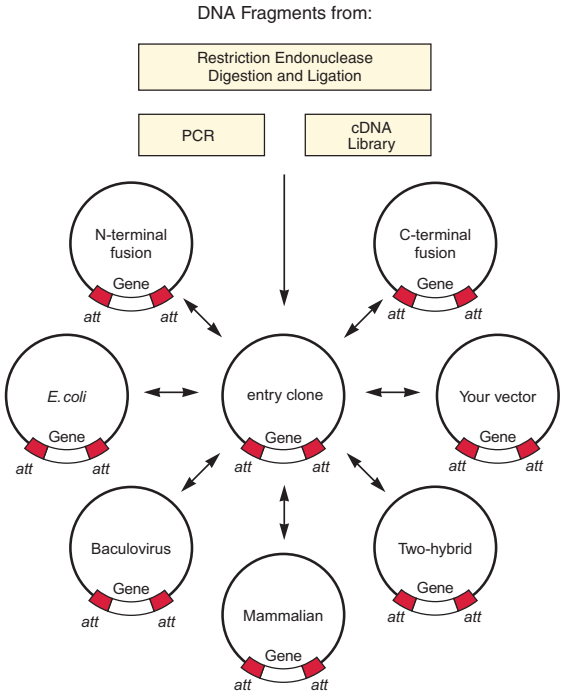
A large selection of Gateway® destination vectors is available from Invitrogen to facilitate expression of your gene of interest in virtually any protein expression system. For more information about the vectors available and their features, see our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

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 diagram below).



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
- Suitable for adaptation to high-throughput (HTP) formats
- Allows easy conversion of your favorite vector into a Gateway® destination vector

continued on next page

Overview, continued

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 Invitrogen destination vector or expression system, refer to the manual for the specific destination vector or system. All Gateway® product manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).

Glossary of Terms

To help you understand the terminology used in the Gateway® Technology, a glossary of terms is provided in the **Appendix**, page 57.

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™ enzyme mix)

These components are discussed below.

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.* Clonase™ 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 below).
- 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 and 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).

continued on next page

The Gateway® Technology, continued

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 table below).

The lysogenic pathway is catalyzed by the bacteriophage λ Integrase (Int) and *E. coli* Integration Host Factor (IHF) proteins (BP Clonase™ 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 (LR Clonase™ 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 x *att*P → *att*L x *att*R	BP Clonase™ (Int, IHF)
Lytic	*att*L x *att*R → *att*B x *att*P	LR Clonase™ (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 diagram below). This reaction is catalyzed by BP Clonase™ 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 diagram below). This reaction is catalyzed by LR Clonase™ 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 above, 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. For example, the pDONR™201 *att*P1 sequence varies slightly from the pDONR™221 *att*P1 sequence. 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

continued on next page

Gateway® BP and LR Recombination Reactions, continued

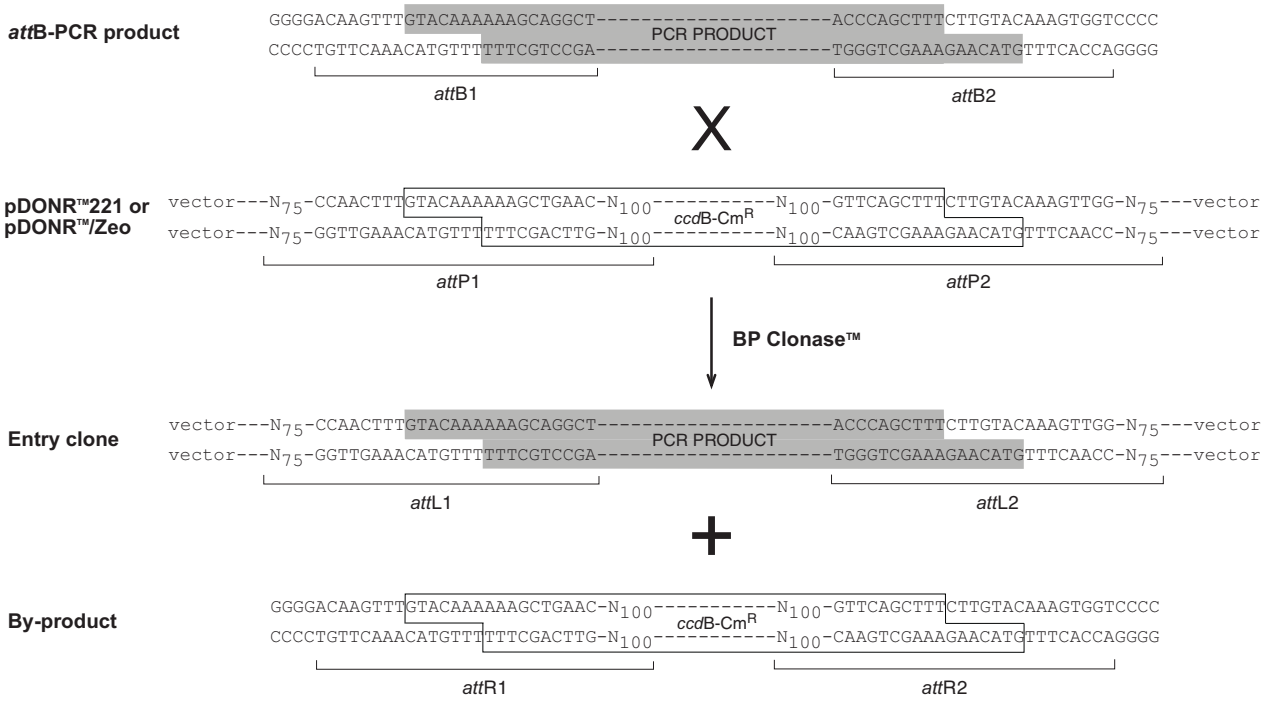
Example of an *att*B x *att*P Recombination Reaction

The diagram below 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.



Gateway® BP and LR Recombination Reactions, continued

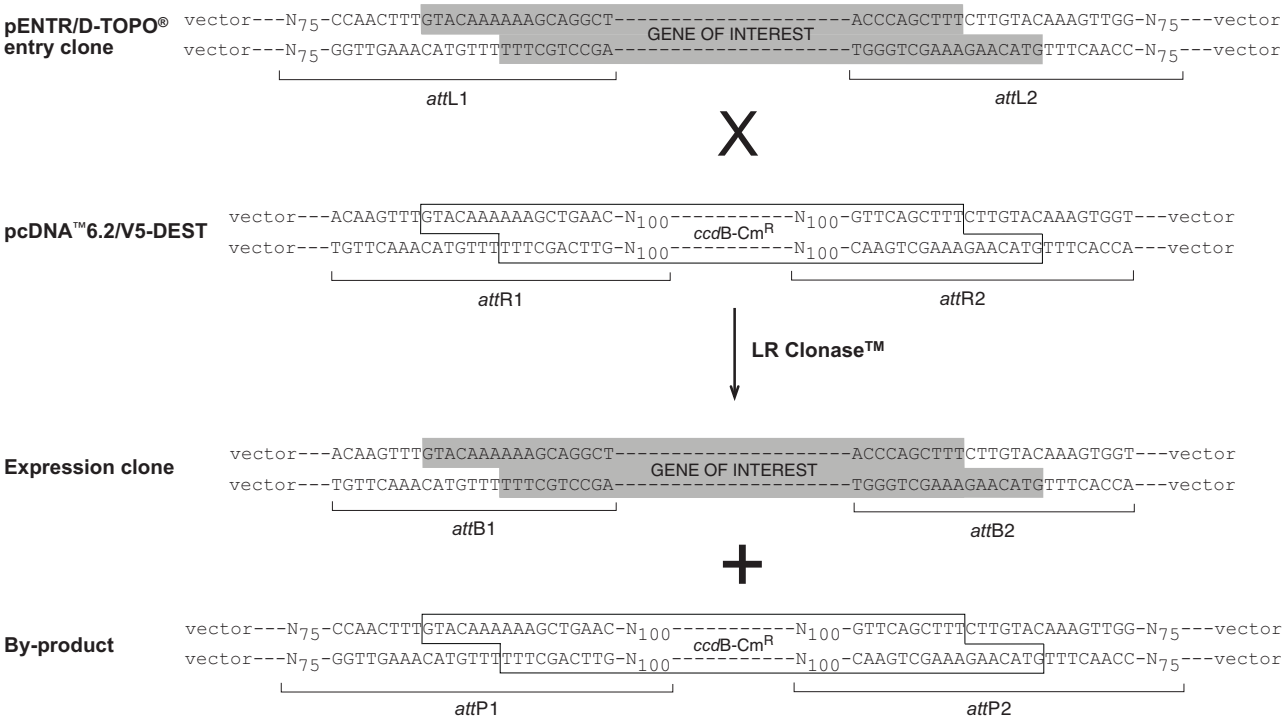
Example of an *att*L x *att*R Recombination Reaction

The diagram below 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

Three different types of Gateway®-adapted vectors are available from Invitrogen:

Gateway® Vector	Characteristics
Donor vector (pDONR™)	Contains *att*P sites Used to clone *att*B-flanked PCR products 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)

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 below) 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 and Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* 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.

Propagating Gateway® Vectors

Because of the lethal effects of the CcdB protein, all Gateway® vectors containing the *ccd*B gene **must** be propagated in an *E. coli* strain that is resistant to CcdB effects. We recommend using the DB3.1™ *E. coli* strain which contains a gyrase mutation (*gyr*A462) that renders it resistant to the CcdB effects (Bernard and Couturier, 1992; Bernard *et al.*, 1993; Miki *et al.*, 1992).

Library Efficiency® DB3.1™ Competent Cells are available from Invitrogen (Catalog no. 11782-018) for transformation. See page 18 for the genotype of DB3.1™.

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	pENTR1, 2,…
*att*L Subclone	Entry Clone	pENTR3-gus,..; pENTR221-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	pDONR201, 221,...

Example: LR Reaction

1. pENTR201-tet x pDEST14 → pEXP14-tet
2. pENTR221-cat x pcDNA-DEST47 → pcDNA/GW-47/cat

Examples: BP Reaction

1. *att*B-p53 PCR product x pDONR221 → pENTR221-p53
2. pEXP14-*lacZ* x pDONR201 → pENTR201-*lacZ*

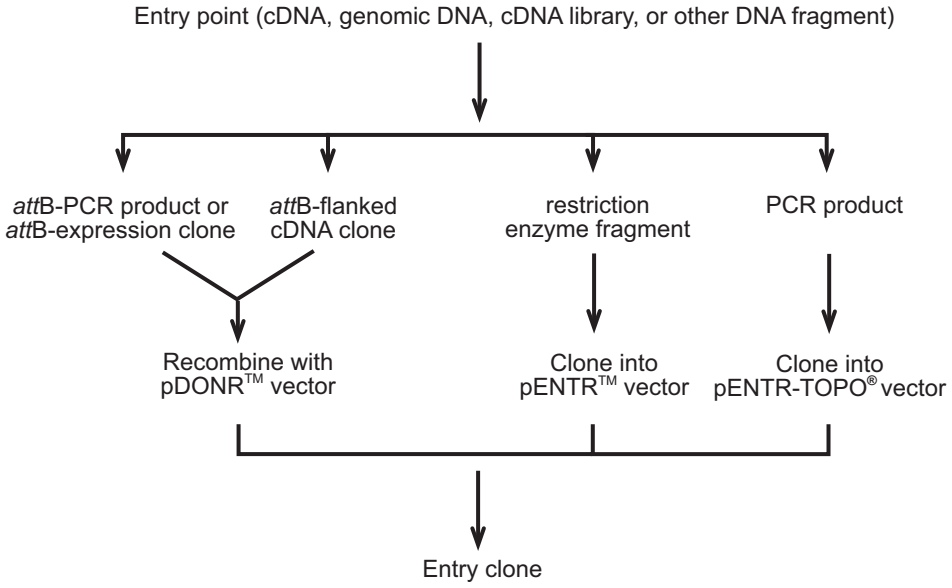
Methods

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 the next page 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 47 for more information).



Note

If you wish to express a particular human or murine gene, we recommend using an Ultimate™ ORF Human or Mouse Clone available from Invitrogen. 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 our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

continued on next page

Options to Create Entry Clones, continued

Entry Vectors

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

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

For more information about the features of each pENTR™ vector, see our Web site (www.invitrogen.com) or contact Technical Service (see page 52).

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

Constructing Entry Clones

To construct an entry clone, refer to the manual for the specific entry vector you are using. All entry vector manuals are available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).

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 wish 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 diagram below and **Examples 1 and 2**, next page for more help.

- To enable efficient Gateway® cloning, the forward primer **MUST** 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 and Dalgarno, 1975) or Kozak consensus sequence (Kozak, 1987; Kozak, 1991; Kozak, 1990), 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 diagram below and **Example 2**, next page). 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
```

continued on next page

Designing *att*B PCR Primers, continued

Example 1: Forward Primer Design for Native Expression

In this 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 this 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 attR1 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, the reverse primer **MUST** 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 wish to fuse your PCR product in frame with a C-terminal tag:
  1. The primer must include one additional nucleotide to maintain the proper reading frame with the *att*B2 region (see diagram below and **Example 2**, next page)
  2. Any in-frame stop codons between the *att*B2 site and your gene of interest must be removed
- If you do not wish to fuse your PCR product in frame with a C-terminal tag, your gene of interest or the primer must include a stop codon (see **Example 1**, next page)

*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 this 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**CCTA(18-25 gene-specific nucleotides)-3′

Example 2: Reverse Primer Design for C-terminal Fusions

In this 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 attR2 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 is sufficient for most applications.
- Dissolve oligonucleotides to 20-50 mM in water or TE Buffer and verify the concentration before use.
- For more efficient cloning of large PCR products (greater than 5 kb), we recommend using HPLC or PAGE-purified oligonucleotides.

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 44-45.

Producing *att*B-PCR Products

DNA Templates

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

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

Recommended Polymerases

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

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

Producing PCR Products

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

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

Checking the PCR Product

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

Note

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

Materials Needed:

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

Protocol:

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

Purifying *att*B-PCR Products

Introduction

After you have generated your *att*B-PCR 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.

Materials Needed

You should have the following materials on hand before beginning:

- *att*B-PCR product (in a 50 µl volume)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 30% PEG 8000/30 mM $MgCl_2$ Solution (supplied with the PCR Cloning System with Gateway® Technology)
- Agarose gel of the appropriate percentage to resolve your *att*B-PCR product

PEG Purification Protocol

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

1. Add 150 µl of TE, pH 8.0 to a 50 µl amplification reaction containing your *att*B-PCR product.
2. Add 100 µl of 30% PEG 8000/30 mM $MgCl_2$. Vortex to mix thoroughly and centrifuge immediately at 10,000 x g for 15 minutes at room temperature.

   Note: In most cases, centrifugation at 10,000 x g for 15 minutes results in efficient recovery of PCR products. To increase the amount of PCR product recovered, the centrifugation time may be extended or the speed of centrifugation increased.
3. Carefully remove the supernatant. The pellet will be clear and nearly invisible.
4. Dissolve the pellet in 50 µl of TE, pH 8.0 (to concentration > 10 ng/µl).
5. Check the quality and quantity of the recovered *att*B-PCR product on an agarose gel.
6. If the PCR product is suitably purified, proceed to **Creating Entry Clones Using the BP Recombination Reaction**, page 17. If the PCR product is not suitably purified (*e.g. att*B primer-dimers are still detectable), see below.

Additional Purification

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

Creating Entry Clones Using the BP Recombination Reaction

Introduction

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 20-22) and **Transforming Competent Cells** (pages 23-25) before beginning.

Note: If you wish to go directly from an *att*B-PCR product or *att*B expression clone into a destination vector, see the **Appendix**, page 43 for a one-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 below)
2. Transform the reaction mixture into a suitable *E. coli* host (see page 23)
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 below)

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 from Invitrogen (see page x for ordering information). For a map and a description of the features of pDONR™221 and pDONR™/Zeo, see the **Appendix**, pages 50-51.

Resuspending the Donor Vectors

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

continued on next page

Creating Entry Clones Using the BP Recombination Reaction, continued

Propagating Donor Vectors

If you wish to propagate and maintain the pDONR™ vectors, we recommend using Library Efficiency® DB3.1™ Competent Cells (Catalog no. 11782-018) from Invitrogen for transformation. The DB3.1™ *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™ and 15 µg/ml chloramphenicol (see page 23 for more information).

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.

Genotype of DB3.1

F$^-$ *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 Δ*leu mtl*1

Linearizing Expression Clones

If you wish 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 to 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

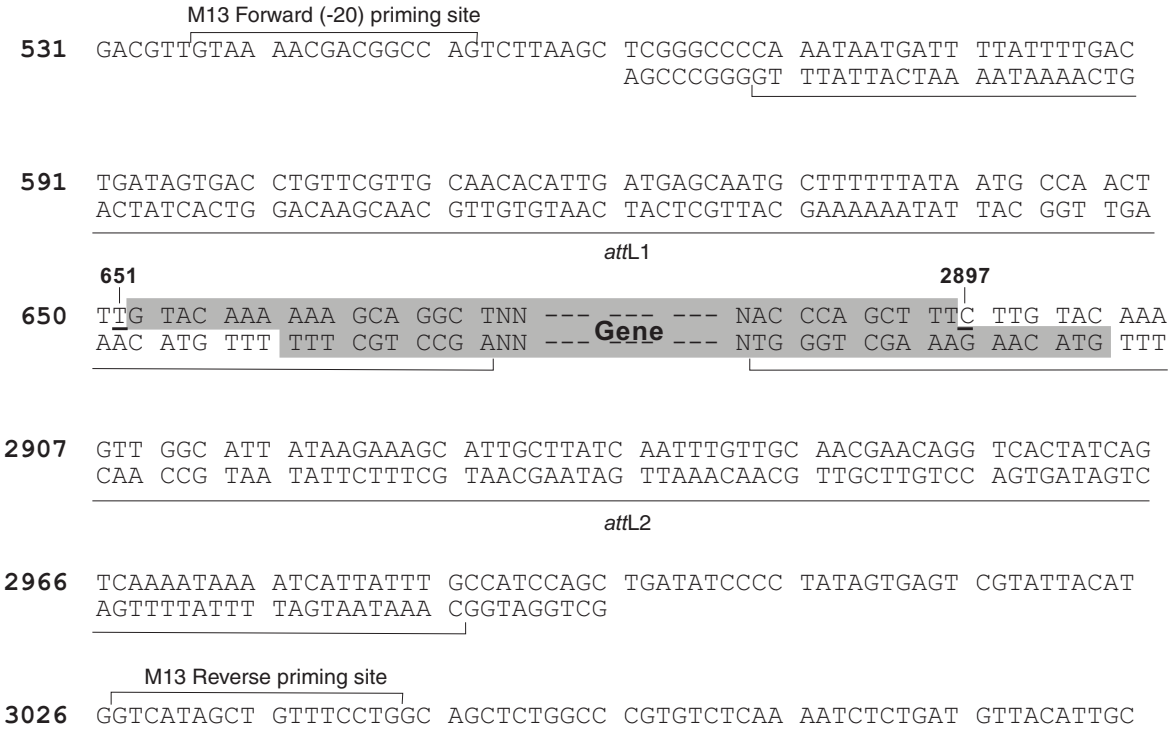
Creating Entry Clones Using the BP Recombination Reaction, continued

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 below and in the next section 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 below) and a negative control (no *att*B substrate) 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
- 100 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 40-100 fmol

  Note: 100 fmol of donor vector (pDONR™201, pDONR™221, or pDONR™/Zeo) is approximately 300 ng
- For large PCR products (>4 kb), use at least 100 fmol of *att*B-PCR product, but no more than 500 ng

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

- Do not use more than 500 ng of donor vector in a 20 µl BP reaction as this will affect the efficiency of the reaction
- Do not exceed more than 1 µg of total DNA (donor vector plus *att*B-PCR product) in a 20 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:

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

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

continued on next page

Performing the BP Recombination Reaction, continued

Example of fmol to ng Conversion

In this example, you need to use 100 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 the previous page:

$$(100\ \text{fmol})(2500\ \text{bp})\left(\frac{660\ \text{fg}}{\text{fmol}}\right)\left(\frac{1\ \text{ng}}{10^6\ \text{fg}}\right) = 165\ \text{ng of PCR product required}$$

Materials Needed

You should have the following materials on hand before beginning:

- *att*B-PCR product or linearized *att*B expression clone (see the previous page to determine the amount of DNA to use)
- pDONR™ vector (supplied with the kit; resuspend to 150 ng/µl with water)
- BP Clonase™ enzyme mix (supplied with the PCR Cloning System; keep at -80°C until immediately before use)
- 5X BP Clonase™ Reaction Buffer (supplied with the BP Clonase™ enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the BP Clonase™ enzyme mix; thaw and keep on ice until use)
- pEXP7-tet positive control (50 ng/µl; supplied with the BP Clonase™ enzyme mix)

continued on next page

Performing the BP Recombination Reaction, continued

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 BP Clonase™ enzyme mix (see Step 4).

Components	Sample	Positive Control
*att*B-PCR product or linearized *att*B expression clone (40-100 fmol)	1-10 µl	--
pDONR™ vector (150 ng/µl)	2 µl	2 µl
pEXP7-tet positive control (50 ng/µl)	--	2 µl
5X BP Clonase™ Reaction Buffer	4 µl	4 µl
TE Buffer, pH 8.0	to 16 µl	8 µl

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

   Reminder: Return BP Clonase™ enzyme mix to -80°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 2 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

Once you have performed the BP recombination reaction, you will transform competent *E. coli* and select for entry clones using the appropriate antibiotic. If you are using the PCR Cloning System with Gateway® Technology, Library Efficiency® DH5α™ chemically competent *E. coli* are included with the kit for use in transformation, however, you may also transform electrocompetent cells. 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 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.

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

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

Selection Media

Refer to the table below 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, you will need to use Low Salt LB agar for selection (see Note below).

Donor Vector	Selection Media
pDONR™201	LB + 50 µg/ml kanamycin
pDONR™221	LB + 50 µg/ml kanamycin
pDONR™/Zeo	**Low Salt LB** + 50 µg/ml Zeocin™

Note

The Zeocin™ resistance gene in pDONR™/Zeo allows selection of *E. coli* transformants using Zeocin™ antibiotic. For selection, use Low Salt LB agar plates containing 50 µg/ml Zeocin™ (see page 49 for a recipe). Note that for Zeocin™ to be active, the salt concentration of the bacterial medium must remain low (<90mM) and the pH must be 7.5. For more information on storing and handling Zeocin™, refer to page 48.

continued on next page

Transforming Competent Cells, continued

Materials Needed

You should have the following materials on hand before beginning:

- BP recombination reaction (from Step 7, page 22)
- Library Efficiency® DH5α™ chemically competent *E. coli* (supplied with the PCR Cloning System; thaw on ice before use) or another suitable *E. coli* strain
- 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 plates containing the appropriate antibiotic, refer to table on the previous page (two for each transformation; warm at 37°C for 30 minutes)
- 42°C water bath (for chemical transformation)
- 37°C shaking and non-shaking incubator

Note

Library Efficiency® DH5α™ competent cells are supplied in 5 tubes containing 0.2 ml of competent cells each. Each tube contains enough competent cells to perform 4 transformations using 50 µl of cells per transformation. Once you have thawed a tube of competent cells, discard any unused cells. **Do not** re-freeze cells as repeated freezing/thawing of cells may result in loss of transformation efficiency.

Chemical Transformation Protocol

1. For each transformation, aliquot 50 µl of Library Efficiency® DH5α™ competent cells into a sterile 1.5 ml microcentrifuge tube.
2. Add 1 µl of the BP recombination reaction (from **Performing the BP Recombination Reaction**, Step 7, page 22) into the tube containing 50 µl of Library Efficiency® DH5α™ competent cells and mix gently. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 450 µl of room temperature S.O.C. medium.
7. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
8. 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 transformation is plated).

continued on next page

Transforming Competent Cells, continued

Verifying pEXP7-tet Entry Clones

If you included the pEXP7-tet control in your BP recombination reaction, you may transform Library Efficiency® DH5α™ competent cells using the protocol on the previous page. The efficiency of the BP reaction may then be assessed by streaking entry clones onto LB agar plates containing 20 µg/ml tetracycline. True entry clones should be tetracycline-resistant.

Transformation by Electroporation

Use **only** electrocompetent cells for electroporation to avoid arcing. **Do not** use the Library Efficiency® DH5α™ chemically competent cells for electroporation.

1. Add 1 µl of the BP recombination reaction (from **Performing the BP Recombination Reaction**, Step 7, page 22) 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 below.
3. Immediately add 450 µl of room temperature S.O.C. medium.
4. Transfer the solution to a 15 ml snap-cap tube (*i.e.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance marker.
5. Spread 50-100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend plating 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

   An efficient BP recombination reaction may produce hundreds of colonies.

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

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

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

Sequencing Entry Clones

Introduction

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

Sequencing Primers

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 19 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 below

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	

DYEnamic™ is a trademark of Amersham Biosciences.

BigDye™ is a trademark of Applied Biosystems.

Creating Expression Clones Using the LR Recombination Reaction

Introduction

After you have generated 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 29-30) 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 below)
2. Transform the reaction mixture into a suitable *E. coli* host (see page 23)
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
- An Invitrogen destination vector **or** your converted destination vector (see below)

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 46 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 at Invitrogen 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

Creating Expression Clones Using the LR Recombination Reaction, continued

Destination Vectors

A large selection of destination vectors is available from Invitrogen to allow expression of your gene of interest in virtually any protein expression system. For more information about the options available, see our Web site (www.invitrogen.com) or call Technical Service (see page 52).

Converting Your Vector to a Destination Vector

You may convert any vector to a destination vector using the Gateway® Vector Conversion System available from Invitrogen. For guidelines and instructions, see **Constructing a Gateway® Destination Vector**, pages 31-36.

Performing the LR Recombination Reaction

Introduction

Once you have obtained an entry clone containing your 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 (see below) to select for an expression clone. We recommend that you include the pENTR™-gus positive control (see below) in your experiments to help you evaluate your results.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, DH10B™or equivalent for transformation (see page 23 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 plan to use Library Efficiency® DH5α™ cells for transformation, see the section entitled **Transforming Competent Cells**, pages 23-25.

Positive Control

The pENTR™-gus plasmid is provided with the LR Clonase™ 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).

Materials Needed

You should have the following materials on hand before beginning:

- 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)
- LR Clonase™ enzyme mix (Catalog no. 11791-019; keep at -80°C until immediately before use)
- 5X LR Clonase™ Reaction Buffer (supplied with the LR Clonase™ enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ enzyme mix; thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µl; supplied with the LR Clonase™ enzyme mix)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates with the appropriate antibiotic (*e.g.* ampicillin) to select for expression clones

continued on next page

Performing the LR Recombination Reaction, continued

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 LR Clonase™ enzyme mix (see Step 4).

Component	Sample	Negative Control	Positive Control
Entry clone (100-300 ng/reaction)	1-10 µl	--	--
Destination vector (300 ng/reaction)	2 µl	2 µl	2 µl
pENTR™-gus (50 ng/µl)	--	--	2 µl
5X LR Clonase™ Reaction Buffer	4 µl	4 µl	4 µl
TE Buffer, pH 8.0	to 16 µl	10 µl	8 µl

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

   Reminder: Return LR Clonase™ enzyme mix to -80°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 2 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to transform a suitable *E. coli* host and select for expression clones. If you are transforming Library Efficiency® DH5α™ competent *E. coli*, follow the protocol on page 24.

   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 x 10^8 cfu/µg, the LR reaction should give >5000 colonies if the entire transformation is plated.

Expressing Your Recombinant Protein

Once you have obtained an expression clone, you are ready to 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 for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).

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 Invitrogen (see page x for ordering information) to facilitate conversion of your vector into a destination vector (see below) 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.

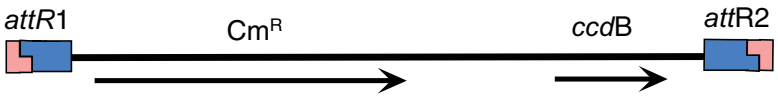
You may use pDONR™/Zeo and your *att*B-flanked gene of interest in a BP recombination reaction to generate an entry clone that confers Zeocin™ resistance. See page x for ordering information.

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 table below).

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, you will:

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 DB3.1™ *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 table below and the diagram on the next page 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 table below and the diagram on the next page 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

Constructing a Gateway® Destination Vector, continued

If you wish 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.

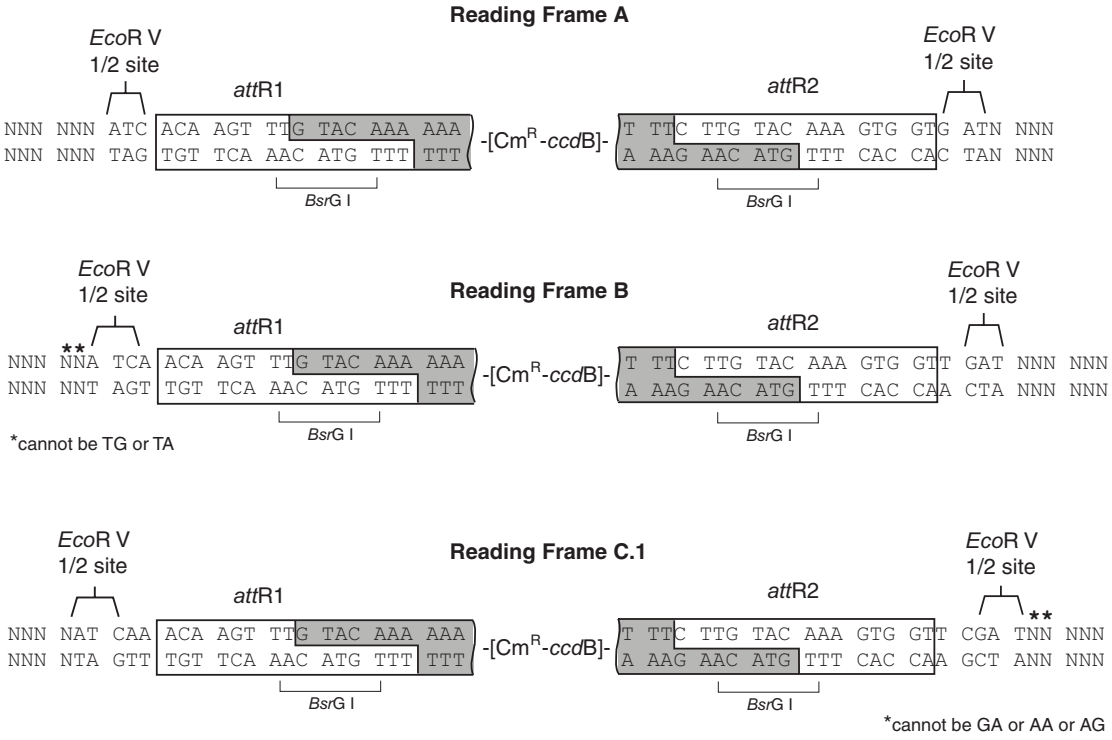
Sequences of the Reading Frame Cassettes

The sequences of the ends of each reading frame cassette are shown below. The complete sequence of each reading frame cassette is available from our Web site (www.invitrogen.com) or by calling Technical Service (see page 52).

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.



continued on next page

Constructing a Gateway® Destination Vector, continued

E. coli Host

To propagate and maintain your destination vector, you must use DB3.1™ *E. coli*. The DB3.1™ *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. Library Efficiency® DB3.1™ Competent Cells are provided with the Gateway® Vector Conversion System and are also available separately from Invitrogen (Catalog no. 11782-018).

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.

To linearize your vector, we recommend that you choose 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 Recommendation above)
- T4 DNA polymerase or Klenow fragment (if necessary to create blunt ends in your vector)
- Calf intestinal alkaline phosphatase (CIAP; Invitrogen, Catalog no. 18009-019)
- 10X CIAP Buffer (supplied with Catalog no. 18009-019)
- Sterile water (autoclaved, distilled)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA)
- T4 DNA ligase (Invitrogen, Catalog no. 15224-017)
- 5X T4 DNA ligase buffer (supplied with Catalog no. 15224-017)
- Appropriate Gateway® reading frame cassette (5 ng/µl)
- Library Efficiency® DB3.1™ competent cells (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

continued on next page

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 DB3.1™ *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

Constructing a Gateway® Destination Vector, continued

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 S.N.A.P.™ MiniPrep Kit (see page x 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 33).

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:

DB3.1™ competent *E. coli*

DH5α™ 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 DH5α™ and DB3.1™ 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 37ºC.
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 DB3.1™ cells than in DH5α™ cells. Any ratio less than 10,000 indicates either an inactive *ccd*B gene or contamination of the plasmid prep with another antibiotic-resistant plasmid.

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 protocol on page 46.), 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 table below for a list of possible restriction enzymes). Be sure to choose a restriction enzyme that does not cut within your vector sequence.

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

Troubleshooting

LR and BP Reactions

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

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Check the antibiotic resistance marker and use the correct antibiotic to select for entry clones or expression clones
	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation
	Used incorrect *att* sites for the reaction	• Use 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™ enzyme mix is inactive or didn't use suggested amount of Clonase™ enzyme mix	• Test another aliquot of the Clonase™ enzyme mix • Make sure that you store the Clonase™ enzyme mix at -80° • Do not freeze/thaw the Clonase™ enzyme mix more than 10 times • Use the recommended amount of Clonase™ enzyme mix (see page 22 or 30 as appropriate)
	Used incorrect Clonase™ enzyme mix	Use the LR Clonase™ enzyme mix for the LR reaction and the BP Clonase™ 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.* ~100 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

Troubleshooting

LR and BP Reactions, continued

Problem	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 100 ng per 20 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* (Invitrogen, Catalog no. 10268-019) 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 23 for more information and page 49 for a recipe.

continued on next page

LR and BP Reactions, continued

Problem	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.* DH5α™, 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 Library Efficiency® DH5α™, follow the protocol on page 24 to transform cells If you are using another *E. coli* strain, follow the manufacturer's instructions
	Loss of transformation efficiency due to repeated freeze/thawing	Once you have thawed a tube of competent cells, discard any unused cells
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated

continued on next page

Troubleshooting

*att*B-PCR Cloning

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

Problem	Reason	Solution
Low yield of *att*B-PCR product obtained after PEG purification	*att*B-PCR product not diluted with TE	Dilute with 150 µl of 1X TE, pH 8.0 before adding the PEG/$MgCl_2$ solution
	Centrifugation step too short or centrifugation speed too low	Increase time and speed of the centrifugation step to 30 minutes and 15,000 x g
	Lost PEG pellet	• When removing the tube from the microcentrifuge, keep track of the orientation of the outer edge of the tube where the pellet is located • When removing the supernatant from the tube, take care not to disturb the pellet
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 12)
	*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

continued on next page

*att*B PCR Cloning, continued

Problem	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 16 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 - a BP reaction followed by an LR reaction without purification of the intermediate entry clone. See page 15 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 20-30; however, fewer expression clones will be obtained (generally 10-20% of the total number of entry clones). If you wish to maximize the number of expression clones generated, **do not** use this protocol. Use the protocols on pages 20-30 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 (100-200 ng)
- *att*P DNA (*i.e.* pDONR™ vector; 150 ng/µl in 1X TE, pH 8.0)
- BP Clonase™ enzyme mix (keep at -80°C until immediately before use)
- 5X BP Reaction Buffer (supplied with the BP Clonase™ enzyme mix)
- TE Buffer, pH 8.0
- Proteinase K solution (supplied with the BP and LR Clonase™ enzyme mixes)
- Destination vector (supercoiled; 150 ng/µl in TE Buffer, pH 8.0)
- 0.75 M NaCl
- LR Clonase™ enzyme mix (keep at -80°C until immediately before use)
- Competent *E. coli* cells (see page 23 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)

continued on next page

"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 25 µl BP reaction:

*att*B DNA (100-200 ng)	5.0 µl
*att*P DNA (pDONR™ vector, 150 ng/µl)	2.5 µl
5X BP Reaction Buffer	5.0 µl
BP Clonase™ enzyme mix	5.0 µl
TE Buffer, pH 8.0	add to a final volume of 20 µl
Final volume	25 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 20 µl reaction, add:

0.75 M NaCl	1.0 µl
Destination vector (150 ng/µl)	3.0 µl
LR Clonase™ enzyme mix	6.0 µl
Final volume	30 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 3 µl of proteinase K solution. Incubate at 37°C for 10 minutes.

7. Transform 100 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 100 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 which 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	50-60°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 16 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 (Invitrogen, Catalog no. 38042-024; use 15 units/µg of total DNA)
- TE Buffer, pH 8.0
- LR Clonase™ enzyme mix (Invitrogen, Catalog no. 11791-019; keep at -80°C until immediately before use)
- 5X LR Reaction Buffer (supplied with the LR Clonase™ enzyme mix)
- Proteinase K solution (supplied with the LR Clonase™; 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 (100-300 ng)	1-9 µl
Destination vector (300 ng)	1-9 µl
5X LR Reaction Buffer	4 µl
Topoisomerase I (15 units/µg total DNA)	0.6-2 µl
TE Buffer, pH 8.0	to 16 µl

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

   Reminder: Return LR Clonase™ enzyme mix to -80°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.
6. Add 2 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 Library Efficiency DH5α™ competent *E. coli*, follow the protocol on page 24.

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 wish 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™

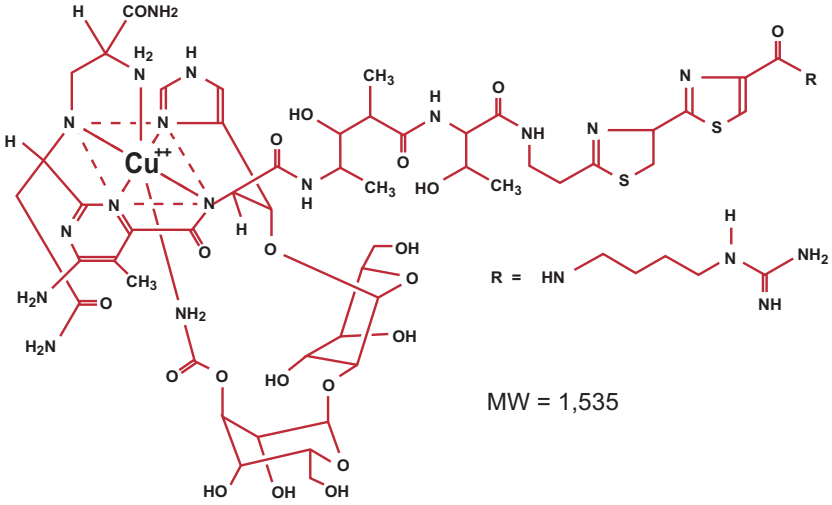
Introduction

Zeocin™ 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™ and inhibits its DNA strand cleavage activity. Expression of this protein in eukaryotic and prokaryotic hosts confers resistance to Zeocin™.

Molecular Weight, Formula, and Structure

The formula for Zeocin™ is $C_{60}H_{89}N_{21}O_{21}S_3$ and the molecular weight is 1,535. The diagram below shows the structure of Zeocin™.



Handling Zeocin™

- High ionic strength and acidity or basicity inhibit the activity of Zeocin™. 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 49 for a recipe).
- Store Zeocin™ at -20°C and thaw on ice before use.
- Zeocin™ 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™

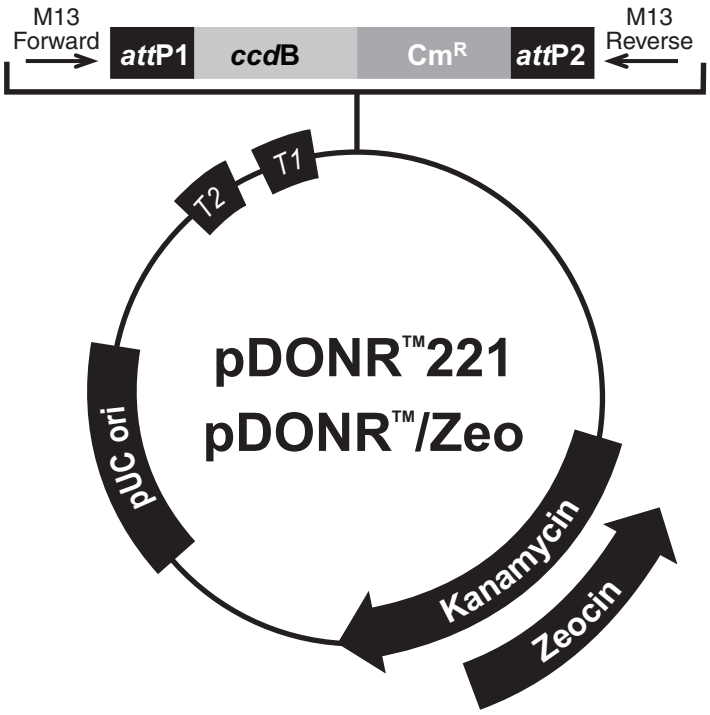
10 g Tryptone
5 g NaCl
5 g Yeast Extract

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™ on ice and vortex before removing an aliquot.
4. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 50 μg/ml final concentration.
5. Store plates at +4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

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

pDONR™221 and pDONR™/Zeo Map

The map below shows the elements of pDONR™221 and pDONR™/Zeo. **The complete sequences of pDONR™221 and pDONR™/Zeo are available from our Web site (www.invitrogen.com) or by contacting Technical Service (see page 52).**



Comments for:	pDONR™221 4762 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):	1847-2506	1847-2506
*att*P2 (c):	2754-2985	2754-2985
M13 Reverse priming site:	3027-3043	3027-3043
Kanamycin resistance gene:	3156-3965	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4086-4759	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 (4762 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*.

Technical Service

World Wide Web

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

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

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

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

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi
Tel: 81 3 3663 7972
Fax: 81 3 3663 8242
E-mail: jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail: eurotech@invitrogen.com

MSDS Requests

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

continued on next page

Technical Service, continued

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Introduction

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

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

The Gateway® Cloning Technology products and their use are the subject of one or more of U.S. Patent Nos. 5,888,732, 6,143,557, 6,171,861, 6,270,969, and 6,277,608 and/or other pending U.S. and foreign patent applications owned by Invitrogen Corporation. 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). No license is conveyed under the foregoing patents to use this product with any recombination sites other than those purchased from Invitrogen Corporation or its authorized distributor. 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 using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use 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. 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. Invitrogen 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 neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200.

continued on next page

Purchaser Notification, continued

Limited Use Label License No. 28: CMV Promoter

The use of the CMV promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned and licensed by the University of Iowa Research Foundation and is sold for research use only. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation (UIRF), 214 Technology Innovation Center, Iowa City, Iowa 52242. For further information, please contact the Associate Director of UIRF, at 319-335-4546.

Limited Use Label License No. 45: Chemically Competent *E. coli* Cells

This product is the subject of one or more of U.S. Patent No. 4,981,797 and foreign equivalents owned by Invitrogen Corporation. 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 buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use 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) to not 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. 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. Invitrogen 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 neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200. Fax (760) 602-6500.

Limited Use Label License No. 86: Zeocin™ Selection Marker

The ble resistance gene is the subject of one or more of U.S. Patent Nos. 5,021,344 and 5,118,620 and foreign equivalents owned by Cayla and licensed to Invitrogen. This product is sold for research purposes only. For commercial license information, please contact: Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008. Phone: 760-603-7200, Fax: 760-602-6500.

Product Qualification

Introduction	This section describes the criteria used to qualify the components of the PCR Cloning System with Gateway® Technology.
pDONR™ Vectors	The structure of each pDONR™ vector is verified by restriction enzyme digestion. In addition, the functionality of each vector is tested in a recombination assay using Gateway® BP Clonase™ enzyme mix. The *ccd*B gene is assayed by transformation using an appropriate *E. coli* strain.
Zeocin™	Zeocin™ is lot qualified by demonstrating the LB media containing 25 µg/ml Zeocin™ prevents growth of the TOP10 *E. coli* strain.
BP Clonase™ Enzyme Mix	Gateway® BP Clonase™ enzyme mix is functionally tested in a 1 hour recombination reaction followed by a transformation assay.
Chemically Competent *E. coli*	1. Library Efficiency® DH5α™ competent cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be greater than 1×10^8 cfu/µg plasmid DNA. 2. To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected. 3. Untransformed cells are plated on LB plates containing 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Glossary of Terms

***att*L, *att*R, *att*B, and *att*P**	The recombination sites from bacteriophage lambda that are utilized in the Gateway® Technology. • *att*L always recombines with *att*R in a reaction mediated by the LR Clonase™ 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 BP Clonase™ 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.
BP Clonase™ 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.
DB3.1™ 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**
LR Clonase™ Enzyme Mix	A proprietary mix of lambda recombination proteins that mediates the *att*L x *att*R recombination reaction.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

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

Bernard, P., Kezdy, K. E., Melderen, L. V., Steyaert, J., Wyns, L., Pato, M. L., Higgins, P. N., and Couturier, M. (1993). The F Plasmid CcdB Protein Induces Efficient ATP-dependent DNA Cleavage by Gyrase. J. Mol. Biol. *234*, 534-541.

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

Calmels, T., Parriche, M., Burand, H., and Tiraby, G. (1991). High Efficiency Transformation of *Tolypocladium geodes* Conidiospores to Phleomycin Resistance. Curr. Genet. *20*, 309-314.

Drocourt, D., Calmels, T. P. G., Reynes, J. P., Baron, M., and Tiraby, G. (1990). Cassettes of the *Streptoalloteichus hindustanus ble* Gene for Transformation of Lower and Higher Eukaryotes to Phleomycin Resistance. Nucleic Acids Res. *18*, 4009.

Gatignol, A., Baron, M., and Tiraby, G. (1987). Phleomycin Resistance Encoded by the *ble* Gene from Transposon Tn5 as a Dominant Selectable Marker in *Saccharomyces cerevisiae*. Molecular and General Genetics *207*, 342-348.

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

Kertbundit, S., Greve, H. d., Deboeck, F., Montagu, M. V., and Hernalsteens, J. P. (1991). *In vivo* Random b-glucuronidase Gene Fusions in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *88*, 5212-5216.

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

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

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

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

Miki, T., Park, J. A., Nagao, K., Murayama, N., and Horiuchi, T. (1992). Control of Segregation of Chromosomal DNA by Sex Factor F in *Escherichia coli*. Mutants of DNA Gyrase Subunit A Suppress *let*D (*ccd*B) Product Growth Inhibition. J. Mol. Biol. *225*, 39-52.

Mulsant, P., Tiraby, G., Kallerhoff, J., and Perret, J. (1988). Phleomycin Resistance as a Dominant Selectable Marker in CHO Cells. Somat. Cell Mol. Genet. *14*, 243-252.

continued on next page

References, continued

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

Perez, P., Tiraby, G., Kallerhoff, J., and Perret, J. (1989). Phleomycin Resistance as a Dominant Selectable Marker for Plant Cell Transformation. Plant Mol. Biol. *13*, 365-373.

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

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

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

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

Weisberg, R. A., and Landy, A. (1983) Site-Specific Recombination in Phage Lambda. In *Lambda II*, R. A. Weisberg, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Press), pp. 211-250.