invitrogen™

BaculoDirect™ GST Gateway® Expression Kit

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

Catalog nos. A10640, A10641

Version A
15 December 2008
A10607

User Manual

Table of Contents

Kit Contents and Storage

Types of Kits

This manual is supplied with the following kits.

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

Kit Components

Each kit contains the components listed below. See the next page for a detailed description of other reagents supplied with each kit.

Component	Cat. no. A10640	Cat. no. A10641
BaculoDirect™ N-GST Linear DNA	✓	✓
Cellfectin® II Reagent	✓	✓
pENTR™-CAT Control Plasmid	✓	✓
Ganciclovir	✓	✓
Gateway® LR Clonase™ II Enzyme Mix for BaculoDirect™		✓
Sf21 Frozen Cells		✓
Grace's Insect Cell Culture Medium, Unsupplemented		✓
BaculoDirect™ GST Gateway® Expression Kit Manual	✓	✓
Insect Cell Lines Manual		✓

Shipping/Storage

The BaculoDirect™ GST Gateway® 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™ GST Gateway® Transfection Kit	Gel ice	4°C, except pENTR™-CAT: –20°C
Gateway® LR Clonase™ II Enzyme Mix for BaculoDirect™	Dry ice	–20°C
Ganciclovir	Dry ice	–20°C
Sf21 Frozen Cells	Dry ice	Liquid nitrogen
Grace's Insect Cell Culture Medium, Unsupplemented	Room Temperature	4°C, protected from light

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Kit Contents and Storage, continued

Transfection Kit Components

The BaculoDirect™ GST Gateway® Transfection Kits include the following components, sufficient to perform 5 reactions. Store components as detailed below.

Item	Composition	Amount	Storage
BaculoDirect™ N-GST Linear DNA, linearized with *Bsu*36 I	300 ng per tube in 10 µl of TE buffer, pH 8.0*	6 tubes	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

***TE buffer, pH 8.0**: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Expression Kit Components

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

Item	Composition	Amount	Storage
BaculoDirect™ N-GST Linear DNA, linearized with *Bsu*36 I	300 ng per tubes in 10 µl of TE buffer, pH 8.0	6 tubes	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
Gateway® LR Clonase™ II Enzyme Mix for BaculoDirect™ Kits	–	40 µl	–20°C for up to 6 months; (–80°C for long-term storage)
Sf21 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

Accessory Products

Additional Products

Many of the reagents supplied with the BaculoDirect™ GST Gateway® Transfection and Expression Kits as well as other products suitable for use with the kits are available separately from Invitrogen. 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	100 µl	10814-010
NuPAGE® LDS Sample Preparation Buffer (4X)	10 ml 250 ml	NP0007 NP0008
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 ml	LC2676

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Accessory Products, continued

Insect Cells

Invitrogen 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™ GST Gateway® 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.invitrogen.com or contact Technical Support (page 45).

Item	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 Invitrogen. Depending on your application, you may choose entry vectors with specific features such as a ribosome binding site (RBS). For more information on the Gateway® cloning technology, as well as the features and vector maps of available Gateway® entry vectors, refer to www.invitrogen.com or contact Technical Support (page 45).

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

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

Continued on next page

Accessory Products, continued

Detection and Purification of Recombinant Proteins

You may use western blot analysis to detect and affinity chromatography on glutathione agarose to purify your recombinant fusion protein that is expressed in frame with the N-terminal peptide containing the GST (glutathione *S*-transferase) tag. You may also use Dynabeads® complexed with anti-GST antibodies to isolate your recombinant protein. For more information, refer to www.invitrogen.com or contact Technical Support (page 45).

Item	Amount	Cat. no.
Anti-glutathione *S*-transferase, rabbit IgG fraction, 3 mg/ml	0.5 ml	A-5800
Anti-glutathione *S*-transferase, rabbit IgG fraction, Alexa Fluor® 488 conjugate, 2 mg/ml	0.5 ml	A-11131
Glutathione Transferase Fusion Protein Purification Kit	5 purifications	G-21801
Glutathione agarose, linked through sulfur (sedimented bead suspension)	10 ml 100 ml	G-2879 G-21800
Purification Columns (10 ml polypropylene columns)	50	R640-50
Dynabeads® M-280 Sheep anti-Rabbit IgG	2 ml	112-03D
Dynabeads® Protein A	2 ml	100-01D
Dynabeads® Protein G	2 ml	100-03D
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

Introduction

Overview

Introduction

The BaculoDirect™ GST Gateway® Transfection and Expression Kits use 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™ GST Gateway® Transfection and Expression Kits the fastest procedure for baculovirus expression.

Advantages of the BaculoDirect™ GST Gateway® Kits

Using the BaculoDirect™ GST Gateway® Transfection and Expression Kits 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 a GST-tagged recombinant protein for easy detection and purification.
- Linearized baculovirus DNA and ganciclovir selection inhibits replication of non-recombinant virus, and eliminates the need for plaque purification.
- The GST tag is helpful for solubilization of the overexpressed protein of interest as it prevents the fusion protein from being sequestered into inclusion bodies.

BaculoDirect™ N-GST Linear DNA

The major features of the BaculoDirect™ N-GST 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 GST fusion tag for detection and purification of recombinant fusion proteins
  Note: If you are planning on expressing a secreted protein, be aware that the presence of the GST-tag may interfere with secretion.

Continued on next page

Overview, continued

The Gateway® Technology

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™ GST Gateway® Expression Kit, 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™ N-GST Linear DNA.
3. Transfect insect cells with recombinant baculovirus DNA and harvest recombinant baculovirus.

For more detailed information about 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.invitrogen.com or by contacting Technical Support (page 45).

Important

The BaculoDirect™ GST Gateway® 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 .

Insect Cell Lines Manual

Before starting baculoviral expression experiments, we recommend that users refer to the Insect Cell Lines manual (part no. 25-0127) for additional information on insect cell culture. 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™ GST Gateway® Expression Kits and is also available from www.invitrogen.com or by contacting Technical Support (page 45).

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.,* LR Clonase™ II Enzyme Mix for BaculoDirect™). 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*P sites are comprised of sequences from *att*R and *att*L sites.
- Strand exchange occurs within a core region that is common to all *att* sites.

att Sites

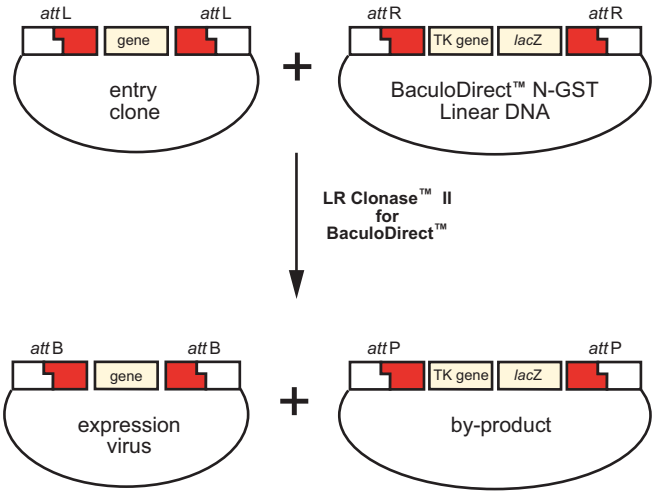
Lambda recombination occurs between site-specific attachment (*att*) sites: *att*B on the *E. coli* chromosome and *att*P on the lambda chromosome. The *att* sites serve as the binding site for recombination proteins and have been well characterized (Weisberg & Landy, 1983). Upon lambda integration, 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).

Continued on next page

The Gateway® Technology, continued

Gateway® LR Recombination Reaction

By using the BaculoDirect™ GST Gateway® Transfection Kit (available separately or as a part of the BaculoDirect™ GST Expression Kit), you will take advantage of the LR reaction to transfer your gene of interest into the BaculoDirect™ N-GST Linear DNA. The LR reaction facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (BaculoDirect™ N-GST Linear DNA) to create an *att*B-containing expression virus (see diagram below). This reaction is catalyzed by LR Clonase™ II Enzyme Mix for BaculoDirect™.



Ganciclovir

Introduction

Ganciclovir is a nucleoside analog used in the BaculoDirect™ GST Gateway® Transfection and Expression Kits 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).

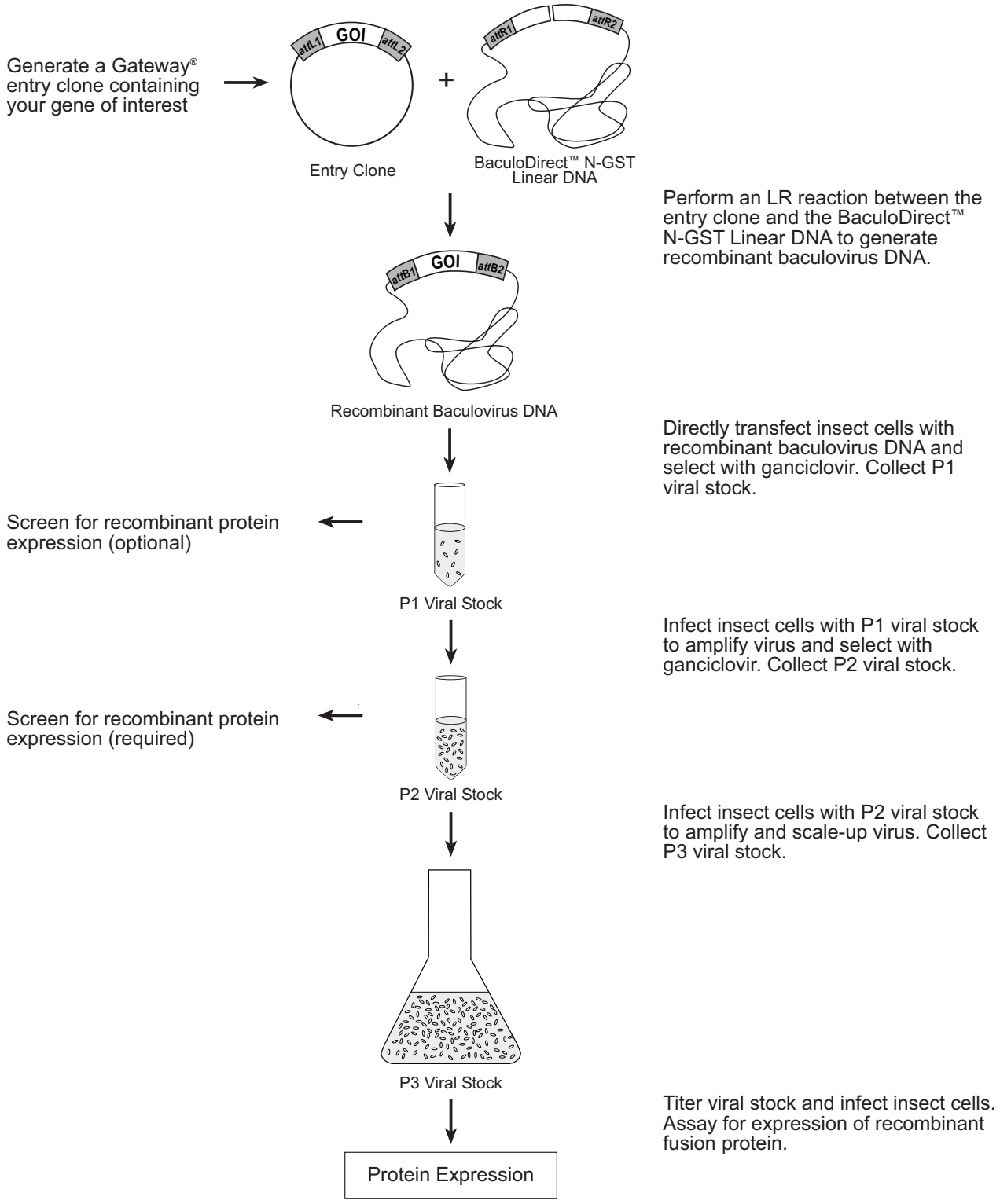
In the BaculoDirect™ GST Gateway® Transfection and Expression Kits, the HSV1 tk gene is under the control of an immediate early promoter ($P_{IE\text{-}1(0)}$) which drives synthesis of the first viral transcript produced in infected cells (Kovacs *et al.*, 1991).

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.invitrogen.com/msds or by contacting Technical Support (page 45).

Experimental Overview

Experimental Summary

The following diagram summarizes the general steps required to express your gene of interest (GOI) using the BaculoDirect™ GST Gateway® Expression Kit.



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Experimental Overview, continued

Experimental Steps

The experimental steps necessary to express your protein of interest using the BaculoDirect™ GST Gateway® Transfection and Expression Kits are outlined below. For more details on each step, refer to the indicated pages.

Step	Action	Page
1	Generate an entry clone containing your gene of interest	8
2	Perform the LR recombination reaction between the BaculoDirect™ N-GST Linear DNA and an entry clone containing your gene of interest using BaculoDirect™ GST Gateway® Transfection Kit	9–10
3	Directly transfect insect cells with recombinant baculovirus DNA and collect P1 viral stock. Screen for recombinant protein expression, if desired.	11–16
4	Infect insect cells with P1 viral stock to generate a high-titer viral stock. Screen for recombinant protein expression.	17–19
5	Determine titer of viral stock by plaque assay.	20–23
6	Isolate recombinant viral DNA and analyze by PCR, if desired.	24–28
7	Infect insect cells and optimize conditions for recombinant protein expression.	29–32
8	Purify recombinant protein, if desired.	33

Methods

Before Starting

Introduction

Before you start your experiments, you will 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™ N-GST Linear DNA, you will need an entry clone containing your gene of interest. For your convenience, Invitrogen offers a variety of Gateway® entry vectors (see page viii). A selection guide for choosing the most appropriate Gateway® entry vector for your application can be found on our website at www.invitrogen.com/Gateway.

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™ N-GST Linear DNA

Keep the following points in mind when constructing your entry clone to be used in the recombination reaction with the BaculoDirect™ N-GST Linear DNA:

- Design your gene of interest to be in frame with the N-terminal GST tag after recombination. Refer to page 28 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.

Recommended Cells

We recommend using Sf9 or Sf21 cells to generate high-titer viral stocks with the BaculoDirect™ GST Gateway® Expression Kits. Note that Sf21 cells are provided with the BaculoDirect™ GST Gateway® 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 viii for ordering information.

Recommended Media

For the highest transfection efficiency, we recommend using Grace's Insect Cell Culture Medium, Unsupplemented (provided with the BaculoDirect™ GST Gateway® 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 40 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.

Performing the LR Recombination Reaction

Introduction

After you have generated an entry clone using most appropriate Gateway® entry vector for your application, perform the LR recombination reaction to transfer the gene of interest into the BaculoDirect™ N-GST 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.

LR Clonase™ II Enzyme Mix for BaculoDirect™

LR Clonase™ II Enzyme Mix for BaculoDirect™ is supplied with the BaculoDirect™ GST Gateway® Expression Kits to catalyze the LR recombination reaction. The LR Clonase™ II Enzyme Mix for BaculoDirect™ 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™.

Note: For the LR recombination reaction, use LR Clonase™ II Enzyme Mix for BaculoDirect™ only, **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)
  Note: Use PureLink™ HiPure Plasmid Prep Kit, **not** a silica-based miniprep kit, for the purification of the entry clone.
- BaculoDirect™ N-GST Linear DNA (300 ng/tube; provided with the BaculoDirect™ GST Gateway® Expression and Transfection Kits)
- pENTR™-CAT control plasmid, optional (100 ng/µl; provided with the kits)
- LR Clonase™ II Enzyme Mix for BaculoDirect™ (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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Performing the LR Recombination Reaction, continued

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™ N-GST Linear DNA tubes 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™ N-GST Linear DNA	10 µl (in tube)	10 µl (in tube)
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™ from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ II Enzyme Mix for BaculoDirect™ briefly twice (~ 2 seconds each time).
4. To each sample above, add 4 µl of LR Clonase™ II Enzyme Mix for BaculoDirect™ 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 up 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.

For the PCR amplification, you may use the Polyhedrin forward primer (5´-AAATGATAACCATCTCGC-3´) and a primer of your own design that binds within your gene of interest.

See page 28 for Polyhedrin forward primer binding site. PCR reaction conditions must be optimized.

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. Sf21 cells are provided with the BaculoDirect™ GST Gateway® Expression Kits.

Cellfectin® II Reagent

Cellfectin® II Reagent is supplied with the BaculoDirect™ GST Gateway® 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 the transfection of 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 resulting 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).

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 40 for instructions on reconstituting and storing ganciclovir.

Important

Ganciclovir provided with the BaculoDirect™ GST Gateway® Transfection and Expression Kits has a concentration of 100 mM and might form a precipitate upon thawing. Incubating the ganciclovir at 37°C for 10 minutes and vortexing will redissolve the ganciclovir and eliminate the precipitate. If you experience ganciclovir precipitation, we recommend diluting the 100 mM stock solution in half with sterile distilled water before refreezing for storage.

Continued on next page

Transfecting Sf9 or Sf21 Cells, continued

Materials Needed

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

Note: All the reagents and cell lines necessary for transfection are also available separately from Invitrogen. For ordering information, see page vii–viii.

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

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.

Continued on next page

Transfecting Sf9 or Sf21 Cells, continued

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

Continued on next page

Transfecting Sf9 or Sf21 Cells, continued

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 11). 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 below). 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, previous page
- 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 3000–5000 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**, next page.

Continued on next page

Isolating P1 Viral Stock, continued

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 below).
- To harvest cells, transfer transfected cells into microcentrifuge tubes and centrifuge. 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 31–32.

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 17). 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 20).

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

- Sf21 cells (provided with the BaculoDirect™ GST Gateway® Expression Kits or available separately, see page viii) or Sf9 cells (available separately, see page viii for ordering information)
- 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.

Continued on next page

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 4). If you see blue-stained cells, we recommend that you perform a plaque assay to isolate a recombinant viral clone (see page 20)

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 below).
  Note: The presence of the GST-tag on your recombinant protein may interfere with its secretion.
- 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 31–32.

Continued on next page

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

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 next page).
- 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 that 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 will 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 Invitrogen, 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

- Sf21 cells (provided with BaculoDirect™ GST Gateway® Expression Kits or available separately, see page viii) or Sf9 cells (available separately, see page viii)
- Sf-900 II, Sf-900™ III SFM, or other appropriate complete growth medium (see **Note** below)
- Sf-900 Medium (1.3X) or other appropriate plaquing medium (see **Note** below)
- 4% Agarose Gel
- Sterile, cell-culture grade, distilled water.
- 100 ml sterile, glass bottle
- Serial dilutions of viral stock (see page 21)
- Bluo-gal (50 mg/ml, see page 41 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

Continued on next page

Performing a Plaque Assay, continued

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 (next page).

Continued on next page

Performing a Plaque Assay, continued

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, previous page), 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**, next page.

Continued on next page

Performing a Plaque Assay, continued

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 29). To plaque purify the virus or to analyze the recombinant DNA, proceed to the next section.

Isolating Virus From a Single Plaque

Introduction

This section provides detailed guidelines for plaque purifying your virus. Isolated virus can be used 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 Recombinant Protein**, page 29.

Materials Needed

- Sf21 cells (provided with the BaculoDirect™ GST Gateway® Expression Kits; also available separately, see page viii) or Sf9 cells (available separately, see page viii)
- Complete growth medium
- Plates containing plaques from Step 5, page 22
- 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**, next page.

Continued on next page

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**, next page.

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 17.

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.

Viral DNA Purification

Invitrogen 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 Invitrogen manuals are available for downloading from our website (www.invitrogen.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 41 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
- Glycogen (2 mg/ml, see page vii for ordering information)
- 100% ethanol
- 70% ethanol
- 50°C water bath

Continued on next page

Analyzing Recombinant Viral DNA, continued

Isolating Viral DNA

Perform the following protocol 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 25, 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
Glycogen (2 mg/ml)	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 will need to optimize PCR conditions for your specific primers and template. For the PCR amplification, you may use the Polyhedrin forward primer (5´-AAATGATAACCATCTCGC-3´) and a primer of your own design that binds within your gene of interest. See page 28 for Polyhedrin forward primer binding site.

Continued on next page

Analyzing Recombinant Viral DNA, continued

Analyzing PCR Results

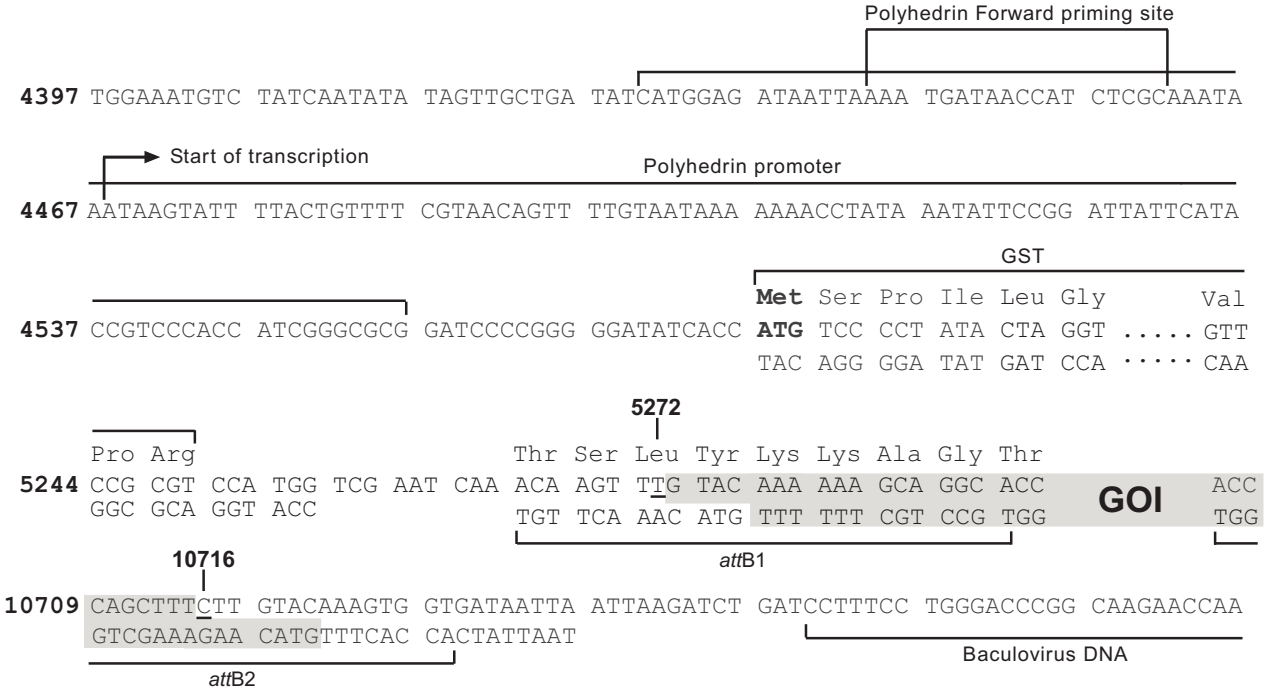
Calculate the expected size of your PCR fragment based on the location of the primer binding sites (see below for a diagram). After running your PCR reactions on a 1% agarose gel, you should see a band of the expected size for recombinant viral DNA and no bands for the negative control.

Recombination Region for BaculoDirect™ N-GST Linear DNA

The recombination region of the recombinant baculovirus resulting from BaculoDirect™ N-GST 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-GST 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 5272 and 10716, respectively, of the BaculoDirect™ N-GST Linear DNA sequence.



Expressing 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 below.

Positive Control

If you generated a high-titer stock from the positive control construct 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 32).

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 as appropriate (see page vii). 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. A dose response should be established for each virus, medium, reactor, and cell line employed to determine the optimal infection parameters to use for protein expression. As a starting point, infect cells using an MOI of 5 and 10. Refer to page 19 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.
- **Secreted proteins:** If you cloned your gene of interest with a secretion signal sequence, be aware that the N-terminal GST-tag may interfere with the secretion of your recombinant protein. We recommend that you verify the expression of protein using samples from both extracellular medium and cells lysates.

Continued on next page

Expressing Recombinant Protein, continued

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 (NuPAGE® LDS Sample Buffer or Novex® Tris-Glycine SDS Sample Buffer, see page vii)
- 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

Expressing Recombinant Protein, continued

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 15 µl sample and add 5 µl of 4X SDS-PAGE Buffer.

   For analysis of secreted protein, remove a 15 µl sample of the cell medium and add 5 µl of 4X SDS-PAGE Buffer.

   Note: The N-terminal GST-tag may interfere with the secretion of your recombinant protein. We recommend that you verify the expression of protein using samples from both the extracellular medium and the cells lysates.
5. Freeze samples at –20ºC or boil samples for at least 3 minutes and separate proteins by SDS-PAGE.

Analyzing Recombinant Protein

Introduction

You may analyze the expression of your recombinant protein by polyacrylamide gel electrophoresis or by Western blot analysis. General information for analyzing your recombinant GST-fusion protein is provided below. For detailed instructions, consult the literature provided with each product.

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 Invitrogen. Invitrogen also carries a large selection of molecular weight protein standards and staining kits. For more information, refer to our website at www.invitrogen.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 N-terminal GST tag available from Invitrogen or an antibody to your protein of interest. The ready-to-use WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of antibodies by colorimetric or chemiluminescent methods. See page ix for ordering information. For more information, refer to our website at www.invitrogen.com or contact Technical Support (page 45).

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 N-terminal peptide containing the GST tag, allowing you to use western blot analysis with an anti-GST antibody. CAT Antiserum is also available separately from Invitrogen (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 53 kDa.

Note

Due to the presence of the *att*B sites, there will be additional amino acids between your gene of interest and the N-terminal GST tag (see page 28 for a diagram). Expression of your protein with the GST tag will increase the size of your recombinant protein by approximately 27.8 kDa.

Purifying Recombinant Protein

Introduction

Once you have optimized expression levels, you may purify your recombinant GST-fusion protein either by affinity chromatography on glutathione agarose or by immunoprecipitation using anti-GST antibodies. General information for purifying your recombinant GST-fusion protein is provided below. For detailed instructions, consult the literature provided with each product, which are also available through our website at www.invitrogen.com or by contacting Technical Support (page 45).

Glutathione Agarose

Invitrogen offers glutathione agarose for the purification of your recombinant GST-fusion protein by affinity chromatography in a single step (see page vii for ordering information). The glutathione agarose consists of glutathione linked via the sulfur atom to crosslinked beaded agarose and has a binding capacity of approximately 5-6 mg of bovine liver GST per ml of gel. Adding excess free glutathione liberates the GST fragment from the matrix, which can then be regenerated by washing with a high salt buffer. If you are using another resin, follow the manufacturer's instructions.

Glutathione agarose is available from Invitrogen either as a sedimented bead suspension (10 ml or 100 ml) or as a part of the Glutathione Transferase Fusion Protein Purification Kit containing anti-GST antibodies and purification columns. For ordering information, see page ix.

Anti-Glutathione *S*-Transferase Antibody

Invitrogen also offers the highly purified rabbit polyclonal anti-GST antibody that can be used to purify GST fusion proteins by immunoprecipitation. This highly specific antibody, which was generated against a 260-amino acid N-terminal fragment of the *Schistosoma japonica* enzyme expressed in *Escherichia coli*, is also useful for detecting GST fusion proteins on western blots (see page 32). The intensely green-fluorescent Alexa Fluor® 488 conjugate of anti-glutathione *S*-transferase is also available for direct detection of GST fusion proteins. For ordering information, see page ix.

Removal of the GST tag

Your purified GST-fusion protein expressed from the recombinant baculovirus (BaculoDirect™ N-GST Linear DNA × entry clone) does not contain a cleavage site for the removal of the N-terminal GST tag. However, you can engineer your Gateway® entry clone to encode a recognition site for a specific protease, such as TEV, thrombin, or factor Xa, between the *att*L1 recombination site and your gene of interest. The LR reaction which facilitates the recombination of your entry clone (*att*L substrate) and the BaculoDirect™ N-GST Linear DNA (*att*R substrate) to create the recombinant expression baculovirus will leave the protease recognition site intact, allowing you to remove the GST tag with the appropriate protease.

For site-specific proteases available from Invitrogen, refer to www.invitrogen.com or contact Technical Support (page 45).

Troubleshooting

Transfection

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

Problem	Possible Cause	Solution
Transfected cells are dead, but Cellfectin® II-only control is fine	Contamination or cytotoxicity from the LR recombination reaction	• Include a no-Cellfectin® II, entry clone-only negative control. • Use PureLink™ HiPure Plasmid Prep Kit, **not** a silica-based miniprep kit, for the purification of the entry clone.
Transfected cells do not show signs of infection	Kinetics of infection are slower than expected	• Virus production can take up to a week after transfection. Observe cells until 8 or 9 days after infection. If no signs of infection appear, investigate other possible causes.
	Low transfection efficiency	• Use Cellfectin® II Reagent that is less than 6 months old and **do not** freeze Cellfectin® II Reagent for storage. • 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 of high passage	Use cells that are between 8 to 15 passages.
	Cells are of too low passage	After reviving cells, grow them for at least 5 passages before transfection.
	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 added the LR Clonase™ II Enzyme Mix for BaculoDirect™ Kits to 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.

Continued on next page

Troubleshooting, continued

Transfection, continued

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

Problem	Possible Cause	Solution
No or little recombinant fusion protein detected in initial expression screen	Entry clone constructed incorrectly	Refer to page 8 of this manual and the instructions in the manual for the specific entry vector you are using for guidelines on constructing your entry clone.
	Insert not in frame with the N-terminal GST tag	• Refer to the diagram on page 28 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 GST tag.

High-titer viral stock production

The table below lists some potential problems and possible solutions to help you troubleshoot your high-titer P2 and P3 viral amplification experiments.

Problem	Possible Cause	Solution
No sign of enlarged cell diameter 72 hours post-infection	MOI is lower than 0.01	Transfect 70% confluent cells on a 6-well plate with a dilution series of P1 viral stock, and monitor the cells every 24 hours. Use the MOI that does not produce any morphological changes within 24 hours in 70–80% of the infected cells.
	Cells are of high passage or passed their logarithmic phase of growth	• Cells used for viral amplification should be younger than 20–25 passages. • If using suspension culture for amplification, cell density should be between 8×10^5 and 1×10^6 cells/ml.
Enlarged cell diameter observed 24 hours post-infection	MOI is higher than 1	High MOI will decrease the viral stock quality. Transfect 70% confluent cells on a six-well plate with a dilution series of P1 viral stock, and monitor the cells every 24 hours. Use the MOI that does not produce any morphological changes within 24 hours in 70–80% of the infected cells.

Continued on next page

Troubleshooting, continued

Plaque Assay

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.

Continued on next page

Troubleshooting, continued

Protein Expression

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
Very little or no recombinant fusion protein detected, but cells are infected and dead	Entry clone constructed incorrectly	Refer to page 8 of this manual and the instructions in the manual for the specific entry vector you are using for guidelines on constructing your entry clone.
	Insert not in frame with N-terminal GST tag	• Refer to the diagram on page 28 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 (page 10). • Sequence PCR product to verify proper reading frame for expression of the GST tag.
	Recombination error during LR reaction, or presence of a premature stop codon in construct	
	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 19. • You may need to test a range of MOIs depending on the kinetics of expression of your recombinant protein.
	Cells harvested too late	Do a time course experiment and harvest cells at different time points (*e.g.*, 48, 60, 72, and 96 hrs).
	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).

Continued on next page

Troubleshooting, continued

Problem	Possible Cause	Solution
Very little or no recombinant fusion protein detected, but cells are infected and dead	Viral stock a mixture of recombinant and non-recombinant virus	Add 100 μM ganciclovir at the end of transfection for P1 viral production. Use the same amount of ganciclovir during P2 viral amplification to select against non-recombinant virus.
	Cell density too low	For protein expression using suspension culture, Sf9 and Sf21 cell density should be between 2.5×10^6 and 3.0×10^6 cells/ml.
	High passage viral stock is used for protein expression	Cells used for protein expression should have less than 4 passages.
	Protein is expressed, but escaped detection	• If expressing a secretion protein, make sure to check cells for the presence of the protein, because secretion will never be 100% efficient and sometimes could be very low. • If your protein of interest is expressed intracellularly, make sure the check the cell lysate pellet for its presence.
Very little or no recombinant fusion protein detected, but cells are healthy and not dying after 72 hours	Viral stock is revived from frozen aliquots	The titer of the frozen viral stock will decrease after reviving. If the titer is too low, amplification may be needed • Do a plaque assay or end-point dilution to accurately determine viral titer stock. • Do an MOI test with different MOI (*e.g.*, 1, 5, and 10).
	MOI is too low	
	Cell density too high or cells are too old	For protein expression using suspension culture, Sf9 and Sf21 cell density should be between 2.5×10^6 and 3.0×10^6 cells/ml.
Protein is expressed but also see degradation	Harvesting time is not optimal	Do a time course experiment and harvest cells at different time points (*e.g.*, 48, 60, 72, and 96 hrs).
	MOI is too low	• Do a plaque assay or end-point dilution to accurately determine viral titer stock. • Do an MOI test with different MOI (*e.g.*, 1, 5, and 10).

Continued on next page

Troubleshooting, continued

Problem	Possible Cause	Solution
Protein is expressed but also see degradation	Premature stop codon in sequence	Check the sequence of your entry clone to verify that it does not contain a premature stop codon or mutations.
Protein is expressed, but is insoluble	A binding partner or other parts of protein may be needed for proper folding	Identify the partner and coexpress.
	Protein is normally secreted, but is cloned without the secretion signal sequence, and is now expressed intracellularly	Express protein as secreted protein by adding secretion signal sequence.
	Protein is not extracted properly	For complete extraction, use sonication with short pulses and DnaseI. Keep samples on ice.
Protein is expressed, but not secreted	N-terminal GST-tag interferes with secretion	Purify your protein from cell lysates
Protein expressed well small scale, but lost expression when scaled up	MOI and harvesting time may need to fine be tuned after scaling up	Using the MOI and harvesting time established during small scale test expression as a guideline, change harvesting time ±6 hours while keeping the MOI constant.
Viral stock worked well initially, but after a couple of months, expression levels decreased considerably	Viral stock was originally amplified using high MOI	Re-amplify virus from lower passage stock using low MOI (0.01–0.1).
	Did not centrifuge and discard cells when harvesting viral supernatant	Re-amplify virus from lower passage stock using low MOI (0.01–0.1). If this viral stock is P2, it can be used in amplification.
	For certain genes, the virus can become very unstable	Freeze the aliquoted viral stock and perform one round of amplification after reviving the virus.

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	100U/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 Stock Solution

Ganciclovir (100 mM in deionized water)

1. Add 26 mg of ganciclovir powder 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.

Ganciclovir Working Solution

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).

Important

Ganciclovir provided with the BaculoDirect™ GST Transfection and Expression Kits has a concentration of 100 mM and might form a precipitate upon thawing. Incubating the ganciclovir at 37°C for 10 minutes and vortexing will redissolve the ganciclovir and eliminate the precipitate. If you experience ganciclovir precipitation, we recommend diluting the 100 mM stock solution in half with sterile distilled water before refreezing for storage.

Continued on next page

Recipes, continued

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.

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™ N-GST Linear DNA

Description

BaculoDirect™ N-GST Linear 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-GST Linear DNA. The first nucleotide of the BaculoDirect™ N-GST 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-GST Linear DNA Gateway® Cassette:

Polyhedrin promoter (P_{PH}): bases 4430-4556
Polyhedrin Forward priming site: bases 4444-4461
Initiation ATG: bases 4577-4579
GST tag: bases 4580-5249
*att*R1 recombination site: bases 5265-5362
Herpes simplex virus thymidine kinase gene (HSV1 tk): bases 5649-6779 (c)
Immediate early promoter [$P_{IE-1(0)}$]: bases 6808-7359 (c)
p10 promoter (P_{p10}): bases 7407-7504
*Bsu*36 I linearization site: base 7755
*lac*Z ORF: bases 7516-10590
*att*R2 recombination site: bases 10606-10730

(c) = complementary strand

Features of the BaculoDirect™ N-GST Linear DNA

Features

Features of the BaculoDirect™ N-GST 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.
GST tag	Allows purification of the recombinant fusion protein by affinity chromatography on sulfur-linked glutathione agarose. Allows detection of the recombinant fusion protein with anti-GST antibodies. Helps solubilize the recombinant fusion protein
*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

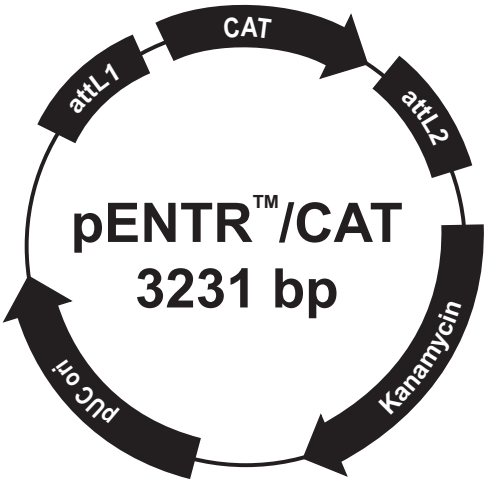
Description

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 control vector and BaculoDirect™ Linear DNA, CAT will be expressed as a fusion to the N-terminal GST tag. The molecular weight of the CAT fusion protein is approximately 53 kDa.

Map

The map below shows the elements of the pENTR™-CAT control vector. The vector sequence of pENTR™-CAT is available at www.invitrogen.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

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
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MSDS

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

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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**, next page.

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.

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