GIBCO BRL®

Instruction Manual

GATEWAY™ Cloning Technology

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LIFE TECHNOLOGIES®

Essential Technologies for the Science of Life™

Choosing Products to Build GATEWAY™ Expression Clones

Step 1: Construct or Select an Entry Clone starting from:

PCR Fragment

Design primers with *att*B sites
Amplify PCR product

↓

Clone *att*B-PCR product into pDONR™201 with BP CLONASE™ Enzyme Mix

PCR Cloning System (BP CLONASE Mix included) Cat. No. 11821-014

↓

Analyze / Propagate Entry Clone

Restriction Fragment

Choose Entry Vector

Individual Entry Vectors Cat. Nos. 11813-011; 11816-014; 11817-012; 11818-010; 11819-018

↓

Ligation of restriction fragment into Entry Vector

↓

Analyze / Propagate Entry Clone

Library Screening

Isolate clone from pCMV•SPORT 6; pSPORT-P; or pEXP-AD502 library

See Catalog or web site for list of GATEWAY Libraries and Custom Libraries

↓

Transfer insert with BP CLONASE Mix into pDONR201

BP CLONASE Cat. No. 11789-013
pDONR201 Cat. No. 11798-014

↓

Analyze / Propagate Entry Clone

Step 2: Construct an Expression Clone

A. Choose or Construct your Destination Vector

Destination Vector **OR** Use Your Own Vector

E. coli

Native: pDEST™14
N-GST: pDEST15
N-His: pDEST17

E. Coli Expression System with BL21-SI™Cells Cat. No. 11823-010 (LR CLONASE Mix included)**

Mammalian

Native: pDEST12.2
N-GST: pDEST27
N-His: pDEST26

Mammalian Expression System

Cat. No. 11826-013 (LR CLONASE Mix included)

Baculovirus

Native: pDEST8
N-GST: pDEST20
N-His: pDEST10

Baculovirus Expression System*

Cat. No. 11827-011 (LR CLONASE Mix included)

Use Your Own Vector

Convert existing vector by cloning Conversion Cassette into MCS

GATEWAY Vector Conversion System Cat. No. 11828-019

Choose a complete Expression System(s) **OR** purchase **Destination Vector(s) individually**

B. Transfer gene from Entry Clone into Destination Vector with LR CLONASE Enzyme Mix to make Expression Clone

LR CLONASE Enzyme Mix Cat. No. 11791-019

↓

Analyze / Propagate Expression Clone - Express Protein

*Baculovirus Expression Systems provide components to construct a transfer vector. User must also purchase **MAX EFFICIENCY® DH10BAC™ Competent Cells, Cat. No. 10361-012,** and **CELLFECTIN® Reagent, Cat. No. 10362-010,** included in BAC-TO-BAC® Baculovirus Expression System.

A second *E. Coli* Expression System is available with DH5α™ competent cells (Cat. No. 11822-012**), suitable for construction of Expression Clone but not for protein expression with pDEST 14, 15, 17.

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Notices to Customer

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Ni-NTA resin may be purchased from QIAGEN, Inc., 9600 De Soto Ave., Chatsworth, CA 91311. (800-426-8157).

2

Overview

cDNAs, partial or complete genes, or genomic DNA (including non-coding sequences) can be transferred from Entry Clones to Destination Vectors using the GATEWAY Cloning System. In the following discussion, "gene" is meant to include all types of DNA sequences.

GATEWAY™ Cloning Technology is a novel universal system for cloning and subcloning DNA sequences, facilitating gene functional analysis, and protein expression (Figure 1). Once in this versatile operating system, DNA segments are transferred between vectors using site-specific recombination. This powerful system can easily transfer one or more DNA sequences into multiple vectors in parallel reactions, while maintaining orientation and reading frame.

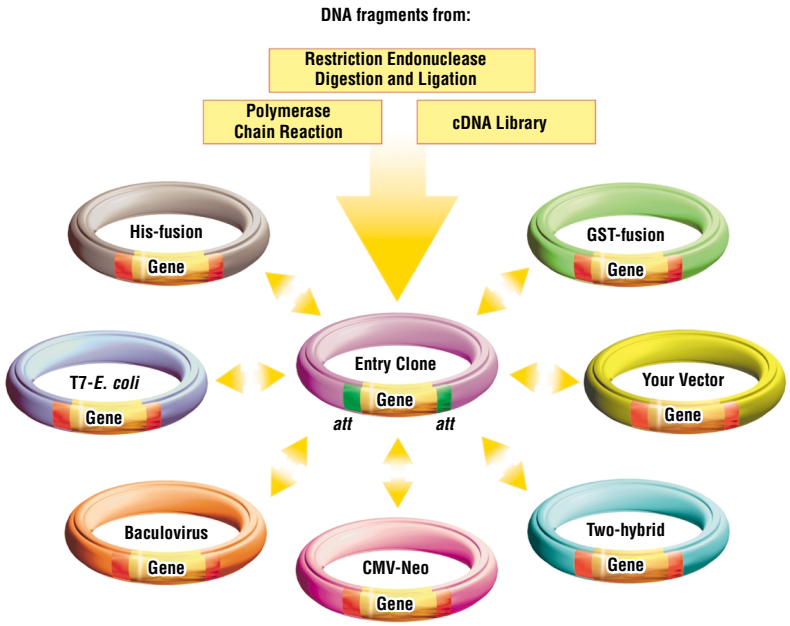

Figure 1. The power of GATEWAY Cloning Technology. The gene of interest can be moved into an Entry Vector via PCR, restriction endonuclease digestion and ligation, or site-specific recombination from a cDNA library constructed in a GATEWAY-compatible vector. A gene in the Entry Clone can then be transferred simultaneously into Destination Vectors. This is done by combining the Entry Clone with a GATEWAY Destination Vector and CLONASE Enzyme Mix in a single tube, incubating for 1 h, transforming *E. coli*, and plating.

The GATEWAY Cloning System uses phage lambda-based site-specific recombination instead of restriction endonucleases and ligase. This recombination system is used by λ during the switch between the lytic and lysogenic pathways (1). The key DNA recombination sequences (*att* sites) and proteins that mediate the recombination reactions are the foundation of GATEWAY Cloning Technology. For a general review of λ recombination, see reference 2.

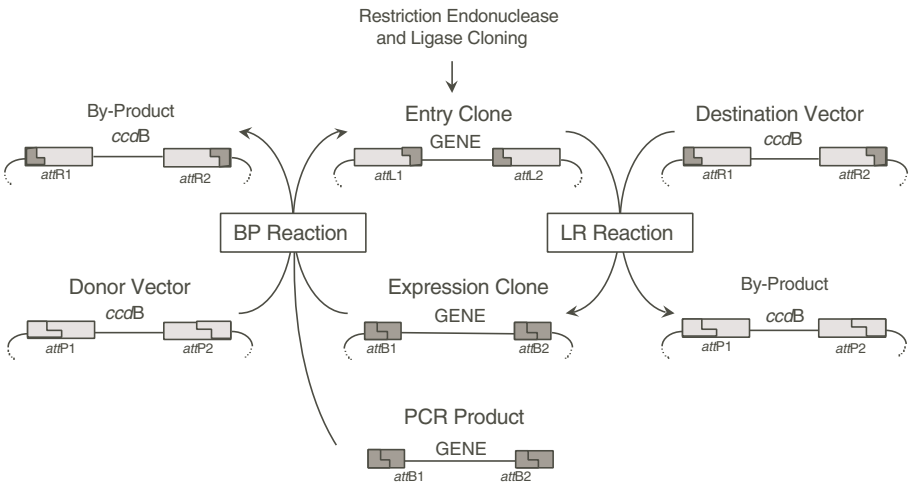

Figure 2. GATEWAY Cloning Technology as an operating system for cloning and subcloning DNA. Genes are transferred between vectors easily using LR or BP reactions.

2.1 Recombination Reactions of the GATEWAY Cloning System

Two reactions constitute the GATEWAY Cloning Technology (Figure 2, Table 1). The LR Reaction is a recombination reaction between an Entry Clone and a Destination (pDEST™) Vector, mediated by a cocktail of recombination proteins, to create an Expression Clone. It is used to move the sequence of interest to one or more Destination Vectors in parallel reactions. The BP Reaction is a recombination reaction between an Expression Clone (or an *att*B-flanked PCR product) and a Donor (pDONR™) Vector to create an Entry Clone.

Table 1. Summary of reactions and nomenclature.

Reaction	Reacting Sites	Catalyzed by	Product	Structure of Product
LR Reaction	*att*L x *att*R	LR CLONASE™ Enzyme Mix	Expression Clone	*att*B1-gene-*att*B2
BP Reaction	*att*B x *att*P	BP CLONASE Enzyme Mix	Entry Clone	*att*L1-gene-*att*L2

The recombination reactions are equivalent to concerted, highly specific, cutting and ligation reactions. The reactions are conservative, *i.e.*, there is no net synthesis or loss of nucleotides. The DNA segments that flank the recombination sites are merely switched. The recombination (*att*) sites of each vector comprise a hybrid sequence, donated by the sites on the parental vectors. The recombination can occur between DNAs of any topology (supercoiled, linear, or relaxed), although efficiency varies.

2.1.1 The GATEWAY LR Cloning Reaction

The LR reaction is used to create an Expression Clone (Figure 3). The recombination proteins cut to the left and right of the gene within the *att*L sites in the Entry Clone and ligate it to the corresponding *att*R site in the Destination Vector, creating an Expression Clone. The resultant 25-bp *att*B sites [*att*B1 on the left (N-

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terminus) and *att*B2 on the right (C-terminus)] created by the LR reaction are derived from the *att*L sites (adjacent to the gene), whereas the distal sequences are derived from the *att*R sites. The LR CLONASE Enzyme Mix mediates the GATEWAY LR Reaction and contains λ recombination proteins Int, Xis, and the *E. coli*-encoded protein IHF.

The wild type λ *att*L and *att*R recombination sites have been modified in the following manner to improve the GATEWAY 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 (*i.e.*, *att*L1 reacts only with *att*R1, *att*L2 reacts only with *att*R2). The *att*L sites are 100 bp.
- A part (43 bp) of *att*R has been removed to make the *in vitro att*L × *att*R reaction irreversible and more efficient (3). The *att*R sites in the Destination Vectors are 125 bp.

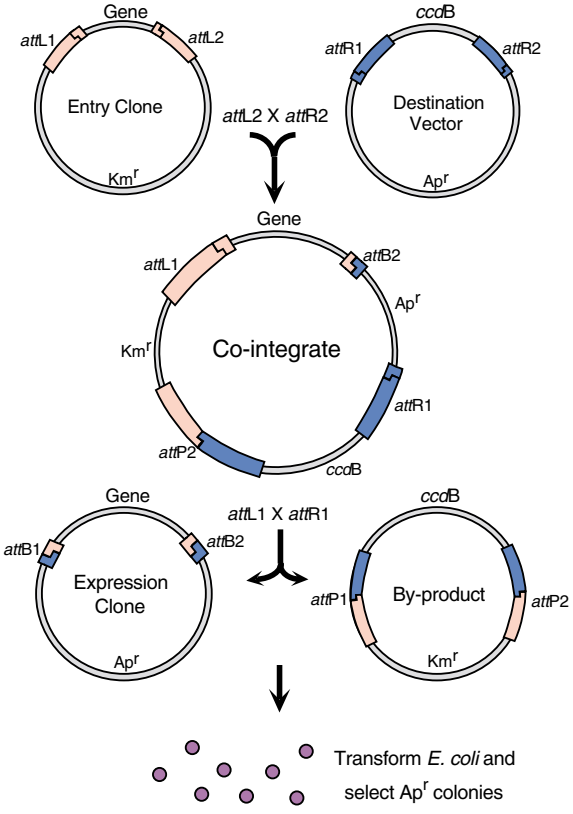


Figure 3. The LR reaction. *att*L1 and *att*R1 (or *att*L2 and *att*R2) recombine to form a co-integrate. The co-integrate resolves to form two daughter molecules by a second recombination reaction. The two daughter molecules have the same structure regardless of which pair of sites, *att*L1 and *att*R1 or *att*L2 and *att*R2, react first to form the co-integrate. Selection of the Expression Clone is achieved by introduction of the mixture into *E. coli* by transformation. Only plasmids without the *ccd*B gene that are ampicillin-resistant (Ap^r) yield colonies.

2.1.2 The GATEWAY BP Cloning Reaction

The BP reaction is used to create an Entry Clone from Expression Clones (Figure 4) or PCR products (Figure 6). Once a gene is flanked by *att*L sites (Entry Clone), it can be transferred into any number of Destination Vectors to generate new Expression Clones. The BP CLONASE Enzyme Mix mediates the BP Reaction and contains λ recombination protein Int and the *E. coli*-encoded protein IHF.

The wild type λ *att*B and *att*P recombination sites have been modified to improve the GATEWAY 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 (*i.e.*, *att*B1 reacts only with *att*P1, *att*B2 reacts only with *att*P2). The *att*P sites are 200 bp.
- 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.*, in phagemid ssDNA or in mRNA. The *att*B sites are 25 bp.

The BP Reaction permits rapid, directional cloning of PCR products synthesized with primers containing terminal 25-bp *att*B sites (+4 Gs). The result is an Entry Clone containing the PCR fragment (Figure 6). Similarly, DNA segments flanked by *att*B sites in Expression Clones can be transferred to generate Entry Clones (Figure 4).

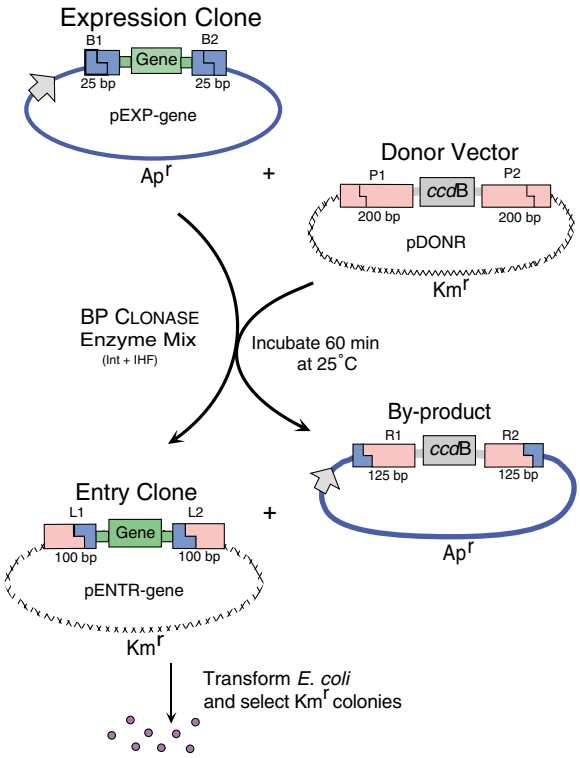


Figure 4. The BP cloning reaction. Only plasmids without the *ccd*B gene that are kanamycin resistant (Km^r) yield colonies.

Overview

2.2 Generating Entry Clones

The design of an Entry Clone is dictated by the particular DNA and what is to be done with it. Because the DNA sequence between the *att*L sites transfers as a unit, all the sequences included between these sites transfer into the Destination Vectors. A variety of Destination Vectors (permitting native or fusion protein expression) can be used, making the choice of whether to include translation start and stop signals an important decision in the planning of Entry Clones. For example, expression of native proteins requires that translation initiation signals (ribosome recognition site and ATG) be included between the *att*L1 sites, whereas Entry Clones used to make N-terminal fusion proteins typically lack these elements since they are donated by the Destination Vector. Note that Entry Clones used to transfer DNA into Destination Vectors for expression require that the encoded N-terminus be oriented proximal to the *att*L1 site. For a more thorough discussion see Section 1.3.

Entry Clones can be made in one of several ways (Figure 5).

- A PCR product made with modified primers can be used to generate and Entry Clone using the BP reaction (Figure 6). Primers consist of the structure GGGG[25 bp *att*B][gene-specific sequence] (see Section 2.3.3).
- An Expression Clone (generated by the LR reaction) can be converted to an Entry Clone using the BP reaction (Figure 4). In addition, clones from cDNA libraries made in vectors in which *att*B sites flank the cDNA (such as pCMV•SPORT6 or pEXP-AD502) can be transferred to generate an Entry Clone.

Considerations in Designing an Entry Clone

The DNA:

—Does it contain a gene?

—Is the sequence known?

—Is the reading frame known?

—Are there 5′ and 3′ untranslated regions?

—Do these regions contain stop codons?

—Does the gene fragment carry its own promoter and/or translation signals?

—Is the DNA a restriction fragment, or a PCR product?

—Are there unique restriction endonuclease sites at the amino and carboxy ends?

How is the gene to be expressed?

—In eukaryotes or in *E. coli*?

—As native protein, or as a fusion protein?

—With or without a protease cleavage site?

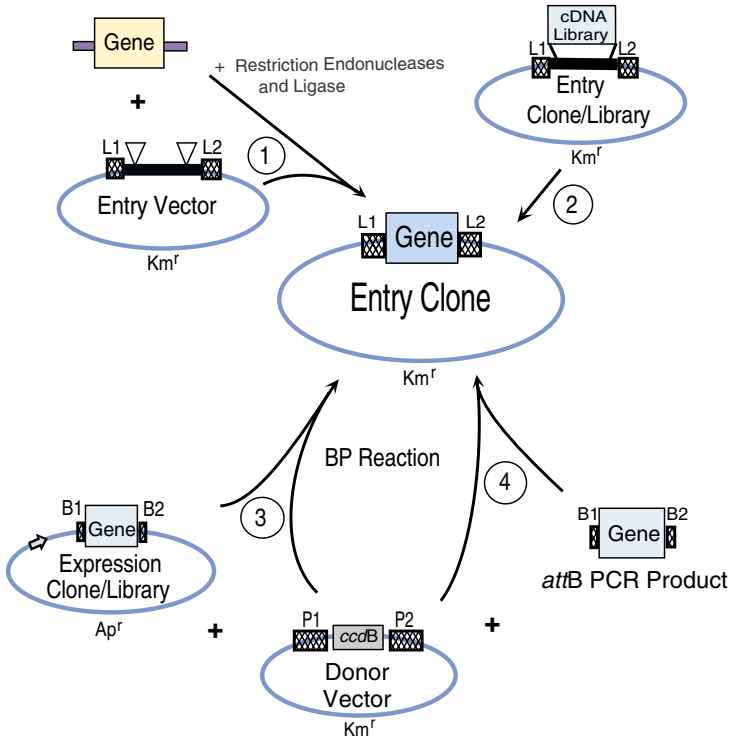


Figure 5. Ways to make Entry Clones. Approaches 3 and 4 utilize recombination with a Donor Vector that provides the Entry Vector backbone carrying Km^r.

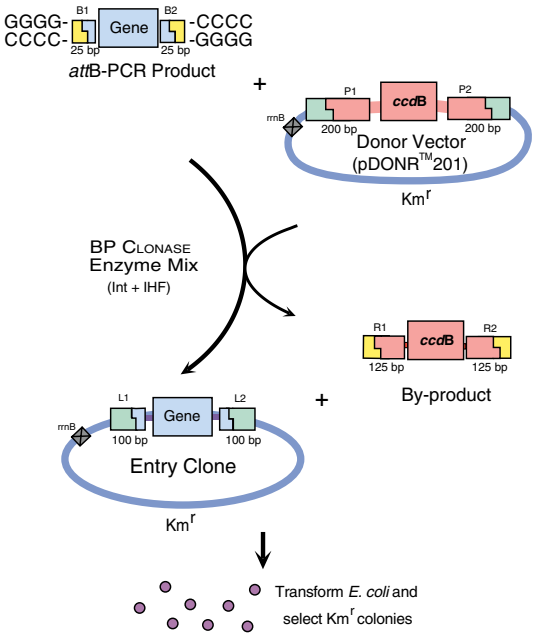


Figure 6. Cloning a PCR product by the BP Reaction.

- A gene can be cloned between the *att*L1 and *att*L2 sites of an Entry Vector using restriction endonucleases and ligase. The starting DNA segment can be generated by restriction digestion or as a PCR product containing restriction sites on the ends. Several Entry Vectors are available (Figure 7, Table 2). These differ as to the translation signals and multiple cloning sites (MCS) available. Detailed vector maps can be found in Section 5.6. Note: Entry Clones made in pENTR1A, 2B, 3C, 4 or 11 are transcriptionally silent and can not be screened with antibodies.

2.3 Designing Entry Clones for Protein Expression

Protein expression consists of transcription (DNA into RNA) and translation (RNA into protein). (For information on protein synthesis see references 4-7.) Both have signal sequences that determine the start sites. In GATEWAY Technology, the promoters typically are provided on the Destination Vectors outside of the *att* sites. The translational start site for nearly all proteins is the AUG (methionine) codon. Ribosomes must be able to distinguish between AUG codons in the middle of proteins from those at the start. Most often ribosomes choose an AUG that is first in the RNA (toward the 5′ end) following the proper sequence context. In *E. coli*, the favored context (8) is a run of purines (As and Gs) from 5 to 12 bases upstream of the initiating AUG, especially AGGAGG or some variant (known as a Shine-Dalgarno sequence). In eukaryotes, the preferred sequence context is --GCC ACC ATG G-- around the initiating methionine, with the A at -3 being most important, and a purine at +4 (where the A of the ATG is +1), preferably a guanine (G), being next most influential (9). Having an A at -3 is enough to make most ribosomes choose the first AUG of an mRNA in plants, insects, yeast, and mammals (known as a Kozak sequence). Shine-Dalgarno and Kozak sequences are referred to here as ribosome recognition sequences (RRS). For a review of initiation of protein synthesis in eukaryotic cells, see reference 10.

Table 2. Entry Vectors. All Entry Vectors carry the kanamycin resistance gene.

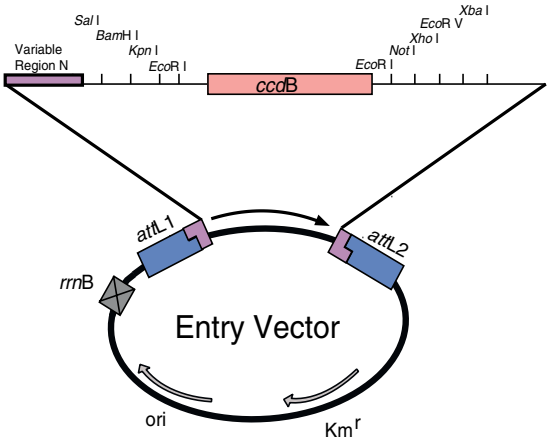
Vector	Cloning Features	Shine-Dalgarno	Kozak	Expression Features
pENTR1A (reading frame 0)	Represent the 3 reading frames for N-terminal fusions.			N-terminal or C-terminal fusions in *E. coli* or eukaryotic cells.
pENTR2B (reading frame+1)	Multiple cloning sites (MCS) immediately follows *att*L1.			Native expression and C-terminal fusions require addition of ribosome recognition sequence and ATG translation initiation codon.
pENTR3C (reading frame+2)	First restriction endonuclease site after *att*L1 yields blunt ends.			C-terminal fusions require that no stop codons precede *att*L2.
pENTR4	Same as pENTR1A, except that the first restriction endonuclease site after *att*L1 is *Nco* I.		•	
pENTR11	*E. coli* and eukaryotic ribosome binding sites (Shine-Dalgarno and Kozak) downstream of *att*L1. Blunt (*Xmn* I) and *Nco* I sites each preceded by Shine-Dalgarno and Kozak.	•	•	Native, N-terminal or C-terminal fusions in *E. coli* or eukaryotic cells. (ATG also needed for native and C-terminal.) C-terminal fusions require that no stop codons precede *att*L2.

In GATEWAY Cloning, the placement of translation signals is determined by whether the protein being expressed is native, or a fusion protein (Figure 8). For native proteins and C-terminal fusions, the translation signals are included downstream of the *att*B1 site. Therefore, these signals must be present in the Entry Clone. In this case the *att*B1 sequence will reside in the 5' untranslated region of the mRNA. (Note: For C-terminal fusions, the stop codon is provided by the Destination Vector and must be absent from the 3'-end of the gene.) In N-terminal or N+C-terminal fusions, the translational signals and the fusion protein sequences are provided by the Destination Vector and will be upstream of the *att*B1 site. Consequently, the 25-bp *att*B1 site becomes part of the coding sequence and inserts 8 amino acids between the fusion domain and the protein encoded by a gene. The *att*B1 sequence has not been observed to affect protein yield in *E. coli*, insect, or mammalian cells.

2.3.1 Location of Translation Start Sequences

For native protein expression, the RRS and the ATG needs to be downstream of the *att*L1 site in the Entry Clone. If the Destination Vector provides a promoter without any N-terminal fusion sequence, protein synthesis will initiate exclusively at the translation start signals of the native open reading frame (ORF).

An Entry Clone containing the RRS and ATG downstream of the *att*L site can be used with a Destination Vector providing an N-terminal fusion peptide if the ATG is in frame with the *att* site. However, protein synthesis will result in production of both N-terminal fusion protein plus some native protein. Even though ribosomes most often initiate protein synthesis at the 5'-most ATG, internal ATGs can serve to initiate protein synthesis. The better the translation context around the internal ATG, the more internal initiation of translation will be seen. Also, the production of native protein can be more pronounced with short N-terminal fusion tags, such as the 6X histidine affinity tag. If the amount of native protein is large or interferes with your applications, construction of different Entry Clones to express native protein may be necessary.



Variable Region N Options:

- Blunt sites [(*Dra* I, *Xmn* I; pENTR1A,3C) (*Ehe* I, *Xmn* I; pENTR2B)] close to *att*L1 site, in the 3 reading frames
- *Nco* I site close to *att*L1 site (pENTR4)
- Blunt (*Xmn* I) and *Nco* I sites each preceded by *E. coli* and eukaryotic ribosome recognition site (pENTR11)

Figure 7. Schematic of Entry Vectors. The *rrn*B transcriptional terminator sequence (11) makes clones transcriptionally silent in contrast to standard *lac* promoter systems. The *ccd*B gene inhibits growth in most *E. coli* strains which facilitates recovery of only the desired clones. The *Xmn* I site has 4 of the 6 most favored bases for the Kozak sequence involved in eukaryotic expression. The restriction sites shown on the figure are in all Entry Vectors. Unique enzymes in the variable Region N are shown below the circle map.

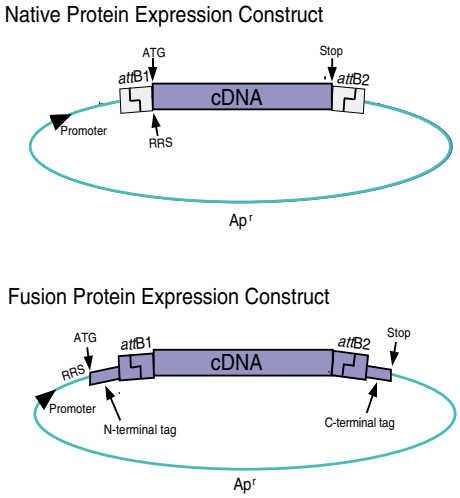


Figure 8. GATEWAY Protein Expression Clones. RRS refers to a ribosome recognition sequence.

2.3.2 Reading Frame

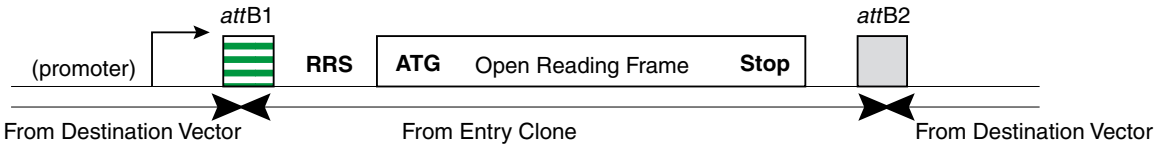
For native expression, reading frame is determined by the translation start site located between the two *att* sites so the reading frame in the Entry Clone relative to that of *att*B1 or *att*B2 is not typically an issue (see example below). For fusion proteins, it is essential to establish the correct reading frame. For N-terminal fusions, construct the Entry Clone so that the DNA sequence is in frame with the two lysine codons (AAA - AAA) found in *att*L1 (or *att*B1 for PCR primer design). For C-terminal fusions, construct Entry Clones so that the DNA sequence is in frame with the Tyr-Lys (TAC-AAA) in *att*L2. (See below and the Entry Vector maps in Section 5) Destination Vectors that make amino-terminal fusions have been constructed with the *att*R1 site in this (AAA - AAA) reading frame, so amino terminal fusions will automatically be correctly phased, for N-terminal fusion tags. For C-terminal fusion Destination Vectors, align the fusion in phase with the -TAC-AAA- (Tyr-Lys) sequence so C-terminal fusions will automatically be in frame.

2.3.3 Examples of Protein Expression Constructs

The following examples of Expression Clone sequences and *att*B-PCR primer sequences (for preparing Entry Clones) have been used successfully to express both native and fusion proteins in *E. coli*, insect, and mammalian cells using GATEWAY Cloning. Other sequence options and motif combinations are possible, and may be preferable in some situations. These examples are a starting point for recombinant protein expression in the GATEWAY Cloning System.

Native Expression

A. Expression clone structure:



B. Expression clone sequence (for *E. coli* and eukaryotic expression):

Shine-Dalgarno Kozak Open reading frame (amino end)

5' - ACA AGT TTG TAC **AAA AAA** GCA GGC TTC GAA GGA GAT AGA ACC **ATG*** NNN NNN NNN ---

3' - TGT TCA AAC ATG **TTT TTT** CGT CCG AAG GTT CCT CTA TCT TGG TAC NNN NNN NNN ---

*att*B1 *Translation start

Open reading frame (carboxy end)

--- NNN NNN NNN **TAG** GAC CCA GCT TTC TTG **TAC AAA** GTG GT - 3'

--- NNN NNN NNN ATC CTG GGT CGA AAG AAC **ATG TTT** CAC CA - 5'

Translation stop (TAG) *att*B2

Note: The ATG in this example is in frame with the *att*B1sequence so this construction can be used in both native and N-terminal fusion Destination Vectors.

C. Oligonucleotides for *att*B-PCR cloning of gene for native expression:

*att*B1 forward oligo: (*att*B1 sequence bold; translation start codon underlined; sequence includes Shine-Dalgarno and Kozak)

*att*B1 Shine-Dalgarno Kozak

5' - GGGG **ACAAGTTTGTACAAAAAAGCAGGCT** TCGAAGGAGATAGAACCATG (18-25 gene-specific nucleotides in frame with start codon) - 3'

*att*B2 reverse oligo: (*att*B2 sequence bold; translation stop codon [complement strand] underlined)

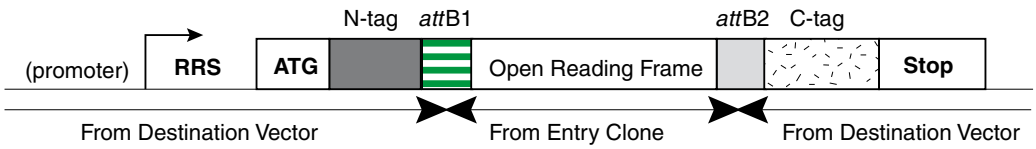
*att*B2 STOP

5' - GGGG **ACCACTTTGTACAAGAAAGCTGGGT** CCTA(18-25 gene-specific nucleotides [complement strand] in frame with stop codon) - 3'

Note: See section 5.2 for adapter-primer method primer design.

Fusion Protein Expression

A. Expression clone structure:



Note: Here the Destination Vector provides the ATG and Stop codon.

B. Expression clone sequence:

Open reading frame (amino end)

5' - ATG NNN --- --- NNN ACA AGT TTG TAC **AAA AAA** GCA GGC TTC NNN NNN NNN ---
3' - TAC NNN --- --- NNN TGT TCA AAC ATG **TTT TTT** CGT CCG AAG NNN NNN NNN ---

N-fusion *att*B1

Open reading frame (carboxy end)

--- NNN NNN NNN GAC CCA GCT TTC TTG **TAC AAA** GTG GTN NNN --- --- NNN (Stop) - 3'
--- NNN NNN NNN CTG GGT CGA AAG AAC **ATG TTT** CAC CAN NNN --- --- NNN NNN - 5'

*att*B2 C-fusion

C. Suggested oligonucleotides for *att*B-PCR cloning of gene for N-terminal and C-terminal fusion expression:

*att*B1 forward oligo: (*att*B1 sequence bold)

*att*B1

5' - GGGG **ACAAGTTTGTACAAAAAAGCAGGCT** TC^{+} (18-25 gene-specific nucleotides in frame with *att*B1) - 3'

*att*B2 reverse oligo: (*att*B2 sequence bold)

*att*B2

5' - GGGG **ACCACTTTGTACAAGAAAGCTGGGT** C^{+} (18-25 gene-specific nucleotides [complement strand] in frame with *att*B2) - 3'

+ Other nucleotides may be substituted for the underlined sequences. For *att*B1, maintain the reading frame and do not create a stop codon. For N-terminal fusion proteins, the *att*B2 primer **must** contain a stop codon in the gene-specific region. For C-terminal or N-terminal plus C-terminal fusion proteins, the *att*B2 primer **must not** contain any in-frame stop codons.

2.4 Destination Vectors

Once a gene is configured as an Entry Clone, it can easily be moved into any Destination Vector using the LR Reaction. The currently available Destination Vectors concentrate on protein expression applications (Table 3). However, it is possible to convert any vector (for maximal compatibility, the Destination Vector should not be kanamycin-resistant) into a GATEWAY Destination Vector using the GATEWAY Vector Conversion System. To convert a vector, a DNA cassette (Figure 10) containing the *ccd*B gene and a chloramphenicol resistance gene flanked by *att*R sites is cloned into your vector (at the multiple cloning site) using restriction endonucleases that generate blunt ends and ligase. The *ccd*B protein interferes with *E. coli* DNA gyrase and thereby inhibits growth of most *E. coli* strains. Since the Destination Vector contains the *ccd*B gene, it must be propagated in the *E. coli* DB3.1 strain (parent strain RR1) containing a gyrase mutation (*gyr*A462) (12-14) Strains of *E. coli* that contain an F' episome also carry the *ccd*A gene which is

Overview

an antidote to *ccd*B protein toxicity. Therefore, do not use strains with F episomes for selection following BP or LR Reactions.

DB3.1 strain genotype:	F^- *gyr*A462 *end*A1 D(*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 λ^- *leu mtl*-1.

Table 3. Destination Vectors. See vector maps (in section 5).

Vector	Expression Host	Protein Expressed	His6 Affinity Tag	GST Affinity Tag	TEV Protease Cleavage Site	Promoter for Expression	Comments
pDEST14	*E. coli*	Native				T7 (*E. coli* strain must express T7 RNA polymerase)	pBR ori to aid in regulation of expression
pDEST15	*E. coli*	N-terminal fusion		•		T7 (*E. coli* strain must express T7 RNA polymerase)	pBR ori to aid in regulation of expression
pDEST17	*E. coli*	N-terminal fusion	•			T7 (*E. coli* strain must express T7 RNA polymerase)	pBR ori to aid in regulation of expression
pDEST8	Insect	Native				polyhedrin (baculovirus)	
pDEST10	Insect	N-terminal fusion	•		•	polyhedrin (baculovirus)	
pDEST20	Insect	Native fusion		•		polyhedrin (baculovirus)	
pDEST12.2	Mammalian	Native				CMV	neo resistant
pDEST26	Mammalian	N-terminal fusion	•			CMV	neo resistant
pDEST27	Mammalian	N-terminal fusion		•		CMV	neo resistant

More Destination Vectors will be available soon. Refer to the GATEWAY website for the most up-to-date listing (www.lifetech.com/gateway).

2.5 GATEWAY Nomenclature

For subclones, the following naming convention has been adopted: the name of the vector is placed first, followed by the name of the transferred gene.

Plasmid Type	Descriptive Name	Individual Vector or Clone Names
*att*L Vector	Entry Vector	pENTR1,2,...
*att*L subclone	Entry Clone	pENTR3-gus, .. ; pENTR201-gus The number 3 refers to the Entry Vector. 201 refers to the Donor Vector used to make the Entry Clone. Gus is the subcloned gene.
*att*R Vector	Destination Vector	pDEST1,2,3,...
*att*B Vector	Expression Vector	pEXP501, 502,… (nos. 501-599). These vectors are used to prepare Expression cDNA libraries. Other nomenclature has also been used for cDNA libraries, *e.g.*, pCMV•SPORT6.
*att*B subclone	Expression Clone	pEXP3-cat,... 3 refers to the Destination Vector (pDEST3) used to make the expression subclone. Cat is the subcloned gene.
*att*P Vector	Donor Vector	pDONR™201

Examples:

An LR Reaction:

pENTR201-tet x pDEST10 → pEXP10-tet

Two BP Reactions:

*att*B-p53 PCR product x pDONR207 → pENTR207-p53

pEXP14-*lac*Z x pDONR201 → pENTR201-*lac*Z

3 Methods

See www.lifetech.com/gateway for current information on additions/modifications to the protocols and an increasing selection of GATEWAY™-compatible vectors and libraries.

3.1 Components

GATEWAY Cloning Technology is the basis for several systems whose components are listed below. Most of the components are also available separately (see section 7). Store the BP CLONASE™ Enzyme Mix, LR CLONASE Enzyme Mix and the competent cells at -70°C. All other components can be stored at -20°C or -70°C.

PCR Cloning System with GATEWAY Technology

(Cat. No. 11821-014; Size: 20 reactions)

Component	Amount
BP CLONASE Enzyme Mix	80 µl
BP CLONASE Reaction Buffer	100 µl
GATEWAY pDONR™201 Vector (150 ng/µl)	40 µl
pEXP7-tet Positive Control (50 ng/µl)	20 µl
proteinase K solution (2 µg/µl)	40 µl
30% PEG/Mg Solution	1 ml
LIBRARY EFFICIENCY® DH5α™ Competent Cells	1 ml
pUC19 DNA	100 µl
manual	one

E. coli Expression Systems with GATEWAY Technology (Size: 20 reactions)

Component	Amount
LR CLONASE Enzyme Mix	80 µl
LR CLONASE Reaction Buffer	100 µl
GATEWAY pDEST™14 Vector (150 ng/µl)	40 µl
GATEWAY pDEST15 Vector (150 ng/µl)	40 µl
GATEWAY pDEST17 Vector (150 ng/µ)	40 µl
pENTR™-gus Positive Control (50 ng/µl)	20 µl
proteinase K solution (2 µg/µl)	40 µl
LIBRARY EFFICIENCY DH5α Competent Cells*	1 ml
BL21-SI™ Competent Cells**	1 ml
pUC19 DNA	100 µl
manual	one

*Included with Cat. No. 11822-012. See section 3.5.1.

**Included with Cat. No. 11823-010. See section 3.5.1.

Baculovirus Expression System with GATEWAY Technology

(Cat. No. 11827-011; Size: 20 reactions)

(Designed for use with the BAC-TO-BAC® technology.)

Component	Amount
LR CLONASE Enzyme Mix	80 µl
LR CLONASE Reaction Buffer	100 µl
GATEWAY pDEST8 Vector (150 ng/µl)	40 µl
GATEWAY pDEST10 Vector (150 ng/µl)	40 µl
GATEWAY pDEST20 Vector (150 ng/µl)	40 µl
pENTR-gus Positive Control (50 ng/µl)	20 µl
proteinase K solution (2 µg/µl)	40 µl
LIBRARY EFFICIENCY DH5α Competent Cells	1 ml
pUC19 DNA	100 µl
manual	one

Refer to section 3.5.1 for additional materials required for protein expression in insect cells.

Mammalian Expression System with GATEWAY Technology

(Cat. No. 11826-013; Size: 20 reactions)

Component	Amount
LR CLONASE Enzyme Mix	80 µl
LR CLONASE Reaction Buffer	100 µl
GATEWAY pDEST12.2 Vector (150 ng/µl)	40 µl
GATEWAY pDEST26 Vector (150 ng/µl)	40 µl
GATEWAY pDEST27 Vector (150 ng/µl)	40 µl
pENTR-gus Positive Control (50 ng/µl)	20 µl
proteinase K solution (2 µg/µl)	40 µl
LIBRARY EFFICIENCY DH5α Competent Cells	1 ml
pUC19 DNA	100 µl
manual	one

3.2 Creating Entry Clones Using Restriction Endonucleases and Ligase

Note: When digesting with two separate restriction endonucleases, the most rigorous procedure is to perform the digests one at a time in the recommended REACT® Buffer and purify the DNA before the second digest. When performing a double digest, choose the buffer that has 100% activity for each enzyme. If no single buffer fulfills these requirements, then choose a buffer that ensures the highest activity possible without causing nonspecific cleavage.

Alternatively, perform a sequential digest by using the restriction endonuclease that requires the lowest salt conditions first. If the enzyme can be heat-inactivated, stop the first reaction by heating for 10 min at 65°C. Adjust the salt with minimal increase in volume to approximate the optimal conditions for the second enzyme. Keep the glycerol concentration ≤5% when both enzymes are present.

Materials:

- Entry Vector
- Restriction endonucleases and buffers
- Calf intestinal alkaline phosphatase
- T4 DNA ligase and buffer
- LIBRARY EFFICIENCY DH5α competent cells
- S.O.C. Medium
- LB plates with 50 µg/ml kanamycin
- TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]
- CONCERT™ Gel Extraction System (or equivalent)
- Prepared DNA restriction fragment(s)

3.2.1 Preparing the Entry Vector

It is necessary to restriction digest the Entry Vector on each side of the *ccd*B gene to remove the gene during cloning. It is recommended that the Entry Vector be dephosphorylated and gel purified after restriction digestion so that there is less competition between the *ccd*B fragment and the DNA of interest for the Entry Vector during ligation.

Methods

1. Digest 1 μg of the Entry Vector with the selected restriction endonucleases.
2. Ethanol precipitate the DNA by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
3. Dephosphorylate the Entry Vector DNA.
   a. Determine the mass of DNA required for 1 pmol of the type of DNA 5′ end.
   b. To a 1.5-ml microcentrifuge tube, add 4 μl of calf intestinal alkaline phosphatase (CIAP) 10X Buffer [500 mM Tris-HCl (pH 8.5), 1 mM EDTA] and 1 pmol of DNA ends.
   c. Add autoclaved, distilled water to 39 μl.
   d. Dilute CIAP in dilution buffer such that 1 μl contains the amount of enzyme required for the appropriate 5′ end (*i.e.*, 1 unit for 5′-recessed and blunt ends and 0.01 units for a 5′ overhang).
   e. For 5′-recessed and blunt-ended DNA, incubate at 50°C for 60 min. For DNA with a 5′ overhang, incubate at 37°C for 30 min.
   f. Heat-inactivate CIAP at 65°C for 15 min.
4. Purify the DNA fragment by agarose gel electrophoresis and extract the DNA from the gel with a silica-based system like CONCERT™ Gel Extraction System (15) (optional).

3.2.2 Preparing the Insert DNA

Restriction endonuclease fragments, cDNA, or PCR products can be cloned into Entry Vectors using restriction endonucleases and ligase.

A. Restriction fragments:
   Digest DNA (0.5 to 1.0 μg) with selected restriction endonucleases (16,17). Purify the DNA fragment by agarose gel electrophoresis and extract the DNA from the gel with a silica-based system like CONCERT™ Gel Extraction System (15) (optional).

B. PCR Products with restriction endonuclease sites in primers:

See Section 5 for information on cloning blunt PCR products.

Materials:

- PCR product
- phenol:chloroform:isoamyl alcohol (25:24:1)
- Restriction endonuclease and buffer
- 2-butanol
- TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]
- 3 M sodium acetate
- ethanol
- 30% PEG 8000/30 mM $MgCl_2$

For the best efficiency, see section 3.3 for cloning *att*B-PCR products.

Efficient cloning of PCR products made using primers containing restriction endonuclease sites on their 5'-ends depends on 3 steps:

1. inactivation or removal of the DNA polymerase (because *Taq* DNA polymerase can fill in sticky ends and add bases to blunt ends of PCR products),
2. efficient restriction endonuclease digestion, and
3. removal of small DNA fragments such as primers and primer-dimers and dNTPs.

B1. Phenol Extraction of PCR Products to Remove the DNA Polymerase
   The DNA polymerase can be removed before restriction endonuclease digestion by phenol extraction (protocol below) or a silica membrane spin cartridge such as the CONCERT Rapid PCR Purification System. (18). Alternatively, TAQUENCH™ PCR Cloning Enhancer can be used to inactivate the DNA polymerase (19).

1. Add TE to the PCR to 200 µl. Add 200 µl of buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex vigorously for 20 s. Centrifuge for 1 min at 15,000 × *g* at room temperature. Remove the upper aqueous phase.
2. Add an equal volume of 2-butanol. Vortex briefly. Centrifuge for 15 s at 15,000 × *g* at room temperature. Remove the lower aqueous phase.
3. Repeat the extraction with 2-butanol. This time the volume of the lower aqueous phase will decrease significantly. Remove the lower aqueous phase.
4. Ethanol precipitate the DNA by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
5. Dissolve in 200 µl of a restriction endonuclease buffer.

Note: When digesting with two separate restriction endonucleases, the most rigorous procedure is to perform the digests one at a time in the recommended REACT® Buffer and purify the DNA before the second digest. When performing a double digest, choose the buffer that has 100% activity for each enzyme. If no single buffer fulfills these requirements, then choose a buffer that ensures the highest activity possible without causing nonspecific cleavage.

Alternatively, perform a sequential digest by using the restriction endonuclease that requires the lowest salt conditions first. If the enzyme can be heat-inactivated, stop the first reaction by heating for 10 min at 65°C. Adjust the salt with minimal increase in volume to approximate the optimal conditions for the second enzyme. Keep the glycerol concentration ≤5% when both enzymes are present.

B2. Restriction Digestion of PCR Products

The efficiency of restriction endonuclease digestion can be improved by adding extra bases on the 5′-end of each PCR primer (20). Depending on the enzyme, the number of nucleotides recommended varies. Also, use 5 times excess restriction endonuclease to ensure complete digestion.

1. Digest with the appropriate restriction endonuclease(s).
2. Inactivate the restriction endonucleases by heat or phenol extraction, depending on the enzyme.
3. Precipitate the DNA by adding 100 µl of 30% PEG 8000/30 mM $MgCl_2$ to the 200 µl reaction mix. Mix well and immediately centrifuge at 10,000 × *g* for 10 min at room temperature. Remove the supernatant (pellet is clear and nearly invisible).
4. Dissolve the pellet in 50 µl TE. Check quality and recovery on a gel.

3.2.3 Ligation of Entry Vectors and Restriction Fragments

1. Ligate the prepared Entry Vector and insert fragments under appropriate conditions.

   For cohesive ends, add the following to a 1.5-ml tube:

Component	**Amount**
5X ligase reaction buffer	4 µl
vector DNA	3 to 30 fmol
insert DNA	9 to 90 fmol
autoclaved distilled water	≤15 µl
T4 DNA ligase	1 unit (in 1 µl)
Final volume	*20 µl*

   Mix gently. Incubate at room temperature for 1 to 2 h.

Note: For blunt-end ligation, increase the amount of insert and vector DNA 2 to 4 times (maintaining a 3:1 molar ratio) and use 5 units of ligase.

2. Transform 2 µl of ligation reaction into LIBRARY EFFICIENCY DH5α Competent Cells according to the instructions on the product profile sheet.
3. Plate transformants on LB plates containing 50 µg/ml **kanamycin**.
4. Isolate miniprep DNA from single colonies (16). Treat the miniprep with RNase A and store in TE. Cut with the appropriate restriction endonuclease to determine the orientation of the PCR fragment. **Choose clones with the *att*L1 site next to the amino end of the open reading frame.** Notes: *Bsr*G I cleaves within all *att* sites and can be used to help characterize clones. To sequence clones in Entry Clones see section 3.4.3.

3.3 Creating Entry Clones from *att*B-flanked PCR Products via the BP Reaction

Materials:

- *att*B-modified GATEWAY primers
- DNA polymerase, reaction buffer, and dNTPs for PCR
- PCR Cloning System with GATEWAY Technology
- TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]
- S.O.C. Medium
- LB plates with 50 μg/ml kanamycin

Addition of 5′-terminal *att*B sequences to PCR primers allows synthesis of a PCR product that is an efficient substrate for recombination with a Donor Vector via a GATEWAY reaction. This is typically more efficient than classic restriction endonuclease cloning of PCR products (see section 3.2.2.B.) and results in directionally cloned PCR products flanked by *att*L1 and *att*L2 sites. For high throughput applications or unusually long primers (>70 nucleotides), the *att*B adapter protocol can be used (section 5.2).

3.3.1 Preparation of *att*B-PCR Products

PCR primers for amplification and subsequent cloning by GATEWAY technology have the structure: 4Gs - 25 bp *att*B site - 18 to 25 bp gene-specific sequence. 50 nmol of standard purity oligonucleotides are adequate for most applications. Dissolve oligonucleotides to 20-50 mM and verify the concentration by spectrophotometry. For cloning of large PCR products (>5 kb), colony output can be increased if oligonucleotides (>65 bases) are further purified (*i.e.*, HPLC or PAGE).

Design primers to contain the *att*B1 and *att*B2 primer sequences (Figure 9). The four guanine (G) residues at the 5′ end are required to make the 25-bp *att*B sequences an efficient substrate for GATEWAY cloning. The *att*B1 primer ends with a thymidine (T). To maintain proper reading frame for N-terminal fusions the primer must donate two additional nucleotides. These two nucleotides cannot be AA, AG, or GA, because these additions would create translation termination codons. Similarly, for C-terminal fusions, the *att*B2 primer requires one nucleotide from the rest of the primer to maintain the proper reading frame into the *att*B2 region. Also, any in-phase termination codons present between the coding region of the PCR sequence and the *att*B2 region need to be eliminated if C-terminal fusions will be generated (see section 2.3.3).

Figure 9. *att*B Sequences to Add to Primers for PCR Cloning into a pDONR Vector.

***att*B1 forward primer (amino-terminal):**

Lys-Lys

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

*att*B1

***att*B2 reverse primer (carboxy terminal):**

Lys-Tyr

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

*att*B2

It is possible to install a protease cleavage sequence to permit the removal of N-terminal or C-terminal peptides from the fusion proteins. Include this sequence between the gene-specific and the *att*B sequences of the primer. For examples of *att*B-PCR primer sequences for native and fusion protein expression clones, refer to Section 2.3.3.)

Standard PCR conditions can be used to prepare the *att*B-PCR product. Genomic DNA, mRNA, cDNA libraries, and cloned DNA sequences have been used successfully for amplification with *att*B-containing primers. In general, the *att*B sequences have not been observed to affect PCR product yield or specificity. The suggested polymerase, if you are cloning PCR products <5-6 kb for protein expression is PLATINUM® *Pfx* DNA Polymerase due to its high fidelity and high specificity (21). For all other applications, PLATINUM *Taq* DNA Polymerase High Fidelity results in high-yield, robust, and high-specificity PCR of products 100 bp to 12 kb.

If the PCR template is a plasmid that contains the Km^r gene, treat the PCR products with *Dpn* I to degrade the plasmid. To a 50-µl reaction, add 5 µl of 10X REACT® 4 Buffer and ≥5 units of *Dpn* I. Incubate for 15 min at 37°C. Heat-inactivate the *Dpn* I at 65°C for 15 min.

Following PCR, analyze 1-2 µl on an agarose gel to assess the yield and purity of the product.

3.3.2 Purification of *att*B-PCR Products

Note: Standard PCR product clean-up protocols with exclusion limits <100 bp don't efficiently remove large primer-dimer products and are therefore not recommended for cleaning up *att*B-PCR products.

Purification of the PCR product is recommended to remove *att*B primers and any *att*B primer-dimers which can clone efficiently into the Entry Vector. The following protocol is fast and will remove DNA <300 bp.

Longer centrifugation times and higher speeds will increase the amount of PCR product recovered.

1. Add 150 µl of TE to a 50-µl amplification reaction.
2. Add 100 µl of 30% PEG 8000/30 mM $MgCl_2$. Mix well and centrifuge immediately at 10,000 × *g* for 15 min at room temperature. Remove the supernatant (pellet is clear and nearly invisible).
3. Dissolve the pellet in 50 µl TE. Check quality and recovery on a gel.
4. Proceed to section 3.4.2.

Note: For some PCR products, agarose gel electrophoresis followed by excision of the PCR product may be needed. Purify the excised product using the CONCERT Rapid Gel Extraction System.

3.4 Creating Entry Clones via the BP Reaction

The BP Reaction transfers a gene present in an *att*B Expression Clone (or *att*B-flanked PCR products) to generate an *att*L-flanked Entry Clone. The gene can then be subcloned into any number of new Expression Vectors using the LR Reaction. See section 5.1 for a one-tube protocol to directly go from a PCR product or Expression Clone into Destination Vectors.

Purify plasmid DNA with the CONCERT™ Rapid Plasmid Systems for best results. Alternatively, DNA can be purified using an alkaline lysis protocol, with or without RNase treatment. During alkaline lysis treatment, keep the NaOH ≤0.125 M to minimize irreversible denaturation of the supercoiled plasmid DNA..

The most efficient *att*B substrates are linear (Expression Clones linearized by restriction endonucleases or *att*B-flanked PCR products). Supercoiled or relaxed Expression Clones (*att*B) react less efficiently than linearized Expression Clones. The *att*P-containing pDONR Vector should be supercoiled.

3.4.1 Preparation of Expression Clone DNA

1. Linearize 1 to 2 µg of the Expression Clone with a unique restriction endonuclease 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. Dissolve DNA in TE.

3.4.2 The BP Reaction

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

See the calculation in the Troubleshooting section for determining the amount of *att*B DNA to use in the reaction.

Component	Negative Control Tube 1	Positive Control Tube 2	Sample Tube 3
*att*B Expression Clone DNA, linearized, ≥10 ng/ml or *att*B PCR product (use 40-100 fmol*; a 1-kb PCR product is ~0.65 ng/fmol)	---	---	1-10 µl
pEXP7-tet Positive Control†, 50 ng/ml	---	2 µl	---
pDONR201 Vector, 150 ng/ml	2 µl	2 µl	2 µl
BP Reaction Buffer (5X)	4 µl	4 µl	4 µl
TE	10 µl	8 µl	To 16 µl

*For PCR products >4 kb, use at least 100 fmol of PCR product, but no more than 500 ng.

†pEXP7-tet is a ~1.4-kb linear DNA encoding the tetracycline resistance gene and its promoter for expression (Tc^r), used to verify the BP reaction. The resulting Entry Clones can be used to estimate Km^r transformants that contain transferred DNA (Tc^r).

2. Remove the BP CLONASE Enzyme Mix from -70°C and thaw on ice (~2 min).
3. Vortex BP CLONASE Enzyme Mix briefly (2 s) twice.
4. Add 4 µl of BP CLONASE Enzyme Mix. Mix well by vortexing briefly twice. Return vial to -70°C.

To convert an even higher percentage of starting plasmid carrying your gene to product, incubate 4 to 18 h. An overnight incubation typically gives 5 to 10 times more product than a 1-h incubation.

5. Incubate reactions at 25°C for 60 min.
6. Add 2 µl of Proteinase K Solution. Incubate for 10 min at 37°C.
7. Transform 1 µl into 50 µl of LIBRARY EFFICIENCY DH5α Competent Cells. Incubate on ice for 30 min. Heat-shock the cells at 42°C for 30 s. Place on ice for 1-2 min. Add 450 µl S.O.C. Medium and incubate at 37°C for 1 h.

   Alternatively, electroporation can be used to transform 1-2 µl of the BP Reaction into 25 to 40 µl electrocompetent *E. coli*. Add 450 µl S.O.C. Medium and incubate at 37°C for 1 h.

Do not use an *E. coli* strain containing an F´ episome, since it contains the *ccd*A gene and prevents negative selection with *ccd*B.

8. Spread 10 µl and 100 µl on LB plates containing 50 µg/ml **kanamycin**. (For *E. coli* cells with a transformation efficiency of 10^8 CFU/µg, the BP Reaction gives ~3,000 colonies if the entire transformation is plated.)

The most common cause of an unsuccessful BP Cloning Reaction is not plating the transformations on plates containing kanamycin.

9. If desired, the percent correct clones in the positive control reaction can be confirmed by streaking the kanamycin-resistant colonies onto LB plates containing 20 µg/ml tetracycline.

3.4.3 Sequencing of pENTR Clones Generated by Recombination with Donor Vectors

pENTR (*att*L) clones can be sequenced with dye-labeled terminator chemistries such as DYEnamic™ energy transfer or BigDye™ reaction chemistries. The primer sequences are:

For Entry Clones derived from recombination with pDONR201:

proximal to *att*L1	TCGCG TTAAC GCTAG CATGG ATCTC
proximal to *att*L2	GTAAC ATCAG AGATT TTGAG ACAC

Use the BigDye chemistry and the following conditions:

5-min at 95°C followed by 30 cycles of PCR:

96°C for 10 s;
50°C for 5 s;
60°C for 4 min.

For small inserts (a couple of hundred bases), the following cycling conditions are recommended:

5-min at 98°C followed by 30 cycles of PCR:

98°C for 10 s;
60°C for 4 min.

3.5 Creating Expression Clones via the LR Reaction

Materials:

- Entry Clone
- Appropriate Expression System with GATEWAY Technology ***or*** your converted Destination Vector and LR CLONASE Enzyme Mix.
- S.O.C. Medium
- LB plates with 100 μg/ml ampicillin
- Appropriate host and cell growth media for expression

The reaction of an Entry Clone (*att*L) with a Destination Vector (*att*R) creates a new Expression Clone (*att*B).

Purify plasmid DNA with the CONCERT Rapid Plasmid Systems for best results. Alternatively, DNA can be purified using an alkaline lysis protocol, with or without RNase treatment. During alkaline lysis treatment, keep the NaOH ≤0.125 M to minimize irreversible denaturation of the supercoiled plasmid DNA.

The efficiency of the LR Reaction depends on the topology of the plasmids in the following order (most efficient to least efficient):

Either or both plasmids linear > both plasmids relaxed >> both plasmids supercoiled

All GIBCO BRL® Destination Vectors are provided linearized. If you have converted a plasmid to a Destination Vector, linearize it by cleaving at a restriction site within the region of the GATEWAY Cassette, taking care to avoid the *ccd*B gene. When suitable restriction sites are unknown, relax the DNA with topoisomerase I treatment (see modified LR Reaction protocol in section 5.4).

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

	Negative Control	Positive Control	Sample
Component	**Tube 1**	**Tube 2**	**Tube 3**
LR Reaction Buffer (5X)	4 μl	4 μl	4 μl
pENTR-gus*, 50 ng/μl	---	2 μl	---
Entry Clone (100-300 ng/reaction)	---	---	1-11 μl
Destination Vector, linearized (~300 ng/reaction)	1-11 μl	1-11 μl	1-11 μl
TE	To 16 μl	To 16 μl	To 16 μl

See the calculation in the Troubleshooting section for determining the amount of Entry Clone to use in the reaction.

*Note: pENTR-gus is a ~1.8 kb plasmid DNA encoding the *gus* gene and is used to verify the LR Reaction. The resulting Expression Clone contains both *E. coli* and eukaryotic translational signals upstream of the *gus* gene, allowing for native expression in *E. coli*, yeast, insect, and mammalian cells when reacted with the appropriate Destination Vector. Also, the ATG of *gus* is in frame with the *att* site for expression of N-terminal fusions.

To convert an even higher percentage of starting plasmid carrying your gene to product, incubate longer, 4 to 18 h. An overnight incubation often gives 5 times more product than a 1-h incubation. Longer incubation times are recommended for large plasmids (≥10 kb).

Do not use an *E. coli* strain containing an F´ episome, since it contains the *ccd*A gene and prevents negative selection with *ccd*B.

2. Remove LR CLONASE Enzyme Mix from -70°C and thaw on ice (~2 min).
3. Vortex LR CLONASE Enzyme Mix briefly (2 s) twice.
4. Add 4 µl of LR CLONASE Enzyme Mix. Mix well by vortexing briefly twice. Return vial to -70°C.
5. Incubate reactions at 25°C for 60 min.
6. Add 2 µl of Proteinase K Solution to all reactions. Incubate for 10 min at 37°C.
7. Transform 1 µl into 50 µl LIBRARY EFFICIENCY DH5α Competent Cells. Incubate on ice for 30 min. Heat-shock the cells at 42°C for 30 s. Add 450 µl S.O.C. Medium and incubate at 37°C for 1 h.

   Alternatively, electroporation can be used to transform 1 to 2 µl of the LR Reaction into 25-40 µl electrocompetent *E. coli*. Add 450 µl S.O.C. Medium and incubate at 37°C for 1 h.
8. Plate 20 µl and 100 µl on LB plates containing 100 µg/ml ampicillin. For *E. coli* cells with a transformation efficiency of 10^8 CFU/µg, the LR Reaction should give about 8,500 colonies if the entire transformation is plated.

   Note: *Bsr*G I cleaves within all *att* sites, and can be used to help characterize clones.

3.5.1 Protein Expression from GATEWAY Expression Clones

The *E. coli* Expression Systems with GATEWAY Technology provide the components to construct *E. coli* Expression Clones from Entry Clones. Use LIBRARY EFFICIENCY DH5α cells to select for Expression Clones. (Note: DH5α cells do not express T7 RNA polymerase and cannot be used for expression from T7 promoters.) Use BL21-SI cells for protein expression. In BL21-SI cells, expression of T7 RNA polymerase is under control of a salt-inducible promoter, allowing for salt induction of expression of proteins from T7 promoters (such as found in GIBCO BRL *E. coli* Destination Vectors).

The Baculovirus Expression System with GATEWAY Technology (cat. no. 11827-011) provides the components necessary to construct the GATEWAY version of the pFASTBAC™ clone. Once constructed, this clone can be used in conjunction with the BAC-TO-BAC® Baculovirus Expression System to generate (by *in vivo* recombination with a bacmid) a recombinant baculovirus for expression in insect cells. In addition to the Baculovirus Expression System with GATEWAY Technology, components from the BAC-TO-BAC system are also required (including DH10BAC™ cells, CELLFECTIN® Reagent, and insect cells for expression.) Refer to the BAC-TO-BAC System manual (on the web site) for more information.

The Mammalian Expression System with GATEWAY Technology (cat. no. 11826-013) supplies the components to construct mammalian Expression Clones from Entry Clones. These Expression Clones contain the *neo*^{r} marker and the CMV promoter for expression. See the related products list in section 6 for cell lines and transfection reagents for mammalian expression.

3.6 Converting a Vector into a GATEWAY Destination Vector

For any vector to serve as a Destination Vector, it must have *att*L sites flanking the *ccd*B gene. Conversion of any vector to a GATEWAY Destination Vector is done by simply ligating a blunt-ended cassette, containing *att*R sites and *ccd*B (and a chloramphenicol resistance marker to select for successful ligation of the cassette) into the multiple cloning site (MCS) of the vector. The GATEWAY Vector Conversion System provides conversion cassettes in all three reading frames (see Table 4, Figures 10 and 11) for N- and C-terminal fusion vectors as well as for native expression vectors.

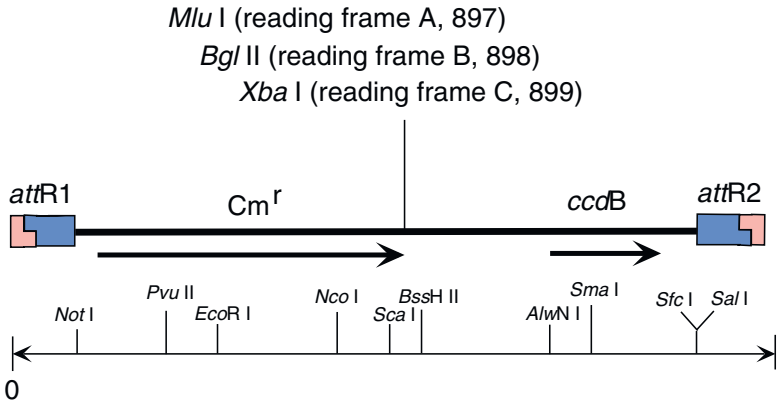


Figure 10. Schematic of the GATEWAY Cloning System Reading Frame Cassettes. Each cassette contains an *att*R1 site at the 5'-end, the chloramphenicol resistance gene (Cm^r), the *ccd*B gene, and the *att*R2 site. Each of the cassettes provides N-terminal and C-terminal fusions in one of three possible reading frames. The unique restriction sites *Mlu* I, *Bgl* II, and *Xba* I distinguish the reading frame cassettes. Restriction endonucleases common to all the cassettes are presented in Table 4.

Table 4. Location of Cleavage Sites for a Selection of Restriction Endonucleases.

	Restriction Endonuclease Cleavage Site									
DNA	*Not* I	*Pvu* II	*Eco*R I	*Nco* I	*Sca* I	*Bss*H II	*Alw*N I	*Sma* I	*Sfc* I	*Sal* I
RfA	129	348	450	751	865	944	1224	1319	1572	1578
RfB	130	349	451	752	866	945	1225	1320	1573	1579
RfC.1	131	350	452	753	867	946	1226	1321	1574	1580

3.6.1 Protocol for Constructing a GATEWAY Destination Vector

Materials:

- GATEWAY Vector Conversion System
- restriction endonucleases
- calf intestinal alkaline phosphatase
- PEG
- TE [10 mM Tris-HCl (pH 7.5), 1 mM EDTA]
- T4 DNA ligase
- LB plates with 30 μg/ml chloramphenicol

- Destination Vectors must be constructed and propagated in DB3.1 cells, a *gyr*A462 strain of *E. coli* because the *ccd*B gene is lethal to other strains.
- If linearizing a vector using restriction endonucleases that generate 5´ overhangs, the ends of the DNA molecules must first be made blunt (by a Klenow fill-in reaction) before the blunt-end cassette may be ligated into the vector.
- Because the reading frame cassettes are blunt-ended, they will clone in both orientations and must be screened to identify the construct with the cassette in the proper orientation.
- If you are converting a vector that encodes kanamycin resistance, use the resulting Destination Vector with Entry Clones that carry a selection marker other than Km^r. You can make this Entry Clone in a BP Reaction using a Donor Vector with a marker such as gentamicin resistance (pDONR207).

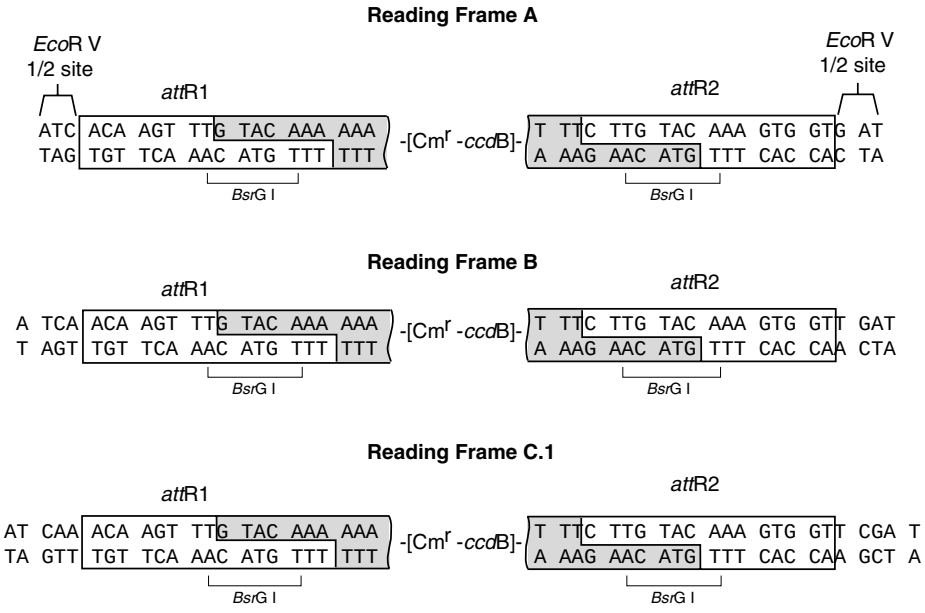


Figure 11. Sequences at ends of GATEWAY Reading Frame Cassettes. The staggered cleavage sites for the CLONASE enzymes are indicated in the boxed regions. Following recombination with an Entry Clone, only the outer (unshaded) sequences in *att*R sites contribute to the resulting *att*B sites in the Expression Clone. *Bsr*G I cleaves within all *att* sites and can be used to help characterize clones.

1. Determine the GATEWAY Reading Frame Cassette to use.
   If the Destination Vector will be used to make a fusion protein, use a GATEWAY Reading Frame Cassette with the correct translation reading frame. For an amino-terminal fusion protein, keep the -AAA-AAA- triplets in *att*R1 (see Figure 11) in phase with the translation reading frame of the fusion protein. This is the reading frame convention used in N-terminal fusion Destination Vectors from Life Technologies. For C-terminal fusion proteins, align the coding sequence in phase with -TAC-AAA- of the *att*R2 sequences.

A. Write out the nucleotide sequence of your vector near the restriction site where the GATEWAY Cassette will be cloned. These must be written in triplets corresponding to the amino acid sequence of the fusion domain.

B. Draw a vertical line through the sequence that corresponds to the restriction site end, after it has been digested and made blunt, (*i.e.*, after filling in a protruding 5′-end or polishing a protruding 3′-end).

For N-terminal fusions:

—If the coding sequence of the blunt end terminates after a complete codon triplet, use the Reading Frame Cassette A. (See Figure 12.)

—If the coding sequence of the blunt end encodes two bases of a complete codon triplet, use the Reading Frame Cassette B.

—If the coding sequence of the blunt end encodes one base of a complete codon triplet, use the Reading Frame Cassette C.1.

For C-terminal fusions:

—If the coding sequence of the blunt end terminates after a complete codon triplet, use the Reading Frame Cassette B. (See Figure 11.)

—If the coding sequence of the blunt end encodes two bases of a complete codon triplet, use the Reading Frame Cassette C.1.

Reading Frame Cassette A

vector sequence------II-----------5´ end Reading Frame Cassette A----------I
--- NNN NNN ATC ACA AGT TTG TAC AAA AAA GCT ---
--- NNN NNN TAG TGT TCA AAC ATG TTT TTT CGA ---
fusion reading frame-I

Reading Frame Cassette B

--vector sequence-----II----------5´ end Reading Frame Cassette B----------I
*
--- NNN NNN NNA TCA ACA AGT TTG TAC AAA AAA GCT ---
--- NNN NNN NNT AGT TGT TCA AAC ATG TTT TTT CGA ---
fusion reading frame---I

*cannot be TG or TA

Reading Frame Cassette C.1

vector sequence------I I----------5´ end Reading Frame Cassette C----------I
--- NNN NNN NAT CAA ACA AGT TTG TAC AAA AAA GCT ---
--- NNN NNN NTA GTT TGT TCA AAC ATG TTT TTT CGA ---
fusion reading frame-I

Figure 12. Choosing the Correct GATEWAY Reading Frame Cassette for N-terminal fusions.

—If the coding sequence of the blunt end encodes one base of a complete codon triplet, use the Reading Frame Cassette A.

For a combined N- and C-terminal fusion, the restriction endonuclease chosen must produce ends (after generating blunt ends) that are compatible with one of the three cassettes.

2. Digest your plasmid vector (1 to 5 μg) with the appropriate restriction endonucleases [where you wish your gene flanked by *att* sites to be after recombination]. Note: It is better to remove as many of the MCS restriction sites as possible to minimize the number of additional amino acids added to the fusion and to increase the number of unique restriction endonuclease sites in the new plasmid, which is important for linearizing the Destination Vector for the LR Reaction.
3. If necessary, convert the ends of the vector to blunt double-stranded DNA using either T4 DNA polymerase or Klenow fragment according the manufacturer's recommendations.
4. Remove the 5′ phosphates with alkaline phosphatase. This increases the probability of success by decreasing background associated with self-ligation of the vector.
   a. Determine the mass of DNA required for 1 pmol of the type of DNA 5′ end.
   b. To a 1.5-ml microcentrifuge tube, add 4 μl of calf intestinal alkaline phosphatase (CIAP) 10X Buffer [500 mM Tris-HCl (pH 8.5), 1 mM EDTA] and 1 pmol of DNA ends.
   c. Add autoclaved, distilled water to 39 μl.
   d. Dilute CIAP in dilution buffer such that 1 μl contains the amount of enzyme required for the appropriate 5′ end (*i.e.*, 1 unit for 5′-recessed and blunt ends and 0.01 units for a 5′ overhang).

e. For 5′-recessed and blunt-ended DNA, incubate at 50°C for 60 min. For DNA with a 5′ overhang, incubate at 37°C for 30 min.
f. Heat-inactivate CIAP at 65°C for 15 min.

5. Adjust the DNA to a final concentration of 20 to 50 ng/µl in TE. Electrophorese 20 to 100 ng on an agarose gel to confirm digestion and recovery.
6. Combine the following at room temperature:

Component	Amount
5X T4 DNA ligase buffer	2 µl
vector	20 - 50 ng
GATEWAY Reading Frame Cassette	10 ng
T4 DNA ligase	1 unit (in 1 µl)
Final volume	*10 µl*

7. Incubate for 1 h at room temperature (or overnight at 16°C, whichever is most convenient).
8. Transform 1 µl into 100 µl DB3.1 Competent Cells. (Note: *E. coli* DB3.1 cells **must** be used. The *ccd*B gene on the GATEWAY Reading Frame Cassette will inhibit growth of other *E. coli* strains.)
9. After expression in S.O.C. Medium, plate 10 µl, 100 µl, and 500 µl on agar plates containing 30 µg/ml chloramphenicol. Incubate at 37°C for 16 to 20 h.
10. Isolate miniprep DNA from single colonies (16).
11. Treat the miniprep with RNase A, ethanol precipitate, and store in TE. Digest with the appropriate restriction endonuclease to determine the orientation of the cassette. **Choose clones with the *att*R1 site next to the amino end of the protein expression function of the plasmid** (see Table 4, Figure 10).

3.6.2 Analysis of Destination Vector

Besides checking for proper orientation of the cassette, it is important to check for the presence of any contaminating ampicillin-resistant plasmid and demonstrate that the *ccd*B gene is functioning in your Destination Vector. Even minute amounts of ampicillin-resistant plasmid result in a high background.

1. Transform equal amounts (10 - 50 pg) of Destination Vector into 100 µl of LIBRARY EFFICIENCY DH5α cells and DB3.1 Competent Cells using the protocol provided with the cells.
2. Plate onto LB plates containing the ampicillin.
3. Transform 50 pg pUC19 into both strains. Plate onto LB plates containing 100 µg/ml ampicillin.
4. Calculate the transformation efficiency of both strains with the pUC19 control to ensure transformation reactions worked well.

Transformation efficiency (CFU/µg) = colonies/pg of DNA × (1 × 10^6 pg/µg) × dilution factor(s)

For example, if 50 pg of pUC19 yields 100 colonies when 100 µl of a 1:10 dilution of the transformation mix is plated, then:

CFU/µg = 100 CFU/50 pg × (1 × 10^6 pg/µg) × (1 ml/0.1 ml plated) × 10 = 2 × 10^8

5. Calculate the number of colonies obtained in both strains from transformations using the Destination Vector.
6. The Destination Vector should give >10,000 times the number of colonies in DB3.1 cells than in LIBRARY EFFICIENCY DH5α Competent Cells. Any ratio <10,000 suggests contamination of the plasmid prep with another ampicillin-resistant plasmid, or an inactive *ccd*B gene. DNA with ratios <10,000 will result in higher background.

3.6.3 Preparing the Destination Vector for Cloning

Linearize the Destination Vector with a restriction endonuclease or relax the DNA with topoisomerase I. About 10 times more colonies result from a GATEWAY reaction if the Destination Vector is linear or relaxed.

The site or sites used for linearization must be within the GATEWAY Reading Frame Cassette, but not within the *ccd*B gene. A sampling of the sites that cut within a cassette is shown in Figure 10. After restriction digestion, ethanol precipitate the DNA by adding 0.1 volume of 3 M sodium acetate, followed by 2.5 volumes of 100% ethanol. The linear Destination Vector is now ready for the LR Reaction.

Troubleshooting Guide

4

Problem	Possible Cause	Suggested Solution
For Both LR and BP Reactions:		
Few or no colonies obtained from sample reaction, and the transformation control with pUC19 gave colonies	Transformation was plated with incorrect antibiotic	Use kanamycin for most Entry Clones. Use ampicillin for most Destination Vectors.
	Reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
	Used incorrect *att* sites for reaction	Use Entry Clone (*att*L) and Destination Vector (*att*R) for the LR Reaction. Use Expression Clone (or *att*B-PCR product) and Donor Vector (*att*P) for BP Reaction.
	DNA topology is not optimal for reaction	For the LR Reaction, linearize the Destination Vector within the *att*R Cassette, avoiding the *ccd*B gene. For the BP Reaction, linearize the *att*B Expression Clone outside the *att*B sites with an appropriate restriction endonuclease or relax with topoisomerase I. Use supercoiled Donor Vector.
	CLONASE™ Enzyme Mix is inactive	Test another aliquot of the CLONASE Enzyme Mix. Check that the CLONASE Enzyme Mix is being stored at -70°C. Do not freeze any aliquot more than 10 times to minimize loss of activity. pENTR-gus can be used in an LR Reaction (section 3.5) to test LR CLONASE Enzyme Mix activity. pEXP7-tet can be used in a BP Reaction (section 3.4) to test BP CLONASE Enzyme Mix activity.
	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 PCR product was used in a BP Reaction	Reduce the amount of PCR product used. Remember to use ~100 fmol of Donor Vector. Therefore, to obtain an equimolar ratio of PCR product and Donor Vector, use 100 fmol of PCR product. If the PCR product is 2.5 kb, convert to nanograms using the following equation: ng = (fmol)(N)(660 fg/fmol)(1 ng/10^6 fg) where N is the size of the DNA in bp. (100 fmol)(2500 bp)(660 fg/fmol)(1 ng/10^6 fg) = 165 ng. Therefore, 165 ng of PCR product are required for this reaction.
	Too much Entry Clone was used in an LR Reaction	Use equal fmol of Destination Vector and Entry Clone.
Two distinct types of colonies appear, large and small	For the LR Reaction, the small colonies can be unreacted Entry Clone that co-transforms with Expression Clone. When small colonies are restreaked onto LB kanamycin (50-100 μg/ml) and LB ampicillin (100 μg/ml) plates, often they only grow on the LB kanamycin.	Reduce the amount of Entry Clone to 100 ng per 20-μl reaction. Reduce the volume of sample used for transformation to 1 μl. Increase the ampicillin to 300 μg/ml.

Problem	Possible Cause	Suggested Solution
	For the BP Reaction, deletions or point mutations of the *ccd*B gene within the Donor (*att*P) Plasmid can allow *E. coli* to grow, although at lower rates. The negative control will give a similar number of colonies.	Obtain a new *att*P Donor Plasmid.
	Plasmids carrying large genes may be deleted during culture, leading to two populations of colonies. Generally, larger colonies contain the deletions.	Incubate plates at 30°C instead of 37°C. Confirm whether a deletion is occurring by analyzing the DNA derived from the colonies. Use STBL2™ cells to help stabilize large genes (22).
For the LR Reaction, high background in absence of Entry Clone	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 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 ampicillin plates.
	Reactions transformed into an F´-containing *E. coli* which has the *ccd*A gene	Use *E. coli* strains without an F´ episome such as DH5α cells.
	Some Destination Vectors have an inherently higher background than others, possibly due to tendency to delete some or all of the *ccd*B gene	Prepare miniprep DNA from one or more background colonies. Unstable Destination Vectors often reveal multiple bands on agarose gels. If this is the case, try using a different vector backbone in the Destination Vector.
Few or no colonies obtained from the transformation control with pUC19	Transformation performed incorrectly, or competent cells stored improperly	Verify that competent cells are stored at -70°C.
	Dilutions were performed incorrectly	Repeat transformation paying special attention to dilution steps.
For *att*B-PCR Cloning: (These are in addition to general BP Reaction problems above.)		
Few or no colonies obtained from BP Reaction with new *att*B-PCR product, and both *att*B-positive control and transformation control gave expected number of colonies	*att*B-PCR primers have a mistake in the *att*B1 or *att*B2 sequences, or are missing the four 5´ terminal Gs	Replace with correct *att*B-PCR primers.
	*att*B primers have high percentage of incomplete sequence	Purify long (>65 nucleotides) *att*B-PCR primers by PAGE, to remove incomplete sequences. Alternatively, use the Adapter PCR protocol (section 5.2).
	For large PCR products (>5 kb), too few PCR molecules added to BP Reaction	Increase the amount (ng) of PCR product to 40 to 80 fmol of PCR DNA/20-µl reaction (*e.g.*, for an 8 kb DNA, 1 fmol ~5 ng.) Note: Do not exceed 400 ng DNA/20-µl reaction.
	Incubation time not sufficient	Increase incubation time to 6 to 18 h.
	PCR products were not purified sufficiently	Gel purify PCR product, making sure to separate product from oligonucleotides.

Problem	Possible Cause	Suggested Solution
Entry Clones migrate as 2.2-kb supercoiled plasmids	BP recombination reaction may have cloned primer-dimers	Purify PCR products >500 bp by precipitating with PEG/$MgCl_2$ solution. Alternatively, excise the correct size DNA product from an agarose gel, and use the eluted, purified DNA in the BP Reaction.
		Use a PLATINUM™ DNA polymerase for automatic hot-start PCR giving higher specificity.
		Redesign primers to minimize potential mutual priming sites leading to primer-dimers.
Low yield of PCR product from PEG precipitation	PCR product not diluted with TE	Dilute with 150 µl TE 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 min and 15,000 x *g*.
	Loss of PEG pellet	Take care when removing the tube from the microcentrifuge and keep track of the orientation of the outer edge of the tube where the pellet is located.
Preparing Entry Clones with Restriction Endonucleases and Ligase:		
Few or no colonies obtained	*ccd*B Cassette still present within Entry Vector	Excise with appropriate restriction endonuclease(s).
	Ligation did not work	Include ligation positive control linearized plasmid, with and without ligase.
	Transformation was plated with incorrect antibiotic	Use kanamycin for most Entry Clones. Use ampicillin for most Destination Vectors.
Protein Synthesis using *att*B Expression Clones:		
No protein of expected molecular weight seen on SDS-PAGE	Protein is being degraded by endogenous proteases, especially for proteins >100 kDa	Use lon^- and $ompT^-$ strains for *E. coli* expression (such as BL21-SI cells).
		Incubate plates at 30°C instead of at 37°C.
		Compare expression using different N-terminal and/or C-terminal fusion tags, and in other types of host cells, such as yeast, insect, or mammalian cells.
		For expression in *E. coli* from T7 promoters (such as pDEST™14, pDEST15, and pDEST17), use a strain such as BL21-SI cells that express T7 RNA polymerase. DH5α cells cannot be used for expression from T7 promoters.
		Expression Clones made from pDEST8, pDEST10, and pDEST20 must first be recombined with bacmid DNA (BAC-TO-BAC® system). The resultant baculovirus can then be used for expression in insect cells.
	Protein contains secondary modifications that increase apparent molecular weight	Compare expression in other types of host cells, such as yeast, insect, or mammalian cells.
No fusion protein of expected molecular weight seen on SDS-PAGE	Incorrect reading frame of Entry Clone	Verify that *att*B-PCR primers were designed with gene in correct reading frame.
		Verify that Entry Clone was constructed with gene in correct reading frame.
		Verify that Destination Vector was constructed with correct reading frame.

Additional Information

5

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

This one-tube protocol moves *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. This protocol is more rapid than the protocol in section 3.3. However, here the Expression Clone is obtained from an Entry Clone that was not unique, so this protocol requires sequence validation of the Expression Clone.

Also, this protocol can transfer a gene from one Expression Clone into another Destination Vector. Linearize the Expression Clone within the plasmid backbone for an optimal BP Reaction and to eliminate false-positive colonies due to co-transformation.

If the PCR template is a plasmid that contains the Km^r or Ap^r gene, treat the PCR products with *Dpn* I to degrade the plasmid. To a 50-µl reaction, add 5 µl of 10X REACT® 4 Buffer and ≥5 units of *Dpn* I, and incubate for 15 min at 37°C. Heat-inactivate the *Dpn* I at 65°C for 15 min.

1. In a 1.5-ml tube, prepare a 25-µl BP Reaction as follows:

Component	Volume (µl)
*att*B DNA (100-200 ng)	5
*att*P DNA (pDONR™201, 150 ng/µl)	2.5
BP Reaction Buffer (5X)	5
TE	sufficient to bring the volume to 20 µl
BP CLONASE™ Enzyme Mix	5
Final volume	25

To convert an even higher percentage of starting plasmid carrying your gene to product, incubate 6 to 20 h. An overnight incubation often gives 5 times more product than a 1-h incubation. Longer incubation times are recommended for large plasmids (≥10 kb) and PCR products (≥5 kb).

2. Mix and incubate for 4 h at 25°C.

3a. Remove 5 µl of the reaction to a separate tube. Add 0.5 µl of proteinase K solution. Incubate for 10 min at 37°C.

When the template for PCR or starting Expression Clone has the same selectable marker as the final Destination Vector (most commonly Ap^r), plate on LB plates containing 100 µg/ml ampicillin to determine the amount of false-positive colonies carried over to the LR Reaction.

3b. Transform 100 µl of competent cells with 1 µl of the mixture. Plate on LB plates containing 50 µg/ml kanamycin. These colonies can be used to isolate Entry Clones and assess the BP Reaction efficiency.

4. To the remaining 20-µl reaction, add:

Component	Volume (µl)
NaCl (0.75 M)	1
Destination Vector linearized (150 ng/ml)	3
LR CLONASE Enzyme Mix	6
Final volume	30

To convert an even higher percentage of starting plasmid carrying your gene to product, incubate 3 h or overnight.

5. Mix and incubate for 2 h at 25°C.

6. Add 3 µl of proteinase K solution. Incubate for 10 min at 37°C.

Do not use an *E. coli* strain containing an F′ episome, since it contains the *ccd*A gene and prevents negative selection with *ccd*B.

7. Transform 100 µl of competent cells with 1 µl of the reaction. Plate on LB plates containing 100 µg/ml ampicillin (for Ap^r Destination Vectors).

   The total number of Expression Clone colonies is usually 10% to 20% of the total number of Entry Clone colonies.

5.2 *att*B Adapter PCR for Preparation of *att*B-flanked PCR Products

Use this protocol to replace the standard protocol (section 3.3.1) to prepare *att*B-flanked PCR products when primers are >70 bases. This protocol requires 2 sets of primers, one for the gene-specific amplification and the second set to install complete *att*B sequences (adapter-primers *att*B1 and *att*B2).

Additional Information

Design template-specific primers with 12 bases of *att*B1 and *att*B2 at their 5'-ends as shown below:

12 *att*B1: AA AAA GCA GGC TNN - forward template-specific primer

12 *att*B2: A GAA AGC TGG GTN - reverse template-specific primer

In addition, the following adapter-primers will be needed to install the full 29-b *att*B sequences:

*att*B1 adapter primer: G GGG ACA AGT TTG TAC AAA AAA GCA GGC T

*att*B2 adapter primer: GGG GAC CAC TTT GTA CAA GAA AGC TGG GT

The addition of greater than 10 pmol of gene-specific primers can decrease the yield of clonable full-*att*B PCR product generated in the second PCR.

1. Prepare a 50-µl PCR containing 10 pmol of each template-specific primer (with 12 *att*B) and the appropriate amount of template DNA.
2. Incubate at 95°C for 2 min. Perform 10 cycles of PCR:
   94°C for 15 s;
   50-60°C for 30 s;
   68°C for 1 min/kb of target.
3. Transfer 10 µl to a 40-µl PCR mixture containing 40 pmol each of the *att*B1 and *att*B2 adapter-primers.
4. Incubate at 95°C for 1 min. Perform 5 cycles of PCR:
   94°C for 15 s;
   45°C for 30 s;
   68°C for 1 min/kb of target.
5. Perform 15-20 cycles of PCR:
   94°C for 15 s;
   55°C for 30 s;
   68°C for 1 min/kb of target.
6. Check quality and recovery on a gel.
7. Refer to section 3.3.2 to purify the *att*B-flanked PCR product.

5.3 Blunt Cloning of PCR Products

Generally PCR products do not have 5′ phosphates (because the primers are usually 5'-OH), and they are not necessarily blunt (23). The following protocol simultaneously creates blunt, 5'-phosphorylated ends.

Materials:

- PCR product
- T4 polynucleotide kinase and buffer
- T4 DNA polymerase
- 30% PEG/30 mM $MgCl_2$
- T4 DNA ligase and buffer
- Dephosphorylated Entry Vector
- LIBRARY EFFICIENCY® competent cells
- S.O.C. medium
- LB plates containing 50 µg/ml kanamycin

1. In a 0.5-ml tube, precipitate ~40 ng of PCR product (as judged from an agarose gel) by adding 0.1 volume of 3 M sodium acetate followed by 2.5 volumes of 100% ethanol.
2. Add the following to the DNA:

Component	Volume (µl)
distilled H_2O	4
10 mM ATP	1
2 mM dNTPs (*i.e.*, 2 mM each dATP, dCTP, dTTP, and dGTP)	1
5X T4 Forward Reaction Buffer [350 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol]	2
T4 polynucleotide kinase (10 units/µl)	1
T4 DNA polymerase	1

3. Incubate at 37°C for 10 min, then at 65°C for 15 min. Cool on ice for 5 min. Centrifuge briefly to bring any condensate to the bottom of the tube.
4. Add 5 µl of 30% PEG 8000/30 mM $MgCl_2$. Mix and centrifuge immediately at room temperature for 10 min.
5. Carefully remove and discard supernatant.
6. Dissolve the invisible pellet in 10 µl containing 2 µl 5X T4 DNA ligase buffer, 0.5 units T4 DNA ligase, and about 50 ng of blunt, dephosphorylated Entry Vector.
7. Incubate at 25°C for 1 h, then at 65°C for 10 min.
8. Add 40 µl TE, transform 2 µl into 100 µl of of LIBRARY EFFICIENCY DH5α Competent Cells.
9. Plate on LB plates containing 50 µg/ml kanamycin.
10. Isolate miniprep DNA from single colonies (16). Treat the miniprep with RNase A and store in TE. Cut with the appropriate restriction endonuclease to determine the orientation of the PCR fragment. **Choose clones with the *att*L1 site next to the amino end of the open reading frame.**

5.4 Modified LR Reaction with Topoisomerase I

Use this protocol to relax Destination Vectors when suitable restriction sites are unavailable. The expected colony output for this modified protocol is ~50% less than when using linear Destination Vectors, and 5-10 times greater than reactions using supercoiled Destination Vectors.

1. Prepare the LR Reaction (described in section 3.5) by adding the following to a 1.5-ml tube at room temperature.

Component	Volume (µl)
LR Reaction Buffer (5X)	4
Supercoiled Entry Clone (100-300 ng)	1-9
Supercoiled Destination Vector (300 ng)	1-9
Topoisomerase I (15 units/µg total DNA)	0.6-2
TE	to 16

2. Remove LR CLONASE Enzyme Mix from -70°C and thaw on ice (~2 min).
3. Vortex LR CLONASE Enzyme Mix briefly (2 s) twice.
4. Add 4 µl of LR CLONASE Enzyme Mix. Mix well by vortexing briefly twice. Return vial to -70°C.
5. Incubate reactions at 25°C for 60 min.
6. Add 2 µl of proteinase K solution. Incubate for 10 min at 37°C.
7. Proceed with transformation of *E. coli*.

5.5 Transferring Clones from cDNA Libraries Made in GATEWAY™ Vectors

There are several things to consider when working with a clone isolated from a cDNA library constructed in a GATEWAY vector, such as SUPERSCRIPT™ cDNA libraries supplied in pCMV•SPORT6 (which contains *att*B sites). These include whether the clone is full-length and whether the protein will be expressed as a native protein or as a fusion protein.

While libraries contain many full-length open reading frames, some clones may be a partial reading frame, or may contain the entire ORF plus 5' untranslated (5' UTR) sequence as well. Contained within the 5' UTR of a cDNA is the ribosome recognition sequence for the organism from which the cDNA was derived. Therefore, a full-length cDNA derived from mammalian cells can be used for native expression in mammalian cells without prior characterization but cannot be used for native expression in *E. coli*, as no Shine-Dalgarno sequence is present. A Shine-Dalgarno sequence can be supplied either by cloning the cDNA into an Entry Vector that contains a Shine-Dalgarno sequence, or by introducing a Shine-Dalgarno

Additional Information

sequence by PCR when amplifying the cDNA with primers containing *att*B sequences and cloning the PCR product by recombination. (See Section 3.3 for cloning of PCR products).

The length and content of the clone is important in expressing fusion proteins. For full-length cDNA, the 5' UTR will be translated as a part of the fusion protein. This may present problems as the additional codons may interfere with the expression or function of the protein, or the 5' UTR may contain stop codons. If the ORF is not full-length, a truncated portion of the protein of interest will be expressed within the fusion. To express any cDNA isolated from a library 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 (see Figure 9). There is one chance in three that the cDNA will be in frame with the *att*B1 site and allow for fusion protein expression. A researcher can construct three Destination Vectors representing the three reading frames through the *att*B1 sites so that any given cDNA clone can be expressed in one of the three vectors. Alternatively, to assure that the ORF encoded by the cDNA will be in frame with an N-terminal fusion protein sequence, use PCR to install *att*B sites, so that the AAA-AAA sequence within *att*B1 is in phase with the ORF.

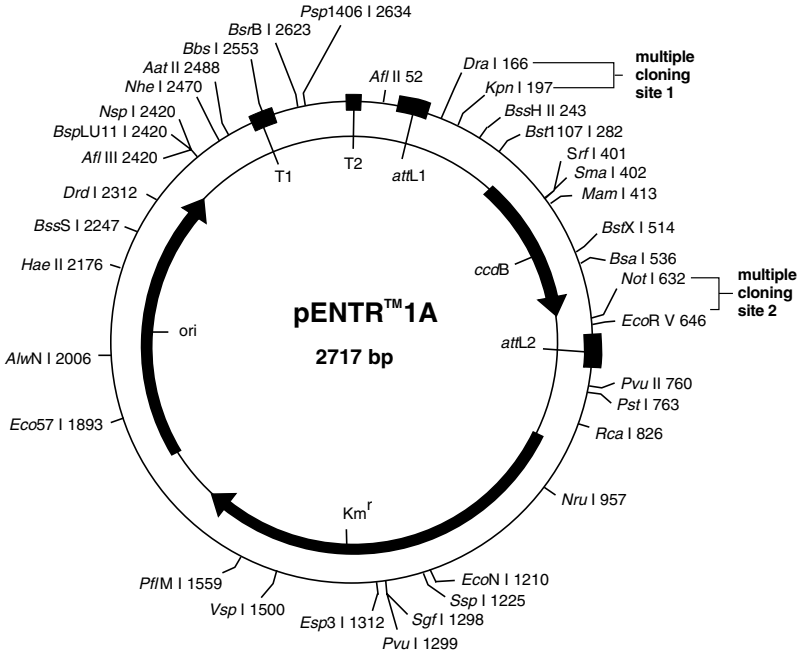
The major consideration in generating C-terminal fusion proteins from cDNAs is that cDNAs contain one or more stop codons, which must be removed before C-terminal fusion expression is possible. This may be done by subcloning the gene into an Entry Vector by classic methods, so that no stop codon is present. Alternatively, it may be done by amplifying the gene by PCR using *att*B primers where the stop codon has been eliminated from the gene-specific sequence.

5.6 GATEWAY™ Vector Restriction Maps

5.6.1 Entry Vectors

Entry Vectors contain a pUC origin of replication and the kanamycin resistance gene (Km^r) for maintenance in *E. coli*.

All Entry Vectors consist of the same vector backbone (outside of the *att*L sites) but differ in the sequences and cloning sites provided between the *att*L sites. Details of the regions between the *att*L1 and *att*L2 sites for each Entry Vector follow the circle map as well as endonucleases that do not cleave the vectors.



Restriction endonucleases that cleave pENTR1A once are shown on the outer circle. The positions refer to the 5´-base of the recognition site.

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

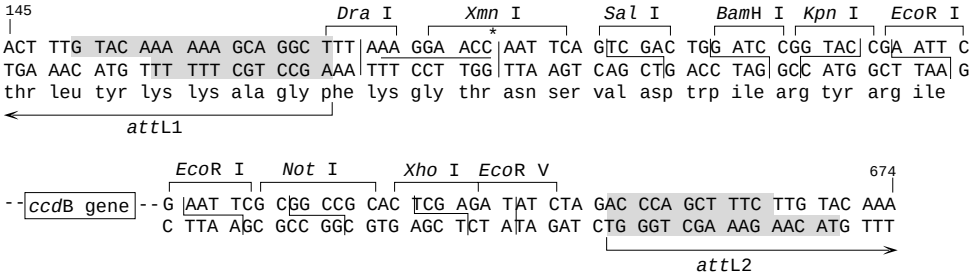
Additional Information

Sequences within the *att*L sites of Entry Vectors:

The amino acids shown before the *ccd*B gene are added to the N-terminus of your protein only if a translation start site is provided in the Destination Vector (such as with an N-terminal fusion). Clone your sequence in frame with the AAA AAA for N-terminal fusion proteins. Clone your sequence in frame with TTT GTA for C-terminal fusion proteins.

If a blunt-ended fragment containing a 5′-ATG is cloned into the *Xmn* I site of pENTR1A, 2B, 3C, or 4, the adenine at position –3 of the underlined ACC sites provides a Kozak eukaryotic ribosome recognition sequence for initiation of translation.

pENTR1A sequence: 145-674 nucleotides

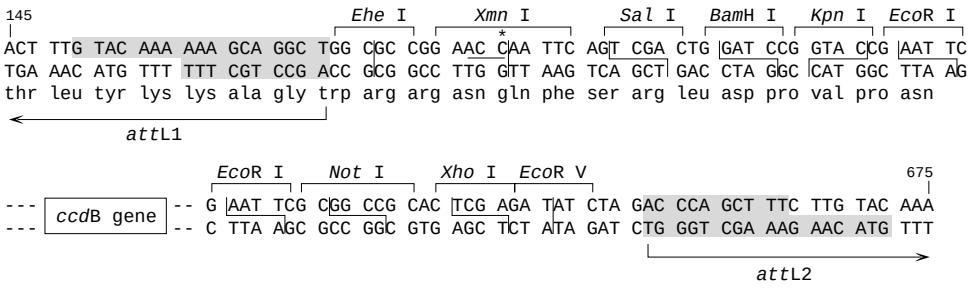


Restriction Endonucleases that do not cleave pENTR1A:

Asc I	*Bst*E II	*Hin*d III	*Pin*A I	*Spe* I
Ava II	*Cla* I	*Hpa* I	*Pme* I	*Sph* I
Avr II	*Cvn* I	*Kpn*2 I	*Psh*A I	*Sse*8387 I
Bcl I	*Dra* III	*Mlu* I	*Psp*5 II	*Sst* I
Bgl I	*Dsa* I	*Mun* I	*Rsr* II	*Sst* II
Bgl II	*Eam*1105 I	*Nar* I	*Sap* I	*Stu* I
*Bpu*1102 I	*Eco*47 III	*Nco* I	*Sca* I	*Sty* I
*Bsa*A I	*Eco*72 I	*Nde* I	*Sex*A I	*Sun* I
*Bse*R I	*Fse* I	*Ngo*A IV	*Sfi* I	*Swa* I
Bsg I	*Fsp* I	*Nsp* V	*Sgr*A I	*Tth*111 I
*Bsp*M I	*Gsu* I	*Pac* I	*Sna*B I	*Xcm* I

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

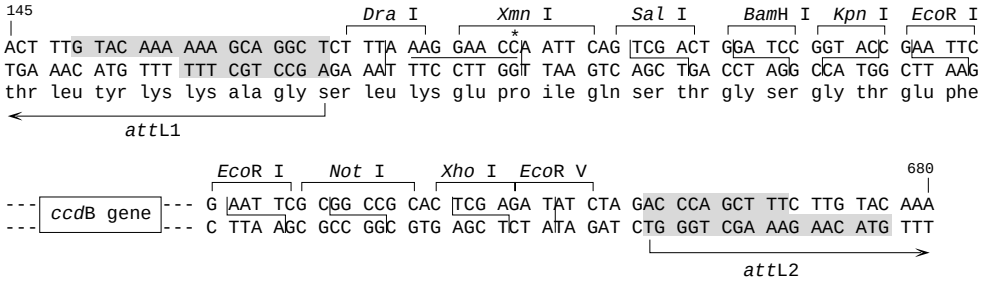
pENTR2B sequence: 145-675 nucleotides



Restriction Endonucleases that do not cleave pENTR2B:

Asc I	*Bst*E II	*Gsu* I	*Pin*A I	*Spe* I
Ava II	*Cla* I	*Hin*d III	*Pme* I	*Sph* I
Avr II	*Cvn* I	*Hpa* I	*Psh*A I	*Sse*8387 I
Bcl I	*Dra* I	*Kpn*2 I	*Psp*5 II	*Sst* I
Bgl I	*Dra* III	*Mlu* I	*Rsr* II	*Sst* II
Bgl II	*Dsa* I	*Mun* I	*Sap* I	*Stu* I
*Bpu*1102 I	*Eam*1105 I	*Nco* I	*Sca* I	*Sty* I
*Bsa*A I	*Eco*47 III	*Nde* I	*Sex*A I	*Sun* I
*Bse*R I	*Eco*72 I	*Ngo*A IV	*Sfi* I	*Swa* I
Bsg I	*Fse* I	*Nsp* V	*Sgr*A I	*Tth*111 I
*Bsp*M I	*Fsp* I	*Pac* I	*Sna*B I	*Xcm* I

pENTR3C sequence: 145-680 nucleotides



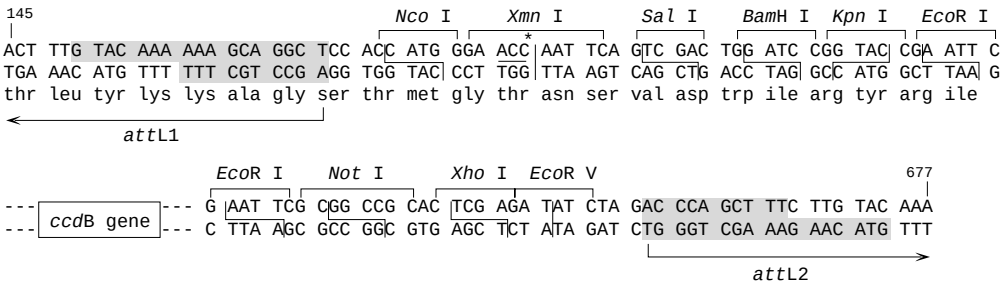
Restriction Endonucleases that do not cleave pENTR3C:

Asc I	*Bst*E II	*Hin*d III	*Pin*A I	*Spe* I
Ava II	*Cla* I	*Hpa* I	*Pme* I	*Sph* I
Avr II	*Cvn* I	*Kpn*2 I	*Psh*A I	*Sse*8387 I
Bcl I	*Dra* III	*Mlu* I	*Psp*5 II	*Sst* I
Bgl I	*Dsa* I	*Mun* I	*Rsr* II	*Sst* II
Bgl II	*Eam*1105 I	*Nar* I	*Sap* I	*Stu* I
*Bpu*1102 I	*Eco*47 III	*Nco* I	*Sca* I	*Sty* I
*Bsa*A I	*Eco*72 I	*Nde* I	*Sex*A I	*Sun* I
*Bse*R I	*Fse* I	*Ngo*A IV	*Sfi* I	*Swa* I
Bsg I	*Fsp* I	*Nsp* V	*Sgr*A I	*Tth*111 I
*Bsp*M I	*Gsu* I	*Pac* I	*Sna*B I	*Xcm* I

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

Additional Information

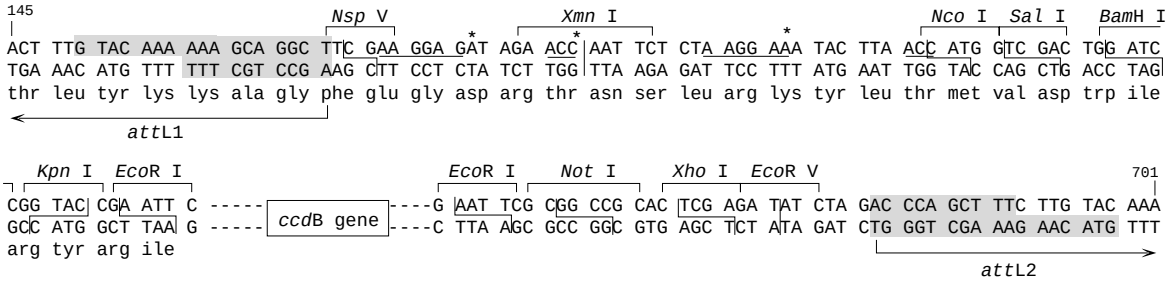
pENTR4 sequence: 145-677 nucleotides



Restriction Endonucleases that do not cleave pENTR4:

Asc I	*Bst*E II	*Hin*d III	*Pme* I	*Sph* I
Ava II	*Cla* I	*Hpa* I	*Psh*A I	*Sse*8387 I
Avr II	*Cvn* I	*Kpn*2 I	*Psp*5 II	*Sst* I
Bcl I	*Dra* I	*Mlu* I	*Rsr* II	*Sst* II
Bgl I	*Dra* III	*Mun* I	*Sap* I	*Stu* I
Bgl II	*Eam*1105 I	*Nar* I	*Sca* I	*Sun* I
*Bpu*1102 I	*Eco*47 III	*Nde* I	*Sex*A I	*Swa* I
*Bsa*A I	*Eco*72 I	*Ngo*A IV	*Sfi* I	*Tth*111 I
*Bse*R I	*Fse* I	*Nsp* V	*Sgr*A I	*Xcm* I
Bsg I	*Fsp* I	*Pac* I	*Sna*B I	
*Bsp*M I	*Gsu* I	*Pin*A I	*Spe* I	

pENTR11 sequence: 145-701 nucleotides



Restriction Endonucleases that do not cleave pENTR11

Asc I	*Bst*E II	*Hin*d III	*Psh*A I	*Sse*8387 I
Ava II	*Cla* I	*Hpa* I	*Psp*5 II	*Sst* I
Avr II	*Cvn* I	*Kpn*2 I	*Rsr* II	*Sst* II
Bcl I	*Dra* I	*Mlu* I	*Sap* I	*Stu* I
Bgl I	*Dra* III	*Mun* I	*Sca* I	*Sun* I
Bgl II	*Eam*1105 I	*Nar* I	*Sex*A I	*Swa* I
*Bpu*1102 I	*Eco*47 III	*Nde* I	*Sfi* I	*Tth*111 I
*Bsa*A I	*Eco*72 I	*Ngo*A IV	*Sgr*A I	*Xcm* I
*Bse*R I	*Fse* I	*Pac* I	*Sna*B I	
Bsg I	*Fsp* I	*Pin*A I	*Spe* I	
*Bsp*M I	*Gsu* I	*Pme* I	*Sph* I	

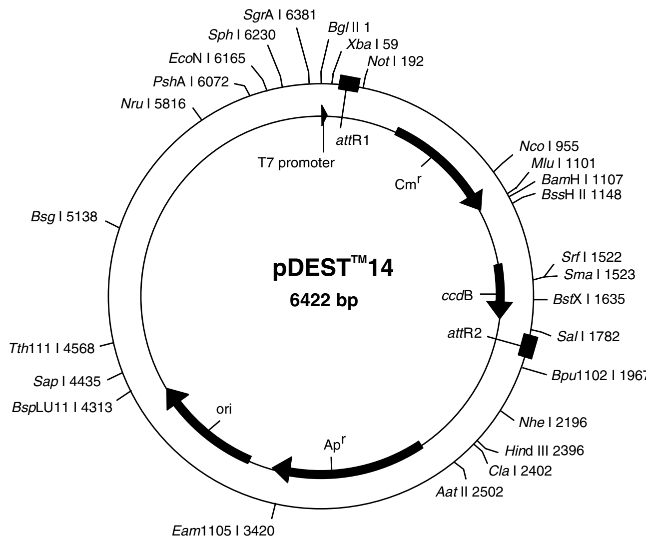
*The AAGGAG/A and ACC sites correspond to the Shine-Dalgarno (prokaryotes) and Kozak eukaryotic ribosome recognition sequences preceding the initiating ATG.

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

5.6.2 *E. coli* Destination Vectors

5

pDEST14 Vector for Native Protein Expression from a T7 Promoter



Recombination Region of the Expression Clone resulting from pDEST14 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 75 and 1897. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST14 by recombination. Non-shaded regions are derived from pDEST14.

```
                                 transcription start
      T7 promoter                                                     74
TTAATACGAC TCACTATAGG GAGACCACAA CGGTTTCCCT CTAGATCACA AGTTTGTACA AAAAAGCAGG CTNN -----
AATTATGCTG AGTGATATCC CTCTGGTGTT GCCAAAGGGA GATCTAGTGT TCAAACATGT TTTTTCGTCC GANN -----
                                                                attB1

                    1898
---------- NACCCAGCTT TCTTGTACAA AGTGGTGATG ATCCGGCTGC TAACAAAGCC CGAAAGGAAG CTGAGTTGGC
   GENE
---------- NTGGGTCGAA AGAACATGTT TCACCACTAC TAGGCCGACG ATTGTTTCGG GCTTTCCTTC GACTCAACCG
                    attB2
```

Restriction endonucleases that do not cleave pDEST14 DNA:

Afl II	*Bst*E II	*Kpn* I	*Pin*A I	*Sna*B I	*Sun* I
Apa I	*Cvn* I	*Mun* I	*Pme* I	*Spe* I	*Swa* I
Asc I	*Dra* III	*Nde* I	*Rsr* II	*Sse*8387 I	*Xcm* I
Avr II	*Eco*72 I	*Nsi* I	*Sex*A I	*Sst* I	*Xho* I
Bcl I	*Fse* I	*Nsp* V	*Sfi* I	*Sst* II	
*Bse*R I	*Hpa* I	*Pac* I	*Sgf* I	*Stu* I	

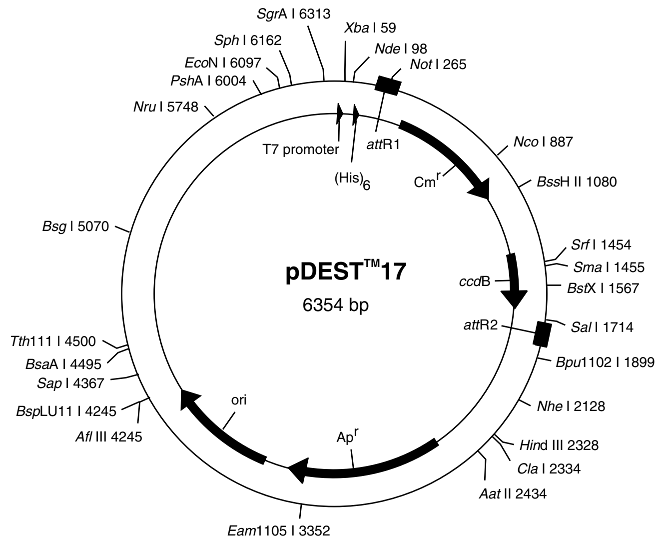
Restriction endonucleases that cleave pDEST14 DNA twice:

Afl III	1101	4313	*Bst*1107 I	1187	4544	*Pvu* I	3053	6139
*Alw*N I	1428	3899	*Drd* I	4205	4620	*Pvu* II	552	4724
Apo I	654	2427	*Ear* I	2631	4435	*Sca* I	1069	2942
Ava I	1523	5363	*Eco*57 I	2738	3786	*Ssp* I	964	2618
Ban II	6307	6321	*Eco*R I	654	2427	*Vsp* I	19	3249
*Bsa*A I	345	4563	*Eco*R V	2049	2240	*Xma* III	193	5849
*Bsp*M I	1778	5725	*Psp*5 II	5307	5349	*Xmn* I	2821	4755
*Bsr*B I	2581	4382	*Pst* I	1776	3179			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

Additional Information

pDEST17 Vector for N-terminal Histidine Fusion Protein Expression from a T7 Promoter



Recombination Region of the Expression Clone resulting from pDEST17 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 148 and 1829.Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST17 by recombination. Non-shaded regions are derived from pDEST17.

```
                         transcription start
   T7 promoter                                                                      RRS
TTAATACGAC TCACTATAGG GAGACCACAA CGGTTTCCCT CTAGAAATAA TTTTGTTTAA CTTTAAGAAG GAGATATACA
AATTATGCTG AGTGATATCC CTCTGGTGTT GCCAAAGGGA GATCTTTATT AAAACAAATT GAAATTCTTC CTCTATATGT

                                                                   147
  met ser tyr tyr his his his his his his leu glu ser thr ser leu tyr lys lys ala gly
T ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC CTC GAA TCA ACA AGT TTG TAC AAA AAA GCA GGC
A TAC AGC ATG ATG GTA GTG GTA GTG GTA GTG GAG CTT AGT TGT TCA AAC ATG TTT TTT CGT CCG
                                                                      attB1

                     1830
TNN ------------- NACCCAGCTT TCTTGTACAA AGTGGTTGAT TCGAGGCTGC TAACAAAGCC CGAAAGGAAG
        GENE
ANN ------------- NTGGGTCGAA AGAACATGTT TCACCAACTA AGCTCCGACG ATTGTTTCGG GCTTTAATTC
                          attB2
```

Restriction endonucleases that do not cleave pDEST17 DNA:

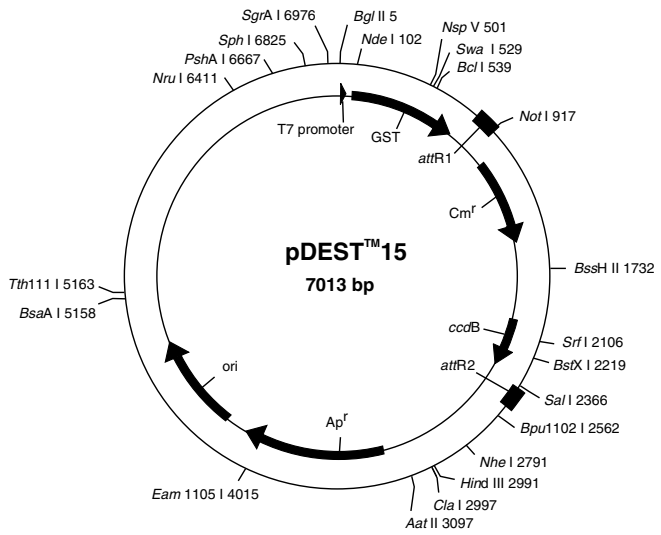
Afl II	*Bst*E II	*Kpn* I	*Pin*A I	*Sna*B I	*Sun* I
Apa I	*Cvn* I	*Mlu* I	*Pme* I	*Spe* I	*Swa* I
Asc I	*Dra* III	*Mun* I	*Rsr* II	*Sse*8387 I	*Xcm* I
Avr II	*Eco*72 I	*Nsi* I	*Sex*A I	*Sst* I	*Xho* I
Bcl I	*Fse* I	*Nsp* V	*Sfi* I	*Sst* II	
*Bse*R I	*Hpa* I	*Pac* I	*Sgf* I	*Stu* I	

Restriction endonucleases that cleave pDEST17 DNA twice:

*Alw*N I	1360	3831	*Bst*1107 I	1119	4476	*Pst* I	1708	3111
Apo I	586	2359	*Drd* I	4137	4552	*Pvu* I	2985	6071
Ava I	1455	5295	*Ear* I	2563	4367	*Pvu* II	484	4656
*Bam*H I	336	1039	*Eco*57 I	2670	3718	*Sca* I	1001	2874
Ban II	6239	6253	*Eco*R I	586	2359	*Ssp* I	896	2550
Bgl II	1	1033	*Eco*R V	1981	2172	*Vsp* I	19	3181
*Bsp*M I	1710	5657	*Esp*3 I	804	4598	*Xma* III	266	5781
*Bsr*B I	2513	4314	*Psp*5 II	5239	5281	*Xmn* I	2753	4687

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

pDEST15 Vector for N-terminal GST Fusion Expression from a T7 Promoter



Recombination Region of the Expression Clone resulting from pDEST15 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 800 and 2481.Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST15 by recombination. Non-shaded regions are derived from pDEST15.

```
    T7 promoter      transcription start                                                    RRS
TAATACGACT CACTATAGGG AGACCACAAC GGTTTCCCTC TAGAAATAAT TTTGTTTAAC TTTAAGAAGG AGATATACAT
ATTATGCTGA GTGATATCCC TCTGGTGTTG CCAAAGGGAG ATCTTTATTA AAACAAATTG AAATTCTTCC TCTATATGTA

                                                                    799
met ser pro ile                          trp ser asn gln thr ser leu tyr lys lys ala gly
ATG TCC CCT ATA      GST                 TGG TCG AAT CAA ACA AGT TTG TAC AAA AAA GCA GGC TNN -------
TAC AGG GGA TAT                          ACC AGC TTA GTT TGT TCA AAC ATG TTT TTT CGT CCG ANN -------
                                                                    attB1
                                2482
                                 •
---------- NACCCAGCTT TCTTGTACAA AGTGGTTTGA TTCGACCCGG GATCCGGCTG CTAACAAAGC CCGAAAGGAA
  GENE     NTGGGTCGAA AGAACATGTT TCACCAAACT AAGCTGGGCC CTAGGCCGAC GATTGTTTCG GGCTTTCCTT
                attB2
```

Restriction endonucleases that do not cleave pDEST15 DNA:

Afl II	*Cvn* I	*Mlu* I	*Rsr* II	*Sse*8387 I	*Xho* I
Apa I	*Dra* III	*Mun* I	*Sex*A I	*Sst* I	
Asc I	*Eco*72 I	*Nsi* I	*Sfi* I	*Sst* II	
Avr II	*Fse* I	*Pac* I	*Sgf* I	*Stu* I	
*Bse*R I	*Hpa* I	*Pin*A I	*Sna*B I	*Sun* I	
*Bst*E II	*Kpn* I	*Pme* I	*Spe* I	*Xcm* I	

Restriction endonucleases that cleave pDEST15 DNA twice:

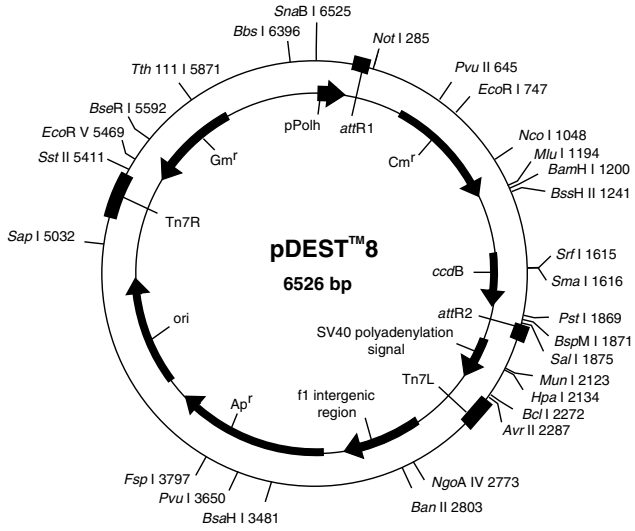
Afl III	343	4908	*Eco*57 I	3333	4381	*Pvu* II	1136	5319
*Alw*N I	2012	4494	*Eco*N I	111	6760	*Sap* I	189	5030
Apo I	1238	3022	*Eco*R I	1238	3022	*Sma* I	2107	2506
Ban II	6902	6916	*Eco*R V	2644	2835	*Ssp* I	1548	3213
Bsg I	370	5733	*Esp*3 I	1456	5261	*Vsp* I	23	3844
*Bsp*LU11 I	343	4908	*Nco* I	777	1539	*Xba* I	63	1685
*Bsp*M I	2362	6320	*Psp*5 II	5902	5944	*Xma* III	918	6444
*Bst*1107 I	1771	5139	*Pst* I	2360	3774			
Drd I	4800	5215	*Pvu* I	3648	6734			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

Additional Information

5.6.3 Baculovirus Destination Vectors

pDEST8 Vector for Native Protein Expression from a Polyhedrin Promoter



Recombination Region of the Expression Clone resulting from pDEST8 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 168 and 1990. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST8 by recombination. Non-shaded regions are derived from pDEST8.

```
24          60          transcription start
                        ATAAATAAGT ATTTTACTGT TTTCGTAACA GTTTTGTAAT AAAAAAACCT ATAAATATTC CGGATTATTC
                        TATTTATTCA TAAAATGACA AAAGCATTGT CAAAACATTA TTTTTTTGGA TATTTATAAG GCCTAATAAG
pPolh
                                              167
ATACCGTCCC ACCATCGGGC GCGGATCATC ACAAGTTTGT ACAAAAAAGC AGGCTNN ------------ NACCCAGCTT
TATGGCAGGG TGGTAGCCCG CGCCTAGTAG TGTTCAAACA TGTTTTTTCG TCCGANN ------------ NTGGGTCGAA
                                                          GENE
                                              attB1
1991                                                                                  2069
TCTTGTACAA AGTGGTGATA GCTTGTCGAG AAGTACTAGA GGATCATAAT CAGCCATACC ACATTTGTAG AGGTTTTACT
AGAACATGTT TCACCACTAT CGAACAGCTC TTCATGATCT CCTAGTATTA GTCGGTATGG TGTAAACATC TCCAAAATGA
     attB2
```

Restriction endonucleases that do not cleave pDEST8 DNA:

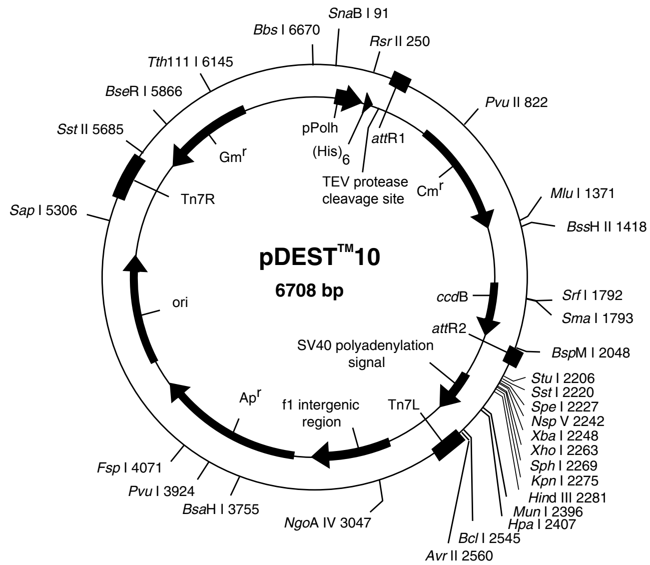
Aat II	*Cla* I	*Hin*d III	*Nsp* V	*Sex*A I	*Sst* I
Afl II	*Cvn* I	*Kpn* I	*Pac* I	*Sfi* I	*Stu* I
Apa I	*Eco*47 III	*Nar* I	*Pin*A I	*Sgf* I	*Sun* I
Asc I	*Eco*72 I	*Nde* I	*Pme* I	*Sgr*A I	*Swa* I
*Bpu*1102 I	*Eco*N I	*Nhe* I	*Psh*A I	*Spe* I	*Xba* I
Bsg I	*Eco*O109 I	*Nru* I	*Psp*5 II	*Sph* I	*Xcm* I
*Bst*E II	*Fse* I	*Nsi* I	*Rsr* II	*Sse*8387 I	*Xho* I

Restriction endonucleases that cleave pDEST8 DNA twice:

*Alw*N I	1521	4496	*Bst*1107 I	2	1280	*Nsp* I	4910	5892
Ban I	2837	4069	*Bst*X I	1728	5356	*Pfl*M I	406	973
Bgl II	5193	5663	*Dra* III	2876	6224	*Rca* I	3182	4190
*Bsp*LU11 I	4910	5892	*Eam*1105 I	2522	4017	*Tfi* I	1097	4936
*Bss*S I	3353	4737	*Gsu* I	848	3932	X*mn* I	3418	6443

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

pDEST10 Vector for N-terminal Histidine Fusion Proteins Expression from a Polyhedrin Promoter



Recombination Region of the Expression Clone resulting from pDEST10 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 345 and 2167. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST10 by recombination. Non-shaded regions are derived from pDEST10.

```
116          155   transcription start
|             •    |→
              AATAAGTATT TTACTGTTTT CGTAACAGTT TTGTAATAAA AAAACCTATA AATATTCCGG ATTATTCATA CCGTCCCACC
              TTATTCATAA AATGACAAAA GCATTGTCAA AACATTATTT TTTTGGATAT TTATAAGGCC TAATAAGTAT GGCAGGGTGG
pPolh

                             met ser tyr tyr his his his his his his asp tyr asp ile pro thr thr
ATCGGGCGCG GATCTCGGTC CGAAACC ATG TCG TAC TAC CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC CCA ACG ACC
TAGCCCGCGC CTAGAGCCAG GCTTTGG TAC AGC ATG ATG GTA GTG GTA GTG GTA GTG CTA ATG CTA TAG GGT TGC TGG

                                             344
                                              •
glu asn leu tyr phe gln gly ile thr ser leu tyr lys lys ala gly
GAA AAC CTG TAT TTT CAG GGC ATC ACA AGT TTG TAC AAA AAA GCA GGC TNN -------------- NACCCAGCTT
CTT TTG GAC ATA AAA GTC CCG TAG TGT TCA AAC ATG TTT TTT CGT CCG ANN -----GENE----- NTGGGTCGAA

   TEV cleavage site                         attB1
2168                                                 2226
 •                                                    |
TCTTGTACAA AGTGGTGATG CCATGGATCC GGAATTCAAA GGCCTACGTC GACGAGCTCA
AGAACATGTT TCACCACTAC GGTACCTAGG CCTTAAGTTT CCGGATGCAG CTGCTCGAGT

   attB2
```

Restriction endonucleases that do not cleave pDEST10 DNA:

Aat II	*Bst*E II	*Eco*O109 I	*Nsi* I	*Sex*A I	*Swa* I
Afl II	*Cla* I	*Fse* I	*Pac* I	*Sfi* I	*Xcm* I
Apa I	*Cvn* I	*Nar* I	*Pin*A I	*Sgf* I	
Asc I	*Eco*47 III	*Nde* I	*Pme* I	*Sgr*A I	
*Bpu*1102 I	*Eco*72 I	*Nhe* I	*Psh*A I	*Sse*8387 I	
Bsg I	*Eco*N I	*Nru* I	*Psp*5 II	*Sun* I	

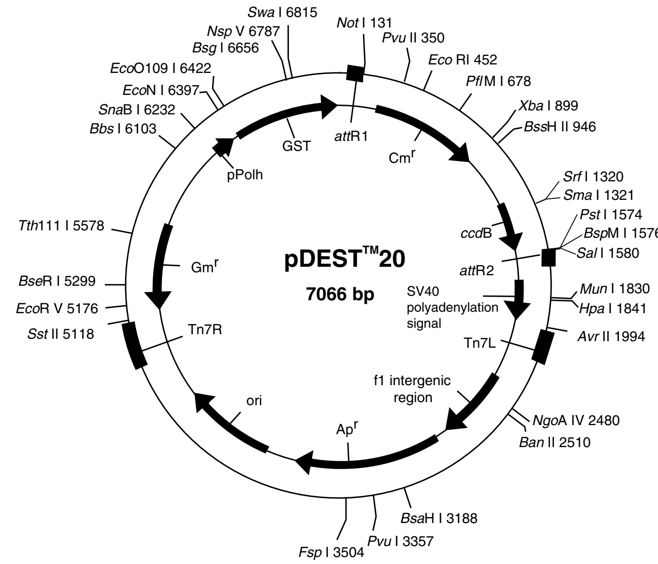
Restriction endonucleases that cleave pDEST10 DNA twice:

*Alw*N I	1698	4770	*Bst*X I	1905	5630	*Not* I	462	2233
*Bam*H I	1377	2191	*Dra* III	3150	6498	*Pfl*M I	583	1150
Ban I	2220	3077	*Eam*1105 I	2795	4291	*Pst* I	2046	2256
Bgl II	5467	5937	*Eco*R I	924	2198	*Rca* I	3456	4464
*Bsp*LU11 I	5184	6166	*Eco*R V	298	5743	*Sal* I	2052	2214
*Bss*S I	3627	5011	*Gsu* I	1025	4206	X*mn* I	9	3692
*Bst*1107 I	94	1457	*Nco* I	1225	2187			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

Additional Information

pDEST20 Vector for N-terminal GST Fusion Protein Expression from a Polyhedrin Promoter



Recombination Region of the Expression Clone resulting from pDEST20 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 14 and 1696. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST20 by recombination. Non-shaded regions are derived from pDEST20.

```
                              transcription start
6257          6295
|              •
[pPolh]        AAATAAGTAT TTTACTGTTT TCGTAACAGT TTTGTAATAA AAAAACCTAT AAATATTCCG GATTATTCAT ACCGTCCCAC
               TTTATTCATA AAATGACAAA AGCATTGTCA AAACATTATT TTTTTGGATA TTTATAAGGC CTAATAAGTA TGGCAGGGTG
pPolh
                                                                     13
                                   6404      7059                    •
                  met ala pro ile   |         | arg his asn gln thr ser leu tyr lys lys ala gly
CATCGGGCGC GGATCC ATG GCC CCT ATA   [  GST  ]   CGT CAT AAT CAA ACA AGT TTG TAC AAA AAA GCA GGC TNN
GTAGCCCGCG CCTAGG TAC CGG GGA TAT               GCA GTA TTA GTT TGT TCA AAC ATG TTT TTT CGT CCG ANN
                                                                        attB1

                1696                                                              1754
                 •                                                                  |
-------------- NACCCAGCTT TCTTGTACAA AGTGGTTTGA TAGCTTGTCG AGAAGTACTA GAGGATCATA ATCAGCCATA
    GENE
-------------- NTGGGTCGAA AGAACATGTT TCACCAAACT ATCGAACAGC TCTTCATGAT CTCCTAGTAT TAGTCGGTAT
                         attB2
```

Restriction endonucleases that do not cleave pDEST20 DNA:

Aat II	*Cvn* I	*Nar* I	*Pme* I	*Sgr*A I	*Xcm* I
Afl II	*Eco*47 III	*Nde* I	*Psh*A I	*Spe* I	*Xho* I
Apa I	*Eco*72 I	*Nhe* I	*Psp*5 II	*Sph* I	
Asc I	*Fse* I	*Nru* I	*Rsr* II	*Sse*8387 I	
*Bpu*1102 I	*Hin*d III	*Nsi* I	*Sex*A I	*Sst* I	
*Bst*E II	*Kpn* I	*Pac* I	*Sfi* I	*Stu* I	
Cla I	*Mlu* I	*Pin*A I	*Sgf* I	*Sun* I	

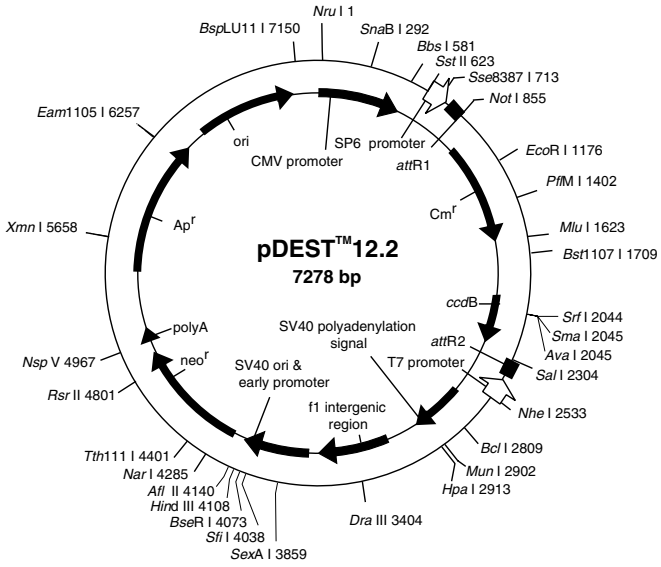
Restriction endonucleases that cleave pDEST20 DNA twice:

*Alw*N I	1226	4203	*Bst*1107 I	985	6235	*Gsu* I	553	3639
Bcl I	1979	6825	*Bst*X I	1433	5063	*Nco* I	753	6389
Bgl II	4900	5370	*Dra* III	2583	5931	*Rca* I	2889	3997
*Bsm*F I	1228	6368	*Eam*1105 I	2229	3724	*Sap* I	4739	6475
*Bss*S I	3060	4444	*Esp*3 I	670	5529	*Tfi* I	802	4643

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

5.6.4 Mammalian Destination Vectors

pDEST12.2 Vector for Native Protein Expression from a CMV Promoter



Recombination Region of the Expression Clone resulting from pDEST12.2 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 738 and 2419. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST12.2 by recombination. Non-shaded regions are derived from pDEST12.2. Transcription starts at nucleotide 537.

```
15        630
          ACGGATAACA ATTTCACACA GGAAACAGCT ATGACCATTA GGCCTTTGCA AAAAGCTATT TAGGTGACAC TATAGAAGGT
          TGCCTATTGT TAAAGTGTGT CCTTTGTCGA TACTGGTAAT CCGGAAACGT TTTTCGATAA ATCCACTGTG ATATCTTCCA
CMV promoter
                              737                                                    2420
ACGCCTGCAG GTACCGGATC ACAAGTTTGT ACAAAAAAGC AGGCTNN---------------NACCCAGCTT TCTTGTACAA AGTGGTGATC
TGCGGACGTC CATGGCCTAG TGTTCAAACA TGTTTTTTCG TCCGANN---------------NTGGGTCGAA AGAACATGTT TCACCACTAG
                                                 GENE
                              attB1                                                  attB2
                                                                2498
GCGTGCATGC GACGTCATAG CTCTCTCCCT ATAGTGAGTC GTATTATAAG CTAGGCACTG
CGCACGTACG CTGCAGTATC GAGAGAGGGA TATCACTCAG CATAATATTC GATCCGTGAC
```

Restriction endonucleases that do not cleave pDEST12.2 DNA:

Apa I	*Bst*E II	*Eco*R V	*Psh*A I	*Sun* I
Asc I	*Cvn* I	*Fse* I	*Psp*5 II	*Swa* I
Bgl II	*Eco*47 III	*Pac* I	*Sgf* I	*Xba* I
*Bpu*1102 I	*Eco*72 I	*Pin*A I	*Sgr*A I	*Xcm* I
Bsg I	*Eco*N I	*Pme* I	*Spe* I	*Xho* I

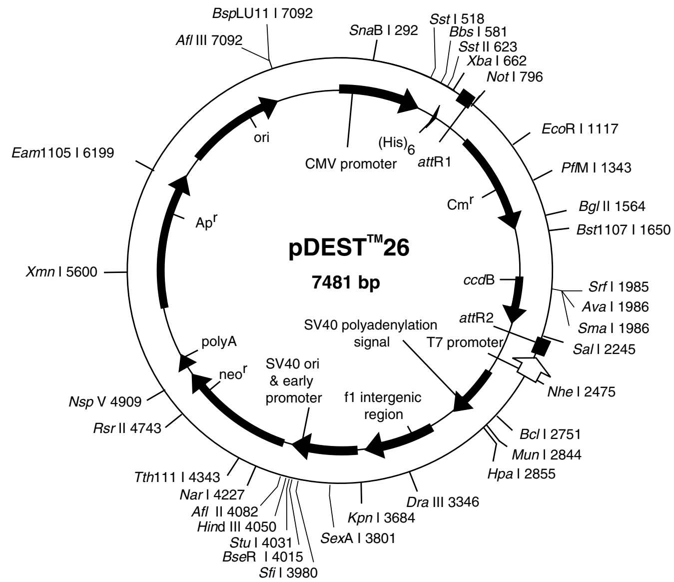
Restriction endonucleases that cleave pDEST12.2 DNA twice:

Acc I	1709	2304	*Cla* I	3048	5066	*Sca* I	1591	5779
Afl III	1623	7150	*Eco*O109 I	2781	5281	*Sst* I	518	7275
*Alw*N I	1950	6736	*Kpn*2 I	605	1172	*Stu* I	669	4089
Avr II	617	4092	*Kpn* I	719	3742	*Vsp* I	3059	6086
Bsa I	2179	6190	*Nde* I	187	2662	*Xma* III	856	4192
*Bss*H II	1670	4683	*Ngo*A IV	3301	4786			
*Bst*X I	2157	5000	*Pvu* I	3132	5890			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

Additional Information

pDEST26 Vector for N-terminal Histidine Fusion Protein Expression from a CMV Promoter



Recombination Region of the Expression Clone resulting from pDEST26 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 679 and 2360. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST26 by recombination. Non-shaded regions are derived from pDEST26. Transcription starts at nucleotide 537.

```
15          615                                                                     678
|            •                                                                        •
                         met ala tyr tyr his his his his his his ser arg ser thr ser leu tyr lys lys ala
            AGCCTAGGCC GCGGACC ATG GCG TAC TAC CAT CAC CAT CAC CAT CAC TCT AGA TCA ACA AGT TTG TAC AAA AAA GCA
            TCGGATCCGG CGCCTGG TAC CGC ATG ATG GTA GTG GTA GTG GTA GTG AGA TCT AGT TGT TCA AAC ATG TTT TTT CGT
CMV promoter
                                                                                   attB1

                          2361                                                         2499
gly                        •                                                              |
GGC TNN ----------- NACCCAGCTT TCTTGTACAA AGTGGTTGAT CGCGTGCATG CGACGTCATA GCTCTCTCCC TATAGTGAGT CGTATTATAA
CCG ANN ----GENE--- NTGGGTCGAA AGAACATGTT TCACCAACTA GCGCACGTAC GCTGCAGTAT CGAGAGAGGG ATATCACTCA GCATAATATT
                          attB2
```

Restriction endonucleases that do not cleave pDEST26 DNA:

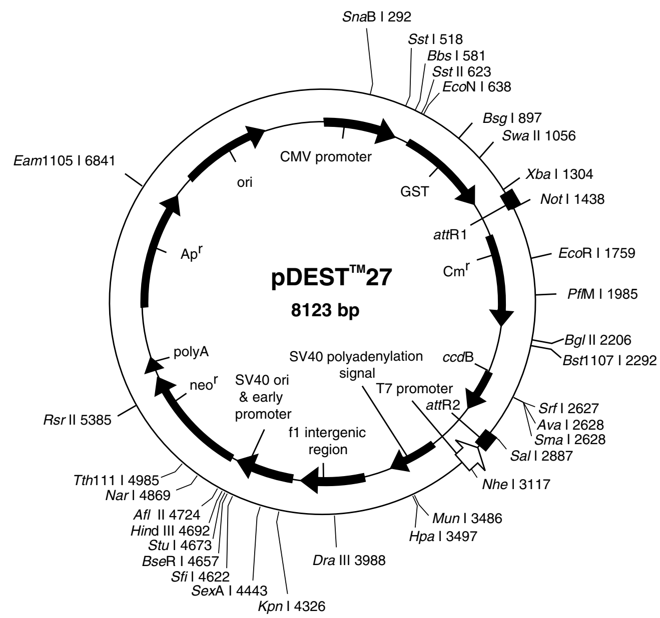
Apa I	*Cvn* I	*Fse* I	*Pme* I	*Spe* I	*Xho* I
Asc I	*Eco*47 III	*Mlu* I	*Psh*A I	*Sse*8387 I	
*Bpu*1102 I	*Eco*72 I	*Nru* I	*Psp*5 II	*Sun* I	
Bsg I	*Eco*N I	*Pac* I	*Sgf* I	*Swa* I	
*Bst*E II	*Eco*R V	*Pin*A I	*Sgr*A I	*Xcm* I	

Restriction endonucleases that cleave pDEST26 DNA twice:

Acc I	1650	2245	*Bst*X I	2098	4942	*Pst* I	2239	4277
*Alw*N I	1891	6678	*Cla* I	2990	5008	*Pvu* I	3074	5832
Avr II	617	4034	*Kpn*2 I	605	1113	*Sca* I	1532	5721
Bsa I	2120	6132	*Nde* I	187	2604	*Xma* III	797	4134
*Bss*H II	1611	4625	*Ngo*A IV	3243	4728			

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

pDEST27 Vector for N-terminal GST Fusion Protein Expression from a CMV Promoter



Recombination Region of the Expression Clone resulting from pDEST27 × Entry Clone. DNA from the Entry Clone replaces the region between nucleotides 1321 and 3002. Shaded regions correspond to those DNA sequences transferred from the Entry Clone into pDEST27 by recombination. Non-shaded regions are derived from pDEST27. Transcription starts at nucleotide 537.

```
                                                                      1320
15       630                                                            •
|        |             met ala pro ile            arg ser arg ser thr ser leu tyr lys lys ala gly
[CMV]    AGGCCGCGGA CC ATG GCC CCT ATA |        |  CGT TCT AGA TCA ACA AGT TTG TAC AAA AAA GCA GGC TNN
         TCCGGCGCCT GG TAC CGG GGA TAT |  GST   |  GCA AGA TCT AGT TGT TCA AAC ATG TTT TTT CGT CCG ANN
CMV promoter                                                       |_______________________________|
                                                                                 attB1

                     3003                                                                          3081
                      •                                                                               |
-------- NACCCAGCTT TCTTGTACAA AGTGGTTGAT CGCGTGCATG CGACGTCATA GCTCTCTCCC TATAGTGAGT CGTATTATAA GCTAGGCACT
 GENE    NTGGGTCGAA AGAACATGTT TCACCAACTA GCGCACGTAC GCTGCAGTAT CGAGAGAGGG ATATCACTCA GCATAATATT CGATCCGTGA
-------- |__________________________|
                    attB2
```

Restriction endonucleases that do not cleave pDEST27 DNA:

Apa I	*Eco*47 III	*Nru* I	*Psp*5 II	*Sun* I
Asc I	*Eco*72 I	*Pac* I	*Sgf* I	*Xcm* I
*Bpu*1102 I	*Eco*R V	*Pin*A I	*Sgr*A I	*Xho* I
*Bst*E II	*Fse* I	*Pme* I	*Spe* I	
Cvn I	*Mlu* I	*Psh*A I	*Sse*8387 I	

Restriction endonucleases that cleave pDEST27 DNA twice:

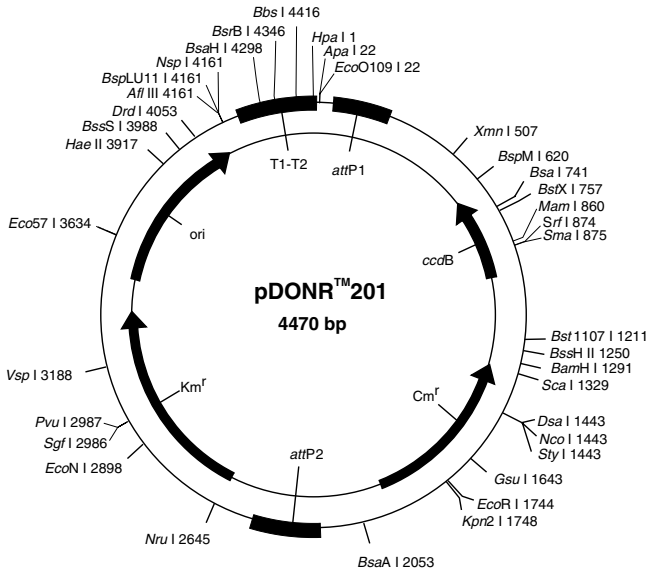
Acc I	2292	2887	*Bsp*LU11 I	870	7734	*Ngo*A IV	3885	5370
Afl III	870	7734	*Bss*H II	2253	5267	*Nsp* V	1028	5551
*Alw*N I	2533	7320	*Bst*X I	2740	5584	*Pst* I	2881	4919
Avr II	617	4676	*Cla* I	3632	5650	*Pvu* I	3716	6474
Bcl I	1066	3393	*Kpn*2 I	605	1755	*Xma* III	1439	4776
Bsa I	2762	6774	*Nde* I	187	3246	*Xmn* I	1021	6242

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

Additional Information

5.6.5 Donor Vector for BP Reactions

pDONR™201 Vector for Production of Km^r Entry Clones



pDONR201 Vector. DNA from the PCR product or Expression Clone replaces the region between nucleotides 111 and 2352. The vector contains T1-T2 transcription terminators to minimize possible toxic effects of cloned genes expressing from vector-encoded promoters. pDONR201 Vector must be propagated in DB3.1™ cells because of the *ccd*B gene.

Restriction Endonucleases that do not cleave pDONR201:

Aat II	*Bsg* I	*Kpn* I	*Psh*A I	*Sst* I
Afl II	*Bst*E II	*Mlu* I	*Psp*5 II	*Sst* II
Asc I	*Cla* I	*Mun* I	*Rsr* II	*Stu* I
Ava II	*Cvn* I	*Nar* I	*Sap* I	*Sun* I
Avr II	*Dra* III	*Nde* I	*Sex*A I	*Swa* I
Ban I	*Eco*47 III	*Ngo*A IV	*Sfi* I	*Tth*111 I
Bcl I	*Eco*72 I	*Not* I	*Sgr*A I	*Xba* I
Bgl I	*Eco*R V	*Nsp* V	*Sna*B I	*Xcm* I
Bgl II	*Fse* I	*Pac* I	*Spe* I	*Xho* I
*Bpu*1102 I	*Fsp* I	*Pin*A I	*Sph* I	*Xma* III
*Bse*R I	*Hin*d III	*Pme* I	*Sse*8387 I	

The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. The sequence and the location of sites for restriction endonucleases that cleave up to 10 times can be found in the TECH-ONLINE℠ section of Life Technologies' web page, http://www.lifetech.com.

References

6

1. Ptashne, M. (1992) *A Genetic Switch: Phage (Lambda) and Higher Organisms*, Cell Press, Cambridge.
2. Landy, A. (1989) *Ann. Rev. Biochem. 58,* 913.
3. Bushman, W., Thompson, J.F., Vargas, L., and Landy, A. (1985), *Science 230,* 906.
4. Coligan, J., Dunn, B., Ploueh, H., Speicher, D., and Wingfield, P. (eds.) (1999) *Current Protocols in Protein Science*. John Wiley and Sons.
5. Ausubel, F., Brent, R., Moore, D., Seidman, J.G., Smith, J., and Struhl, K. (eds.) (1999) *Current Protocols in Molecular Biology*. John Wiley and Sons.
6. Makrides, S.C. (1996) *Microbiological Reviews 60*, 512.
7. Kost, T.A. (1999) *Curr. Op. Biotech. 10*, 409.
8. Shine, J. and Dalgarno, L. (1975) *Eur. J. Biochem. 57,* 221.
9. Kozak, M. (1991) *J. Biol. Chem. 266,* 19867.
10. Pain, V.M. (1996) *Eur. J. Biochem. 236,* 747.
11. Orosz, A., Boros, I., and Venetianer, P. (1991) *Eur. J. Biochem. 201,* 653.
12. Miki, T., Park, J.A., Nagao, K., Murayama, N., and Horiuchi, T. (1992) *J. Mol. Biol. 225,* 39.
13. Bernard, P. and Couturier, M. (1992) *J. Mol. Biol. 226,* 735.
14. Bernard, P., Kezdy, K.E., Van Melderen, L., Steyaert, J., Wyns, L., Pato, M.L., Higgins, P.N., Couturier, M. (1993) *J. Mol. Biol. 234,* 534.
15. Young, A.C., Xu, L., and Goldsborough, M.D. (1999) *Focus® 21,* 40.
16. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
17. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (1994) *Current Protocols in Molecular Biology,* Greene Publishing Associates and Wiley-Interscience.
18. Young, A.C., Xu, L., Goldsborough, M.D., and Blakesley, R. (1999) *Focus 21*, 11.
19. Fox, D., Nathan, M., Natarajan, P., Bracete, A., and Mertz, L. (1998) *Focus 20,* 15.
20. Zimmerman, K., Schögl, D, and Mannhalter, J.W. (1998) *BioTechniques 24:4,* 582.
21. Westfall, B., Sitaraman, K., Lee, J.E., Borman, J., and Rashtchian, A. (1999) *Focus 21,* 46.
22. Trinh, T., Jessee, J., and Bloom, F.R. (1994) *Focus 16*, 78.
23. Brownstein, M.J., Carpten, J.D., and Smith, J.R. (1996) *BioTechniques 20*, 1004.

7 Related Products

Product	Size	Cat. No.
GATEWAY™ Products		
Systems (see Section 3.1 for components)		
PCR Cloning System (with GATEWAY Technology	20 reactions	11821-014
E. coli Expression System (with GATEWAY Technology) (with LIBRARY EFFICIENCY® DH5α™ Competent Cells)	20 reactions	11822-012
E. coli Expression System (with GATEWAY Technology) (with BL21-SI™ Competent Cells)	20 reactions	11823-010
Baculovirus Expression System (with GATEWAY Technology)	20 reactions	11827-011
Mammalian Expression System (with GATEWAY Technology)	20 reactions	11826-013
GATEWAY Vector Conversion System	20 reactions	11828-019
Enzymes		
GATEWAY BP CLONASE Enzyme Mix	20 reactions	11789-013
GATEWAY LR CLONASE Enzyme Mix	20 reactions	11791-019
Entry Vectors (see Table 2)		
GATEWAY pENTR™1A Vector (500 ng/µl)	20 µl	11813-011
GATEWAY pENTR2B Vector (500 ng/µl)	20 µl	11816-014
GATEWAY pENTR3C Vector (500 ng/µl)	20 µl	11817-012
GATEWAY pENTR4 Vector (500 ng/µl)	20 µl	11818-010
GATEWAY pENTR11 Vector (500 ng/µl)	20 µl	11819-018
Destination Vectors (see Table 3)		
GATEWAY pDEST™14 Vector (150 ng/µl)	40 µl	11801-016
GATEWAY pDEST15 Vector (150 ng/µl)	40 µl	11802-014
GATEWAY pDEST17 Vector (150 ng/µl)	40 µl	11803-012
GATEWAY pDEST8 Vector (150 ng/µl)	40 µl	11804-010
GATEWAY pDEST10 Vector (150 ng/µl)	40 µl	11806-015
GATEWAY pDEST20 Vector (150 ng/µl)	40 µl	11807-013
GATEWAY pDEST12.2 Vector (150 ng/µl)	40 µl	11808-011
GATEWAY pDEST26 Vector (150 ng/µl)	40 µl	11809-019
GATEWAY pDEST27 Vector (150 ng/µl)	40 µl	11812-013
Donor Vectors		
GATEWAY pDONR™201 Vector (150 ng/µl)	40 µl	11798-014
Competent Cells		
LIBRARY EFFICIENCY DB3.1™ Competent Cells	5 × 0.2 ml	11782-018
LIBRARY EFFICIENCY DH5α Competent Cells	5 × 0.2 ml	18263-012
BL21-SI Competent Cells	5 × 0.2 ml	11665-015
MAX EFFICIENCY® DH10BAC™ Competent Cells	5 × 0.1 ml	10361-012
Other Related Products:		
Bacterial Expression:		
Bluo-gal	100 mg	15519-010
X-gal	100 mg	15520-034
IPTG	1 g	15529-019
S.O.C. Medium	10 × 10 ml	15544-034
Ampicillin Sodium salt, lyophilized	5 ml	13075-015
Kanamycin Sulfate	1 g	11815-016
LB Broth (1X), liquid	500 ml	10855-021
LB Agar, powder (Lennox L Agar)	500 g	22700-025

Product	Size	Cat. No
Mammalian and Insect Expression:		
BAC-TO-BAC® Baculovirus Expression System	5 reactions	10359-016
LIPOFECTAMINE™ 2000 Reagent	1.5 ml	11668-019
CELLFECTIN® Reagent	1 ml	10362-010
Sf-900 II SFM (1X), liquid	500 ml	10902-096
Sf9 Cells, SFM Adapted	3 ml	11496-015
Sf21 Cells, SFM Adapted	3 ml	11497-013
CD-CHO Medium	500 ml	10743-011
CHO-S Cells	3 ml	11619-012
293 SFM II	500 ml	11686-011
293-F Cells	3 ml	11625-019
VP SFM	1,000 ml	11681-020
COS-7L Cells	3 ml	11622-016
GENETICIN® Selective Antibiotic, liquid	20 ml	10131-035
PCR/RT-PCR Products:		
Custom Primers-GATEWAY *att*B modifications*		
PLATINUM® *Pfx* DNA Polymerase	50 units	11708-047
PLATINUM *Taq* DNA Polymerase High Fidelity	500 units	11304-029
TAQUENCH™ PCR Cloning Enhancer	100 units	11265-014
THERMOSCRIPT™ RT-PCR System plus PLATINUM *Taq* DNA Polymerase High Fidelity	100 reactions	11146-040
DNA Purification:		
CONCERT™ High Purity Plasmid Miniprep System	25 reactions	11449-014
CONCERT High Purity Plasmid Midiprep System	25 reactions	11451-010
CONCERT High Purity Plasmid Maxiprep System	10 reactions	11452-018
Nucleic Acid Purification Rack	each	11494-010
CONCERT Rapid Plasmid Miniprep System	50 reactions	11453-016
CONCERT Rapid Plasmid Midiprep System	25 reactions	11454-014
CONCERT Rapid Plasmid Maxiprep System	10 reactions	11455-011
CONCERT Rapid Gel Extraction System	50 reactions	11456-019
CONCERT Matrix Gel Extraction System	150 reactions	11457-017
Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)	100 ml	15593-031
Cloning Reagents:		
SUPERSCRIPT™ II RNase H^- Reverse Transcriptase	10,000 units	18064-014
SUPERSCRIPT Plasmid System for cDNA Synthesis and Plasmid Cloning	3 reactions	18248-013
PROQUEST™ Two-Hybrid cDNA Libraries**		
SUPERSCRIPT cDNA Libraries**		
Calf Intestinal Alkaline Phosphatase (CIAP)	1,000 units	18009-019
Dpn I	100 units	15242-019
Nco I	200 units	15421-019
Thermosensitive Alkaline Phosphatase (TsAP)	1,000 units	10534-014
T4 DNA Ligase	100 units	15224-017
T4 DNA Polymerase	50 units	18005-017
T4 Polynucleotide Kinase	200 units	18004-010
Topoisomerase I	200 units	38042-016
Proteinase K	100 mg	25530-015
Plasmid pUC19	10 µg	15364-011
DNA Analysis Products:		
CLONECHECKER™ System	100 reactions	11666-013
1 Kb PLUS DNA LADDER	250 µg	10787-018
Low DNA MASS Ladder	200 µl	10068-013
High DNA MASS Ladder	200 µl	10496-016
Low Melting Point Agarose	50 g	15517-014
Kodak Digital Science™ EDAS 120 System,		
Windows version	each	10947-042
Macintosh version	each	10947-059

Related Products

Protein Analysis Products:		
BENCHMARK™ Protein Ladder	2 × 250 µl	10747-012
BENCHMARK Prestained Protein Ladder	2 × 250 µl	10748-010

*See our website (lifetech.com) for information about Custom Primers.
**See our website for an updated list of GATEWAY-compatible libraries.

Additional sizes of these products are also available.

Part No. 53266
Lot No. XXXXXXXX