USER GUIDE

invitrogen™
by life technologies™

BaculoDirect™ Baculovirus Expression System

For cloning and high-level expression of recombinant proteins using Gateway®-adapted Baculovirus DNA

Catalog numbers 12562-013, 12562-039, 12562-054, 12562-062

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For Research Use Only. Not for use in diagnostic procedures.

life technologies™

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

Product	Cat. no.
BaculoDirect™ C-Term Expression Kit	12562-013
BaculoDirect™ C-Term Transfection Kit	12562-039
BaculoDirect™ N-Term Expression Kit	12562-054
BaculoDirect™ N-Term Transfection Kit	12562-062

Shipping/Storage

The BaculoDirect™ Transfection and Expression Kits are shipped as described below. Upon receipt, store the components as detailed. All components are guaranteed for six months if stored properly.

Item	Shipping	Storage
BaculoDirect™ Transfection Kit	Blue ice	BaculoDirect™ DNA and Cellfectin® II Reagent: 4°C pENTR™/CAT: –20°C
LR Clonase® II Enzyme Mix for BaculoDirect™ Kits	Dry ice	–20°C
Ganciclovir	Dry ice	–20°C
Sf9 Frozen Cells	Dry ice	Liquid nitrogen
Grace's Insect Cell Culture Medium, Unsupplemented	Room Temperature	4°C, protected from light

Transfection Kit Components

The BaculoDirect™ Transfection Kits include the following components. Store components as detailed below.

Item	Composition	Amount	Storage
BaculoDirect™ Linear DNA, linearized with *Bsu*36 I	300 ng per vial in 10 µl of TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)	6 vials	4°C
Cellfectin® II Reagent	1 mg/ml in membrane-filtered water	125 µl	4°C
pENTR™/CAT Control Plasmid	40 µl of 0.5 ng/µl vector in TE buffer, pH 8.0	20 µg	–20°C
Ganciclovir	100 mM in deionized water	50 µl	–20°C, protected from light

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Kit Contents and Storage, continued

Expression Kit Components

The BaculoDirect™ Expression Kits include the following components. Store components as detailed below.

Item	Composition	Amount	Storage
BaculoDirect™ Linear DNA, linearized with *Bsu*36 I	300 ng per vial in 10 µl of TE buffer, pH 8.0	6 vials	4°C
Cellfectin® II Reagent	1 mg/ml in membrane-filtered water	125 µl	4°C
pENTR™/CAT Control Plasmid	40 µl of 0.5 ng/µl vector in TE buffer, pH 8.0	20 µg	–20°C
Ganciclovir	100 mM in deionized water	50 µl	–20°C, protected from light
LR Clonase® II Enzyme Mix for BaculoDirect™ Kits	Proprietary	40 µl	–20°C for up to 6 months; (–80°C for long-term storage)
Sf9 Frozen Cells	1×10^7 cells/ml in: 60% complete TNM-FH 30% FBS 10% DMSO	1 ml	Liquid nitrogen
Grace's Insect Cell Culture Medium, Unsupplemented	Sterile-filtered medium contains L-glutamine	500 ml	4°C, protected from light

Product Use

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

Accessory Products

Additional Products

Many of the reagents supplied with the BaculoDirect™ Transfection and Expression Kits as well as other products suitable for use with the kits are available separately from Life Technologies. Ordering information is provided below.

Product	Amount	Cat. no.
Gateway® LR Clonase II Enzyme Mix for BaculoDirect™	10 reactions	11791-023
Cellfectin® II Reagent	1 ml	10362-100
Grace's Insect Cell Culture Medium, Unsupplemented	500 ml	11595-030
Grace's Insect Cell Culture Medium, Supplemented	500 ml	11605-094
Sf-900™ II SFM	500 ml	10902-096
Sf-900™ III SFM	500 ml	12658-019
Sf-900™ Medium (1.3X)	100 ml	10967-032
Express Five® SFM	1000 ml	10486-025
Penicillin-Streptomycin	20 ml	15140-148
Fungizone® Antimycotic	20 ml	15290-018
Fetal Bovine Serum Qualified, Heat-Inactivated	100 ml	16140-063
Easy-DNA™ Kit	15–200 reactions	K1800-01
PureLink® Genomic DNA Mini Kit	10 preps 50 preps 250 preps	K1820-00 K1820-01 K1820-02
PureLink® HiPure Plasmid Miniprep Kit	25 preps	K2100-02
BaculoTiter™ Assay Kit	30 titers	K1270
4% Agarose Gel	40 ml	18300-012
β-Gal Staining Kit	1 kit	K1465-01
Bluo-gal	1 g	15519-028
CAT Antiserum	50 µl	R902-25
Sterile, cell culture grade, distilled water	500 ml	15230-162
Proteinase K	100 mg	25530-015
UltraPure™ Glycogen (20 µg/µl)	100 µl	10814-010
NuPAGE® LDS Sample Preparation Buffer (4X)	10 ml 250 ml	NP0007 NP0008
NuPAGE® Sample Reducing Agent (10X)	250 µl 10 ml	NP0004 NP0009
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 ml	LC2676
AcTEV™ Protease	1,000 Units	12575-015

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Accessory Products, continued

Insect Cells

Life Technologies offers a variety of insect cell lines for protein expression studies. We recommend using Sf9 or Sf21 cells to generate high-titer viral stocks with the BaculoDirect Transfection and Expression Kits. Once you have generated high-titer viral stocks, you may use Sf9, Sf21, High Five™, or Mimic™Sf9 cells for protein expression studies. For more information, refer to **www.lifetechnologies.com** or contact Technical Support (page 45).

Product	Amount	Cat. no.
Sf9 Frozen Cells	1 ml tube, 1×10^7 cells/ml	B825-01
Sf21 Frozen Cells	1 ml tube, 1×10^7 cells/ml	B821-01
High Five™ Cells	1 ml tube, 3×10^6 cells/ml	B855-02
Mimic™ Sf9 Insect Cells	1 ml tube, 1×10^7 cells/ml	12552-014

Gateway® Entry Vectors

A variety of Gateway® entry vectors are available from Life Technologies. Depending on your application, you may choose entry vectors with specific features such as a ribosome binding site (RBS). For more information and about the features of the entry vectors and plasmid maps, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 45).

Product	Amount	Cat. no.
pENTR™/TEV/D-TOPO® Cloning Kit	20 reactions	K2535-20
pENTR™/D-TOPO® Cloning Kit	20 reactions	K2400-20
pENTR™/SD/D-TOPO® Cloning Kit	20 reactions	K2420-20

Note: A selection guide for choosing the most appropriate Gateway® entry vector for your application can be found on our website at **www.lifetechnologies.com/Gateway**.

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Detecting Recombinant Protein

The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Life Technologies to facilitate detection of antibodies by colorimetric or chemiluminescent methods. In addition, the Positope™ Control Protein is available from Life Technologies for use as a positive control for detection of fusion proteins containing a V5, HisG or C-terminal 6×His epitope.

Product	Amount	Cat. no.
Positope™ Control Protein	5 µg	R900-50
WesternBreeze® Chromogenic Kit, Anti-Mouse	1 kit	WB7103
WesternBreeze® Chromogenic Kit, Anti-Goat	1 kit	WB7107
WesternBreeze® Chromogenic Kit, Anti-Rabbit	1 kit	WB7105
WesternBreeze® Chemiluminescent Kit, Anti-Mouse	1 kit	WB7104
WesternBreeze® Chemiluminescent Kit, Anti-Goat	1 kit	WB7106
WesternBreeze® Chemiluminescent Kit, Anti-Rabbit	1 kit	WB7108
WesternBreeze® Blocker/Diluent (part A and B)	80 ml each	WB7050
WesternBreeze® Wash Solution (16X)	2 × 100ml	WB7003

Antibodies for Detecting Recombinant Protein

Expression of your recombinant fusion protein can be detected using an antibody to the appropriate epitope. The amount of antibody supplied is sufficient for 25 Western blots.

Product	Epitope	Cat. no.
Anti-V5 Antibody	Detects 14 amino acid epitope derived from the P and V proteins of the paramyxovirus, SV5 (Southern *et al.*, 1991) GKPIPNPLLGLDST	R960-25
Anti-V5-HRP Antibody		R961-25
Anti-V5-AP Antibody		R962-25
Anti-HisG Antibody	Detects the N-terminal polyhistidine (6×His) tag followed by glycine: HHHHHHG	R940-25
Anti-HisG-HRP Antibody		R941-25
Anti-HisG-AP Antibody		R942-25
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6×His) tag, requires the free carboxyl group for detection (Lindner *et al.*, 1997) HHHHHH-COOH	R930-25
Anti-His(C-term)-HRP Antibody		R931-25
Anti-His(C-term)-AP Antibody		R932-25

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Purifying Recombinant Protein

If your gene of interest in is frame with the C-terminal peptide containing a polyhistidine (6×His) tag, you may use Life Technologies' ProBond™ or Ni-NTA Purification System to purify your recombinant fusion protein. See the table below for ordering information.

Product	Amount	Cat. no.
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Nickel-Chelating Resin	50 ml 150 ml	R801-01 R801-15
Ni-NTA Purification System	6 purifications	K950-01
Ni-NTA Agarose	10 ml 25 ml	R901-01 R901-15
Purification Columns (10 ml polypropylene columns)	50	R640-50

BaculoDirect™ GST Gateway® Transfection and Expression Kits

The BaculoDirect™ GST Gateway® Transfection and Expression Kits, available separately from Life Technologies, are designed to help you construct a recombinant baculovirus to deliver and express your GST-tagged gene of interest in insect cells. The N-terminal GST fusion tag facilitates easy detection and purification of your protein of interest, and it is reported to be helpful for solubilizing overexpressed proteins by preventing them from being sequestered into inclusion bodies. For more information on the BaculoDirect™ GST Gateway® Transfection and Expression Kits, as well as other baculoviral expression systems available from Life Technologies, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 45).

Product	Amount	Cat. no.
BaculoDirect™ GST Gateway® Transfection Kit	5 reactions	A10640
BaculoDirect™ GST Gateway® Expression Kit	5 reactions	A10641

Introduction

Overview

Introduction

The BaculoDirect™ Baculovirus Expression System uses Gateway® Technology to facilitate direct transfer of the gene of interest into the baculovirus genome *in vitro* without the need for additional cloning or recombination in bacterial or insect cells. The resulting recombinant baculovirus DNA is transfected directly into insect cells to generate recombinant virus and to screen for expression. The ability to clone and express genes from baculovirus without plaque purification or selection in bacteria makes the BaculoDirect™ System ideal for baculovirus expression.

Advantages of the BaculoDirect™ System

Using the BaculoDirect™ System to obtain purified recombinant virus suitable for production of high titer stocks offers the following advantages:

- Saves time by allowing rapid cloning of the gene of interest into the baculovirus genome without the need for traditional homologous recombination or site-specific transposition methods.
- Produces high-level expression of an epitope-tagged recombinant protein for easy detection and purification.
- Eliminates need for plaque purification. Linearized baculovirus DNA and ganciclovir selection inhibits replication of non-recombinant virus.

BaculoDirect™ Linear DNA

The major features of the BaculoDirect™ Linear DNA include:

- *att*R1 and *att*R2 sites for recombinational cloning of the gene of interest from a Gateway® entry clone
- Herpes simplex virus thymidine kinase gene (HSV1 tk) located between the two *att*R sites for negative selection using ganciclovir
- *lac*Z gene located between the two *att*R sites for determination of viral purity using β-galactosidase staining
- N-terminal or C-terminal V5–6×His fusion tag for detection and purification of recombinant fusion proteins
- Recognition site for cleavage of the fusion tag from the recombinant protein of interest using the AcTEV™ Protease (BaculoDirect™ N-Term Linear DNA only)

Note

In addition to the BaculoDirect™ Linear DNA containing N- or C-terminal V5–6×His fusion tag, Life Technologies also offers the BaculoDirect™ N-GST Linear DNA containing an N-terminal GST fusion tag for detection and purification of recombinant fusion proteins. The BaculoDirect™ N-GST Linear DNA is available in BaculoDirect™ GST Gateway® Transfection and Expression Kits (see page x for ordering information).

Continued on next page

The Gateway Technology

The Gateway® Technology is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). The Gateway® Technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression. To produce recombinant baculovirus using the BaculoDirect™ Baculovirus Expression System, simply:

1. Clone the gene of interest into a Gateway® entry vector of choice to create an entry clone.
2. Perform an LR recombination reaction to transfer the gene of interest from the entry clone to the BaculoDirect™ Linear DNA.
3. Transfect insect cells with recombinant baculovirus DNA and harvest recombinant baculovirus.

For more detailed information about the Gateway® Technology, generating an entry clone, and performing the LR recombination reaction, refer to the Gateway® Technology with Clonase® II manual (part no. 25-0749). This manual is available for downloading from **www.lifetechnologies.com** or by contacting Technical Support (page 45).

Important

The BaculoDirect™ Baculovirus Expression System is designed to help you construct a recombinant baculovirus to deliver and express a gene of interest in insect cells. Use of this system is geared towards those users who are familiar with the principles of baculovirus expression systems and Gateway® Technology. We highly recommend that users possess a working knowledge of viral and insect cell culture techniques.

For more information about the baculovirus life cycle, viral structure, and laboratory techniques, refer to the following published reviews: King and Possee, 1992; O'Reilly *et. al.*, 1992; and Richardson *et. al.*, 1995.

The Gateway® Technology

Introduction

The Gateway® Technology is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). In the Gateway® Technology, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman *et al.*, 1985). This section provides a brief overview of the Gateway® Technology. For detailed information, refer to the Gateway® Technology with Clonase® II manual (part no. 25-0749).

Characteristics of Recombination Reactions

Lambda integration into the *E. coli* chromosome occurs via intermolecular DNA recombination that is mediated by a mixture of lambda and *E. coli*-encoded proteins (i.e., Clonase® II Enzyme Mix for BaculoDirect™ Kits). The hallmarks of lambda recombination are listed below. For more detailed information about lambda recombination, see published references and reviews (Landy, 1989; Ptashne, 1992).

- Recombination occurs between specific (*att*) sites on interacting DNA molecules.
- Recombination is conservative (i.e., there is no net gain or loss of nucleotides) and does not require DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector. For example, *att*L sites are comprised of sequences from *att*B and *att*P sites.
- Strand exchange occurs within a core region that is common to all *att* sites.

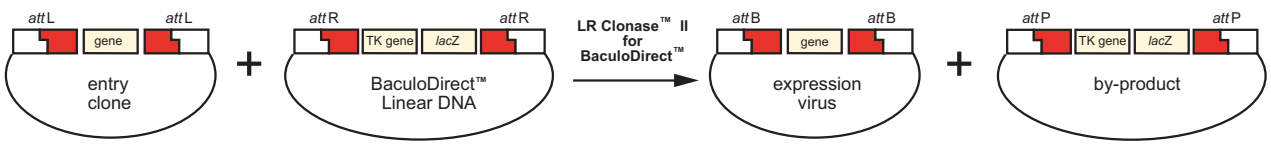
att Sites

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

In the BaculoDirect™ Baculovirus Expression System, the wild-type *att*R1 and *att*R2 sites encoded by the BaculoDirect™ Linear DNA have been modified to improve the efficiency and specificity of the Gateway® LR recombination reaction.

Gateway® LR Recombination Reaction

By using the BaculoDirect™ System, you will take advantage of the LR reaction to transfer your gene of interest into the BaculoDirect™ Linear DNA. The LR reaction facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (BaculoDirect™ Linear DNA) to create an *att*B-containing expression virus (see the following diagram). This reaction is catalyzed by LR Clonase® II Enzyme Mix for BaculoDirect™ Kits.



Ganciclovir

Introduction

Ganciclovir is a nucleoside analog used in the BaculoDirect™ System to negatively select against non-recombinant baculovirus. Ganciclovir selection, which begins immediately after transfection and continues through infection, reduces background levels and eliminates the need for plaque purification.

Ganciclovir Selection

Ganciclovir is a nucleoside analog [9-(1,3-Dihydroxy-2-propoxymethyl) guanine] that is enzymatically phosphorylated by Herpes Simplex Virus type 1 thymidine kinase (HSV1 tk). Once phosphorylated, the active analog incorporates into DNA and inhibits DNA replication (Rubsam *et al.*, 1999). Ganciclovir selection has been used in Sf9 cells to purify recombinant viruses that have lost the counter-selectable gene marker (HSV1 tk) due to homologous recombination (Godeau *et al.*, 1992).

In the BaculoDirect™ System, the HSV1 tk gene is under the control of an immediate early promoter (PIE-1(0)) which drives synthesis of the first viral transcript produced in infected cells (Kovacs *et al.*, 1991).

Ganciclovir is a hazardous material that may cause harm if ingested, inhaled, or absorbed through the skin. Exercise caution and wear suitable protective clothing, gloves, and safety goggles while handling solutions containing ganciclovir. Before handling ganciclovir, review the Material Safety Data Sheet available from our website at **www.lifetechnologies.com/sds** or by contacting Technical Support (page 45).

Preparing and Storing Ganciclovir

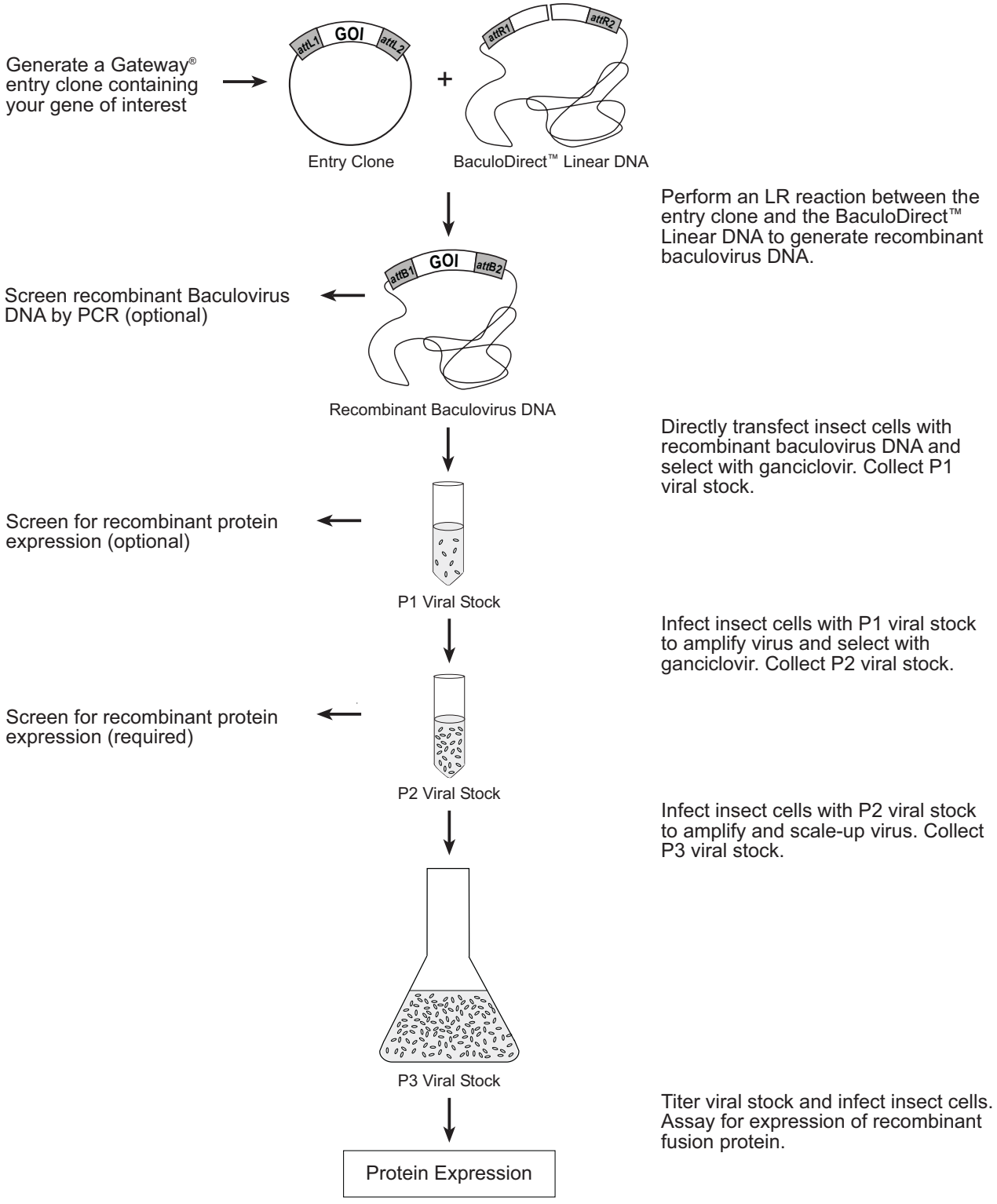
We recommend setting aside the amount of complete growth medium needed for the experiment requiring ganciclovir selection and adding the appropriate amount of ganciclovir to a final concentration of 100 µM. Aliquot the remaining ganciclovir into multiple tubes to reduce the number of freeze/thaw cycles.

Additional ganciclovir may be purchased in powder form from InvivoGen (Cat. no. sud-gcv). Refer to page 39 for instructions on reconstituting and storing ganciclovir.

Experimental Overview

Experimental Summary

The following diagram summarizes the general steps required to express your gene of interest using the BaculoDirect™ Baculovirus Expression System.



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Experimental Steps

The experimental steps necessary to express your protein of interest using the BaculoDirect™ Baculovirus Expression System are outlined below. For more details on each step, refer to the indicated pages.

Note: Before you perform the LR recombination reaction (Step 1), you need to have an entry clone containing your gene of interest. Refer to the next page for general guidelines on generating an entry clone.

Step	Action	Page
1	Perform the LR recombination reaction between the BaculoDirect™ Linear DNA and an entry clone containing your gene of interest using LR Clonase® II for BaculoDirect™ Kits.	9–11
2	Directly transfect insect cells with recombinant baculovirus DNA and collect P1 viral stock. Screen for recombinant protein expression, if desired.	12–17
3	Infect insect cells with P1 viral stock to generate a high-titer viral stock. Screen for recombinant protein expression.	18–20
4	Determine titer of viral stock by plaque assay.	21–24
5	Isolate recombinant viral DNA and analyze by PCR, if desired.	26–30
6	Infect insect cells and optimize conditions for recombinant protein expression.	32–36
7	Purify recombinant protein, if desired.	37

Methods

Before Starting

Introduction

Before you start your experiments, you need to have an entry clone containing your gene of interest, cultures of Sf9 or Sf21 cells growing, and frozen master stocks available. Refer to the guidelines below for more information.

Gateway® Entry Vectors

To recombine your gene of interest into the BaculoDirect™ Linear DNA, you need an entry clone containing your gene of interest. For your convenience, Life Technologies offers a variety of Gateway® entry vectors (see page viii for ordering information). For detailed information on constructing an entry clone, refer to the manual for the specific entry vector you are using.

Points to Consider for BaculoDirect™ C-Term Linear DNA

To recombine your entry clone with BaculoDirect™ C-Term Linear DNA, keep the following points in mind when constructing your entry clone:

- Make sure your insert contains an ATG start codon for initiation of translation.
- If you wish to include the V5 epitope and 6×His tag, make sure your insert **does not** contain a stop codon. Design the gene of interest to be in frame with the C-terminal epitope tag after recombination. Refer to page 30 for a diagram of the recombinant baculovirus DNA.

  Tip: Keep the translation reading frame of your protein of interest in frame with the TAC AAA triplet in the *att*L2 site of the entry clone.
- If you wish to express native protein and **not** include the V5 epitope and 6×His tag, make sure your insert contains a stop codon.

Points to Consider for BaculoDirect™ N-Term Linear DNA

To recombine your entry clone with BaculoDirect™ N-Term Linear DNA, keep the following points in mind when constructing your entry clone:

- Design your gene of interest to be in frame with the N-terminal 6×His-V5 epitope tag after recombination. Refer to page 31 for a diagram of the recombinant baculovirus DNA.

  Tip: Keep the translation reading frame of your protein of interest in frame with the AAA AAA triplet in the *att*L1 site of the entry clone.
- Make sure your insert contains a stop codon.

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Recommended Cells

We recommend using Sf9 or Sf21 cells to generate high-titer viral stocks with the BaculoDirect™ System. Note that Sf9 cells are provided with the BaculoDirect™ Expression Kits. **We do not recommend using High Five™ cells to generate viral stocks due to lower transfection efficiency.** Once you have generated high-titer viral stocks, you may use Sf9, Sf21, High Five™, or Mimic™ Sf9 cells for protein expression studies. See page vii for ordering information.

Recommended Media

For the highest transfection efficiency, we recommend using Grace's Insect Cell Culture Medium, Unsupplemented (provided with the BaculoDirect™ Expression Kits) for the transfection experiment. For infection, expression studies, and general culturing of insect cells, you may use any complete growth medium (e.g., Sf-900™ II SFM, Sf-900™ III SFM, complete TNM-FH, or other suitable medium). Refer to page 39 for a recipe for complete TNM-FH.

When working with recombinant or wild-type viral stocks, always maintain separate media bottles for cell culture and for virus work. Baculovirus particles can survive and be maintained in media at 4°C and will contaminate your cell cultures if added to tissue culture plates or flasks during passaging.

Insect Cell Lines Manual

For additional information on insect cell culture, refer to the Insect Cell Lines manual. This manual contains information on:

- Thawing frozen cells
- Maintaining and passaging cells
- Freezing cells
- Scaling up cell culture

This manual is provided with the BaculoDirect™ Expression Kits and is also available from our website (**www.lifetechnologies.com**) or by contacting Technical Support (page 45).

Performing the LR Recombination Reaction

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into the BaculoDirect™ Linear DNA. We recommend that you include the pENTR™/CAT positive control supplied with the kit in your experiments to help you evaluate your results.

Important

The LR recombination reaction protocol provided on the next page contains optimized amounts of each reagent. To obtain the best possible results, follow the protocol exactly as described.

Resuspending pENTR™/CAT

The positive control plasmid pENTR™/CAT is provided lyophilized in TE buffer, pH 8.0. Resuspend the plasmid in 200 µl of sterile water to a final concentration of 100 ng/µl.

LR Clonase® II Enzyme Mix for BaculoDirect™ Kits

LR Clonase® II Enzyme Mix for BaculoDirect™ Kits is supplied with the BaculoDirect™ Expression Kits to catalyze the LR recombination reaction. The LR Clonase® II Enzyme Mix for BaculoDirect™ Kits combines the proprietary enzyme formulation and 5X LR Clonase® Reaction Buffer. Use the protocol provided on the next page to perform the LR recombination reaction using LR Clonase® II Enzyme Mix for BaculoDirect™ Kits.

Note: For the LR recombination reaction, only use the LR Clonase® II Enzyme Mix for BaculoDirect™ Kits, do **not** use LR Clonase® II or LR Clonase® from other kits.

Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/µl in TE buffer, pH 8.0)
- BaculoDirect™ Linear DNA (300 ng/vial; provided with the BaculoDirect™ Expression and Transfection Kits)
- pENTR™/CAT control plasmid, optional (100 ng/µl; provided with the BaculoDirect™ Expression and Transfection Kits)
- LR Clonase® II Enzyme Mix for BaculoDirect™ Kits (provided with the kits; keep at –20°C until immediately before use)
- 1X TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 25ºC water bath

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LR Recombination Reaction Protocol

Perform Steps 1–4 in a **sterile laminar flow hood** to reduce the chances of contamination.

1. To set up your sample and positive control reaction, add the following components **directly** to the BaculoDirect™ Linear DNA vials containing 10 µl (300 ng) of DNA at room temperature and mix the contents. **Do not vortex or pipette up and down as this will shear the baculovirus DNA and reduce transfection efficiency.**

Component	Sample	Positive Control
BaculoDirect™ Linear DNA	10 µl (in vial)	10 µl (in vial)
pENTR™/CAT control (100 ng/µl)	--	1 µl
Entry clone (100–300 ng/reaction)	1–2 µl	--
1X TE Buffer, pH 8.0	4–5 µl	5 µl
Total volume	**16 µl**	**16 µl**

   Note: To include a negative control, set up a third sample reaction and substitute 4 µl of 1X TE Buffer, pH 8.0 for the enzyme mix (see step 4).

2. Remove the LR Clonase® II Enzyme Mix for BaculoDirect™ Kits from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase® II Enzyme Mix for BaculoDirect™ Kits briefly twice (2 seconds each time).
4. To each sample above, add 4 µl of LR Clonase® II Enzyme Mix for BaculoDirect™ Kits or 4 µl of 1X TE Buffer, pH 8.0 (if preparing a negative control) for a total reaction volume of 20 µl.
5. Mix well by tapping the tube several times. **Do not vortex or pipette up and down as this will shear the baculovirus DNA and reduce transfection efficiency.**
6. Incubate the reactions at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically increases the efficiency of the LR recombination reaction.

Note

After incubation, you may analyze the LR reaction by PCR. Dilute a 2 µl aliquot of the LR reaction 200-fold and use 2 µl of the dilution in a 25 µl PCR reaction.

- If you are using the C-terminal BaculoDirect™ vector, you may use the Polyhedrin forward primer and the V5 reverse primer (see page 30 for primer binding sites). Use the PCR conditions described on page 29.

BaculoDirect™ C-Term DNA	Primer Sequence
Polyhedrin Forward Primer	5′-AAATGATAACCATCTCGC-3′
V5 Reverse Primer	5′-ACCGAGGAGAGGGTTAGGGAT-3′

- If you are using the N-terminal BaculoDirect™ vector, you may use the Polyhedrin forward primer and a primer of your own design that binds within your gene of interest. See page 31 for Polyhedrin forward promoter binding site. You must optimize the PCR conditions.

BaculoDirect™ N-Term DNA	Primer Sequence
Polyhedrin Forward Primer	5′-AAATGATAACCATCTCGC-3′

The Next Step

Once the LR reaction is completed, you are ready to directly transfect the recombinant baculovirus DNA into insect cells. Proceed to the next section for transfection guidelines.

Transfecting Sf9 or Sf21 Cells

Introduction

This section provides detailed guidelines for transfecting your LR recombination reaction into Sf9 or Sf21 insect cells.

Cellfectin® II Reagent

Cellfectin® II Reagent is supplied with the BaculoDirect™ Transfection and Expression Kits for lipid-mediated transfection of your insect cells. Cellfectin® II Reagent is a proprietary liposome formulation of a cationic lipid in membrane-filtered water, and is ideally suited for transfecting Sf9 and Sf21 insect cells.

Serum-Free Medium

We recommend using Grace's Insect Cell Culture Medium, Unsupplemented; however, you may use serum-free medium during the transfection experiment. Note that components in serum-free medium may interfere with transfection and result in a decrease in transfection efficiency.

Note: If you are already culturing Sf9 or Sf21 cells in Sf-900™ II SFM or Sf-900™ III SFM, you can perform the transfection in Grace's Insect Cell Culture Medium, Unsupplemented, then easily switch back to Sf-900™ II SFM or Sf-900™ III SFM after transfection.

Note

Use of complete growth medium that contains antibiotics and antimycotics in addition to ganciclovir for the **last step** of the transfection protocol (see next page) is optional. If so desired, you can use 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B (i.e., Fungizone® Antimycotic) in this last step (see page vii for ordering information).

Controls

We recommend that you include the following controls in your experiments:

- LR recombination reaction using pENTR™/CAT plasmid as a positive control
- Cellfectin® II Reagent only (mock transfection) as a negative control

Materials Needed

- Sf9 cells (provided with the BaculoDirect™ Expression Kits) or Sf21 cells
- Complete growth medium (e.g., Sf-900™ II SFM, Sf-900™ III SFM, complete TNM-FH, or other suitable medium)
- LR reaction from **LR Recombination Reaction Protocol**, page 10
- Grace's Insect Cell Culture Medium, Unsupplemented (provided with the BaculoDirect™ Expression Kits; also available separately, see page vii)
- Cellfectin® II Reagent (provided with the BaculoDirect™ Expression and Transfection Kits; also available separately, see page vii)
- Complete growth medium with 100 µM ganciclovir and antibiotics (see **Note** above)
- Six-well tissue culture plates
- 27ºC incubator
- Inverted microscope
- Air-tight bags or containers

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Transfecting Sf9 or Sf21 Cells, continued

Important

Use Grace's Insect Medium, Unsupplemented which does not contain any supplements, FBS, or antibiotics for the transfection procedure. The proteins in the FBS and supplements will interfere with the Cellfectin® II Reagent, causing the transfection efficiency to decrease.

Transfection Procedure

For Sf9 or Sf21 insect cells cultured in Grace's Insect Medium, Supplemented containing 10%FBS, use the following protocol to transfect your cells in a 6-well format. All amounts and volumes are given on a per well basis.

1. Verify that the Sf9 or Sf21 cells are in the log phase (1.5–2.5 × 10^6 cells/ml) with greater than 95% viability.
2. If the cell density is in range of 1.5–2.5 × 10^6 cells/ml and the culture is without antibiotics, proceed to step 2a. If the cell density is **not** in this range or the cell culture contains antibiotics, follow steps 2b–2c:
   a. Add 2 ml of Grace's Insect Medium, Unsupplemented (without antibiotics and serum) in each well. Seed 8 × 10^5 Sf9 or Sf21 cells from Step 1 per well. **Do not change medium or wash the cells. The medium carried over will enhance the transfection efficiency.** Allow cells to attach for 15 minutes at room temperature in the hood. Proceed to step 3.
   b. Prepare 10ml plating medium by mixing 1.5 ml Grace's Insect Medium, Supplemented containing 10%FBS (without antibiotics) and 8.5 ml Grace's Insect Medium, Unsupplemented (without FBS and antibiotics).
   c. Plate 8 × 10^5 Sf9 or Sf21 cells from Step 1 per well. Allow cells to attach for 15 minutes at room temperature in the hood. Remove the medium. Add 2.5 ml plating medium from step 2b per well. Proceed to step 3.
3. Prepare the following solutions in 1.5 ml microcentrifuge tubes **for each transfection sample**. Cellfectin® II Reagent in the Transfection Mixture A can be left at room temperature for up to 30 minutes.

Transfection Mixture A:	
Cellfectin® II Reagent	8 µl
Grace's Insect Medium, Unsupplemented **(without supplements, serum, or antibiotics)**	100 µl
Transfection Mixture B:	
LR recombination reaction	10 µl
Grace's Insect Medium, Unsupplemented **(without supplements, FBS, or antibiotics)**	100 µl

Procedure continued on next page

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Transfection Procedure, continued

Procedure continued from previous page

4. Combine Transfection Mixture A and Transfection Mixture B. Mix gently by tapping the tube and incubate at room temperature for 25–35 minutes.
5. After 25–35 minutes incubation, add the transfection mix from Step 4 (total volume ~210 µl) **dropwise** onto the cells from step 2. Repeat for all transfections.

   Note: With Cellfectin® II, you do not have to remove the medium from cells and wash cells prior to adding the DNA:lipid complex to cells.
6. Incubate the cells in a 27ºC incubator for 3 to 5 hours.
7. Remove the transfection mixture and replace with 2 ml of complete growth medium (e.g., Grace's Insect Medium, Supplemented and 10% FBS) **with 100 µM ganciclovir** to each well. Addition of antibiotics is optional (see page 12). Repeat for all transfections.

   Note: Distribute the drops evenly to avoid disturbing the monolayer.
8. Place the plates in a sealed plastic bag with moist paper towels to prevent evaporation. Incubate the cells at 27°C for 72 hours or until you start to see signs of viral infection.

Isolating P1 Viral Stock

Introduction

Budded virus should be released into the medium 72 hours after transfection. However, depending on transfection efficiency, cells may not show all of the signs of viral infection for up to a week. Beginning at 72 hours after transfection, visually inspect the cells daily for signs of infection (see Characteristics of Infected Cells). Once the cells appear infected, harvest the virus from the cell culture medium using the procedure below. You may also perform an initial screen for expression of your recombinant fusion protein, if desired.

Materials Needed

- Transfected insect cells from Step 8, page 14
- Inverted microscope
- 15 ml tubes

Characteristics of Infected Cells

Virus infected insect cells typically display the following characteristics as observed from visual inspection using an inverted phase microscope at 250–400X magnification.

Early (first 24 hours)	Increased cell diameter	A 25–50% increase in cell diameter may be seen.
	Increased size of cell nuclei	Nuclei may appear to "fill" the cells.
Late (24–72 hours)	Cessation of cell growth	Cells appear to stop growing when compared to a cell-only control.
	Detachment	Cells release from the plate or flask.
Very Late (>72 hours)	Cell lysis	Cells appear lysed, and there are signs of clearing in the monolayer.

Isolating P1 Viral Stock

1. Once the transfected cells demonstrate signs of very late stage infection (e.g., 72 hours post-transfection) collect 2 ml of medium from each well and transfer to sterile 15 ml tubes. Centrifuge the tubes at 3,000–5,000 rpm for 5 minutes to remove cells and large debris.
2. Transfer the supernatant to fresh 15 ml tubes. **This is the P1 viral stock**. Store at 4ºC, protected from light. See the next page for additional storage information.
3. If you wish to screen for expression of your recombinant fusion protein, proceed to **Screening for Expression**, page 16.

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Screening for Expression

You may perform a small-scale or preliminary expression experiment on the transfected cells to verify expression of your recombinant protein. Follow the general guidelines below to assay for expression.

- If you are expressing a secreted protein, remove a sample from the medium to analyze protein expression and secretion. You may also harvest cells to analyze intracellular levels of your recombinant protein (see page 34).
- To harvest cells, transfer transfected cells into microcentrifuge tubes and centrifuge to collect cells. Wash cells 2X with PBS to remove traces of serum.
- Assay for expression by Western blot analysis. For information on preparing protein samples and detecting expression, refer to pages 34–35.

Storing Viral Stocks

Store viral stocks as follows:

- If medium is serum-free, add serum to 10%. Serum proteins act as substrates for proteases and therefore prevent degradation of viral coat proteins.
- Store viral stock at 4ºC, protected from light.
- Store an aliquot of the viral stock at –80ºC.
- Do not store routinely used viral stocks at temperatures below 4ºC. Repeated freeze/thaw cycles can result in a 10- to 100-fold decrease in viral titer.

The Next Step

Once you have obtained your P1 viral stock, you may:

- Amplify the viral stock by infecting Sf9 or Sf21 cells (refer to **Preparing a High-Titer Viral Stock and Screening for Protein Expression**, page 18). We recommend this procedure to obtain the highest viral titers and optimal results in your expression studies.
- Perform a plaque assay to amplify your viral stock from a single viral clone or to determine the titer of your P1 viral stock (refer to **Performing a Plaque Assay**, page 21).

Continued on next page

Troubleshooting

The table below lists some potential problems and possible solutions to help you troubleshoot your transfection and protein expression experiments.

Problem	Possible Cause	Solution
Transfected cells do not show signs of infection	Low transfection efficiency	• Use Cellfectin® II Reagent that is less than 6 months old. • Perform transfection in Grace's Insect Medium Unsupplemented that **does not** contain supplements, antibiotics, or FBS.
	Cells are not viable	Cells should be in log phase and 95–98% viable. Refer to the Insect Cell Lines manual for tips on culturing Sf9 and Sf21 cells.
	Cells are not confluent enough	Plate cells at 50–70% confluence.
	Cells are too dense	Plate 8×10^5 cells per well for six-well plates. Split the cells if they are too confluent 3 days after transfection. Add complete growth medium containing 100 µM ganciclovir and incubate for 1 to 2 more days at 27ºC.
	LR recombination reaction unsuccessful	• Make sure you used LR Clonase® II Enzyme Mix for BaculoDirect™ Kits for the LR reaction. • Check the pENTR™/CAT positive control transfection to verify that the LR reaction was successful. • Check your LR reaction using PCR as described on page 10. • Incubate LR reactions for up to 18 hours to increase recombinational efficiency.
No or little recombinant fusion protein detected in initial expression screen	Entry clone constructed incorrectly	Refer to page 7 for guidelines on constructing your entry clone.
	Insert not in frame with N-terminal or C-terminal tag	• Refer to the diagram on page 30 or page 31 to verify the correct reading frame of the resulting recombinant baculovirus DNA following the LR reaction. • Analyze recombinant viral DNA by PCR to confirm correct size and orientation. • Sequence PCR product to verify proper reading frame for expression of the V5 and 6×His epitope tag.

Preparing a High-Titer Viral Stock and Screening for Protein Expression

Introduction

The P1 viral stock is a low-titer stock (1×10^5 to 1×10^6 pfu/ml). You will infect cells with the P1 stock to generate a high-titer P2 stock of approximately 5×10^7 to 1×10^8 pfu/ml. This P2 viral stock can then be used to generate a large-scale, high-titer viral stock suitable for expression studies. Guidelines are provided in this section to amplify the recombinant baculovirus and to screen for recombinant protein expression.

Materials Needed

- Sf9 cells (provided with the BaculoDirect™ Expression Kits ; also available separately, see pagevii) or Sf21 cells
- Complete growth medium with 100 µM ganciclovir
- P1 viral stock (from Step 2, page 15)
- β-Gal Staining Kit (recommended; see page vii for ordering information) or other suitable kit
- Six-well tissue culture plates
- 27ºC incubator
- Inverted microscope
- Air-tight bags or containers

Note

You will infect duplicate samples of Sf9 or Sf21 cells with P1 viral stock. One set of cells will be assayed for the presence of non-recombinant virus by β-galactosidase staining. The other set will be assayed for expression of your recombinant protein.

Preparing Cells

Use log phase Sf9 or Sf21 cells with greater than 95% viability.

1. Seed 8×10^5 Sf9 or Sf21 cells per well in 2 ml of complete growth medium **with 100 µM ganciclovir** in a six-well tissue culture plate. Remember to seed duplicate wells (see **Note** above). Gently tip the plate from side to side 4–6 times to evenly distribute the cells.
2. Incubate the cells at 27ºC for one hour to allow the cells to fully attach to the bottom of the plate.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

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Preparing a High-Titer Viral Stock and Screening for Protein Expression, continued

Isolating P2 Viral Stock

1. Add 5 µl of the P1 viral stock to each well. Place the plates in a sealed plastic bag with moist paper towels to prevent evaporation. Incubate infected cells for 72 hours at 27ºC.
2. At 72 hours post-infection, collect 2 ml of medium from each well and transfer to sterile 15 ml tubes. Centrifuge the tubes at 3,000–5,000 rpm for 5 minutes to remove debris.
3. Transfer the supernatant to fresh 15 ml tubes. **This is the P2 viral stock**. Store at 4ºC, protected from light. Refer to page 16 for additional storage information.
4. With one set of infected cells, proceed to **β-Galactosidase Staining**. With the other set of infected cells, proceed to **Screening for Expression**. We recommend performing both procedures before scaling up your viral stock and performing expression experiments.

β-Galactosidase Staining

Because the BaculoDirect™ Linear DNA contains the *lac*Z gene, you may assay for the presence of non-recombinant virus by staining the infected cells for β-galactosidase expression. Recombinant virus will not stain blue because the gene of interest replaces the *lac*Z gene after the LR recombination reaction (see diagram on page 3). If you see blue-stained cells, we recommend that you perform a plaque assay to isolate a recombinant viral clone (see page 21).

Screening for Expression

You will need to verify expression of your recombinant protein before further amplifying your viral stock. Follow the general guidelines below to assay for expression:

- If you are expressing a secreted protein, remove a sample from the medium to analyze protein expression and secretion. You may also harvest cells to analyze intracellular levels of your recombinant protein (see page 34).
- To harvest cells, transfer transfected cells into microcentrifuge tubes and centrifuge to collect cells. Wash cells 2X with PBS to remove traces of serum.
- Assay for expression by Western blot analysis. For information on preparing protein samples and detecting expression, refer to pages 34–35.

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Scaling Up the Amplification Procedure

If you are satisfied with the purity of the viral stock and have confirmed expression of your recombinant protein, you may scale-up the amplification procedure to any volume of your choice. To produce a large-scale, high-titer P3 stock, we recommend doing the following:

- Perform a plaque assay to determine the titer of the P2 viral stock (see page 21).
- Use the equation provided below to determine the amount of P2 viral stock to use to infect at a specific MOI.
- Scale up the amount of cells and volume of virus appropriately and follow the guidelines outlined in this section.

  Note: The ganciclovir selection is not required for generation of the P3 viral stock.

Multiplicity of Infection (MOI)

To amplify your viral stock, infect cells at a multiplicity of infection (MOI) ranging from 0.1 to 1.0. MOI is defined as the number of virus particles per cell. Use the following formula to calculate how much viral stock to add to obtain a specific MOI.

$$\text{Inoculum required (ml)} = \frac{\text{MOI (pfu/cell)} \times \text{number of cells}}{\text{viral titer (pfu/ml)}}$$

Note: If you have not determined the titer of your P2 viral stock, you may assume that the titer ranges from 5×10^7 to 1×10^8 pfu/ml.

Generating High-Titer Stocks From Frozen Master Stock

If you start with a frozen viral master stock, we recommend generating a new high-titer stock as viral titer generally decreases from storage at –80ºC. To generate another high-titer stock from the master stock, re-infect insect cells and amplify the viral stock using the guidelines outlined in this section.

The Next Step

Now that you have a high-titer viral stock, you need to determine the titer of your viral inoculum. Proceed to the next section to perform a plaque assay and calculate viral titer.

Performing a Plaque Assay

Introduction

We recommend you perform a plaque assay to determine the titer of your viral stock. You may also perform a plaque assay to purify a single viral clone, if desired. In this procedure, you will infect cells with dilutions of your viral stock and identify focal points of infection (plaques) on an agarose overlay. You may also titer your viral stock by the end-point dilution method described in O'Reilly *et. al.*, 1992.

BaculoTiter™ Assay Kit

We recommend using the BaculoTiter™ Assay Kit, available separately from Life Technologies, to determine the titer of your baculoviral stock. The BaculoTiter™ Assay Kit rapidly determines the titer of an unknown baculovirus sample with minimal handling steps, providing both accuracy and convenience in an easy-to-use kit format in two days as opposed to ten days with the serial dilution assays. See page vii for ordering information.

Blue/White Screening

You will use a chromogenic substrate to distinguish colorless plaques containing recombinant virus from blue plaques containing non-recombinant virus. We recommend using Bluo-gal instead of X-gal for blue/white screening because Bluo-gal generally produces a darker blue color than X-gal. Add Bluo-gal directly to the overlay solution before pouring over the infected cells.

Materials Needed

- Sf9 cells (provided with the BaculoDirect™ Expression Kits ; also available separately, see page vii) or Sf21 cells
- Sf-900™ II SFM, Sf-900™ III SFM, or other appropriate complete growth medium (see **Note** on page 22)
- Sf-900™ Medium (1.3X) (see page vii) or other appropriate plaquing medium (see **Note** on page 22)
- 4% Agarose Gel (see page vii)
- Sterile, cell-culture grade, distilled water (see page vii)
- 100 ml sterile, glass bottle
- Serial dilutions of viral stock (see page 22)
- Bluo-gal (50 mg/ml, see page 40 for a recipe)
- Six-well tissue culture plates
- Sterile hood
- Water baths at 47°C and 70°C
- 27ºC incubator
- Inverted microscope
- Air-tight bags or containers

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Note

If you are culturing your Sf9 or Sf21 cells in serum-supplemented media (i.e., complete TNM-FH), you should have the following reagents on hand (see page vii for ordering information):

- Grace's Insect Cell Culture Medium, Supplemented
- Grace's Insect Cell Culture Medium (2X)
- Fetal Bovine Serum (FBS), Qualified, Heat-Inactivated

Diluting Virus

You will be infecting cells with serial dilutions of your viral stock. Keep in mind the following points when preparing the 10-fold serial dilutions:

- Prepare dilutions in complete growth medium
- Vortex viral stocks or dilutions before making the next dilution to ensure virus is evenly resuspended
- Prepare 3 ml (for duplicate wells) or 4 ml (for triplicate wells) of each viral dilution
- Make sure to return your viral stock to 4ºC
- Prepare dilutions according to the viral stock you are using to perform the plaque assay (see recommended dilutions below)

Viral Stock	Dilution
P1	10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}
P2	10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}
P3	10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

Infecting Cells with Virus

The quality of the cell monolayer is critical for a successful plaque assay. Be sure to include a cells-only control to assess cell viability, contamination, and monolayer quality.

1. Seed 8×10^5 Sf9 or Sf21 cells per well in 2 ml complete growth medium in a six-well plate. Use 2 to 3 wells for **each** viral dilution to be tested (see **Diluting Virus**, above). Gently tip the plate from side to side 4–6 times to evenly distribute the cells.
2. Incubate the cells at 27ºC for one hour to allow the cells to fully attach to the bottom of the plate.
3. Verify that the cells have attached by inspecting them under an inverted microscope.
4. Aspirate the medium from the wells. Carefully add 1 ml of each viral dilution **dropwise** to the appropriately labeled well. Be careful not to disturb the monolayer.
5. Incubate the cells at 27ºC for 1 hour. While cells are incubating, prepare the Plaquing Medium (page 23).

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Preparing the Plaquing Medium

Plaquing medium, a mixture of culture medium and agarose, is used to immobilize the infected cells for the plaque assay. Prepare plaquing medium immediately before use. If you are culturing your cells in Sf-900™ II SFM or Sf-900™ III SFM, prepare Sf-900™ Plaquing Medium. If you are culturing cells in TNM-FH, prepare Grace's Plaquing Medium.

Note: This procedure provides instructions to prepare 40 or 50 ml of Sf-900™ and Grace's Plaquing Medium, respectively. You will need 2 ml of Plaquing Medium per well. To prepare more Plaquing Medium, scale up the volume of reagents used accordingly.

1. Melt the 4% Agarose Gel by placing the bottle in a 70°C water bath for 20 to 30 minutes **or** heating the agarose in a microwave oven. While the 4% agarose gel is melting, place the following in the 45°C water bath:
   - Empty, sterile 100 ml bottle
   - Sf-900™ Medium (1.3X) or Grace's Insect Cell Culture Medium (2X), as appropriate
2. Once the 4% agarose gel has liquefied, move the agarose gel, medium, and empty 100 ml bottle to a sterile hood.
3. Working quickly, prepare the plaquing medium as follow:

   Sf-900™ Plaquing Medium: Combine 30 ml of Sf-900™ Medium (1.3X) and 10 ml of the melted 4% Agarose Gel in the empty 100 ml bottle and mix gently.

   Grace's Plaquing Medium: Add 20 ml of heat-inactivated FBS to the 100 ml bottle of Grace's Insect Medium (2X) and mix. Combine 25 ml of the Grace's Insect Medium (2X) containing serum with 12.5 ml of cell-culture grade, sterile, distilled water and 12.5 ml of the melted 4% Agarose Gel in the empty 100 ml bottle and mix gently.
4. Add Bluo-gal to a final concentration of 150 µg/ml. Mix immediately by pipetting up and down. Place the bottle in a 47°C water bath until use.

Agarose Overlay

Pouring the agarose overlay may require some practice if you are unfamiliar with this technique. You should already have your plaquing medium prepared (see above; remove from water bath). It is important to work quickly and efficiently.

1. After the 1 hour incubation period (Step 5, Infecting Cells with Virus page 22), remove the cells from the incubator and completely aspirate the medium from each well containing cells and virus. If you have multiple plates, follow this protocol for one plate before proceeding to the next. Do not let the cells dry out.
2. Withdraw 2 ml of the plaquing medium and slowly stream the solution down the side of the well. Repeat for all wells. **Do not move the plate until the agarose overlay has set.**
3. Repeat Steps 1–2 until all plates have been completed.
4. Place the plates in a sealed plastic bag with moist paper towels to prevent evaporation.
   Note: Once condensation appears on the plastic bag or container, open the bag or container. Moisture can destroy the monolayer, preventing plaque formation.
5. Incubate the cells at 27°C for 4–6 days or until plaques are well formed. Proceed to **Calculating Viral Titer**, page 24.

Continued on next page

Neutral Red Overlay

To improve visualization of plaques, you may perform a neutral red overlay 4 days post-infection. Do not use this procedure if you plan to plaque purify your virus as neutral red is a known mutagen that can alter your recombinant virus.

1. Prepare a 1 mg/ml Neutral Red solution in complete growth medium and filter sterilize.
2. Combine the following reagents in a 50 ml tube and place in a 37ºC water bath.

Neutral Red (1 mg/ml)	1.5 ml
Complete growth medium	16.5 ml

3. Microwave 4% Agarose Gel until melted, then place in a 47°C water bath for 5 minutes.
4. Move the 50 ml tube of neutral red solution and the 4% Agarose Gel to a sterile hood. Add 6 ml of 4% Agarose Gel to the neutral red solution.
5. Add 1 ml of the Neutral Red overlay to each well containing plaquing overlay. Once the agarose has hardened, return plates to a 27°C incubator until plaques are ready to count. Plaques will appear as clear spots on a red monolayer.

Calculating Viral Titer

Use the equation below to calculate your viral titer.

$$\text{pfu/ml} = \frac{\text{number of plaques (pfu)}}{\text{dilution factor} \times \text{ml of inoculum}}$$

Example

A well with a viral dilution of 10^{-8} contains 18 white plaques. The viral titer is:

$$\text{pfu/ml} = \frac{18 \text{ pfu}}{10^{-8} \times 1 \text{ ml}}$$

$$= 1.8 \times 10^{9} \text{ pfu/ml}$$

The Next Step

Once you have a viral stock of suitable titer ($\geq 1 \times 10^{8}$ pfu/ml), you may infect cells and perform expression studies (see page 31). To plaque purify the virus or to analyze the recombinant DNA, proceed to the next section.

Continued on next page

Troubleshooting

The following table lists some potential problems and possible solutions to help you troubleshoot your plaque assay.

Problem	Possible Cause	Solution
No plaques	Kinetics of infection are slower than expected	Observe plates until 8 or 9 days after infection. If no plaques appear, investigate other possible causes.
	No confluent monolayer on Day 2 or Day 3 post-infection	Seed 8×10^5 cells in a six-well plate with 70% confluence. Cells should double at least once before infection stops growth.
	Excessive condensation during incubation at 27ºC	Remove paper towels or open the container containing plates as soon as condensation appears.
	Viral titer too low	Use higher concentrations of viral titer. You may need to re-infect your cells and collect a higher titer of your viral stock.
Small plaques that are difficult to visualize	Too many cells seeded	Seed fewer cells. We recommend seeding 8×10^5 cells per well for a six-well plate.
Too many plaques or complete cell lysis	Viral titer not dilute enough	Prepare additional dilutions of your viral stock for infection.
Cells are dead	Temperature of the plaquing medium is too high	Prepare plaquing medium, then place in a 47°C water bath until use.
Cracks in the agarose overlay	Growth medium not completely removed	Completely aspirate the growth medium before adding the plaquing medium. Any remaining growth medium can interfere with the gelling process.

Isolating Virus From a Single Plaque

Introduction

This section provides detailed guidelines for plaque purifying your virus. You can use the isolated virus to generate a viral stock from a single viral clone or for PCR analysis of the recombinant baculovirus DNA. If you do not wish to plaque purify your virus, proceed to **Expressing and Analyzing Recombinant Protein**, page 32.

Materials Needed

- Sf9 cells (provided with the BaculoDirect™ Expression Kits ; also available separately, see page viii) or Sf21 cells (available separately, see page viii)
- Complete growth medium
- Plates containing plaques from Step 5, page 23
- Six-well tissue culture plates
- 27ºC incubator
- Inverted microscope
- Sterile Pasteur pipette and bulb
- Air-tight bags or containers

Preparing Cells

Use log phase Sf9 or Sf21 cells with greater than 95% viability.

1. Seed 8×10^5 Sf9 or Sf21 cells per well in 2 ml of complete growth medium in a six-well tissue culture plate. Gently tip the plate from side to side 4–6 times to evenly distribute the cells.
2. Incubate the cells at 27ºC for one hour to allow the cells to fully attach to the bottom of the plate.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

Infecting Cells

1. Using a sterile Pasteur pipette and bulb, carefully penetrate and remove the agarose containing the desired plaque.
2. Transfer the agarose plug containing the plaque to a 1.5 ml microcentrifuge tube containing 500 µl of complete growth medium. Mix well by vortexing.
3. Add 100 µl of the agarose plug solution from Step 2 to each well.
4. Place the plates in a sealed plastic bag with moist paper towels to prevent evaporation. Incubate at 27ºC for 72 hours.
5. If you wish to isolate viral DNA, proceed to **Isolating Viral DNA for PCR Analysis** next page. If you wish to amplify your viral stock, proceed to **Isolating Virus for Amplification**, page 27.

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Isolating Virus From a Single Plaque, continued

Isolating Viral DNA for PCR Analysis

1. Collect 2 ml of medium from each well from Step 4, previous page, and transfer to sterile 15 ml tubes. Centrifuge the tubes at 3,000–5,000 rpm for 5 minutes to remove cells and large debris.
2. Transfer the supernatant to fresh 15 ml tubes.
3. Proceed to **Analyzing Recombinant Viral DNA**, page 28.

Isolating Virus for Amplification

1. Incubate the cells for 2 more days. At 5 days post-infection, collect 2 ml of medium from each well and transfer to sterile 15 ml tubes. Centrifuge the tubes at 3,000–5,000 rpm for 5 minutes to remove cells and large debris.
2. Transfer the supernatant to fresh 15 ml tubes. Store 1 ml of the viral clone stock at –80ºC as a frozen stock and 1 ml at 4ºC as a reserve stock. Refer to page 16 for additional storage information.
3. Proceed to **Preparing a High-Titer Viral Stock and Screening for Expression,** page 18.

Analyzing Recombinant Viral DNA

Introduction

You may analyze your recombinant viral DNA by PCR to verify the presence and orientation of your gene of interest. You may also use the PCR procedure on the next page to confirm your recombinant baculovirus DNA after the LR reaction. We recommend including a negative control (no DNA template) in your experiments to help you evaluate your results.

Easy-DNA Kit

Life Technologies offers a variety of products that enable high-yield, high-purity DNA extraction from a wide range of sample types. For fast and easy isolation of DNA from baculoviruses, we recommend using the PureLink® Genomic DNA Mini Kit or the Easy-DNA™ Kit (see page vii for ordering information). Follow the protocol provided with the kit manual for isolating baculovirus DNA. All Life Technologies manuals are available for downloading from our website (**www.lifetechnologies.com**) or by contacting Technical Support (page 45). An alternative protocol is also provided on the next page to isolate your baculovirus DNA.

Materials Needed

- Viral supernatant from **Isolating Viral DNA for PCR Analysis**, previous page
- 20% PEG 8000 in 1 M NaCl at 4ºC (see page 39 for a recipe)
- Lysis buffer (0.1% Triton X-100 in PBS or TBS)
- Proteinase K (5–10 mg/ml, see page vii for ordering information)
- Phenol:chloroform:isoamyl alcohol (25:24:1)
- 3 M sodium acetate
- UltraPure™ Glycogen (20 µg/µl, see page vii for ordering information)
- 100% ethanol
- 70% ethanol
- 50°C water bath

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Isolating Viral DNA

Follow the protocol below to lyse cells and extract the viral DNA.

1. Transfer 750 µl of your viral supernatant from Step 2 of **Isolating Viral DNA for PCR Analysis**, page 27, to a fresh 1.5 ml microcentrifuge tube.
2. Add 750 µl of cold (4°C) 20% PEG 8000 in 1 M NaCl. Invert the tube twice to mix and incubate at room temperature for 30 minutes.
3. Centrifuge at maximum speed for 10 minutes at room temperature to spin down the virus particles. Remove all medium from the pellet.

   Note: An additional quick spin may be required to remove trace amounts of medium. The pellet may not be visible at this point.
4. Add 100 µl of lysis buffer (0.1% Triton X-100 in PBS or TBS) to the pellet. Carefully wash the sides of the tubes to ensure that all of the viral particles are resuspended.
5. Add 10 µl of Proteinase K (5–10 mg/ml) and mix gently by inverting the tube. Incubate at 50°C for 1 hour.
6. Add 110 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and mix gently by inverting the tube. Centrifuge at maximum speed for 5 minutes at room temperature. Transfer the upper aqueous phase to a fresh microcentrifuge tube.
7. Add the following reagents to the aqueous phase:

3 M sodium acetate	10 µl
UltraPure™ Glycogen (20 µg/µl)	0.5 µl
100% ethanol	250 µl

   Incubate tubes at –20°C for at least 20 minutes.
8. Centrifuge at maximum speed for 15 minutes at 4°C. Wash the pellet with 70% ethanol. Centrifuge again and remove all traces of ethanol.
9. Resuspend the pellet in 10 µl of sterile water. Proceed to **PCR Procedure**, below.

PCR Procedure

You need to optimize PCR conditions for your specific primers and template. If you are using the Polyhedrin forward and V5 reverse primers (see page 11) **and not** an internal gene-specific primer, or if you are performing the PCR on your positive control CAT DNA, you may use the following parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	5 minutes	95ºC	1X
Denaturation	45 seconds	94ºC	25X
Annealing	1 minutes	52ºC	
Extension	1.5 minutes	72ºC	
Final Extension	10 minutes	72ºC	1X

Continued on next page

Analyzing PCR Results

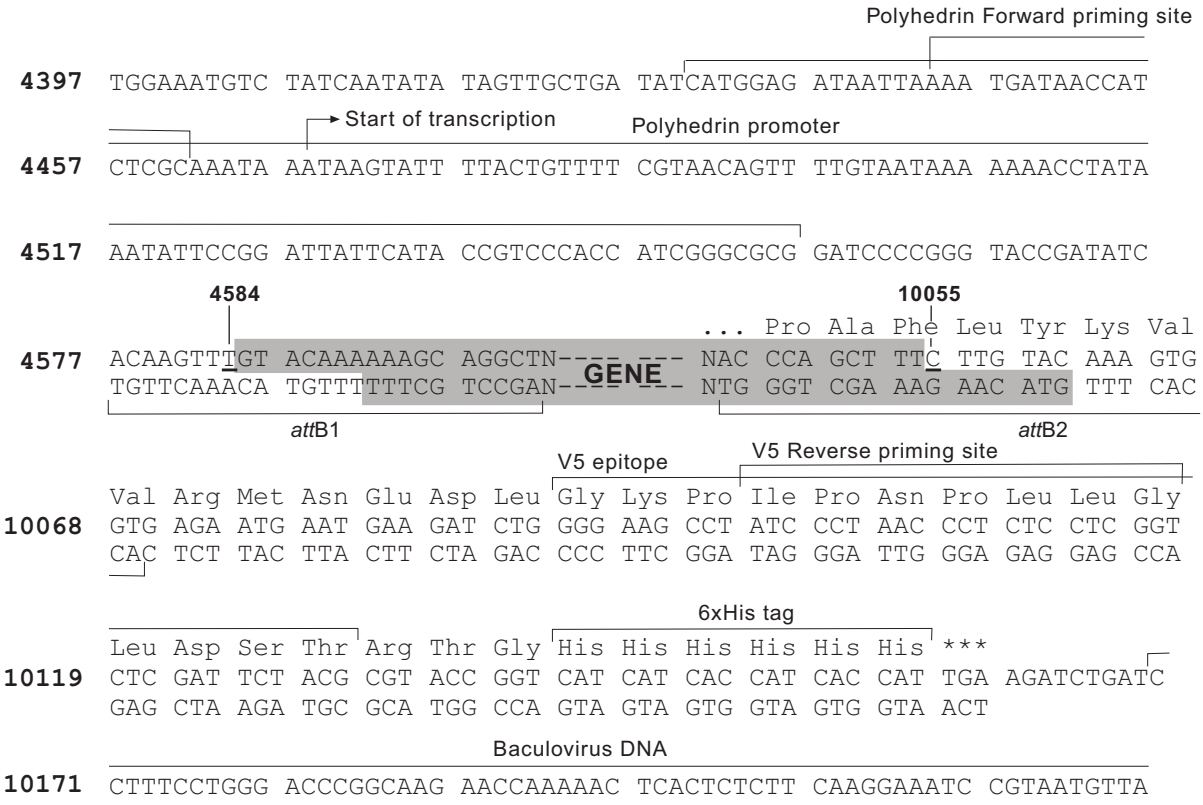
Calculate the expected size of your PCR fragment based on the location of the primer binding sites (see below or the next page for a diagram). After running your PCR reactions on a 1% agarose gel, you should see a band of the expected size for recombinant viral DNA and no bands for the negative control.

Recombination Region for BaculoDirect™ C-Term Linear DNA

The recombination region of the recombinant baculovirus resulting from BaculoDirect™ C-Term Linear DNA × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequence transferred from the entry clone into the BaculoDirect™ C-Term Linear DNA by recombination. Non-shaded regions are derived from the BaculoDirect™ C-Term Linear DNA.
- The underlined nucleotides flanking the shaded region correspond to bases 4584 and 10055, respectively, of the BaculoDirect™ C-Term Linear DNA sequence.



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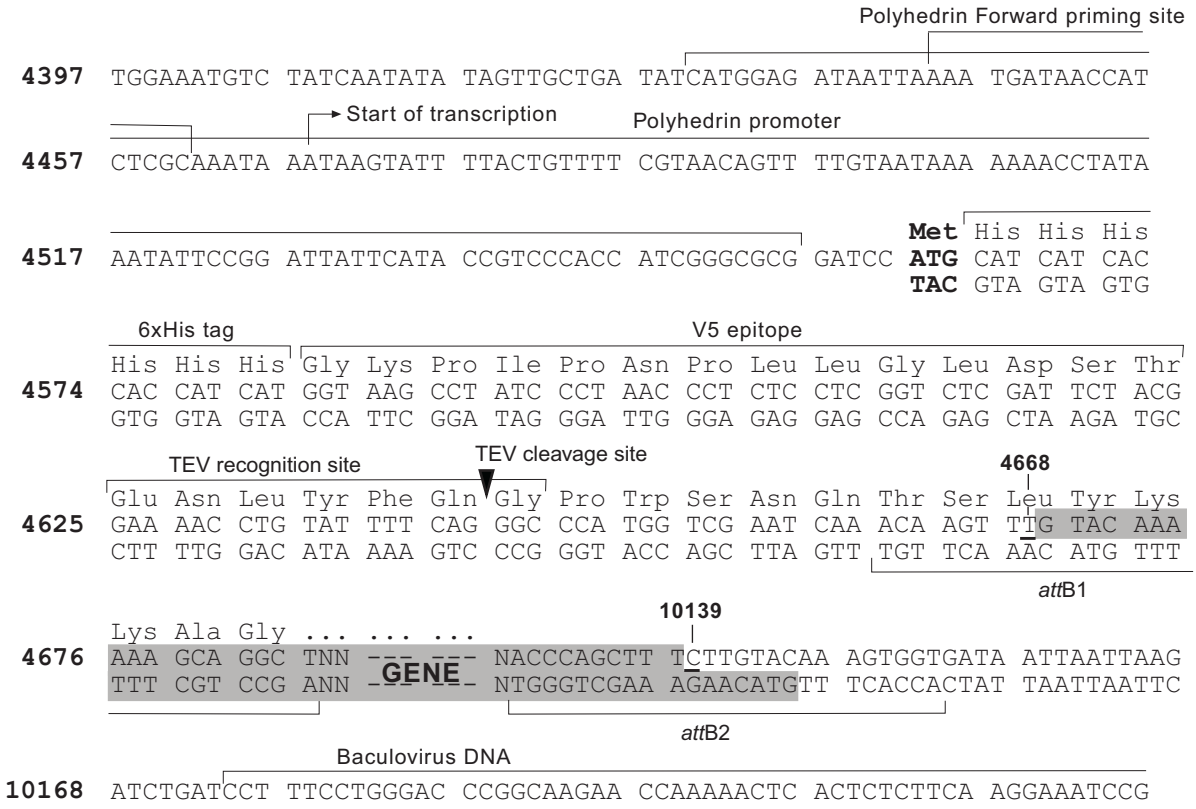
Recombination Region for BaculoDirect™ N-Term Linear DNA

The recombination region of the recombinant baculovirus resulting from BaculoDirect™ N-Term Linear DNA × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequence transferred from the entry clone into the BaculoDirect™ N-Term Linear DNA by recombination. Non-shaded regions are derived from the BaculoDirect™ N-Term Linear DNA.
- The underlined nucleotides flanking the shaded region correspond to bases 4668 and 10139, respectively, of the BaculoDirect™ N-Term Linear DNA sequence.



Expressing and Analyzing Recombinant Protein

Introduction

Once you have generated a viral stock of suitable titer (e.g., 1×10^8 pfu/ml), you are ready to use the viral stock to infect insect cells and assay for expression of your recombinant protein. Guidelines for infection and expression are provided in **Guidelines for Expression** below.

Positive Control

If you have generated a high-titer stock from the positive control construct (i.e., pENTR™/CAT), we recommend infecting cells with this viral stock to help determine the optimal MOI for your particular cell line and application. Once you have infected cells with the positive control virus, the gene encoding chloramphenicol acetyltransferase (CAT) will be constitutively expressed and can be easily assayed (see page 35).

Guidelines for Expression

General guidelines are provided below to infect insect cells with the recombinant baculovirus to express your protein of interest.

- **Cell line:** Depending on your application and gene of interest, you may use any insect cell line (e.g., Sf9, Sf21, High Five™, Mimic™ Sf9) for expression. Cells may be grown in adherent or suspension culture in the culture vessel of choice.

  Note: If you are expressing a secreted protein, you may improve expression by using High Five™ cells.

- **Culture Conditions:** We generally culture cells in serum-free conditions using Sf-900™ II SFM, Sf-900™ III SFM, or Express Five® SFM (see page vii) as appropriate. Depending on your application and the protein of interest, note that it may be necessary to supplement the culture post-infection with 0.1% to 0.5% FBS or BSA to protect the recombinant protein from proteolysis. Protein-based protease inhibitors are generally less expensive and more effective than many synthetic protease inhibitors.

- **Infection Conditions:** We recommend infecting cultures while cells are in the mid-logarithmic phase of growth at a density of 1.5×10^6 to 2.5×10^6 cells/ml. Make sure that the culture is not rate-limited by nutritional (i.e., amino acid or carbohydrate utilization) or environmental factors (i.e., pH, dissolved O_2, or temperature) during infection.

- **MOI:** Optimal MOI will vary between cell lines and the relative infection kinetics of the virus isolate or clone used. You should establish a dose response for each virus, medium, reactor, and cell line employed to determine the optimal infection parameters for protein expression. As a starting point, infect cells using an MOI of 5 and 10. Refer to page 20 for an equation to determine how much virus stock to add to obtain a specific MOI.

- **Time course:** We recommend performing a time course to determine the expression kinetics for your recombinant protein as many proteins may be degraded by cellular proteases released in cell culture.

  Note: Maximum expression of secreted proteins is generally observed between 30–72 hours and non-secreted proteins between 48–96 hours post-infection.

Continued on next page

Optimizing Expression

You may perform the following to determine the optimal conditions to use to express your recombinant protein of interest:

- **Cell line:** Infect Sf9, Sf21, High Five™, or Mimic™ Sf9 cells at a constant MOI. Assay for recombinant protein expression at different times post-infection (e.g., 24, 48, 72, 96 hours post-infection). Choose the cell line that provides the optimal level of recombinant protein expression.
- **MOI:** Infect a population of cells at varying MOIs (e.g., 1, 2, 5, 10, 20) and assay for protein expression. Use the MOI that provides the optimal level of recombinant protein expression.
- **Time course:** Infect cells at a constant MOI and assay for recombinant protein expression at different times post-infection (e.g., 24, 48, 72, 96 hours post-infection). Choose the time point at which optimal recombinant protein expression is obtained.

Materials Needed

- Insect cells of choice
- Complete growth medium
- Viral stock of known titer, $\geq 1 \times 10^8$ pfu/ml
- SDS-PAGE Loading Buffer (We recommend NuPAGE® LDS Sample Buffer or Novex® Tris-Glycine SDS Sample Buffer; see page vii for ordering information)
- NuPAGE® Sample Reducing Agent
- Six-well tissue culture plate
- 27ºC incubator
- Inverted microscope

Preparing Cells

1. Seed 1×10^6–2×10^6 insect cells per well in 2 ml complete growth medium in a six-well tissue culture plate.
2. Incubate the cells at 27ºC for one hour to allow the cells to fully attach to the bottom of the plate.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

Continued on next page

Preparing Protein Samples

Use the following procedure to prepare samples of your recombinant protein. This procedure is designed to allow expression analysis in a six-well format from cells harvested 24 to 96 hours post-infection. Other protocols are suitable.

1. Add the viral stock to each well at the desired MOI. Include the appropriate controls (e.g., mock-infected (uninfected) cells, positive control baculovirus, previously characterized recombinant baculoviruses).
2. Incubate infected cells at 27ºC.
3. At the appropriate time (e.g., 24, 48, 72, 96 hours post-infection), harvest the cells and media and place in a 15 ml tube. Gently spin to pellet the cells. Transfer the cell medium to a fresh tube.
4. For analysis of intracellular protein, wash the cells 2X with PBS and resuspend the cells in 2 ml of PBS. Remove a 13-µl sample and add 5 µl of 4X NuPAGE® LDS Sample Buffer and 2 µl of 10X NuPAGE® Sample Reducing Agent.

   For analysis of secreted protein, remove a 13-µl sample and add 5 µl of 4X NuPAGE® LDS Sample Buffer and 2 µl of 10X NuPAGE® Sample Reducing Agent.
5. Freeze samples at –20ºC or boil samples for at least 3 minutes and separate proteins by SDS-PAGE (see Polyacrylamide Gel Electrophoresis).

Polyacrylamide Gel Electrophoresis

To facilitate separation and visualization of your recombinant fusion protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Life Technologies. Life Technologies also carries a large selection of molecular weight protein standards and staining kits. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 45).

Detecting Recombinant Proteins

To detect expression of your recombinant fusion protein by Western blot analysis, you may use antibodies against the appropriate epitope available from Life Technologies or an antibody to your protein of interest. In addition, the Positope™ Control Protein is available from Life Technologies for use as a positive control for detection of fusion proteins containing a V5, HisG or C-terminal 6×His epitope. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Life Technologies to facilitate detection of antibodies by colorimetric or chemiluminescent methods. See page ix for ordering information. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 45).

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Assay for CAT Protein

If you used the control plasmid pENTR™/CAT to produce baculovirus expressing the CAT protein, you may assay for CAT expression using your method of choice. CAT will be fused to the C-terminal or N-terminal peptide containing the V5 and 6×His epitope tags, allowing you to use Western blot analysis with an antibody against the appropriate epitope. CAT Antiserum is also available separately from Life Technologies (see page vii for ordering information). Other commercial kits are available for assaying CAT expression. The molecular weight of the CAT fusion protein is approximately 30 kDa.

Note

Due to the presence of the *att*B sites, there will be additional amino acids between your gene of interest and the V5 and 6×His epitope (see pages 30 and 31 for a diagram). Expression of your protein with the C-terminal tag will increase the size of your recombinant protein by 4 kDa. Expression of your protein with the C-terminal tag will increase the size of your recombinant protein by 5 kDa

Continued on next page

Troubleshooting

The following table lists some potential problems and possible solutions to help you troubleshoot your expression studies. We recommend including both positive and negative controls in your experiments to verify that correct reagents and protocols were used and to narrow down potential causes of the problem.

Problem	Possible Cause	Solution
No recombinant fusion protein detected	Entry clone constructed incorrectly	Refer to page 7 for guidelines on constructing your entry clone.
	Insert not in frame with N-terminal or C-terminal tag	• Refer to the diagrams on pages 30 and 31 to verify the correct reading frame of the resulting recombinant baculovirus DNA after the LR reaction. • Analyze recombinant viral DNA by PCR to confirm correct size and orientation (page 10). • Sequence PCR product to verify proper reading frame for expression of the epitope tag.
Little recombinant fusion protein detected	Incorrect MOI used	• Run initial expression studies with an MOI of 5 and 10. • Recalculate the amount of viral stock needed to infect cells using the equation on page 20. • You may need to test a range of MOIs depending on the kinetics of expression of your recombinant protein.
	Protein is lost during cell lysis	If you are trying to detect an intracellular protein, analyze the supernatant to determine if the protein is being lost due to cell lysis.
	Protein is degraded or unstable	• Add protease inhibitors to your cell lysates. • Check mRNA levels.
	Protein is toxic to cells	Harvest cells at earlier time points (e.g., 18–24 hours post-infection).

Purifying Recombinant Protein and Removing the N-terminal Fusion Tag

Introduction

Once you have optimized expression levels, you may purify your recombinant protein. If you expressed your recombinant protein from BaculoDirect™ N-Term Linear DNA, the presence of the TEV recognition site allows removal of the N-terminal fusion tag using the AcTEV™ Protease (see page vii for ordering information). General guidelines for protein purification and removal of the N-terminal fusion tag are provided below.

Metal-Chelating Resin

You may use the ProBond™ Purification System, the Ni-NTA Purification System, or a similar product to purify your 6×His-tagged protein (see page x for ordering information). Both purification systems contain a metal-chelating resin specifically designed to purify 6×His-tagged proteins. Before starting, be sure to consult the ProBond™ or Ni-NTA Purification System manual to familiarize yourself with the buffers and the binding and elution conditions. If you are using another resin, follow the manufacturer's instructions.

If you plan to use a metal-chelating resin such as ProBond™ to purify your secreted protein from serum-free medium, **note that adding serum-free medium directly to the column will strip the nickel ions from the resin.** See the following section for a general recommendation to address this issue.

Purifying 6×His-Tagged Secreted Proteins

To purify 6×His-tagged recombinant proteins from the culture medium, we recommend that you perform dialysis or ion exchange chromatography 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).

Continued on next page

Note

Many insect cell proteins are naturally rich in histidines, with some containing stretches of six histidines. When using the ProBond™ Purification System or other similar products to purify 6×His-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.

Removal of the N-terminal Fusion Tag Using the AcTEV™ Protease

If your recombinant protein is being expressed from BaculoDirect™ N-Term Linear DNA, you may use the recombinant AcTEV™ Protease available from Life Technologies (see page vii for ordering information) to remove the N-terminal fusion tag. Instructions for digestion are included with the product. For more information, refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 45).

Note: After digestion with AcTEV™ Protease, fifteen amino acids will remain at the N-terminus of your protein (see diagram on page 31).

We recommend purifying your protein using both ion exchange chromatography and affinity chromatography on metal-chelating resins (see page 37) before removing the N-terminal fusion tag using the AcTEV™ Protease. Performing ion exchange chromatography will help reduce background contamination that may interfere with AcTEV™ Protease activity.

Appendix

Recipes

Complete TNM-FH Medium

Complete TNM-FH medium is Grace's Insect Medium with supplements (lactalbumin hydrolysate, L-glutamine, TC-yeastolate) and 10% fetal bovine serum (FBS).

1. If you are using Grace's Insect Medium, Supplemented, add 55 ml of FBS. Mix well.
2. To include antibiotics and antimycotics, add the following at the recommended concentration:

Penicillin	100 U/ml
Streptomycin	100 µg/ml
Amphotericin	0.25 µg/ml

3. Filter-sterilize the solution through a 0.2 µm filter into a sterile container. A pre-filter may be required.
4. Store at 4°C and warm to 27°C before use.

Ganciclovir

Ganciclovir (100 mM in deionized water)

1. Add 26 mg of ganciclovir powder (InvivoGen Cat. no. sud-gcv) to 800 µl of deionized water.
2. Add 1 M NaOH dropwise until the solution reaches pH 12 and the ganciclovir dissolves into solution.
3. Add HCl dropwise until the solution reaches pH 11.
4. Bring up the final volume to 1 ml with deionized water.
5. Filter-sterilize the solution through a 0.2 µm filter.
6. Aliquot the solution into multiple tubes, and thaw each aliquot only once. Store at –20ºC, protected from light, for up to 6 months. Thawed aliquots are stable at 4ºC for up to 1 month.

PEG/NaCl Solution

20% Polyethylene glycol (PEG) 8000
1 M NaCl

1. Add the following reagents to 80 ml of deionized water:

PEG 8000	20 g
NaCl	5.84 g

2. Bring the final volume to 100 ml with deionized water.
3. Autoclave 20 minutes on liquid cycle.
4. While the solution is still warm (~55°C), swirl carefully to mix thoroughly.

Continued on next page

Bluo-gal

Follow the guidelines below to prepare a 50 mg/ml stock solution of Bluo-gal.

1. Dissolve the Bluo-gal in dimethylformamide or dimethyl sulfoxide (DMSO) to make a 50 mg/ml stock solution. Use a glass or polypropylene tube.

   Important: Exercise caution when working with dimethylformamide. Dispense solutions in a vented chemical hood only.

2. Do not filter the stock solution.
3. Store at –20°C, protected from light.

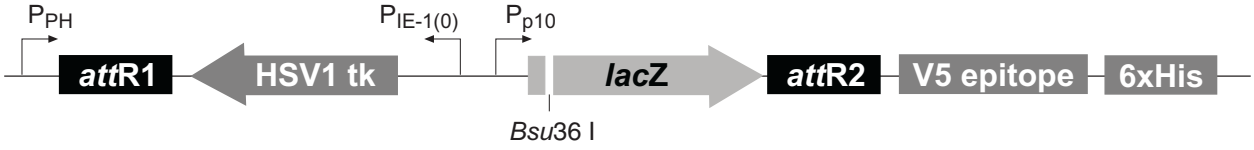
Map of BaculoDirect™ C-Term Linear DNA

Description

BaculoDirect™ C-Term DNA was constructed by homologous recombination between wild type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) DNA and a transfer plasmid containing a Gateway® cassette (see map below). After recombination, the Gateway® cassette replaces the native polyhedrin gene resulting in β-galactosidase positive, polyhedrin negative recombinant virus. The modified baculovirus genome is linearized at the *Bsu*36 I site located at the 5′ end of the *lac*Z gene to produce BaculoDirect™ C-Term Linear DNA.

Map

The map below shows the Gateway® cassette elements of the BaculoDirect™ C-Term Linear DNA. The first nucleotide of the BaculoDirect™ C-Term Linear DNA sequence corresponds to the first *Eco*R I site in Homologous Region 1 (hr1). For the complete sequence of *Autographa californica* nuclear polyhedrosis virus, refer to Genbank Accession #NC 001623 or Ayres, M.D. *et. al.*, 1994.



BaculoDirect™ C-term Linear DNA Gateway® Cassette:

Polyhedrin promoter (P_{PH}): bases 4428-4556
Polyhedrin Forward priming site: bases 4444-4461
*att*R1 recombination site: bases 4577-4701
Herpes simplex virus thymidine kinase gene (HSV1 tk): bases 4988-6118 (c)
Immediate early promoter [$P_{IE-1(0)}$]: bases 6147-6698 (c)
p10 promoter (P_{p10}): bases 6746-6843
*Bsu*36 I linearization site: base 7094
*lac*Z ORF: bases 6855-9929
*att*R2 recombination site: bases 9945-10069
V5 epitope: bases 10089-10130
V5 Reverse priming site: bases 10098-10118
6xHis tag: bases 10140-10157

(c) = complementary strand

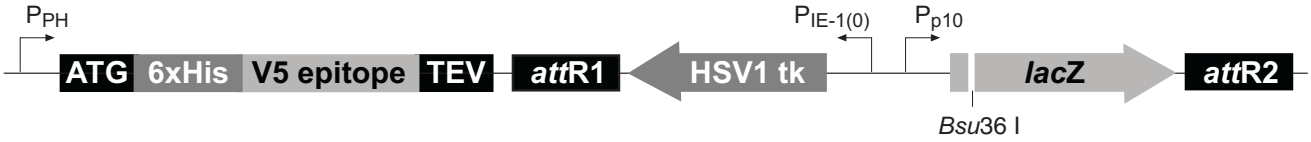
Map of BaculoDirect™ N-Term Linear DNA

Description

BaculoDirect™ N-Term DNA was constructed by homologous recombination between wild type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) DNA and a transfer plasmid containing a Gateway® cassette (see map below). After recombination, the Gateway® cassette replaces the native polyhedrin gene resulting in β-galactosidase positive, polyhedra negative recombinant virus. The modified baculovirus genome is linearized at the *Bsu*36 I site located at the 5´ end of the *lac*Z gene to produce BaculoDirect™ N-Term Linear DNA.

Map

The map below shows the Gateway® cassette elements of the BaculoDirect™ N-Term Linear DNA. The first nucleotide of the BaculoDirect™ N-Term Linear DNA sequence corresponds to the first *Eco*R I site in Homologous Region 1 (hr1). For the complete sequence of *Autographa californica* nuclear polyhedrosis virus, refer to Genbank Accession #NC 001623 or Ayres, M.D. *et. al.*, 1994.



BaculoDirect™ N-Term Linear DNA Gateway® Cassette:

Polyhedrin promoter (P_{PH}): bases 4428-4556
Polyhedrin Forward priming site: bases 4444-4461
Initiation ATG: bases 4562-4564
6xHis tag: bases 4565-4582
V5 epitope: bases 4583-4624
TEV recognition site: bases 4625-4645
*att*R1 recombination site: bases 4661-4785
Herpes simplex virus thymidine kinase gene (HSV1 tk): bases 5072-6202 (c)
Immediate early promoter [$P_{IE-1(0)}$]: bases 6231-6782 (c)
p10 promoter (P_{p10}): bases 6830-6927
*Bsu*36 I linearization site: base 7178
*lac*Z ORF: bases 6939-10013
*att*R2 recombination site: bases 10029-10153

(c) = complementary strand

Features of the BaculoDirect™ Linear DNA

Features

Features of the BaculoDirect™ Linear DNA Gateway® cassettes are described below. All features have been functionally tested.

Feature	Benefit
Polyhedrin promoter	Allows efficient, high-level expression of your recombinant protein.
Polyhedrin Forward priming site	Allows PCR detection and sequencing of the insert.
6×His tag	Allows purification of the recombinant fusion protein on metal-chelating resins. Allows detection of the recombinant fusion protein with the Anti-His Antibodies (Lindner *et al.*, 1997).
V5 epitope	Allows detection of the recombinant fusion using the Anti-V5 Antibodies (Southern *et al.*, 1991).
V5 Reverse priming site	Allows PCR detection and sequencing of the insert.
TEV recognition site **(BaculoDirect™ N-Term Linear DNA only)**	Allows removal of the N-terminal tag from your recombinant protein using the AcTEV™ Protease (Carrington & Dougherty, 1988; Dougherty *et al.*, 1988).
*att*R1 and *att*R2 sites	Allows recombination cloning of the gene of interest from an entry clone.
Immediate-early promoter ($P_{IE\text{-}1(0)}$)	Allows expression of the herpes simplex virus thymidine kinase gene (Kovacs *et al.*, 1991).
Herpes simplex virus thymidine kinase gene (HSV1 tk)	Allows negative selection of non-recombinant virus in the presence of ganciclovir (Godeau *et al.*, 1992).
p10 promoter	Allows expression of the *lac*Z gene.
*lac*Z gene	Allows detection non-recombinant virus through blue/white screening.

Map of pENTR™/CAT

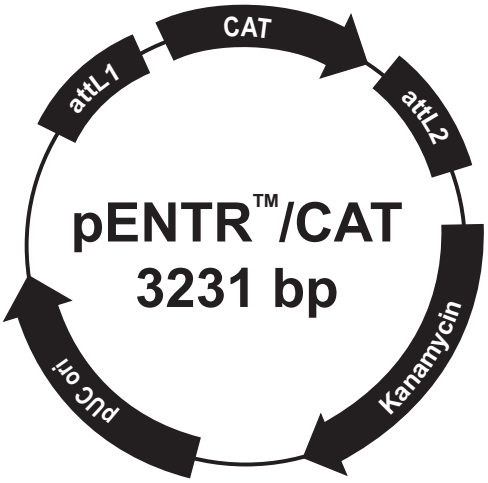
Description

pENTR™/CAT (3231 bp) is a control vector containing the chloramphenicol acetyltransferase (CAT) gene. The CAT 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™221 to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway® Technology with Clonase® II manual (part no. 25-0749).

Following an LR recombination reaction between pENTR™/CAT and BaculoDirect™ Linear DNA, CAT will be expressed as a fusion to the C-terminal V5 epitope and 6×His tag. The molecular weight of the CAT fusion protein is approximately 30 kDa.

Map

The map below shows the elements of the pENTR™/CAT control vector. The vector sequence of pENTR™/CAT is available from our website (**www.lifetechnologies.com**) or by contacting Technical Support (page 45).



Comments for pENTR™/CAT
3231 nucleotides

*att*L1 recombination site: bases 569-668
CAT ORF: bases 698-1354
*att*L2 recombination site: bases 1356-1455
Kanamycin resistance gene: bases 1625-2434
pUC origin: bases 2555-3228

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- Download software updates and patches

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Gateway® Clone Distribution Policy

For additional information about Life Technologies' policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 47.

Continued on next page

Gateway® Clone Distribution Policy

Introduction

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

Gateway® Entry Clones

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

Gateway® Expression Clones

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

Additional Terms and Conditions

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

References

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