invitrogen™

Baculovirus Expression System with Gateway® Technology

Gateway®-adapted destination vectors for cloning and high-level expression of recombinant proteins in Baculovirus

Catalog nos. 11827-011, 11806-015, 11804-010, 11807-013

Version F

1 July 2008

25-0516

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following products:

Kit	Cat. no.
Baculovirus Expression System with Gateway® Technology	11827-011
Gateway® pDEST™8 Vector	11804-010
Gateway® pDEST™10 Vector	11806-015
Gateway® pDEST™20 Vector	11807-013

Kit Components

Each product contains the following components. For a detailed description of the contents of each component, see the next page.

Component	Cat. no.			
	11827-011	11804-010	11806-015	11807-013
pDEST™8 Vector	√	√		
pDEST™10 Vector	√		√	
pDEST™20 Vector	√			√
Gateway® LR Clonase™ II Enzyme Mix	√			
Library Efficiency® DH5α Competent *E. coli*	√			

Shipping/Storage

The Baculovirus Expression System with Gateway® Technology is shipped as described below. Upon receipt, store each box as detailed below.

Box	Component	Shipping	Storage
1	pDEST™ Vectors	Wet ice	–20°C
2	Gateway® LR Clonase™ II Enzyme Mix	Dry ice	–20°C
3	Library Efficiency® DH5α™ Chemically Competent *E. coli* Kit	Dry ice	–80°C

Note: The individual Gateway® pDEST™ vectors (Cat. nos. 11804-010, 11806-015, 11807-013) are shipped on wet ice. Upon receipt, store at –20°C.

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Kit Contents and Storage, continued

Destination Vectors

The following destination vectors (Box 1) are supplied with the Baculovirus Expression System with Gateway® Technology. **Store the vectors at –20°C.**

Note: Catalog nos. 11804-010, 11806-015, 11807-013 contain 40 µl of 150 ng/µl of the appropriate pDEST™ vector in 10 mM TrisHCl, 1 mM EDTA, pH 8.0 **only**.

Reagent	Composition	Amount
pDEST™8 Vector	40 µl of vector at 150 ng/µl in TE Buffer, pH 8.0(10 mM TrisHCl, 1 mM EDTA, pH 8.0)	6 µg
pDEST™10 Vector	40 µl of vector at 150 ng/µl in TE Buffer, pH 8.0	6 µg
pDEST™20 Vector	40 µl of vector at 150 ng/µl in TE Buffer, pH 8.0	6 µg

LR Clonase™ II Enzyme Mix

The following reagents are supplied with the Gateway® LR Clonase™ II Enzyme Mix (Box 2). **Store Box 2 at –20°C for up to 6 months.** For long-term storage, keep at –80°C.

Reagent	Composition	Amount
LR Clonase™ II enzyme mix	Proprietary	40 µl
Proteinase K	2 mg/ml in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µl
pENTR™-gus Positive Control	20 µl of vector at 50 ng/µl in TE Buffer, pH 8.0	1 µg

DH5α™ Competent *E. coli*

The Library Efficiency® DH5α™ Competent *E. coli* kit (Box 3) includes the following items. Transformation efficiency is ≥ 1 x 10^8 cfu/µg DNA. **Store Box 3 at –80°C.**

Reagent	Composition	Amount
Library Efficiency® Chemically Competent DH5α™	--	5 × 200 µl
S.O.C. Medium (may be stored at room temperature or 4°C)	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	2 × 6 ml
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µl

Genotype of DH5α™

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

Accessory Products

Introduction

The products listed in this section are intended for use with the Baculovirus Expression System with Gateway® Technology. For more information, refer to our website at www.invitrogen.com or call Technical Support (see page 28).

Additional Products

The following products are available separately from Invitrogen.

Product	Amount	Cat. no.
Library Efficiency® DH5α™ Chemically Competent Cells	5 × 0.2 ml	18263-012
LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Cellfectin® II Reagent	1 ml	10362-100
MAX Efficiency® DH10Bac™Competent *E. coli*	5 × 100 µl	10361-012
Express Five® SFM	1000 ml	10486-025
Sf-900 II SFM (1X) liquid	500 ml	10902-096
High Five™ Frozen Cells	3×10^6 cells/ml	B855-02
Sf9 Frozen Cells	1 ml, 10^7 cells/ml	B825-01
Sf21 Frozen Cells	1 ml, 10^7 cells/ml	B821-01
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin	50 ml	R801-01
Purification Columns (10 ml polypropylene columns)	50 columns	R640-50
Ni-NTA Purification System	6 purifications	K950-01
AcTEV™ Protease	1,000 units 10,000 units	12575-015 12575-023

Introduction

Overview

Introduction

The Baculovirus Expression System with Gateway® Technology allows you to express your gene of interest in insect cell lines using the Bac-to-Bac® Baculovirus Expression System. For more information on the Bac-to-Bac® Baculovirus Expression System, see page 3.

The kit uses Gateway® Technology to create an expression clone by recombining an entry clone containing your gene of interest with a destination vector (pDEST™) of choice. For more information on the Gateway® Technology, see the next page. Depending on the vector chosen, the pDEST™ vectors allow production of native or N-terminal-tagged recombinant proteins (see table below).

Vector	Fusion Peptide	Fusion Tag
pDEST™8	–	–
pDEST™10	N-terminal	6×His
pDEST™20	N-terminal	Glutathione *S*-transferase (GST) (Smith *et al.*, 1986)

Features of the Destination Vectors

pDEST™8, pDEST™10, and pDEST™20 have the following features:

- The polyhedrin gene promoter from *Autographa californica* multi nuclear polyhedrosis virus (AcMNPV) for high-level expression of the gene of interest (Possee and Howard, 1987)
- Mini-Tn*7* elements for site-specific transposition into the bacmid DNA propagated in *E. coli* (Craig, 1989; Luckow *et al.*, 1993)
- N-terminal fusion tags for detection and purification of recombinant fusion proteins (choice of tag depends on the particular vector; see above)
- Two recombination sites, *att*R1 and *att*R2, for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- The *ccd*B gene located between the two *att*R sites for negative selection
- The SV40 polyadenylation signal for efficient transcription termination and polyadenylation of mRNA
- Ampicillin resistance gene for selection of transformants in *E. coli*
- Gentamicin resistance gene for selection of transformants containing recombinant bacmid DNA
- The pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*

For more information and maps of these vectors, see pages 23–26.

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Overview, continued

Gateway® Technology

The Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your gene of interest into multiple expression systems. To express your gene of interest using the Gateway® Technology:

1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone.
2. Perform an LR recombination reaction between the entry clone and a Gateway® destination vector (*e.g.* pDEST™8, pDEST™10, pDEST™20).
3. Transform Library Efficiency® DH5α *E. coli* and select for an expression clone.
4. Use your expression clone in the Bac-to-Bac® Baculovirus Expression System to generate a recombinant baculovirus that expresses your recombinant protein.

For more detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase™ II manual. This manual is supplied with the Baculovirus Expression System with Gateway® Technology and is also available for downloading at www.invitrogen.com or by contacting Technical Support (see page 28).

LR Recombination Reaction

You will perform an LR recombination reaction between the entry clone and your destination vector of choice to generate an expression clone. The LR recombination reaction is mediated by LR Clonase™ II Enzyme Mix, a mixture of the bacteriophage λ Integrase (Int) and Excisionase (Xis) proteins, and the *E. coli* Integration Host Factor (IHF) protein. For more information about the LR recombination reaction, see the Gateway® Technology with Clonase™ II manual.

Bac-to-Bac® Baculovirus Expression System

Introduction

The Bac-to-Bac® Baculovirus Expression System is a rapid and efficient method to generate recombinant baculoviruses. This method is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* (Ciccarone *et al.*, 1997; Luckow *et al.*, 1993). For more details on this system, refer to the Bac-to-Bac® Baculovirus Expression System manual and the Guide to Baculovirus Expression Vector Systems. These manuals are available for downloading at www.invitrogen.com or by contacting Technical Support (see page 28).

Advantages of Using Site-Specific Transposition

Using site-specific transposition to insert foreign genes into a bacmid propagated in *E. coli* has the following advantages over the generation of recombinant baculoviruses in insect cells using homologous recombination:

- Eliminates the need for multiple rounds of plaque purification as the recombinant virus DNA isolated from selected colonies is not mixed with parental, nonrecombinant virus
- Requires less than 2 weeks to identify and purify a recombinant virus as compared to the 4–6 weeks required to generate a recombinant baculovirus using homologous recombination
- Permits rapid and simultaneous isolation of multiple recombinant viruses and is suited for the expression of protein variants for structure/function studies

Baculovirus Shuttle Vector

The baculovirus shuttle vector (bacmid), bMON14272 (136 kb) is used in the Bac-to-Bac® Baculovirus Expression System. The bacmid contains a low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the LacZα peptide from a pUC-based cloning vector.

A short segment containing the attachment site for the bacterial transposon Tn*7* (mini-*att*Tn*7*) is inserted into the N-terminus of the *lacZα* gene of the bacmid. This insertion does not disrupt the reading frame of the LacZα peptide. The bacmid propagates in *E. coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG.

Recombinant bacmids (composite bacmids) are generated by transposing a mini-Tn*7* element from a donor plasmid (pDEST™ vectors) to the mini-*att*Tn*7* attachment site on the bacmid. The Tn*7* transposition functions are provided by a helper plasmid (see below).

Refer to the diagram on the next page for a schematic representation of the Bac-to-Bac® Baculovirus Expression System.

Helper Plasmid

The helper plasmid, pMON7124 (13.2 kb) provides the Tn*7* transposition function *in trans* (Barry, 1988). The helper plasmid is present in DH10Bac™ and confers resistance to tetracycline and encodes the transposase.

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Bac-to-Bac® Baculovirus Expression System, continued

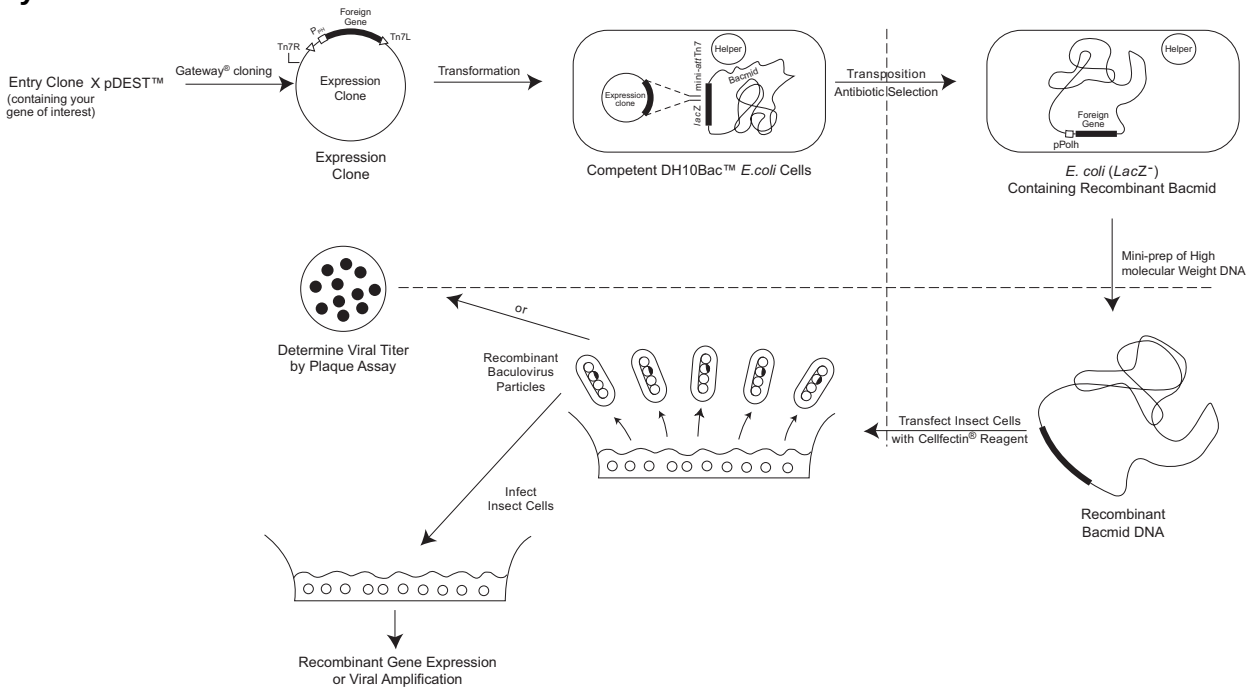
pDEST™ Vectors

Each pDEST™ vector has a mini-Tn*7* containing the expression cassette. Each expression cassette consists of a gentamicin resistance gene, the polyhedrin promoter from AcMNPV for expression of proteins in insect cells, a Gateway® cloning cassette (*att*R1, Chloramphenicol resistance gene, *ccd*B gene and *att*R2), and an SV40 poly (A) signal inserted between the left and right arms of Tn*7*. The important features of the pDEST™ vectors are described on pages 1 and 26. The gene of interest is cloned into the Gateway® cloning cassette of the pDEST™ vectors using an entry vector (see page 8). Transposition of the mini-Tn*7* from the pDEST™ vector into the mini-*att*Tn*7* attachment site on the bacmid disrupts expression of the *lacZ*α gene resulting in white colonies of the recombinant bacmid in a background of blue colonies containing the unaltered parent bacmid.

The recombinant bacmid DNA is rapidly isolated from small-scale cultures of the white colonies and then used to transfect insect cells. Viral stocks (>10^6 pfu/ml) are harvested from the transfected cells and used to infect fresh insect cells for protein expression, purification, and analysis (see diagram below).

Diagram of the Bac-to-Bac® Expression System

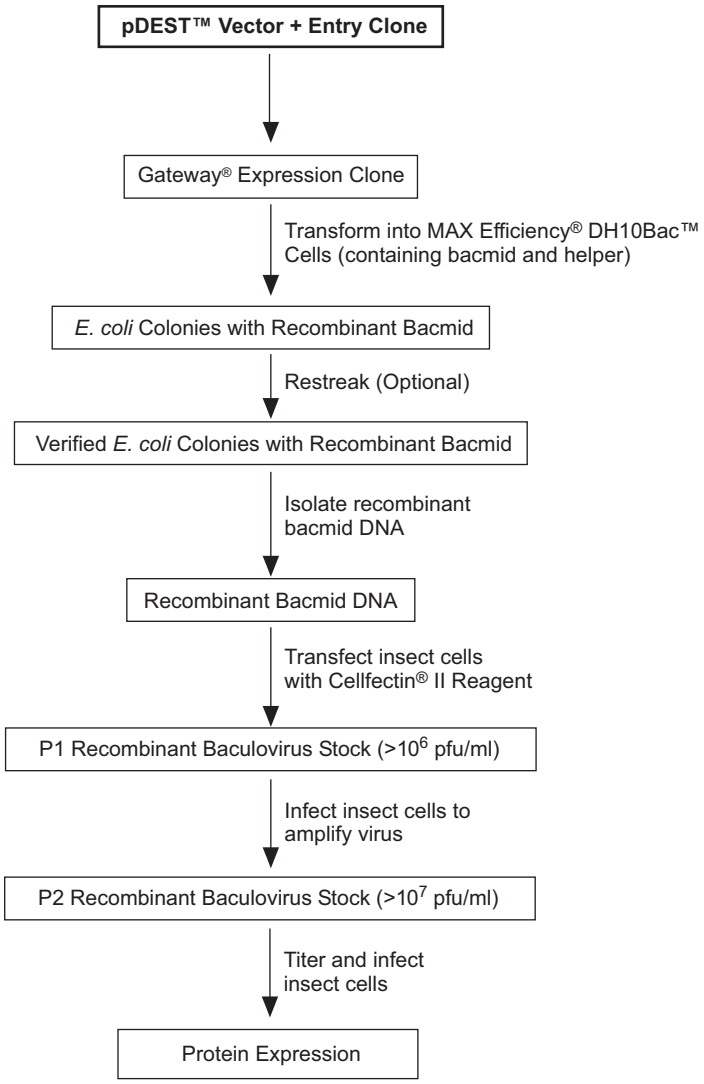
The figure below depicts the generation of recombinant baculovirus and the expression of your gene of interest using the Bac-to-Bac® Baculovirus Expression System.



Experimental Overview

Experimental Outline

The figure below describes the steps necessary to clone and express your gene of interest using pDEST™8, pDEST™10, or pDEST™20.



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Experimental Overview, continued

Materials Needed

- Entry clone containing your gene of interest (see page 8)
- Insect cell lines (see page 7)
- Media for insect cells
- Cellfectin® II Reagent
- Appropriate tissue culture plates and flasks
- Sterile microcentrifuge tubes (1.5 ml)
- MAX Efficiency® DH10Bac™ Chemically Competent *E. coli* (see page vii for ordering information)

If you have ordered the individual Gateway® pDEST™ vectors, you will also need:

- LR Clonase™ II enzyme mix (see page vii)
- Library Efficiency® DH5α™ Chemically Competent Cells or appropriate competent cells (see page vii).

Methods

Culturing Insect Cells

Introduction

Before you start your cloning experiments, be sure to have cultures of Sf9, Sf21, or High Five™ cells growing and have frozen master stocks available.

Cells for Transfection

You will need log-phase cells with >95% viability to perform a successful transfection. Refer to the Bac-to-Bac® Baculovirus Expression System manual to determine how many cells you will need for transfection.

Insect Cell Lines Manual

For additional information on insect cell culture, refer to the Insect Cell Lines manual and the Guide to Baculovirus Expression Vector Systems and Insect Cell Culture Techniques. These manuals contain information on:

- Thawing frozen cells
- Maintaining and passaging cells
- Freezing cells
- Using serum-free medium
- Growing cells in suspension
- Scaling up cell culture

These manuals are available for downloading at www.invitrogen.com or by contacting Technical Support (see page 28).

Generating an Entry Clone

Introduction

To recombine your gene of interest into pDEST™8, pDEST™10, or pDEST™20, you will need an entry clone containing the gene of interest. Many entry vectors are available from Invitrogen to facilitate generation of entry clones (see table below). For more information about each vector, see our website (www.invitrogen.com) or contact Technical Support (see page 28).

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

Once you have selected an entry vector, refer to the manual for the specific entry vector you are using for instructions to construct an entry clone.
All entry vector manuals are available for downloading at www.invitrogen.com or by contacting Technical Support (see page 28).

Cloning Considerations

It is important to have a properly designed entry clone before recombining with the destination vector. Refer to the table below and the recombination region on pages 10–12.

If you wish to…..	Then….
recombine your entry clone with pDEST™8	your insert should contain an ATG start codon for proper initiation of translation and a stop codon.
include the 6×His tag (pDEST™10)	the entry clone must be designed to ensure that your gene of interest is in frame with the ATG and the 6×His tag after recombination and must contain a stop codon.
include the GST fusion tag (pDEST™ 20)	the entry clone must be designed to ensure that your gene of interest is in frame with the ATG and the GST tag after recombination and must contain a stop codon.

Creating an Expression Clone

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into the pDEST™ vector to create your expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Performing the LR Recombination Reaction**, pages 13–14 before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pDEST™ vector. **Note:** Both the entry clone and the destination vector should be supercoiled (see **Important Note** below).
2. Transform the reaction mixture into a suitable *E. coli* host (see page 15).
3. Select for expression clones (see pages 10–12 for illustrations of the recombination region of expression clones in pDEST™8, pDEST™10, or pDEST™20).

Important

The pDEST™8, pDEST™10, and pDEST™20 vectors are supplied as supercoiled plasmids. Although Invitrogen has previously recommended using a linearized destination vector for more efficient recombination, further testing has found that linearization of these vectors is **NOT** required to obtain optimal results for any downstream application.

Propagating the Vectors

If you wish to propagate and maintain the pDEST™8, pDEST™10, or pDEST™20 vectors prior to recombination, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (Cat. no. A10460) from Invitrogen. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50–100 µg/ml ampicillin and 15–30 µg/ml chloramphenicol.

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

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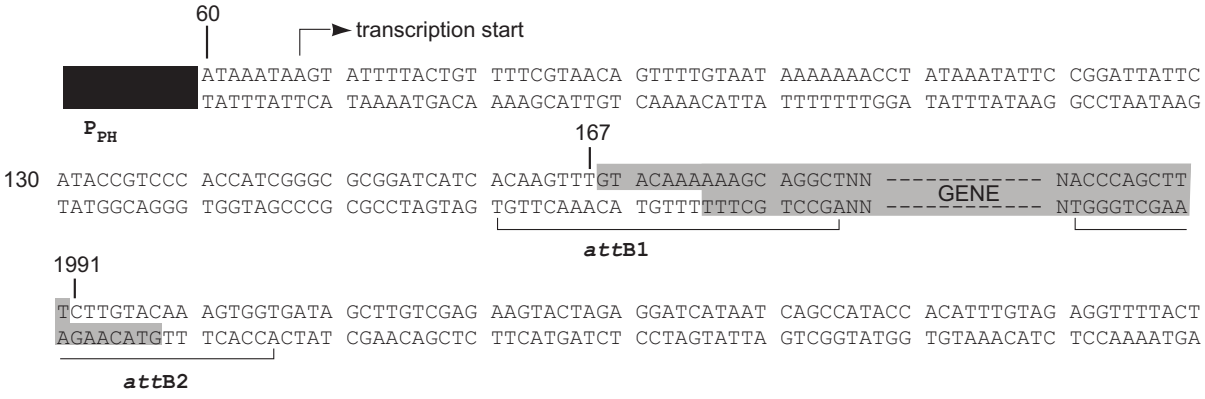
Creating an Expression Clone, continued

Recombination Region of pDEST™8

The recombination region of the expression clone resulting from pDEST™8 × entry clone is shown below. **The complete sequence of pDEST™8 is available for downloading at www.invitrogen.com or from Technical Support (see page 28).** For a map and a description of the features of pDEST™8, refer to pages 23 and 26.

Features of the Recombination Region:

- Shaded regions correspond to the DNA sequences transferred from the entry clone into pDEST™8 by recombination. Non-shaded regions are derived from pDEST™8.
- The nucleotides flanking the shaded region correspond to bases 167 and 1991, respectively of pDEST™8.



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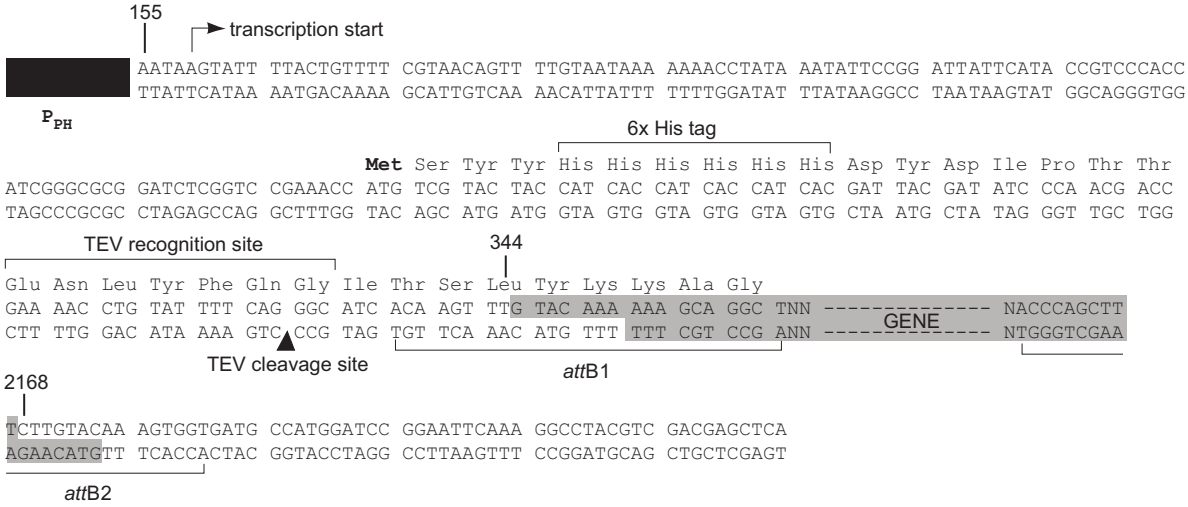
Creating an Expression Clone, continued

Recombination Region of pDEST™10

The recombination region of the expression clone resulting from pDEST™10 × entry clone is shown below. **The complete sequence of pDEST™10 is available for downloading at www.invitrogen.com or from Technical Support (see page 28).** For a map and a description of the features of pDEST™10, refer to pages 24 and 26.

Features of the Recombination Region:

- Shaded regions correspond to the DNA sequences transferred from the entry clone into pDEST™10 by recombination. Non-shaded regions are derived from pDEST™10.
- The nucleotides flanking the shaded region correspond to bases 344 and 2168, respectively of pDEST™10.



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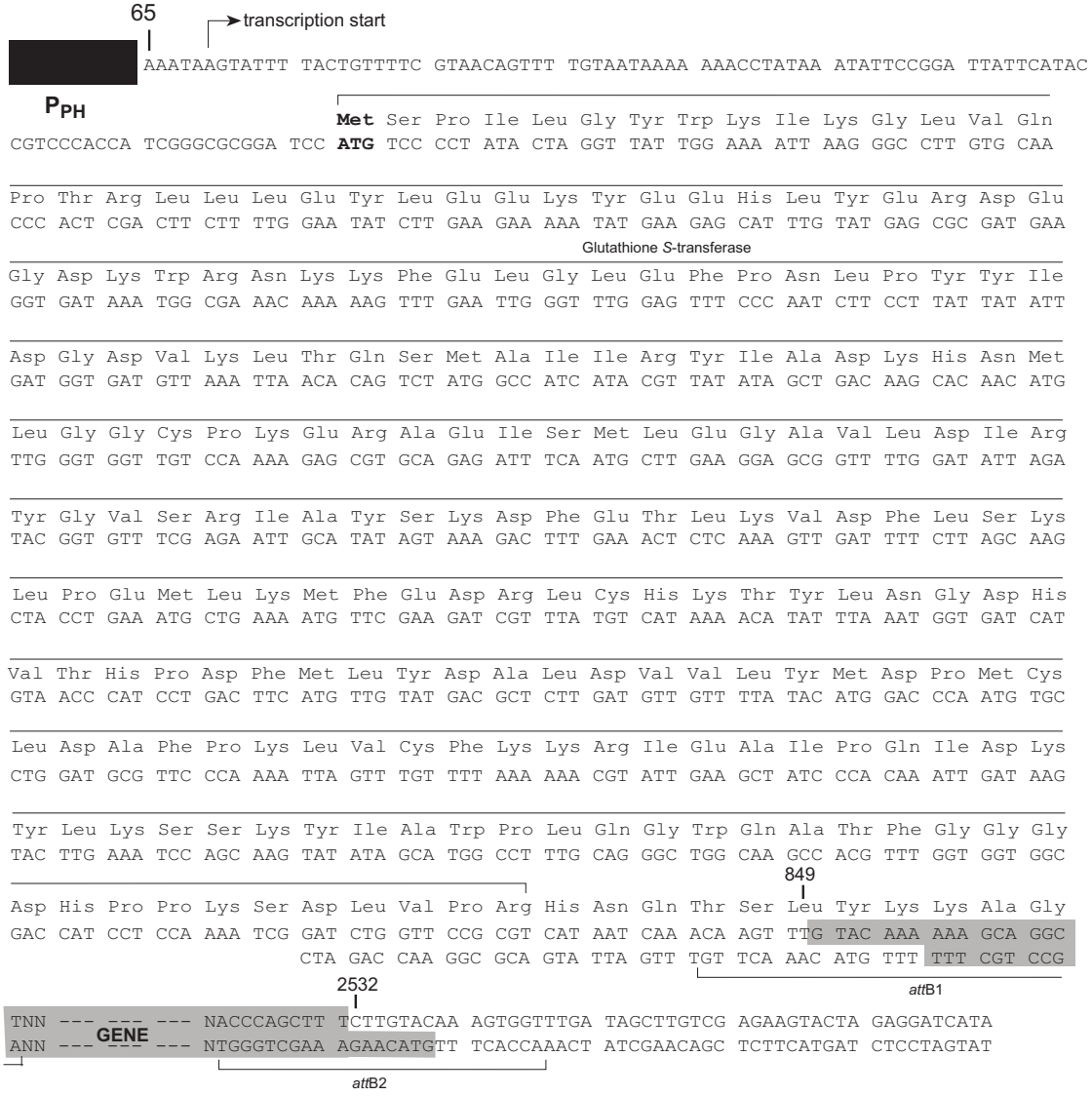
Creating an Expression Clone, continued

Recombination Region of pDEST™20

The recombination region of the expression clone resulting from pDEST™ 20 × entry clone is shown below. **The complete sequence of pDEST™20 is available for downloading at www.invitrogen.com or from Technical Support (see page 28).** For a map and a description of the features of pDEST™20, refer to pages 25–26.

Features of the Recombination Region:

- Shaded regions correspond to the DNA sequences transferred from the entry clone into pDEST™20 by recombination. Non-shaded regions are derived from pDEST™20.
- The nucleotides flanking the shaded region correspond to bases 849 and 2532, respectively of pDEST™20.
- The glutathione S-transferase (GST) gene is indicated.



Performing the LR Recombination Reaction

Introduction

Once you have produced an entry clone containing your gene of interest, you are ready to perform an LR recombination reaction between the entry clone and the appropriate pDEST™ vector, and to transform the reaction mixture into Library Efficiency® DH5α™ to select for an expression clone. It is important to have everything you need set up and ready to use to ensure that you obtain the best results. We suggest that you read this section and the one entitled **Transforming Library Efficiency® DH5α™ Cells**, page 15 before beginning. We also recommend that you include a positive control (see below) and a negative control (no LR Clonase™ II) in your experiment.

Positive Control

The pENTR™-gus plasmid is included in the Baculovirus Expression System with Gateway® Technology for use as a positive control for LR recombination and expression. Use of the pENTR™-gus entry clone in an LR recombination reaction with any pDEST™ vector will allow you to generate an expression clone containing the gene encoding β-glucuronidase (*gus*).

LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is supplied with the kit (Cat. no. 11827-011 only) or available separately from Invitrogen to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 14 to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix provided in this manual as reaction conditions differ.

Materials Needed

- Entry clone containing your gene of interest (50–150 ng/µl in TE, pH 8.0)
- pDEST™ vector (150 ng/µl in TE, pH 8.0)
- pENTR™-gus positive control (if desired, supplied with the LR Clonase™ II enzyme mix, Box 2; 50 ng/µl in TE, pH 8.0)
- LR Clonase™ II enzyme mix (Box 2, keep at –20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Proteinase K solution (supplied with the LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- Water bath set at 37°C
- 1.5 ml microcentrifuge tubes

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Performing the LR Recombination Reaction, continued

LR Recombination Reaction

Follow this procedure to perform the LR recombination reaction between your entry clone and the destination vector. If you want to include a negative control, set up a separate reaction but omit the LR Clonase™ II enzyme mix.

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

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

2. Remove the LR Clonase™ II enzyme mix from –20°C and thaw on ice (2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. To each sample, add 2 µl of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

   Reminder: Immediately return the LR Clonase™ II enzyme mix to –20°C.
5. Incubate reactions at 25°C for 1 hour.

   Note: For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation will yield more colonies.
6. Add 1 µl of Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming Library Efficiency® DH5α™ Cells**, next page.

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

Transforming Library Efficiency® DH5α™ Cells

Introduction

Once you have performed the LR recombination reaction, you will transform competent *E. coli*. Library Efficiency® DH5α™ Chemically Competent *E. coli* (Box 3) are included with the Baculovirus Expression System to facilitate transformation.

E. coli Host

If you have ordered the individual Gateway® pDEST™ vectors, you will need competent *E. coli*. We recommend that you propagate vectors containing inserts in *E. coli* strains that are *rec*A and *end*A deficient such as TOP10 (Cat. no. C4040-03) or DH5α™-T1^R (Cat. no. 12297-016). Avoid using an *E. coli* strain containing an F′ episome. The F′ episome contains the *ccd*A gene and prevents negative selection of the clone with *ccd*B.

Materials Needed

- LB plates containing 100 µg/ml ampicillin (two for each transformation; warm at 37°C for 30 minutes)
- 42°C water bath
- 37°C shaking and non-shaking incubator
- Library Efficiency® DH5α™ Chemically Competent *E. coli* (see page vii) or appropriate competent cells (see above)
- S.O.C. Medium

Note

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

Transformation Protocol

1. For each transformation, aliquot 50 µl of Library Efficiency® DH5α™ Chemically Competent cells into a sterile microcentrifuge tube.
2. Add 1 µl of the LR recombination reaction (from Step 7, previous page) into the tube containing 50 µl of Library Efficiency® DH5α™ competent cells and mix gently. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 450 µl of room temperature S.O.C. Medium.
7. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
8. Spread 20 µl and 100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We generally plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

An efficient LR recombination reaction should produce hundreds of colonies (> 5000 colonies if the entire LR reaction is transformed and plated).

Analyzing Transformants

Analyzing Positive Clones

1. Pick 5 colonies (from Step 8, previous page) and culture them overnight in LB or SOB medium containing 100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice. We recommend using the S.N.A.P.™ MiniPrep Kit (Cat. no. K1900-01) or the PureLink™ HQ Mini Plasmid Purification Kit (Cat. no. K2100-01) available from Invitrogen.
3. Analyze the plasmids by restriction analysis to confirm the presence of the insert.

Analyzing Transformants by PCR

You may also analyze positive transformants using PCR. For PCR primers, use a primer that hybridizes within the vector and one that hybridizes within your insert. You will have to determine the amplification conditions. If you are using this technique for the first time, you may want to perform restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol below is provided for your convenience. Other protocols are suitable.

Materials Needed:

PCR SuperMix High Fidelity (Invitrogen, Cat. no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure:

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 5 colonies and resuspend them individually in 50 µl of the PCR SuperMix containing primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at 4°C.
6. Visualize by agarose gel electrophoresis.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

Sequencing

To confirm that your gene of interest is in frame with the appropriate tags, you may want to sequence your expression construct.

continued on next page

Analyzing Transformants, continued

Long-Term Storage

Once you have confirmed that you have the correct expression clone, prepare a glycerol stock for long-term storage. We also recommend keeping a stock of plasmid DNA at –20°C.

To prepare a glycerol stock:

1. Grow the *E. coli* strain containing the plasmid overnight in selective medium.
2. Combine 0.85 ml of the overnight culture with 0.15 ml of sterile glycerol.
3. Vortex and transfer to a labeled cryovial.
4. Freeze the tube in liquid nitrogen or dry ice/ethanol bath and store at –80°C.

Expressing Your Protein Using the Bac-to-Bac® Baculovirus Expression System

Introduction

Once you have your expression clone, you are ready to transform your clone into MAX Efficiency® DH10Bac™ Chemically Competent *E. coli* and express your protein in the desired insect cell line using the Bac-to-Bac® Baculovirus Expression System.

Preparing Plasmid DNA

Prepare plasmid DNA from the selected expression clone for transformation. We recommend isolating plasmid DNA using the S.N.A.P.™ MiniPrep Kit (Cat. no. K1900-01), PureLink™ HQ Mini Plasmid Purification Kit (Cat. no. K2100-01), or CsCl gradient centrifugation.

Materials Supplied by the User

You will need the following items before starting:

- Insect cell line (see page 7)
- Appropriate cell culture media
- Cellfectin® II Reagent (see page vii for ordering information)
- MAX Efficiency® DH10Bac™ Chemically Competent *E. coli* (see page vii for ordering information)

Bac-to-Bac® Baculovirus Expression

Refer to the Bac-to-Bac® Baculovirus Expression System manual for detailed protocols to perform the steps outlined below. For more information on the Bac-to-Bac® Baculovirus Expression System and insect cell culture techniques, refer to the Guide to Baculovirus Expression Vector Systems and Insect Cell Culture Techniques. These manuals are available from our website at www.invitrogen.com or by contacting Technical Support (see page 28).

You will need to perform the following steps to express your protein of interest from the expression clone using the Bac-to-Bac® Baculovirus Expression System.

1. Transform plasmid DNA from the expression clone into MAX Efficiency® DH10Bac™ Chemically Competent *E. coli*.
2. Isolate recombinant bacmid DNA. Verify transposition to the bacmid using PCR analysis.
3. Transfect the desired insect cell line with the recombinant bacmid DNA using Cellfectin® II Reagent.
4. Harvest the recombinant baculovirus. Remember to store the virus stocks at 4°C, protected from light. For long-term storage, store at –80 C.
5. Amplify viral stocks.
6. Titer the viral stock and infect insect cells with recombinant baculovirus particles using an optimal MOI.
7. Harvest cells or media at 24, 48, 72 , and 96 hours post-infection and assay for expression (see next page).

Testing for Expression

Introduction

Guidelines are provided in this section for testing the expression of your protein and the protein expressed from the positive control vector, pENTR™-gus.

Polyacrylamide Gel Electrophoresis

To facilitate separation of your expressed protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels are available from Invitrogen. In addition, Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our website (www.invitrogen.com) or call Technical Support (see page 28).

Western Analysis

To detect expression of your protein by western blot analysis, you may use an antibody to your protein of interest. WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. For more information, refer to our website (www.invitrogen.com) or call Technical Support (see page 28).

Analyzing Expression by Recombinant Viruses

Analysis of recombinant virus expression is performed in 24-well plates using the virus stock harvested 72 hours post-infection.

1. Seed 6 x 10^5 insect cells per well in a 24-well plate. Allow the cells to attach for at least 30 minutes.
2. Wash the cells once with fresh media and replace with 300 µl of fresh media.
3. Add 200 µl of viral stock to each well. Include controls that contain uninfected cells, wild type infected cells.
4. Incubate the plate at 27°C for 48 hours.
5. Remove the viral supernatant and save for analysis.
6. Wash the cells with SFM and lyse cells with 400 µl of 1X SDS-PAGE sample buffer.
7. Boil the samples for 3 minutes. Load 20 µl of the sample on an appropriate polyacrylamide gel and perform electrophoresis.

β-Glucuronidase Assay

An assay for analyzing β-glucuronidase (*gus*) activity from the positive control vector pENTR™-gus is described in the Bac-to-Bac® Baculovirus Expression System manual to verify the recombination reaction and expression.

continued on next page

Testing for Expression, continued

Note

Expression of your protein with the N-terminal tag will increase the size of your recombinant protein. The table below lists increase in the molecular weight of your recombinant fusion protein that you should expect from the tag in each pDEST™ vector. Be sure to account for any additional amino acids between the fusion tag and the start of your protein.

Vector	Fusion Tag	Expected Size Increase (kDa)
pDEST™10	N-terminal	4.3
pDEST™20	N-terminal	27.8

Purifying the Recombinant Protein

Introduction

The presence of the N-terminal 6×His tag in pDEST™10 allows purification of recombinant fusion protein using a nickel-chelating resin such as ProBond™ or Ni-NTA, while the presence of the N-terminal GST tag in pDEST™20 allows purification of recombinant fusion protein using glutathione agarose.

Purifying 6×His-Tagged Recombinant Proteins

ProBond™ and Ni-NTA resin are available separately from Invitrogen (see page vii for ordering information). Other metal-chelating resins are suitable.

- To purify your fusion protein using ProBond™ or Ni-NTA, refer to the ProBond™ Purification System or Ni-NTA Purification System manuals as appropriate. Both manuals are available for downloading from our website (www.invitrogen.com) or by contacting Technical Support (see page 28).
- To purify your fusion protein using another metal-chelating resin, refer to the manufacturer's instructions.

Purifying 6×His-tagged Proteins from Medium

To purify 6×His-tagged recombinant proteins from the culture medium, we recommend a dialysis or ion exchange chromatography step prior to affinity chromatography on metal-chelating resins.

Dialysis allows:

- Removal of media components that strip Ni^{+2} from metal-chelating resins

Ion exchange chromatography allows:

- Removal of media components that strip Ni^{+2} from metal-chelating resins
- Concentration of your sample for easier manipulation in subsequent purification steps

Conditions for successful ion exchange chromatography will vary depending on the protein. For more information, refer to *Current Protocols in Protein Science* (Coligan *et al.*, 1998), *Current Protocols in Molecular Biology*, Unit 10 (Ausubel *et al.*, 1994) or the *Guide to Protein Purification* (Deutscher, 1990).

Note

Many insect cell proteins are naturally rich in histidines, with some containing stretches of six histidines. When using a metal chelating resin to purify 6xHis-tagged proteins, these histidine-rich proteins may co-purify with your protein of interest. The contamination can be significant if your protein is expressed at low levels. We recommend that you add 5 mM imidazole to the binding buffer prior to addition of the protein mixture to the column. Addition of imidazole may help to reduce background contamination by preventing proteins with low specificity from binding to the metal-chelating resin.

continued on next page

Purifying the Recombinant Protein, continued

Purification Using Glutathione Agarose

If you express your recombinant protein as a fusion to the GST tag in pDEST™20 you can affinity purify your protein using glutathione agarose. Refer to the manufacturer's instructions to purify your protein.

Using AcTEV™ Protease

AcTEV™ Protease is a site-specific protease recognizing the seven amino acid sequence; Glu-As-Le-Tyr-Ph-Gln-Gly. The cleavage site is between Gln and Gly (Dougherty *et al.*, 1989). Recombinant AcTEV™ Protease is available from Invitrogen (see page vii).

Use the AcTEV™ Protease to cleave the 6×His tag from the fusion protein generated using pDEST™10 after purifying the recombinant protein on a nickel-chelating resin. The Recombinant AcTEV™ Protease is engineered with a 6×His tag to facilitate removal of the enzyme from the protein sample after digestion.

Note: After TEV cleavage, at least 10 amino acids will remain at the N-terminus of your protein (see diagram on page 11).

For detailed protocols, refer to the AcTEV™ Protease manual available at www.invitrogen.com or by contacting Technical Support (see page 28).

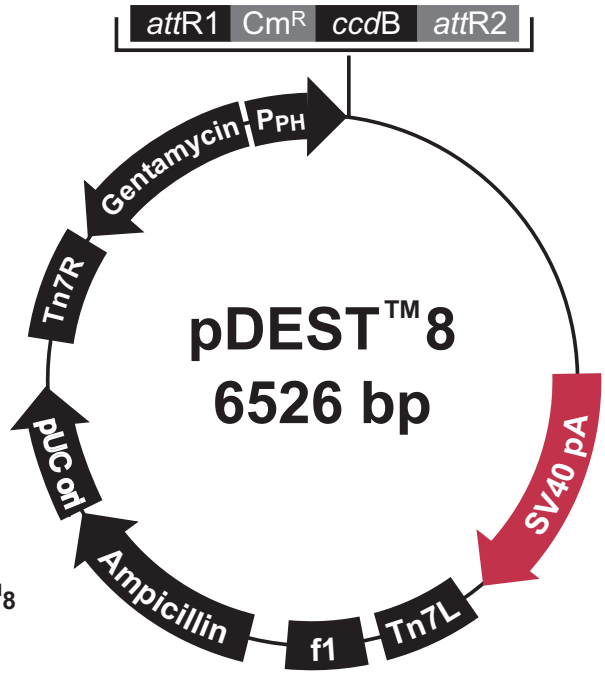
Appendix

Map of pDEST™8

pDEST™8 Map

The figure below summarizes the features of the pDEST™8 vector (6526 bp). For a more detailed explanation of each feature, see page 26. **The sequence of pDEST™8 is available from our website (www.invitrogen.com) or from Technical Support (see page 28).**

This vector has not been completely sequenced. It was compiled from published sequence data and actual sequence data. If you suspect an error, contact Technical Support (see page 28).



Features of pDEST™8
6526 nucleotides

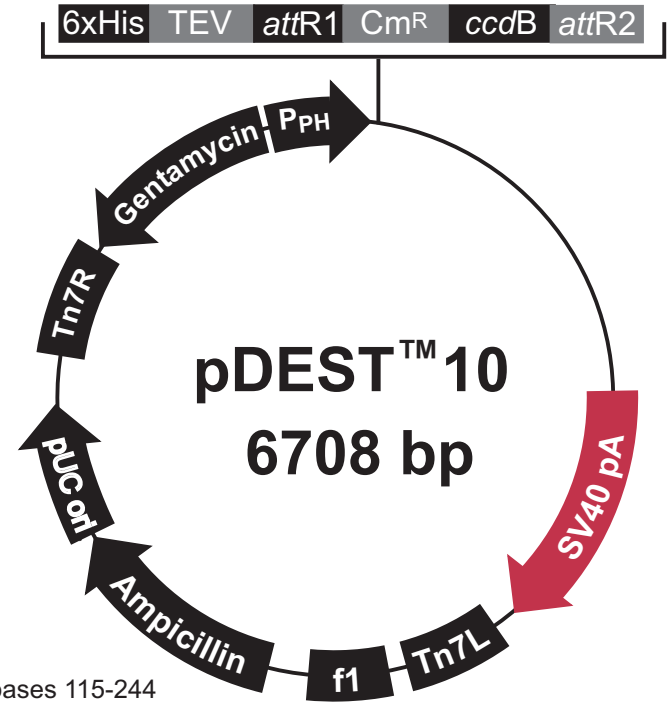
AcMNPV polyhedrin promoter: bases 43-152
*att*R1 recombination site: bases 160-284
Chloramphenicol resistance gene: bases 534-1193
*ccd*B gene: bases 1535-1840
*att*R2 recombination site: bases 1881-2005
SV40 late polyadenylation signal: bases 2093-2271
Tn7 left arm: bases 2300-2484
f1 intergenic region: bases 2648-3103
bla promoter: bases 3136-3240
Ampicillin resistance ORF (*bla*): bases 3235-4095
pUC origin: bases 4470-4750
Tn7 right arm: bases 5157-5381
Gentamicin resistance ORF: bases 5448-5981 (C)
Prokaryotic promoter: bases 6170-6197 (C)
(C) = Complementary strand

Map of pDEST™10

pDEST™10 Map

The figure below summarizes the features of the pDEST™10 vector (6708 bp). For a more detailed explanation of each feature, see page 26. **The sequence of pDEST™10 is available from our website (www.invitrogen.com) or from Technical Support (see page 28).**

This vector has not been completely sequenced. It was compiled from published sequence data and actual sequence data. If you suspect an error, contact Technical Support (see page 28).



Features of pDEST™10
6708 nucleotides

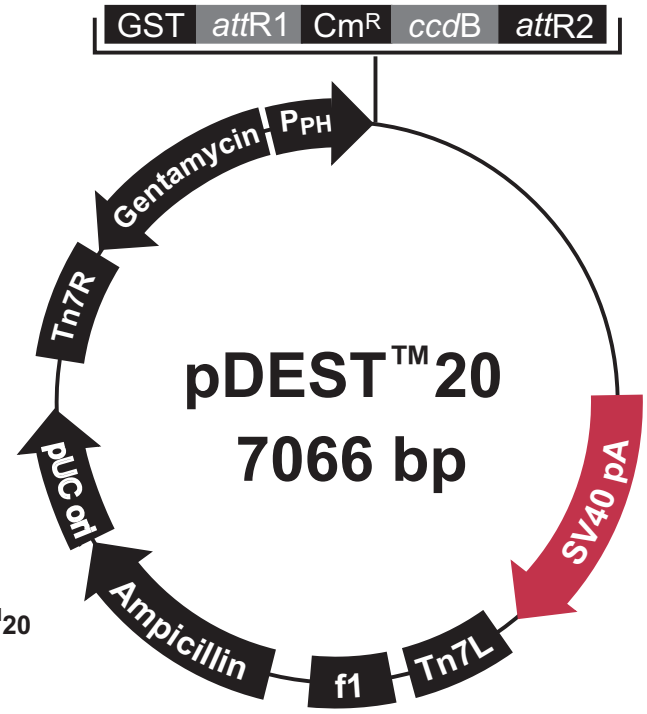
AcMNPV polyhedrin promoter: bases 115-244
6xHis tag: bases 274-291
TEV cleavage site: bases 313-333
*att*R1 recombination site: bases 337-461
Chloramphenicol resistance gene: bases 711-1370
*ccd*B gene: bases 1712-2017
*att*R2 recombination site: bases 2058-2182
SV40 late polyadenylation signal: bases 2304-2540
Tn7 left arm: bases 2563-2757
F1 intergenic region: bases 2922-3377
bla promoter: bases 3410-3514
Ampicillin resistance ORF (*bla*): bases 3509-4369
pUC origin: bases 4510-5164
Tn7 right arm: bases 5432-5655
Gentamicin resistance ORF: bases 5722-6255 (C)
Prokaryotic promoter: bases 6444-6471 (C)
(C) = Complementary strand

Map of pDEST™20

pDEST™20 Map

The figure below summarizes the features of the pDEST™20 vector (7066 bp). For a more detailed explanation of each feature, see the next page. **The sequence of pDEST™20 is available from our website (www.invitrogen.com) or from Technical Support (see page 28).**

This vector has not been completely sequenced. It was compiled from published sequence data and actual sequence data. If you suspect an error, contact Technical Support (see page 28).



Features of pDEST™20
7066 nucleotides

AcMNPV polyhedrin promoter: bases 46-153
GST ORF: bases 161-832
*att*R1 recombination site: bases 842-966
Chloramphenicol resistance gene: bases 1075-1734
*ccd*B gene: bases 2076-2381
*att*R2 recombination site: bases 2422-2546
SV40 late polyadenylation signal: bases 2636-2814
Tn7 left arm: bases 2846-3027
f1 intergenic region: bases 3191-3646
bla promoter: bases 3679-3783
Ampicillin resistance ORF (*bla*): bases 3778-4638
pUC origin: bases 5013-5293
Tn7 right arm: bases 5701-5924
Gentamicin resistance ORF: bases 5991-6524 (C)
Prokaryotic promoter: bases 6713-6740 (C)
(C) = Complementary strand

Features of pDEST™8, pDEST™10, and pDEST™20

Features

The features of pDEST™8 (6526 bp), pDEST™ 10(6708 bp), and pDEST™20 (7066 bp) are described below. All features have been functionally tested.

Features	Function
Polyhedrin promoter	Allows efficient, high-level expression of your recombinant protein (Possee and Howard, 1987).
Mini-Tn7 element (Tn7R and Tn7L)	Allows site-specific transposition of your gene of interest into a bacmid propagated in *E. coli* (Craig, 1989; Luckow *et al.*, 1993).
N-terminal 6×His tag (in pDEST™10 only)	Permits purification of your recombinant protein on metal-chelating resins such as ProBond™.
N-terminal glutathione *S*-transferase (GST) tag (in pDEST™20 only)	Allows affinity purification of recombinant fusion protein using glutathione agarose.
TEV cleavage site (in pDEST™10 only)	Allows removal of the N-terminal polyhistidine tag from your recombinant protein using AcTEV™ protease (Carrington and Dougherty, 1988).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that allow recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene	Permits counterselection of the expression clone.
*ccd*B gene	Permits negative selection.
SV40 polyadenylation sequence	Efficient transcription termination and polyadenylation of mRNA.
pUC origin	Permits high copy replication and maintenance in *E. coli*.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (*bla*)	Allows selection of transformants in *E. coli*.
Gentamicin resistance gene	Allows selection of transformants containing recombinant bacmid DNA.

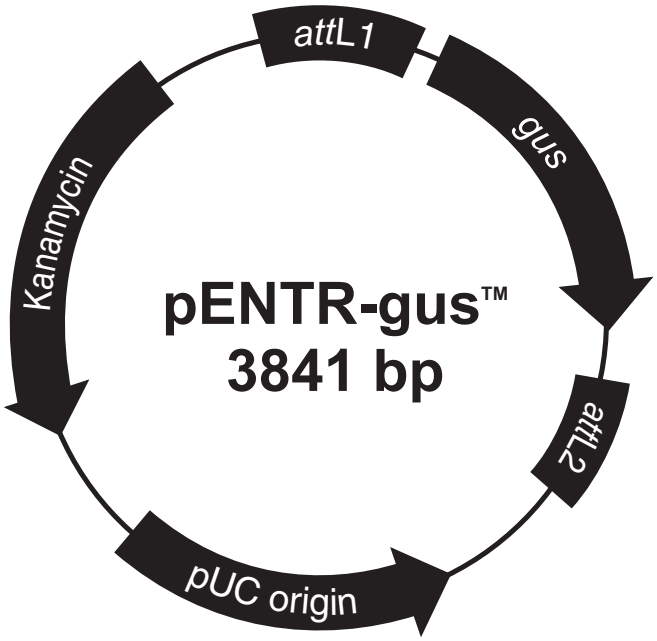
Map of pENTR™-gus

Description

pENTR™-gus is a 3841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*) (Kertbundit *et al.*, 1991).The *gus* gene was amplified using PCR primers containing *att*B recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR™201 to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase™ II manual.

Map of Control Vector

The figure below summarizes the features of the pENTR™-gus vector.
The complete sequence and restriction enzyme cleavage sites for pENTR™-gus are available from our Web (www.invitrogen.com) or by contacting Technical Support (see page 28).



Comments for pENTR-gus™
3841 nucleotides

*att*L1: bases 99-198 (complementary strand)
gus gene: bases 228-2039
*att*L2: bases 2041-2140
pUC origin: bases 2200-2873 (C)
Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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

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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

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Technical Support, continued

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 33.

continued on next page

Purchaser Notification, continued

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Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

References

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