USER GUIDE

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Bac-N-Blue™ Transfection and Expression Guide

A Guide for Baculovirus Transfection, Expression, and Purification Using the Bac-N-Blue™ Baculovirus Expression System

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Contents and Storage

Kit Contents

The Bac-N-Blue™ Kit is shipped on blue ice. Upon receipt, store components of the kit at 4°C **(do not freeze).** The Bac-N-Blue™ Transfection Kit contains enough reagents for 5 transfections in 60 mm dishes.

Item	Composition	Amount	Storage
Bac-N-Blue™ DNA	10 mL per vial at 50 ng/mL in TE buffer, pH 8.0*	5 vials	4°C
Cellfectin® II Reagent	125 mL per vial at 1 mg/mL in membrane-filtered water	2 × 125 mL	4°C

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

Cellfectin® II Reagent

Cellfectin® II Reagent is also available separately from Life Technologies at 1 mg/mL concentration as a 1 mL aliquot or as a 125 µL sample size (see page vi for ordering information). The amount of Cellfectin® II Reagent supplied in the Bac-N-Blue™ Kit is sufficient for 5 transfections in 60 mm dishes.

Acknowledgments

This manual has its origins in the Insect Virology Laboratory at the University of Texas, Austin, in the early 1970's. We would like to acknowledge Max D. Summers, Gale E. Smith, Ms. Dana Broussard, Ms. Nancy Webb and all other members of that laboratory for their contributions.

Portions of this manual were reproduced with permission from:

Texas Agricultural Experiment Station, Bulletin No. 1555
First printing May, 1987, Second printing February, 1988

Expression of Proteins using Recombinant Baculoviruses
N. R. Webb and M. D. Summers
Technique 2: 173–188 (1990)

Intended Use

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

Accessory Products

Introduction

The products listed in the table below are intended for use with the Bac-N-Blue™ Baculovirus Expression System. For more information, refer to our website at www.lifetechnologies.com or contact Technical Support (see page 76).

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 Bac-N-Blue™ Baculovirus 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 (see page 76).

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

Accessory Products, continued

Additional Products

The following products, available separately from Life Technologies, are intended for use with the Bac-N-Blue™ Baculovirus Expression System.

Product	Quantity	Cat. no.
Cellfectin® II Reagent	1 mL 125 µL	10362-100 10362-125
pMelBac A, B, and C	20 µg each	V1950-20
Grace's Insect Cell Culture Medium, Unsupplemented	500 mL	11595-030
Grace's Insect Cell Culture Medium, Supplemented	500 mL	11605-094
GIBCO® Fetal Bovine Serum, Qualified, Heat-Inactivated	100 mL	16140-063
Phosphate-Buffered Saline (PBS), pH 7.4 (1X)	500 mL	10010-023
Phosphate-Buffered Saline (PBS), pH 7.4 (10X)	500 mL	70011-044
4% Agarose Gel	40 mL	18300-012
Bluo-gal	1 g	15519-028
X-gal	1 g	15520-018
PureLink® HiPure Plasmid Miniprep Kit	25 preps 100 preps	K2100-02 K2100-03
PureLink® HiPure Plasmid Midiprep Kit	25 preps 50 preps	K2100-04 K2100-05
Easy-DNA™ Kit	15–200 reactions	K1800-01
NuPAGE® LDS Sample Preparation Buffer (4X)	10 mL 250 mL	NP0007 NP0008
Novex® Tris-Glycine SDS Sample Buffer (2X)	20 mL	LC2676
UltraPure™ Sodium Dodecyl Sulfate (SDS)	500 g	15525-017
Gentamicin Reagent Solution, liquid (50 mg/mL)	10 mL 10 × 10 mL	15750-060 15750-078
Trypan Blue Stain	100 mL	15250-061
Neutral Red (high purity)	25 mg	N-3246
Novex® AP Chromogenic Substrate (BCIP/NBT)	250 mL	WP20001
Countess® Automated Cell Counter (includes 50 Countess® cell counting chamber slides and 2 mL of Trypan Blue Stain)	1 unit	C10227
BaculoTiter™ Assay Kit	30 titers	K1270
Anti-Xpress™ Antibodies	50 µL	R910-25

Introduction

Overview

Description

The Bac-N-Blue™ Transfection Kit contains triple-cut, linearized AcMNPV (*Autographa californica* multiple nuclear polyhedrosis virus) DNA for the production of >90% recombinant virus and Cellfectin® II Reagent for efficient transfection of insect cells. Our triple-cut, linearized AcMNPV DNA (Bac-N-Blue™ DNA) may be used with any polyhedrin promoter-based transfer vector. Recombinant plaques may either be detected as blue plaques on Bluo-gal or X-gal or as occlusion body negative (occ^-) plaques depending on the transfer vector used.

Cellfectin® II Reagent is supplied with the Bac-N-Blue™ Transfection Kit 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 a variety of insect cells, including Sf9 and Sf21 cells. Liposome-mediated transfection of insect cells is the most efficient transfection method available and provides greater reproducibility than other methods.

Purpose of this Manual

The following topics are covered in this manual:

- Kit contents
- Description and benefits of Bac-N-Blue™ DNA
- Cellfectin® II-mediated transfection of your insect cell line with a baculovirus transfer vector containing your gene and linearized Bac-N-Blue™ DNA
- Isolation of recombinant plaques (Plaque Assay)
- PCR verification that a pure plaque contains your gene of interest
- Generation of a high-titer stock
- Expression of recombinant protein
- Analyzing recombinant protein

For details on the transfer vector, refer to the manual included with the transfer vector.

Information on culturing, maintaining, and freezing insect cell lines is included in the Insect Cell Line Manual available for downloading from our website at www.lifetechnologies.com or by contacting Technical Support (see page 76).

Wild-type Definition

The term "wild-type" refers to uncut Bac-N-Blue™ DNA that will produce occlusion body positive (occ^+) plaques. Note that the frequency of wild-type plaques is less than 20%.

Continued on next page

Background Information

In the **Appendix**, we have included:

- A brief overview of the baculovirus life cycle and biology, pages 66.
- Regulation of the polyhedrin and p10 promoters, pages 71.
- Information on different types of posttranslational modifications observed in insect cells, pages 73.
- Examples of various types of proteins expressed using the baculovirus expression system, pages 75.

In addition, there is a large body of information that is available to the researcher on the baculovirus expression system. We recommend the following texts:

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. E., Seidman, J. G., Smith, J. A., and Struhl, K., eds (1994) *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., New York, NY. Unit 16.9-16.11.
- King, L. A. and Possee, R. D. (1992) *The Baculovirus System: A Laboratory Guide.* Chapman and Hall, New York, NY.
- O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. W. H. Freeman and Company, New York, NY.
- Richardson, C. D., ed. (1995) *Baculovirus Expression Protocols*. Methods in Molecular Biology, Vol. 39 (J. M. Walker, ed.) Humana Press, Totowa, NJ.

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Bac-N-Blue™ DNA

Bac-N-Blue™ DNA was engineered to contain three unique *Bsu*36 I restriction sites for complete linearization and removal of sequence essential for propagation of the virus (see diagram next page). ORF1629 has been shown to be essential for viral propagation (Kitts and Possee, 1993). Specifically, insertions in the *Sna*B I site at the 3´ end of the ORF1629 gene are lethal. Sequence at the 3´ end of ORF1629 is removed during digestion with *Bsu*36 I. The only way to isolate viable virus is if the essential ORF1629 sequence is supplied by the transfer vector. Recombination between homologous sequences, usually ORF603 (Gearing and Possee, 1990) and ORF1629, in the transfer vector and the linearized DNA results in the propagation of only recombinant virus.

A 3´ fragment of the *lac*Z gene was included in the Bac-N-Blue™ DNA to generate blue, recombinant plaques if desired (see diagram next page). The 3´ *lac*Z fragment is located between ORF603 and polyhedrin promoter. Note there is no promoter for direct expression of this fragment; therefore, non-recombinant virus will not express β-galactosidase.

Bac-N-Blue™ DNA has the following features:

Features	Benefits
Triple-cut linear viral DNA	Decreases background of uncut or non-recombinant viral DNA
Produces blue, recombinant plaques with pBlueBac and pMelBac vectors	Makes identification of recombinant plaques very easy
Elimination of sequences essential for propagation of virus (ORF1629)	Prevents production of non-recombinant wild-type virus
Uncut viral DNA will produce an occlusion body positive (occ^+) plaque	Differentiates between uncut (occ^+) and recombinant (occ^-) plaques

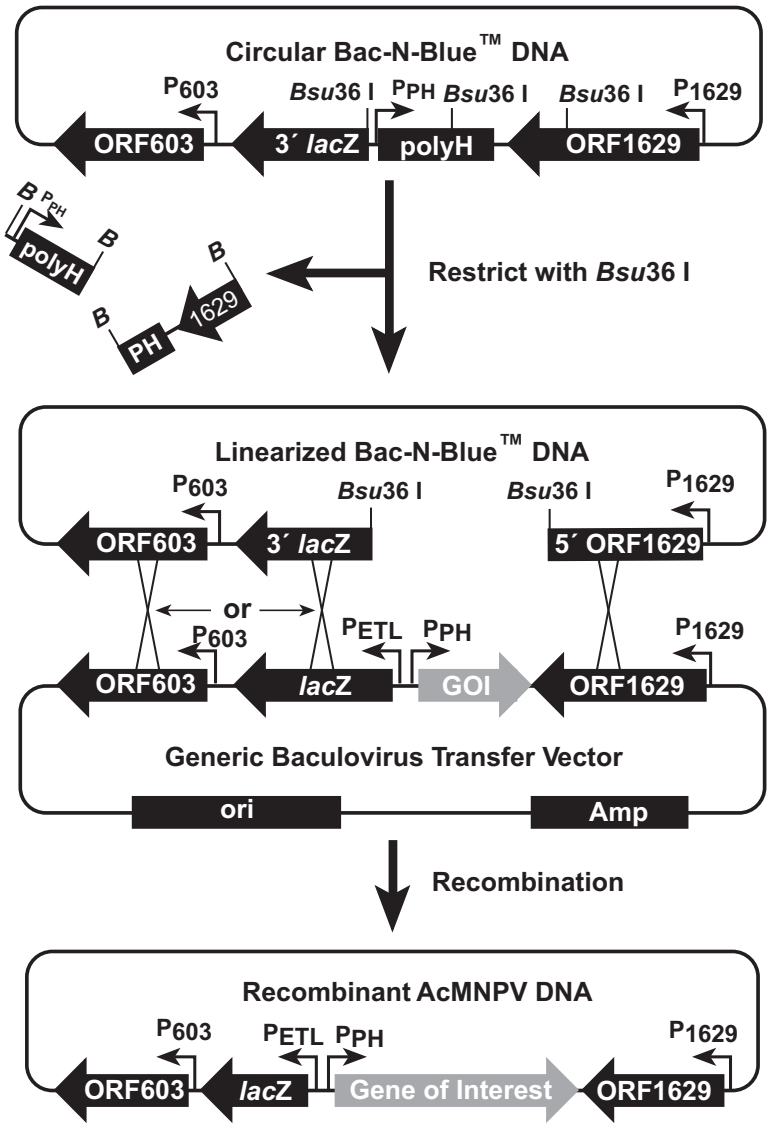
Continued on next page

General Homologous Recombination

Digestion of Bac-N-Blue™ DNA with *Bsu*36 I results in removal of essential C-terminal ORF1629 sequence, the polyhedrin promoter, and the polyhedrin ORF. During transfection, recombination between homologous sequences in the viral DNA and the transfer vector supply the essential sequence needed for replication of recombinant virus. In addition, cutting the DNA at three sites increases the chances of obtaining completely linearized DNA and removing the essential part of ORF1629. The result is an increase in the percent of recombinant virus produced following co-transfection.

Note: GOI = gene of interest.

See **Recombination between Bac-N-Blue™ DNA and Transfer Vector**, page 63, for more details.



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Important

The transfer vectors pMelBac, pBlueBac4.5 (discontinued), and pBlueBacHis2 (discontinued) from Life Technologies will not recombine with Life Technologies' Linear AcMNPV DNA, BD BaculoGold™ (BD Biosciences), or BacPAK6 (Clontech). These vectors do not contain ORF603, and the *lac*Z sequences found in other linear DNAs are in the opposite orientation for recombination.

Vector/AcMNPV DNA Recombination Table

The table below summarizes which Life Technologies vectors recombine with Bac-N-Blue™ DNA, BD BaculoGold™ DNA (BD Biosciences), or BacPAK6 DNA (Clontech) and the phenotype of the recombinants.

Vector	Recombines with Bac-N-Blue™?	Phenotype of Recombinants	Recombines with BD BaculoGold™ or BacPAK6?	Phenotype of Recombinants
pBlueBacIII*	Yes	Blue, occ⁻	Yes	Blue, occ⁻
pBlueBac4*	Yes	Blue, occ⁻	No	N/A
pBlueBac4.5*	Yes	Blue, occ⁻	No	N/A
pBlueBacHis*	Yes	Blue, occ⁻	Yes	Blue, occ⁻
pBlueBacHis2*	Yes	Blue, occ⁻	No	N/A
pMelBac	Yes	Blue, occ⁻	No	N/A
pVL1392*	Yes	occ⁻	Yes	occ⁻
pVL1393*	Yes	occ⁻	Yes	occ⁻
p2Bac*	Yes	occ⁻	Yes	occ⁻

*vectors no longer available from Life Technologies

Use of Other Transfer Vectors

Any baculovirus transfer vector that contains ORF603 and ORF1629 sequences may be used with Bac-N-Blue™ DNA.

Experimental Overview

Experimental Outline

The table below describes the steps required to express the desired protein using the baculovirus expression system.

For help with Step 1: Refer to the **Growth and Maintenance of Insect Cell Lines Manual** (part no. 25-0127), which provides you with the information necessary to grow and maintain adherent as well as suspension insect cell cultures. This manual is available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 76).

For help with Step 2: Refer to the manual supplied with your transfer vector for guidelines for cloning your gene of interest into the transfer vector to express the desired recombinant protein. If you are using a transfer vector from Life Technologies, you can find the vector maps and multiple cloning sites in the appropriate vector manuals, all of which are available for downloading from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 76).

For help with Steps 3–11: These steps are all covered in this manual. Once you have established the insect cells in culture and have cloned the desired gene into a baculovirus transfer vector, you are now ready to co-transfect with Bac-N-Blue™ linear AcMNPV DNA and isolate recombinant virus.

Step	Action	Time
1	Establish an insect cell line (Sf9, Sf21, or High Five™ cell lines).	3–4 weeks
2	Select the appropriate transfer plasmid, insert your gene into the vector, and prepare plasmid DNA.	variable
3	Co-transfect *S. frugiperda* cells with Bac-N-Blue™ DNA and plasmid DNA.	2–4 days
4	Purify recombinant virus from transfection supernatant by plaque assay.	7–10 days
5	Verify recombinant plaques by PCR.	3–5 days
6	Generate a high-titer stock.	7–10 days
7	Verify titer by plaque assay.	7–10 days
8	Perform a time course of expression.	6 days
9	Analyze time course by SDS-PAGE/western blot.	1 day
10	Optimize recombinant protein expression levels.	2 weeks
11	Scale-up expression.	1–5 days

Methods

Culturing Insect Cells

Introduction

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

Cells for Transfection

You will need log-phase cells with >95% viability to perform a successful transfection. Refer to page 10 to determine how many cells you will need for transfection.

Insect Cell Culture Reference Guide

For guidelines and detailed information on insect cell culture, refer to the *Guide to Baculovirus Expression Vector Systems (BEVS) and Insect Cell Culture Techniques*. This guide is available from our website (www.lifetechnologies.com) or by contacting Technical Support (see page 76), and contains information on:

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

Transfection

Purpose

The purpose of this section is to co-transfect insect cells with Bac-N-Blue™ DNA and a baculovirus transfer vector containing your gene. Recombination occurs between homologous sequences in the viral DNA and the transfer vector to yield recombinant viral DNA that is circular and will replicate and infect cells (refer to the graphics on page 4).

Method of Transfection

Plasmid containing your gene is co-transfected with the Bac-N-Blue™ DNA by the technique of cationic liposome mediated transfection. In this technique, Bac-N-Blue™ DNA and your transfer vector are mixed with the Cellfectin® II Reagent in serum-free medium. Serum contains proteins that interfere with liposomes. A positively-charged liposome-DNA complex forms and binds to the negatively-charged plasma membrane. The liposomes fuse with the cell membrane and the DNA is taken up by the cells.

Cellfectin® II Reagent

Cellfectin® II Reagent is a proprietary cationic lipid formulation that offers the highest transfection efficiencies and protein expression levels on the widest variety of adherent and suspension insect cell lines, including Sf9 and Sf21 cells.

Important

The quality of the plasmid DNA is critical to generation of recombinant virus. *Spodoptera frugiperda* cells are sensitive to contaminants found in crude plasmid preparations that cannot be removed by phenol extraction or ethanol precipitation. Impure preparations of plasmid DNA are toxic to the cells, and may cause cells to lyse shortly after transfection. This results in an apparently lower titer of recombinant virus or no virus at all.

We recommend isolating plasmid using resin-based anion-exchange DNA isolation systems such as Life Technologies' PureLink™ HiPure Plasmid Prep Kits (see page vi), or CsCl-ethidium bromide gradient centrifugation. We do **not** recommend using silica-based miniprep kits to purify plasmids for cationic liposome mediated transfections. If using CsCl gradient centrifugation, be sure to wash the nucleic acid pellet thoroughly to remove any traces of salt. Salt and other highly-charged molecules lower the efficiency of transfection by interacting with the cationic liposomes.

Control for Plasmid Quality

To test the quality of a plasmid DNA preparation, include a cells-only control in all transfection experiments and a control of cells with the transfer vector containing your gene (no viral DNA). At about 24 hours post-transfection, compare the controls with those transfected with Bac-N-Blue™ DNA and the plasmid DNA. If the plasmid preparation contained contaminants, then the cells exposed to the plasmid will look unhealthy and start to lyse. This should not be confused with viral infection which will show some occ^+ cells at 72 hours.

Continued on next page

We recommend using Sf9 or Sf21 cells for transfection and identification of recombinant plaques. Sf9 cells are more regular in shape and form a better monolayer. Sf21 cells are larger and more easily infected, but they are irregularly shaped, making it difficult to identify signs of infection using vectors that do not produce blue plaques on X-gal. While it is possible to use High Five™ cells for plaque assays, they are more fibroblastic and have a tendency to overlap one another, making it difficult to identify recombinant plaques. Once you have generated high-titer viral stocks, you may use Sf9, Sf21, High Five™, or Mimic™ Sf9 cells for protein expression studies. We recommend that you use High Five™ cells for expression of your heterologous protein (see page 47 for more information).

Media for Transfection

For the highest transfection efficiency, we recommend performing the transfection in Grace's Insect Cell Culture Medium, Unsupplemented (see page vi). Note that the Grace's Insect Cell Culture Medium **should not** contain supplements or fetal bovine serum (FBS) as the supplements and the proteins in the FBS will interfere with the Cellfectin® II Reagent, inhibiting the transfection.
Note: If you are culturing Sf9 or Sf21 cells in Sf-900 II SFM or Sf-900™ III SFM, you can perform the transfection in unsupplemented Grace's Medium, and then easily switch back to Sf-900 II SFM or Sf-900™ III SFM after transfection.

Before Starting

You will need 10 µg of highly purified plasmid DNA (~1 µg/µL in TE buffer) for each transfection experiment and the following materials:

- Sf9 or Sf21 cells (see page v for ordering information)
- Grace's Insect Cell Culture Medium (see page vi for ordering information)
- GIBCO® Fetal Bovine Serum (see page vi for ordering information)
- 60 mm tissue-culture dishes
- 1.5 mL sterile microcentrifuge tubes
- 27°C incubator
- Inverted microscope
- Air-tight bags or containers
- Paper towels

Continued on next page

Transfection, continued

Prepare Cells

For each transfection, use log phase Sf9 or Sf21 cells (1.5–2.5 × 10^6 cells/mL) cultured in complete TNM-FH medium (see **Recipes**, page 61) with greater than 98% viability.

Note: Use Grace's Insect Medium, Unsupplemented, without FBS to seed cells in plate for Sf9 and Sf21 cells grown in complete TNM-FH medium.

1. Plate 2 × 10^6 Sf9 or Sf21 cells in a total volume of 5 mL of Grace's Insect Medium (without supplements or FBS) in a 60 mm dish. For example, if the cell density is 2 × 10^6 cells/mL, add 1 mL of cells to 4 mL of unsupplemented Grace's Medium. Rock gently side to side to evenly distribute the cells.
2. Allow the cells to fully attach to the bottom of the dish to form a monolayer of cells for at least 15 minutes.
3. Verify that the cells have attached by inspecting them under an inverted microscope.

Transfection Procedure

In this procedure, Bac-N-Blue™ DNA, recombinant transfer DNA, and Cellfectin® II Reagent are mixed together in Grace's Insect Medium **(without supplements or FBS)** and incubated with freshly seeded insect cells. The amount of cells, Cellfectin® II Reagent, and viral DNA has been optimized for the conditions described below. It is very important to optimize transfection conditions if you are using plates other than 60 mm plates.

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.
- Do **not** add antibiotics to media during transfection as this causes cell death.

1. To prepare each transfection mixture, use one of the 1.5 mL microcentrifuge tubes containing 10 µL (0.5 µg) of the Bac-N-Blue™ DNA. Centrifuge the tube, and add the following reagents:

Recombinant transfer plasmid (1 µg/µL)	4 µL
Grace's Insect Medium (**without supplements or FBS**)	250 µL

   Vortex briefly to mix.
2. Mix the Cellfectin® II Reagent before use, and dilute 30 µL of the reagent in 250 µL of Grace's Insect Medium (without supplements or FBS). Vortex briefly to mix and incubate at room temperature for 15–30 minutes.
3. To prepare the transfection mixture, combine the diluted DNA with diluted Cellfectin® II. Mix gently and incubate at room temperature for 15 minutes.
4. Add the DNA-lipid mixture (*i.e.*, the transfection mixture) **dropwise** onto the cells (previous page). Repeat for all transfections.
5. Incubate the cells at 27°C for 4 hours.
6. Following the 4-hour incubation period, remove the transfection mixture and replace with 2 mL of complete TNM-FH medium. Place the dishes in a sealed plastic bag and incubate at 27°C for 72 hours. Proceed to **Post-transfection Procedure**, next page.

Continued on next page

Post-transfection Procedure

Around 72 hours, budded virus particles are released into the medium. This transfection supernatant is harvested at this time and assayed for recombinant plaques (the transfection viral stock). However, the cells may not show all of the signs of infection until day 4 or 5. You will want to continue to incubate the cells and inspect daily to confirm that the transfection was successful.

1. Harvest 2 mL of medium from each dish of transfected cells by pipetting off the medium and transferring to a sterile 15 mL, snap-cap, polypropylene tube.

   Note: This is the transfection viral stock and can be stored at 4°C until required. You will use this stock to identify recombinant virus by plaque assay.

2. Meanwhile, add 3 mL of fresh complete TNM-FH medium to the transfected cells and incubate the cells at 27°C for an additional 48 hours.

3. Check the cells 4–7 days post-transfection for visual confirmation of a successful transfection. This is accomplished using an inverted phase microscope at 250–400X magnification. Signs of viral infection are classified as early (within the first 24 hours), late (24–72 hours) and very late (> 72 hours).

   Early

   Increased cell diameter. A 25–50% increase in the diameter of the cells may be observed.

   Increased size of cell nuclei. The nuclei may appear to "fill" the cells.

   Late

   Cessation of cell growth. Cells appear to stop growing when compared to a cell-only control.

   Granular appearance. Signs of viral budding; vesicular appearance to cells.

   Viral occlusions. A few cells will contain occlusion bodies, which appear as refractive crystals in the nucleus of the insect cell.

   Detachment. Cells release from the dish or flask.

   Very Late

   Cell lysis. A few cells may fill with occluded virus, die, and burst leaving signs of clearing in the monolayer.

4. Once you have confirmed that your transfection was successful, you need to purify recombinant baculovirus by plaque assay of the transfected virus. Proceed to **Plaque Assay**, page 13.

Important

It is very important to purify recombinant virus away from any uncut or non-recombinant viral DNA. Contamination of your recombinant DNA with uncut (occ^+) DNA will lead to dilution of your recombinant virus over time because, in general, uncut (wild-type, occ^+) virus infects and replicates at higher efficiency than recombinant virus. Also, initiating expression studies with a pure, single virus population will ensure reproducible results.

Continued on next page

Troubleshooting

By Day 4 post-infection, you should see, using a microscope, signs of viral infection (nuclear swelling, detachment from the plate, viral budding, and lysis). If you do not, check the cells-only plate. The uninfected cells should appear overgrown when compared to infected cells as transfection inhibits cell growth. If cell growth appears to be inhibited, keep checking the infected cells daily for other signs of infection. It may be that the kinetics of infection is slower than expected. If cell growth does not appear to be inhibited, the cells were not successfully transfected and you should consider the following factors:

Method of DNA Preparation

The most common cause of failed transfection experiments is the method of DNA preparation. Resin-based DNA purification systems such as Life Technologies' PureLink™ HiPure Plasmid Prep Kits work very well (see page vi for ordering information). If you use CsCl-purified DNA, you must precipitate the DNA and wash thoroughly with 70% ethanol to remove residual salts. Salt interferes with the positively charged liposomes and decreases transfection efficiency. Also, any trace of phenol is detrimental to cell growth. Compare infected cells with cells exposed to only plasmid DNA and the cells-only control. If the plasmid DNA contains contaminants toxic to the cells, the cells exposed to only plasmid DNA will have signs of poor growth and cell lysis while the cells-only control will remain healthy.

Cell Viability

Cells must be in log-phase and 95–98% viable for successful transfection experiments.

Liposomes

Using liposomes that are greater than 6 months old may result in lower transfection efficiencies. Be sure that your Cellfectin® II Reagent is less than 6 months old.

Density of Cells

It is very important to plate cells at 50–70% confluence to ensure there is enough surface area for the Cellfectin® II Reagent to bind. If cells are plated too densely, transfection efficiency will decrease. This most commonly occurs when plating cells into 6-well microtiter plates instead of 60 mm plates. Note that the transfection experiment has been optimized for 60 mm plates.

Plaque Assay

Introduction

At this point, you should have a supernatant containing active, viable virus, 90% of which will be recombinant. It is very important to purify your recombinant virus away from any uncut viral DNA background (occlusion body plus, occ^+) and/or illegitimate (non-homologous) recombinants that do not contain your gene. In particular, failure to purify your recombinant virus away from uncut, wild-type virus will result in dilution of your recombinant virus over time as, in general, the wild-type virus infects and replicates more efficiently than recombinant virus. Purification of a recombinant plaque is done by infecting cells with dilutions of the transfection stock obtained in the **Post-transfection Procedure**, Step 1, page 11 and isolating focal points of infection (plaques) from an agarose overlay.

Outline

The table below outlines the steps needed to perform a plaque assay.

Step	Action
1	Seed 100 mm plates at 5×10^6 cells/plate and 50% confluence; this allows cells to double before growth ceases.
2	Infect cells with 10^{-2}, 10^{-3}, and 10^{-4} dilutions of transfection viral stock for 1 hour.
3	Remove medium and overlay with medium/agarose mix to "pin down" the infected monolayers.
4	Incubate plates until plaques are formed (5–10 days).

Materials Needed

The following materials are needed for the plaque assay:

- 100 mm tissue culture grade plates (3–8 plates per viral dilution)
- 47°C water bath
- 15 mL sterile, polypropylene, snap-cap culture tubes
- Log-phase Sf9 or Sf21 cells
- TNM-FH complete medium (see **Recipes**, page 61)
- Rocking platform (no orbital shakers)
- Low temperature melting agarose
- 5 and 10 mL sterile pipettes
- 50 mg/mL X-Gal, Blu-gal, or Bluo-gal in DMF (see **Recipes**, page 61)
- Dissecting microscope
- Light source
- Inverted phase microscope
- Sterile Pasteur pipettes (without cotton plugs)
- 5 mM EDTA
- Sterile 1.5 mL microcentrifuge tubes

Continued on next page

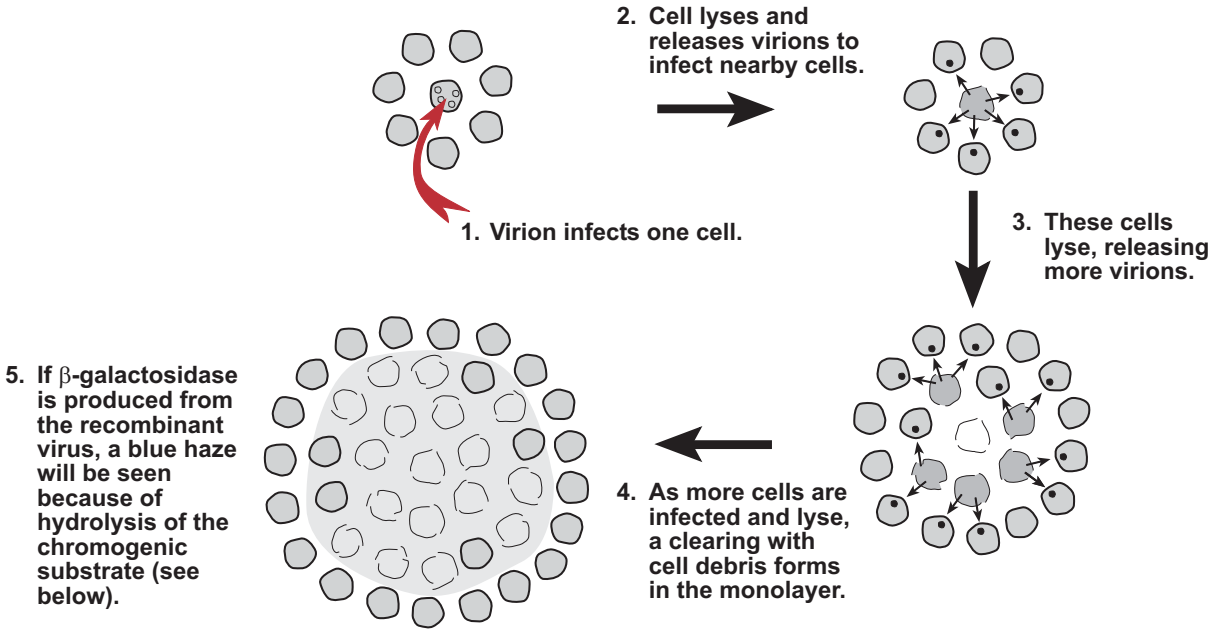
Note

Always use cells that are in log phase (doubling every 24 hours or less) and 95% viable. Do not use cells that have been in culture longer than 3 to 4 months as they gradually lose the ability to be infected by virus even though they are viable and healthy.

Plaque Formation

The most important factor is the quality of your cell monolayer. If the cells are not evenly distributed or are not in log-phase growth, plaques will be very difficult to identify.

The figure below shows how a plaque is formed. Viral plaques are observed as an area in the cell monolayer which is ringed by infected cells. The infected cells are morphologically distinct from the uninfected cells. They are generally larger in diameter, display a marked increase in the size of the nuclei relative to the total cell volume and show signs of cell lysis. This will eventually lead to a clearing in the monolayer. Note that if a transfer vector with *lac*Z is used that recombinant plaques will be blue if a chromogenic substrate is included. This allows you to easily distinguish recombinant plaques from non-recombinant.



Screening for Blue Recombinant Plaques

To detect blue, recombinant plaques, the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) or halogenated indolyl-β-D-galactosidase (Bluo-gal) is incorporated into the medium at a concentration of 150 µg/mL. Bluo-gal will give deep blue plaques while X-gal will give blue-green plaques.

For example: add 150 µL of a 50 mg/mL X-gal or Bluo-gal stock solution to 50 mL of complete TNM-FH medium.

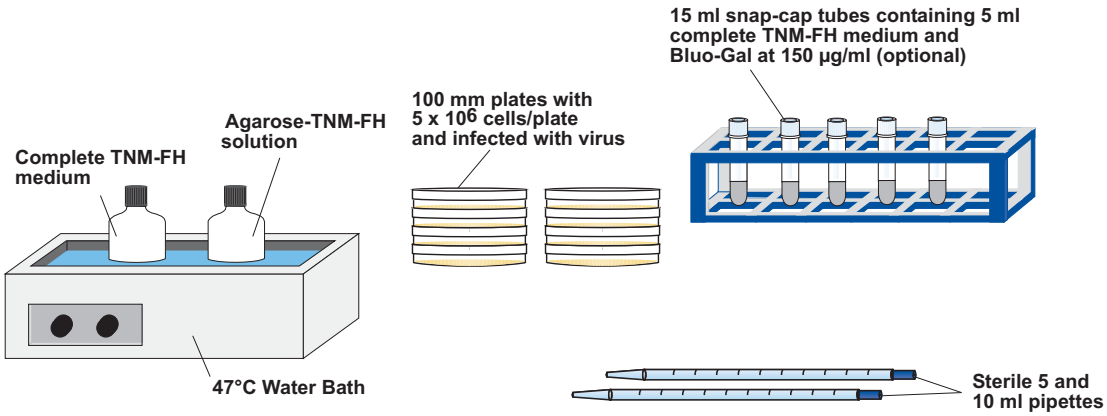
Note: The intensity of the color will increase over time due to cell lysis. Hydrolysis of the chromogenic substrate does not occur until the cells lyse, releasing β-galactosidase.

Continued on next page

Setting up the Tissue Culture Hood

You may wish to refer to the diagram below to set up your tissue culture hood for a plaque assay. It is very important to be organized and have everything you need before starting.

- Decontaminate a water bath by wiping it down with alcohol and place it in the hood. Set at 47°C.
- Prepare agarose-TNM-FH solution (see **Recipes**, page 61) and place in a 47°C water bath.
- Aliquot 50 mL of complete TNM-FH into a sterile, 100 mL bottle. Place in a 47°C water bath until needed.
- For each 100 mm plate, place 5 mL of complete TNM-FH medium (plus chromogenic substrate if required, see previous page) into a sterile 15 mL Falcon tube. Set aside at room temperature until needed.
- Be sure to have enough 5 mL and 10 mL sterile pipettes.



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Plaque Assay, continued

Preparing Cells

The quality of the cell monolayer is critical for a successful plaque assay. You will see plaque formation only if the cells are evenly distributed and are in log-phase growth when infected. Be sure to include a cells-only control to assess cell viability, contamination, and monolayer quality.

1. Pre-wet the plates with TNM-FH medium and seed log phase Sf9 or Sf21 cells (98% viable) at a density of 5×10^6 cells/100 mm plate in complete TNM-FH. Use 3 to 8 plates for **each** viral dilution to be tested (10^{-2}, 10^{-3}, and 10^{-4}, see **Diluting Virus** below).
2. Rock plates (~8 side to side motions per minute) for 10 minutes at room temperature on a side/side rocking platform to distribute the cells evenly.

   Do not use orbital shakers. Cells will be swirled around and will be distributed around the edges instead of evenly across the plate.
3. Stop rocker and let the plates stand for at least 10 minutes. Be sure to level the rocker to produce an evenly distributed monolayer.
4. Remove plates from the rocker and set at room temperature for at least 30 minutes before using or until cells have attached.
5. Examine the plates to confirm that cells have attached. Cells should look 50% confluent on the plate.

Diluting Virus

Take your transfection viral stock, vortex vigorously, and prepare 10-fold serial dilutions in complete TNM-FH. Prepare approximately 1 mL of diluted virus for each plate. We recommend 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Vortex viral stock or dilution before making the next dilution to ensure virus is evenly suspended.

Be sure to return your transfection viral stock to 4°C.

Procedure for Viral Infection and Agarose Overlay

Read this procedure carefully before starting. This procedure involves infecting the cells with your viral dilutions and then overlaying the monolayer with agarose. It is very important to add the virus carefully to ensure that the monolayer is not disturbed and to evenly distribute the virus over the monolayer. Also, pouring the agarose overlay may require some practice if you are new to the baculovirus system. Make sure you have everything set up and ready as it is important to work efficiently (see previous page).

1. Start with one viral dilution at a time. Take the 3–8 plates you have labeled for one viral dilution and remove all but 2 mL of medium from the cells once they have firmly attached. We generally plate cells in about 5 mL so you would remove 3 mL in this case.
2. Carefully add 1 mL of a viral dilution dropwise to the appropriately labeled plate. Be careful not to disturb the monolayer. A sterile, 1 mL pipette is ideal for this step.
3. Incubate the plates at room temperature on a slowly rocking platform (~2 side to side motions per minute) for 1 hour. Note the time that you start the incubation on the first set of plates.

Continued on next page

Procedure for Agarose Overlay, continued

4. Repeat Steps 1–3 for each remaining dilutions of virus, noting the time you start each incubation.

   Note: The incubation times for each viral dilution are now staggered to allow enough time to pour the agarose overlay starting in Step 6, below.

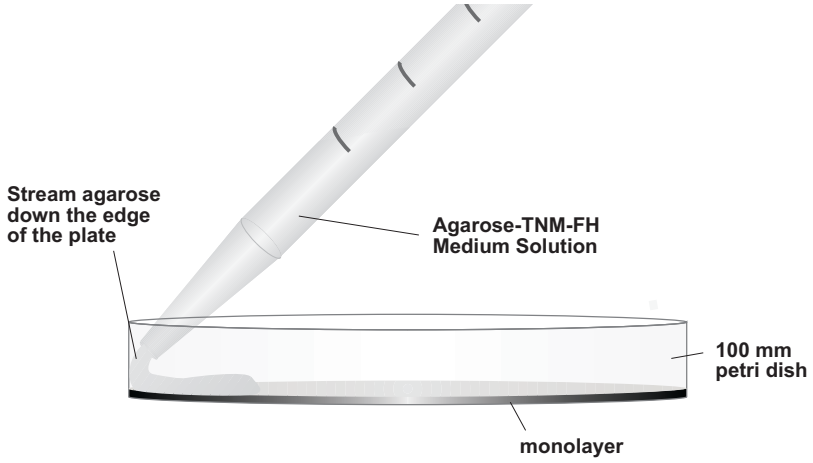
5. During the 1 hour incubation period, adjust the water bath in the hood to a temperature of 47°C. **Temperature is very important.** If the agarose-TNM-FH solution is too hot, it can kill your cells. If the agarose is too cool, it will gel and clump, forming an uneven overlay.

6. At the completion of the 1 hour incubation period begin working with the first set of plates infected. **Completely** aspirate the medium from the plates. **It is very important not to aspirate too many plates at a time as the cells will dry out without medium.**

   Note: We use a sterile Pasteur pipette with a plastic pipette tip over the end to aspirate all of the medium. Tip the plate slightly and remove medium from the edge. Do **not** drag the tip over the monolayer. If the plates are too wet, infected cells will not stay localized, making it difficult to isolate recombinant plaques.

7. Working quickly and maintaining sterile technique, withdraw 5 mL of the (47°C) agarose-medium mix and add to a tube containing the 5 mL of **room temperature** pre-measured medium with or without chromogenic substrate. Mix immediately by pipetting up and down once, and then draw up the agarose mixture into the pipette.

8. Working from the edge of the plate, hold the pipette so the tip does not touch the side edge and slowly stream the agarose mixture against the side edge so that it flows down the side and across the plate. **You must take care not to move the plates until the agarose has set.**



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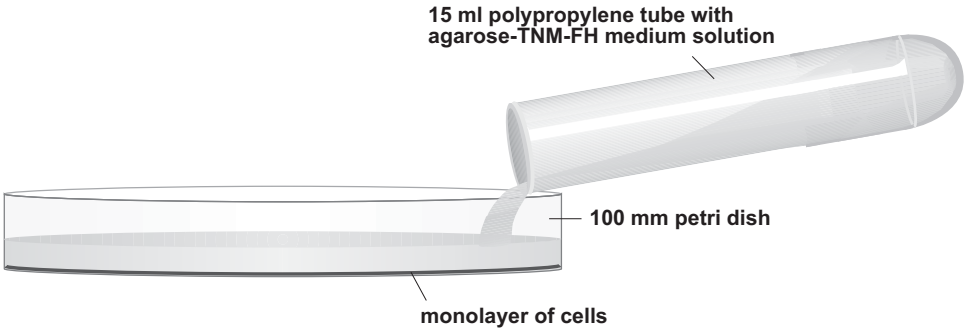
Procedure for Agarose Overlay, continued

You may also gently pour the agarose across the plate from one edge; however, be sure not to disturb the monolayer.



9. Repeat Steps 6–8 until all plates have been completed. Make sure the agarose is set before moving the plates.
10. Seal the plates in a plastic bag or sealed container with paper towels slightly dampened with 5 mM EDTA to prevent plates from drying out. Using EDTA prevents the growth of mildew and bacteria on the paper towels.

    Note: Once condensation appears on the plastic bag or container, open the bag or container. Moisture can destroy the monolayer, preventing recombinant plaque formation.
11. Incubate at 27°C for 5–6 days, or until plaques are well-formed. Proceed to **Visual Screening for Plaques**, next page.

Continued on next page

Visual Screening for Plaques

The purpose of this procedure is to identify putative recombinant plaques prior to purification and DNA isolation for PCR analysis. The following procedure to visually screen plaques is described by Summers and Smith, 1988 and Webb and Summers, 1990 .

1. When plaques are distinct (at least 6 days post-infection), examine plates using a dissecting microscope with a magnification of 30–40X.
2. Place the plate upside down on a non-reflective dark surface and illuminate the plate from the side using an intense light source (e.g., a fiber optic light).
3. Adjust the angle of the light until the plaques can be observed (usually, a 45° angle or greater is best). Against a black, non-reflective background, the occ^+ plaques will look shiny, almost crystal-like, while the recombinants will be a dull milky-white color.
4. If you have used a transfer vector containing the *lacZ* gene (e.g., pBlueBac, pBlueBacHis, or pMelBac) to generate the recombinant virus and included a chromogenic substrate in the agarose, the recombinants will be blue in color and clearly distinguishable by eye. Blue plaques may appear as early as Day 4 or 5 post-infection.
5. Look at the plates which have been infected with a 10^{-4} dilution of virus first. This dilution should result in plaques that are well-separated from each other.
6. Scan the plate at a magnification of 30–40X and circle any plaques you suspect may be recombinant (occ^-).
7. Re-examine each circled plaque under an inverted phase microscope at 200–400X. Examine the entire plaque area for the presence or absence of occlusion bodies. To save time, it is important that only recombinants that are totally free of occlusion bodies are selected. However, locate one occ^+ plaque as a control for wild-type Bac-N-Blue™ DNA. You will need this control for the PCR analysis, page 21.
8. When you have located several putative recombinant plaques and one wild-type plaque, check the circled plaques again using a dissection microscope. Place a tiny dot within each circle, directly over the plaque to be picked. Proceed to **PCR Analysis of Recombinant Virus**, page 21.

Continued on next page

Plaque Assay, continued

Visualizing Plaques

If you have problems distinguishing between occ$^+$ and occ$^-$ plaques, you may overlay one or two of your plaque assay plates with a second overlay containing Neutral Red or Trypan Blue to better visualize plaques (see page 32).

Do not select recombinant plaques from plates stained with Neutral Red or Trypan Blue. These dyes are mutagens that may damage your recombinant viral DNA. Use these dyes **only** for visualizing the plaques. Be sure to perform the plaque assay in triplicate to have an extra plate or two to stain with Neutral Red or Trypan Blue. **Pick your recombinant plaque only from unstained plates.**

Troubleshooting

The following table provides solutions to common problems with the plaque assay.

Problem	Reason	Solution
No plaques	The kinetics of infection varies for each recombinant virus.	If it is less than Day 6 post-plating, be patient. If no plaques appear after Day 8 or 9, proceed to investigate other possibilities listed below.
	There was not a confluent monolayer on Day 2 or Day 3 post-infection	Seed 5×10^{-6} cells in a 100 mm plate (50% confluence). Cells should double at least once before infection stops growth.
		Agar was too hot. You will see more cells growing on one side of the plate where the agar cooled compared to side where the agar was hot when poured.
		Cell viability was not > 95% or cells were not in log phase. Check viability of cells before seeding plates.
	Incubation in a humidified incubator. This can cause condensation that runs down the side of the agar and onto the monolayer. This will interfere with plaque formation.	Incubate in a sealed container or bag with damp paper towels. Remove towels or open container if condensation appears.
	Virus titer too low	Do not dilute virus as much. Use undiluted, 10^{-1} or 10^{-2} dilutions. You may have to repeat the transfection to increase viral titer.
Small plaques, difficult to visualize.	Too many cells seeded over area used.	Seed fewer cells and redo the plaque assay.
Too many plaques or complete cell lysis	Virus titer too high	Dilute virus to 10^{-5} or 10^{-6} and repeat plaque assay.

PCR Analysis of Recombinant Virus

Purpose

Polymerase chain reaction (PCR) technology allows a quick, safe, nonradioactive method to determine the presence of an insert in a putative recombinant virus and confirm the isolation of a pure, recombinant plaque. PCR analysis is a fast and efficient way to rule out false positives at an early stage. Since the size of the foreign gene insert is known, you can easily determine the size of the expected PCR product and detect wild-type contamination.

Outline of PCR Analysis

The table below outlines the steps needed to perform PCR analysis to confirm isolation of recombinant virus.

Step	Action
1	Design appropriate PCR primers for use in verifying the presence of an insert in a putative recombinant virus.
2	Calculate the expected size of your PCR fragment based on the primers you are using.
3	Grow putative recombinants in 12-well microtiter plates.
4	Inspect wells and select only those that are occ$^-$.
5	Resuspend the cells into the medium and harvest 0.75 mL of the suspension for PCR analysis.
6	Continue to incubate the cells in the microtiter plates until they lyse.
7	Isolate viral DNA from the 0.75 mL sample and perform PCR on the viral DNA.
8	Analyze your results on a 1% agarose gel and select only recombinant virus that contain your expected fragment (there should be no contamination with wild-type virus).
9	Return to the microtiter plates when the cells have lysed and harvest the entire medium from wells containing only pure recombinant virus for the P1 viral stock.

Designing PCR Primers

To perform PCR analysis, you need to design appropriate PCR primers to allow verification of recombinant viruses containing inserts. We recommend designing forward and reverse primers with the following sequence:

Forward primer: 5´-TTTACTGTTTTCGTAACAGTTTTG-3´ T_m = 62°C

Reverse primer: 5´-CAACAACGCACAGAATCTAGC-3´ T_m = 58°C

These primers flank the polyhedrin region and are compatible with all polyhedrin promoter based baculovirus transfer vectors. The forward PCR primer binds from –44 (nt 4049) to –21 (nt 4072) in front of the start of the polyhedrin gene, using the nomenclature of O'Reilly, *et al.*, 1992. The reverse PCR primer binds at +794 (nt 4886) to +774 (nt 4866) 3´ to the polyhedrin gene.

Continued on next page

PCR Analysis of Recombinant Virus, continued

Controls for PCR Analysis

The following controls are recommended for PCR analysis.

Control	Reason
Wild-type plaque	Control for wild-type background
Transfer vector with insert	Positive control for recombinant virus

Expected PCR Fragments

If you design forward and reverse PCR primers with the sequences recommended on the previous page, you should generate PCR fragments of the following sizes:

Note: pVL1392, pVL1393, pBlueBacIII, pBlueBac4, pBlueBac4.5, pBlueBacHis A, B, and C, pBlueBacHis2 A, B, and C, pAc360, p2Bac vectors are no longer available from Life Technologies.

Viral or Vector DNA	Size of Fragment (bp)
wild-type Bac-N-Blue™ DNA	839
pVL1392, pVL1393	749
pBlueBacIII	672
pBlueBac4	296
pBlueBac4.5	435
pBlueBacHis A, B, and C	735 (A), 725 (B), 733 (C)
pBlueBacHis2 A, B, and C	338 (A), 328 (B), 336 (C)
pMelBac A, B, and C	326 (A), 316 (B), 324 (C)
pAc360	703
p2Bac	672

Add the size of the fragment contributed by the vector to the size of your insert to yield the predicted PCR product. For example, a 1.1 kb insert cloned into the *Pst* I site of pBlueBac4.5 will yield a ~1.5 kb PCR fragment.

Continued on next page

PCR Analysis of Recombinant Virus, continued

Materials Needed

You will need the following materials:

- Log-phase Sf9 or Sf21 cells
- 12-well microtiter plates
- 65°C heat block
- Complete TNM-FH medium
- Inverted phase microscope
- Sterile Pasteur pipettes
- Sterile 1.5 mL microcentrifuge tubes
- Easy-DNA™ Kit (see page vi for ordering information)
- Proteinase K, 5–10 mg/mL
- Phenol:chloroform (1:1)
- 3 M sodium acetate
- Glycogen (2 mg/mL)
- PCR Thermocycler
- Thermostable polymerase
- 5X or 10X PCR buffer
- dNTPs (25 mM; 6.25 mM of each nucleotide)
- Chloroform
- 100% and 70% ethanol
- Agarose and Agarose gel apparatus
- Appropriate forward and reverse PCR primers
- Sterile water
- TE buffer, pH 8.0
- 20% PEG 8000 in 1 M NaCl

Continued on next page

PCR Analysis of Recombinant Virus, continued

Growth of Recombinant Virus for PCR Analysis and the P1 Viral Stock

The purpose of the following procedure is to amplify your putative recombinant plaques to isolate viral DNA for PCR analysis and generate a P1 stock. PCR analysis is used to identify pure, recombinant plaques and the P1 stock from a pure recombinant plaque will be used to generate a large scale, high-titer stock (HTS).

1. Pre-wet the wells of a 12-well microtiter plate with 2 mL of complete TNM-FH medium.
2. Seed 5×10^5 log-phase Sf9 or Sf21 cells in each well. The total volume of the wells should not exceed 3 mL.
3. Using a sterile Pasteur pipette and bulb carefully penetrate the agarose and remove the cell monolayer containing the recombinant plaque or the wild-type plaque.
4. Transfer the agarose plug containing the plaque to one of the wells in the microtiter plate.
5. Repeat steps 3 and 4 until 10 of the 12 wells have been infected. Use one well for the occ^+ plaque (wild-type Bac-N-Blue™ DNA) and the other as a cells-only control.
6. Cover and seal the microtiter plate with parafilm or place the plate in a sealed plastic bag. Incubate at 27°C for 3 days.
7. On day 3, screen the wells visually using the inverted microscope for the presence of occlusion bodies.

   Note: Compare the 10 infected wells with the occ^+ well and the cells-only control. Infected, occ^- cells should appear swollen and growth arrested.

   Any of the putative recombinant wells containing occlusion bodies (occ^+) are not plaque pure. Do not use these wells or wells that appear uninfected for PCR analysis.
8. Using only occ^- wells and the one occ^+ well (wild-type Bac-N-Blue™ control), gently pipette the medium in the microtiter plate well up and down to dislodge the cells.
9. Remove 0.75 mL of the suspension and transfer to a sterile microcentrifuge tube. This is used to isolate DNA and perform PCR analysis of the putative recombinant virus.
10. The microtiter plates should be kept and incubated at 27°C in a humid environment until all of the cells lyse (5–7 days). The medium from each occ^- well is then harvested and stored at 4°C. These are the purified P1 virus stocks, and one will be used to generate a large-scale, high-titer virus stock on page 29. The other P1 stocks may be tested, if desired.

Continued on next page

Isolating Viral DNA

The following protocol for isolating viral DNA uses the Easy-DNA™ Kit available from Life Technologies (see page vi for ordering information). It is faster and easier to use than other methods to isolate DNA from baculovirus. If you do not have the Easy-DNA™ kit, see the alternative protocol on the next page.

Before starting:

- Prepare sterile 20% PEG 8000 in 1 M NaCl and chill at 4°C (See **Recipes**, page 61)
- Chill 100% and 70% ethanol in a –20°C freezer
- Equilibrate a heat block at 65°C

1. Take the occ⁻ samples generated in **Growth of Recombinant Virus for PCR Analysis and the P1 Viral Stock**, Step 9, previous page, and pellet the cell debris by centrifugation in a microcentrifuge at 5,000 rpm for 3 minutes.
2. Transfer the supernatant to a fresh tube and add 0.75 mL cold (4°C) 20% polyethylene glycol (PEG) in 1 M NaCl. Mix three times by inversion, and incubate on ice for 30 minutes.
3. Centrifuge at maximum speed for 10 minutes at 4°C to pellet the viral particles. Carefully 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. Resuspend the viral pellet in 100 µL of sterile water. Carefully wash the sides of the tubes to ensure that all of the viral particles are resuspended.
5. Add 143 µL of Solution A to the viral particles and vortex 1 second to mix.
6. Incubate at 65°C for 6 minutes.
7. Add 58 µL of Solution B and vortex vigorously for 5 seconds or until mixture is uniform and there is no white plug in the bottom of the tube.
8. Add 258 µL chloroform and vortex until evenly mixed.
9. Centrifuge at maximum speed for 10 minutes at 4°C to separate phases and create the interface. Pipette the upper, aqueous phase to a fresh microcentrifuge tube to precipitate the DNA.

Viral DNA Precipitation

1. Add 500 µL of 100% ethanol (–20°C) to the DNA solution. Invert the tube eight times to precipitate the DNA.
2. Centrifuge at maximum speed for 5 minutes at 4°C. Keep the pellet and decant the ethanol.
3. Wash the pellet with 500 µL of 70% ethanol (–20°C) and centrifuge at maximum speed for 5 minutes at 4°C. Remove all the ethanol.
4. Centrifuge again at maximum speed for 2–3 minutes at 4°C. Carefully remove residual ethanol and air dry the nucleic acid pellet for 5 minutes.
5. Resuspend the pellet in 20 µL sterile water and proceed to **PCR Procedure,** page 27. Alternatively, the DNA may be stored at 4°C or –20°C until ready for use.

Continued on next page

Alternative Protocol to Isolate Viral DNA

Use this protocol to isolate DNA for PCR if you do not have the Easy-DNA™ Kit. Prepare the following solutions:

- Proteinase K (5–10 mg/mL)
- Phenol:chloroform (1:1)
- 3 M sodium acetate
- Glycogen (2 mg/mL)

1. Take the sample generated in **Growth of Recombinant Virus for PCR Analysis and the P1 Viral Stock**, Step 9, page 24, and pellet the cell debris by centrifugation at 5,000 rpm for 3 minutes at room temperature.
2. Transfer the supernatant to a fresh microcentrifuge tube and add 0.75 mL cold (4°C) 20% polyethylene glycol (PEG) in 1 M NaCl. Invert twice to mix and incubate at room temperature for 30 minutes.
3. Centrifuge at maximum speed for 10 minutes at room temperature. 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 sterile water 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 incubate at 50°C for 1 hour.
6. Extract with an equal volume (110 µL) of phenol-chloroform (1:1). Centrifuge at maximum speed in a microcentrifuge for 5 minutes at room temperature. Transfer the upper aqueous phase to a fresh, sterile tube.
7. Precipitate the DNA by adding 1/10 volume 3 M sodium acetate, 5 µL of glycogen, and 2 volumes of 100% ethanol. Incubate at –20°C for at least 20 minutes.
8. Centrifuge at maximum speed for 15 minutes at 4°C. Wash pellet with 70% ethanol. Centrifuge again and remove all traces of ethanol.
9. Resuspend the pellet in 10 µL of sterile water and proceed to **PCR Procedure**, next page. Store the DNA at 4°C or –20°C until ready for use.

Continued on next page

PCR Analysis of Recombinant Virus, continued

PCR Procedure

The PCR protocol below allows you to generate a PCR product using the recommended forward and reverse PCR primers described on page 21. If you use other PCR primers you should optimize PCR conditions for your primers.

1. Set up your 50 µL PCR reaction as described below. Be sure to include the wild-type Bac-N-Blue™ DNA as a negative control and your recombinant transfer plasmid as a positive control.

Viral DNA	5 µL
10X PCR Buffer	5 µL
25 mM dNTPs	1 µL
Forward PCR Primer (100 ng/µL)	1 µL
Reverse PCR Primer (100 ng/µL)	1 µL
Taq Polymerase	1.5 units
Sterile water	to a final volume of 50 µL

2. Overlay each reaction with 50 µL of mineral oil.
3. Place in the thermocycler and cycle using the following parameters:

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	2 minutes	1X
Denaturation	94°C	1 minute	30X
Annealing	55°C	2 minutes	
Extension	72°C	3 minutes	
Final Extension	72°C	7 minutes	1X

4. Cool samples to 30°C, and analyze 5 µL of the PCR reaction on a 1% agarose gel.

Analysis of PCR

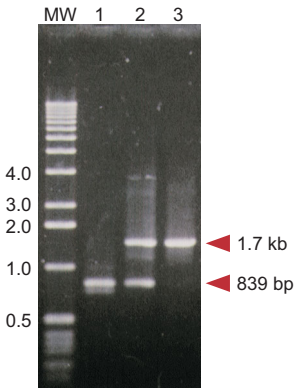
First, calculate the expected size of your PCR fragment based on the size of the insert and the size of the DNA contributed by the transfer vector (see page 22 for a list of vectors and the PCR fragments they produce). Select 3–6 recombinant clones that **only** have the recombinant PCR fragment. There should be no recombinant PCR fragment generated from wild-type Bac-N-Blue™ DNA. Keep the pure P1 viral stocks from Step 10, page 24 on hand for each of the positive clones. Select the clone that gave you the best PCR result and prepare a large-scale, high-titer stock for expression studies (see page 29).

Continued on next page

PCR Analysis of Recombinant Virus, continued

Typical PCR Analysis of Recombinant Viral Clones

A typical PCR analysis is shown below. Two samples were analyzed for the recombinant PCR fragment (1.7 kb) and the wild-type Bac-N-Blue™ DNA PCR fragment (839 bp). DNA was isolated from 0.75 mL of the P1 viral stock and 5 µL was used as a template in a PCR reaction with forward and reverse PCR primers containing the sequence listed on page 21. Ten microliters from each sample were analyzed on a 1% gel. DNA was also isolated from a wild-type (occ^+) plaque as a negative control. Lane 1 is the wild-type (occ^+) control; lane 2 demonstrates that a putative recombinant plaque is actually a mixture of recombinant and wild-type virus; and lane 3 is a pure, recombinant plaque.



Troubleshooting

Most difficulty with PCR is usually related to the quality of the template. Be sure to use qualified, DNase-free reagents when isolating viral DNA. You may wish to quantitate the amount of DNA in your starting material. You will need 50 ng to 1 µg template DNA for PCR.

If There Are No Pure Plaques

If there is contamination with wild-type virus in all of your samples, take a P1 viral stock and redo the plaque assay. Be sure to select well-spaced, occ^- plaques. If you are having difficulty distinguishing a recombinant plaque from a non-recombinant plaque, try using one of the pBlueBac vectors. Recombinant plaques will be blue when chromogenic substrate is incorporated into the medium during the agarose overlay (see page 14 for more information).

Preparing High-Titer Viral Stocks

Purpose

Once a recombinant virus has been identified, you will return to the P1 viral stocks (see **Recombinant Virus for PCR Analysis and the P1 Viral Stock**, Step 10, page 24) and identify the stock corresponding to the positive clone. You will need to generate a high-titer viral stock to initiate expression studies. The P1 viral stock is only 1.5 mL and the titer is unknown. Expression studies are best performed using a high, known multiplicity of infection (MOI). Therefore, you must generate a viral stock of high, known titer, ~1×10^8 pfu/mL.
Note: pfu = plaque forming units

Materials Needed

You will need the following materials:

- P1 viral stock
- Log-phase Sf9 or Sf21 cells, in suspension (98% viable, doubling every 18–24 hours)
- 25 cm^2 flasks
- TNM-FH medium
- 1 liter spinner flask
- *Optional*: 10 mg/mL Neutral Red solution
- *Optional*: 2.5% low melting agarose solution

Before Starting

Read the following protocol carefully, so you can have a 500 mL suspension culture of Sf9 or Sf21 cells at 1.8–2.2 × 10^6 cell/mL and 98% viability ready for infection at Step 4.

Procedure

In this procedure you will take the P1 viral stock, a small-scale, low-titer stock, and generate a large-scale, high-titer viral stock, suitable for expression experiments.

1. Seed two 25 cm^2 flasks with 2 × 10^6 log-phase Sf9 or Sf21 cells in 5 mL complete TNM-FH medium.
2. Add 20 µL of the P1 viral stock to each flask and incubate at 27°C for 5–10 days, or until the cells are 100% lysed.
3. Using a sterile Pasteur pipette, slough the cells and remove the entire medium and the lysed cells (~10 mL). This is the P2 viral stock, a small-scale, high-titer stock. Take 1 mL of this stock and store as is at –80°C for long-term storage. Take another 4 mL aliquot and store at 4°C as a reserve stock.
4. Add the remaining 5 mL of P2 viral stock to a 500 mL suspension of log-phase Sf9 or Sf21 cells seeded at a density of 1.8–2.2 × 10^6 cells/mL. Return flask to the spinner plate for 5 minutes to insure proper mixing.

Procedure continued on next page

Continued on next page

Preparing High-Titer Viral Stocks, continued

Procedure, continued

Procedure continued from previous page

5. Transfer 5 mL of the cell/virus suspension from the 500 mL suspension culture to a fresh 25 cm^2 flasks to monitor the infection process.
6. Incubate the 500 mL culture at 27°C with constant stirring (80–90 rpm for cells without Pluronic® F-68 or up to 120 rpm for cells with Pluronic® F-68 BASF) for 7–10 days.

   Check the progress of the infection regularly by observing the 25 cm^2 flasks, or by using the Trypan Blue exclusion assay (see **Recipes**, page 61) to determine extent of cell lysis. Harvest when $\geq$ 90% of the cells have lysed.
7. To harvest the high-titer stock for future inoculum or protein purification, pellet cells at 1,000 × *g* for 20 minutes and transfer the supernatant to a sterile bottle. This is the P3 viral stock, a large-scale, high-titer stock that can be assayed for plaque forming units per mL (see next page) and used in a time course to optimize protein expression. Store at 4°C.

Storing Viral Stocks

We recommend storing viral stocks as follows:

1. Centrifuge viral stock at 4,000 × *g* to remove cellular debris and prevent proteolysis.
2. If medium is serum-free, add serum to 10%. Serum proteins act as substrates for proteases.
3. Store viral stocks at 4°C. Viral stocks stored at 4°C for up to two years show virtually no change in titer.
4. Protect all stocks from light to ensure maintenance of titer (Jarvis and Garcia, 1994)
5. Do **not** store routinely used viral stocks at temperatures below 4°C. Repeated freezing and thawing of stocks of AcMNPV and its derivatives can result in 10- to 100-fold decrease in virus titer.
6. Store small aliquots of important isolates at –70°C as emergency stock.

Important

Do not store routinely used viral stocks at temperatures below 4°C. Repeated freezing and thawing of stocks of AcMNPV and its derivatives can result in 10- to 100-fold decrease in virus titer. In contrast, virus stored at 4°C in excess of 2 years show virtually no change in titer. However, we recommend storing small aliquots of important isolates at –70°C as emergency stock. Protect all stock from light to ensure maintenance of titer (Jarvis and Garcia, 1994).

Continued on next page

Preparing High-Titer Viral Stocks, continued

Determining Viral Titer

To obtain a synchronous infection, or to infect cells at a particular multiplicity of infection (MOI), it is essential to know the concentration of virus in the inoculum. Viral titers also allow you to evaluate your cell handling and infection techniques.

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 vi for ordering information.

Procedure for Determining Viral Titer

Follow the procedure below to determine the concentration of virus in the inoculum.

1. Prepare 10-fold serial dilutions in complete TNM-FH medium of the viral stock to be tested. Dilute viral stock to 10^{-6} and 10^{-7}.
2. Perform a plaque assay using these two dilutions of virus as described on page 16.
3. Count plaques on a plate that has between 50–100 plaques. The plaques will be well-spaced, making them easy to count.

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^{-6} contains 124 plaques. The viral titer is:

$$\text{pfu/mL} = \frac{124 \text{ pfu}}{10^{-6} \times 1 \text{ mL}}$$

$$= 1.24 \times 10^{8} \text{ pfu/mL}$$

Visualizing Plaques

To better view plaques, you can use Neutral Red or Trypan Blue overlays. These methods are recommended only for assaying viral titers as both compounds are known mutagens that could alter your recombinant virus. We recommend using Neutral Red as the plaques will appear whitish on a red background of cells.

Continued on next page

Neutral Red Overlay

1. On day 6–7 of the plaque assay, prepare a second agarose overly. You will need 1–1.5 mL of the Neutral Red-agarose solution per 100 mm plate.
2. Prepare 50 mL of 2.5% low-melting agarose solution in distilled water. Microwave on medium for 3 minutes to dissolve the agarose and sterilize the solution. Cool to 47–50°C and place in a 47°C water bath.
3. Place 50 mL TNM-FH medium in a 47°C water bath.
4. Prepare 1% Neutral Red Stock (10 mg/mL) in distilled water and filter sterilize.
5. Combine 16 mL of TNM-FH medium, 3.25 mL of 2.5% agarose, and 0.5 mL of 1% Neutral Red, mix, and keep at 47°C until ready to use.
6. Overlay the 100 mm plates with 1–1.5 mL of the Neutral Red-agarose solution. Rotate the plate to evenly cover the plate.
7. Incubate the cells at room temperature and allow the cells to take up the Neutral Red for 3 hours or overnight before inspecting the plaques. The plaques will appear as morphologically distinct opaque areas on the pink/red monolayer.

Trypan Blue Overlay

This procedure is described in Ausubel, *et al.*, 1994 pp. 16.10.5-16.10.7.

1. On day 3–5 of the plaque assay, prepare a second agarose overlay. You will need 1–1.5 mL of the Trypan Blue-agarose solution per 100 mm plate.
2. Prepare a 1% Trypan Blue solution in distilled water and filter sterilize. Equilibrate to 40–42°C.
3. Microwave a 1% agarose solution until dissolved and equilibrate to 40–42°C.
4. Add 1–1.5 mL of the Trypan blue solution to 12.5 mL of 1% agarose and mix well.
5. Overlay the plates with 1 mL of the Trypan Blue-agarose solution.
6. Incubate the plates overnight at 27°C to allow the dye to diffuse into the dead cells.
7. Count the number of blue plaques and determine the viral titer.

Master Stock

Once you have determined the titer of your HTS, store this master stock at 4°C, wrapped in foil to protect from light (Jarvis and Garcia, 1994). Use this stock to initiate expression experiments.

You may wish to store a portion at –80°C, but the titer will drop and you will need to generate another HTS if you start from the frozen stock.

To generate another HTS from a master stock, use the plaque assay to generate fresh, recombinant plaques. Select recombinant plaques and generate a P1 viral stock. Use this P1 viral stock to generate a new master HTS as described on page 30.

Continued on next page

Preparing High-Titer Viral Stocks, continued

Defective Interfering Particles

Re-amplifying HTS stocks without performing a plaque assay can lead to the accumulation of defective interfering particles (DIPS). These are defective virions that are not infective and lead to a lack of reproducibility in recombinant protein production (Wickham *et al.*, 1991). These defective virions:

- Contain only a part of the viral genome
- Contain normal viral structural proteins
- Reproduce only in the presence of "helper virus" (they do not plaque)
- Interfere specifically with the replication of homologous standard virus
- Produced following serial passaging of virus

DIPS are readily avoided by starting with plaque-purified, low-passage virus inocula (P1 viral stocks) and amplifying to an HTS; see **Master Stock**, previous page.

Using a High-Titer Stock

To infect insect cells for protein expression experiments, use an MOI of 5–10. This will synchronously infect your cells to produce protein at the same time.

To infect insect cells to generate another HTS, use an MOI of 0.5. A low MOI is used here to allow for exponential increase in viral titer. If you use too high a MOI, you will kill the cells without increasing the viral titer.

Time Course for Expression of Recombinant Protein

Introduction

At this point, you should have a high-titer stock of known titer of the recombinant virus (page 31). You are now ready to optimize expression of the recombinant protein. The following section provides you with protocols for carrying out a time course in both adherent and suspension cell cultures. It is essential that you carry out an initial time course of expression. The time course will enable you to determine the optimal multiplicity of infection and time of harvest for your recombinant protein. You must do a separate expression time course for each recombinant protein and each cell line you wish to use.

Cell Lines

Expression of the recombinant protein may be done in Sf9, Sf21, High Five™, or Mimic™ Sf9 cell lines. For each cell line, you must perform a time course to determine the point of maximum expression. Each cell line has slightly different growth characteristics, and both posttranslational modifications and the kinetics of infection may vary.

Multiplicity of Infection (MOI)

Range: A broad range of MOIs (multiplicity of infection) can be tested to determine which will induce the best kinetics of infection for maximal protein expression. An MOI of 5–10 is generally recommended for protein expression.

Note: This is unlike the generation of a high-titer stock where a low MOI of 0.5–1.0 is recommended.

High MOIs: Higher MOIs are preferable because they result in synchronous infection where all the cells become infected at time zero. As a result, all cells should be expressing protein at the same time so that the maximal amount of recombinant protein can be harvested at a given time point.

Low MOIs: Lower MOIs may result in only a percentage of the cell population being infected, while the rest of the cells continue to divide. These cells in turn become infected later on when the first group of cells lyse. This results in a protein expression curve that is spread out over the time course, rather than peaking at a given time point.

Recommended MOI: In the following time course protocols, we recommend testing an MOI of 5 and an MOI of 10 initially. MOIs of 5–10 are generally the most successful in expression work. By testing both 5 and 10, you are likely to be able to achieve synchronous infection.

Synchronous infection: Synchronous infection is defined as the infection of all cells in a culture at the same time point. Therefore, a true time = zero is established. Achieving synchronous infection will result in the maximum amount of protein being harvested at a given time point post-infection. This is because all cells in the culture will be expressing protein at the same time. The maximum time point must be determined for each protein and for each cell line used. Different MOIs may be tested after the initial time course to achieve synchronous infection.

Calculating Virus Volumes: It is very simple to calculate the volume of viral stock needed to achieve a given MOI using the following formula:

$$\text{Volume of Virus} = \frac{(\text{MOI desired})(\text{Total Number of Cells})}{\text{Titer of the Virus Stock}}$$

Continued on next page

Time Course for Expression of Recombinant Protein, continued

Example for MOI

A high-titer virus stock that is 1×10^8 pfu (virions)/mL will be used to infect a spinner with 50 mL of culture at a cell density of 2×10^6 cells/mL. Therefore, a total of (50 mL) (2×10^6 cells/mL) = 1×10^8 cells will be infected. To achieve an MOI of 5, you would need:

$$\frac{(5 \text{ virions/cell})\ (1 \times 10^8 \text{ cells})}{1 \times 10^8 \text{ virions/mL})} = 5 \text{ mL of your viral stock}$$

Time Points

Time points taken at 24 hour intervals are recommended initially to get a general idea of when your protein is being expressed. Once you have determined a time frame where optimal protein expression occurs (e.g., between 48 and 72 hours), you may want to perform a second time course with selected intermediate time points (e.g., 52, 60 and 68 hours). This will allow you to further optimize your expression levels (see **Optimization of Protein Expression Levels,** page 47).

Detecting Recombinant Protein

In the initial phases of expression (e.g., the time course), when you are attempting to determine the optimal conditions for maximal expression, you should not rely only on SDS-PAGE analysis for detection of protein. Levels may be suboptimal and other more sensitive assays may be necessary. For additional information regarding detection of your protein, refer to **Identification of Recombinant Protein**, page 45.

Materials Needed

- High-titer stock of known titer (10^8 pfu/mL or greater).
- Complete TNM-FH medium (if using High Five™ cells, EXCELL-400)

For each MOI to be tested:

- one 100 mL spinner flask (if using suspension culture)

  or
- one 6-well plate (if using adherent culture)
- Microcentrifuge tubes

Continued on next page

Time Course for Expression of Recombinant Protein, continued

Summary of Samples to be Taken

The following table summarizes the samples you will be taking for each of two MOIs, 5 and 10. Any convenient labeling system can be used. For the purposes of this protocol we will use a label that indicates:

1. MOI
2. Supernatant or Pellet sample
3. Time point of harvest (hours)

A typical label might be "5S24", which would represent infection at a MOI of 5, supernatant sample, 24 hours post-infection.

MOI	Time Point	Supernatant	Cell Pellet
5	T=24	5S24	5P24
	T=48	5S48	5P48
	T=72	5S72	5P72
	T=96	5S96	5P96
	T=120	5S120	5P120
10	T=24	10S24	10P24
	T=48	10S48	10P48
	T=72	10S72	10P72
	T=96	10S96	10P96
	T=120	10S120	10P120
0	Uninfected	control (S)	control (P)

Controls

When performing a time course for expression of your protein, it is very important to include controls. The controls will enable you to analyze the amount of background proteins that are being expressed at the same time as your protein of interest. The two controls that can be used are:

1. **Uninfected cells** (to assay for cellular protein background)

2. **Wild-type AcMNPV** (to assay for viral protein background)

You may use one or both controls. It may be more convenient to use one or the other.

Continued on next page

Time Course for Expression of Recombinant Protein, continued

Adherent Culture

The following protocol will enable you to carry out a time course in a 6-well dish. You will be testing two different MOIs, 5 and 10. You can perform this assay in any size flask. We recommend that you use plates as they are easier to harvest in time point studies. For other plate and/or flask sizes, see page 39 for the recommended cell seeding densities and volumes.

1. Seed two 6-well plates with 10^6 cells in each well. The final volume in each well should be 1–1.5 mL. Maintaining a low volume will keep the virus concentrated on the cells and increase infection efficiency.
2. Label Plate #1 "MOI = 5" and Plate #2 "MOI = 10".
3. For each plate, label wells #1–5 with each of the following time points: 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours. These will be the times of harvest (post-infection) for each well. See **Time Points,** page 35.
4. **Controls:** Label the last well as a control well. Perform one or both of the following controls (for more information, see **Controls**, previous page.

   Uninfected Control: This will contain uninfected cells and can be harvested at 24–48 hours after the other wells have been infected. You can process this control and run alongside your time point samples on SDS-PAGE. It will provide information on background cellular protein levels. **Do not infect this well with virus.**

   Wild-type Infected Control: This well will contain cells infected with wild-type AcMNPV at an MOI of 5–10, and can be harvested at 24–48 hours post-infection. The wild-type control can be processed and run alongside your time point samples on SDS-PAGE. It will provide information on background viral protein levels.
5. Take one microtiter plate and infect each well with an MOI of 5. Then, infect each well in Plate #2 with an MOI of 10. To calculate the amount of virus to add, see **Multiplicity of Infection**, page 34.
6. Cover both plates and incubate at 27°C.
7. Harvest each well at the designated time point. Scrape the cells from each well and transfer the entire solution (approximately 1–1.5 mL) from each well to a microcentrifuge tube.
8. Pellet cells at 800 × *g* for 10 minutes at 4°C. It is best to keep samples at 4°C or on ice to prevent proteolysis.
9. Transfer supernatant to a new tube. Label each tube.
10. Store the cell pellet and the supernatant at –70°C. The cell pellet will be lysed later when all time points have been collected.
11. Proceed to **Processing of Time Point Samples,** page 40.

Continued on next page

Time Course for Expression of Recombinant Protein, continued

Suspension Culture

The following protocol will enable you to carry out a time course in a 100 mL spinner. However, you can perform this assay in any size spinner. It is very easy to take time points from a suspension cell culture. Only one spinner per MOI to be tested is needed. If you are not set up for spinner culture, see **Adherent Culture**, previous page.

1. To begin, you will need two 100 mL spinner flasks with 50 mL of insect cells at a density of 2×10^6 cells/mL. Cells should have a doubling time of 18–24 hours and a viability of 98%, and be of passage number less than 30 (i.e., should not have been in spinner culture for more than 2 months).
2. Label Spinner #1 as "MOI = 5" and Spinner #2 as "MOI = 10."

   Note: It is helpful to have a background control for SDS-PAGE and/or western blot analysis. We recommend that you take a 1 mL sample of uninfected cells from the spinner **before infection** and process it as you would your time point samples (see Steps 5–8).
3. Infect Spinner #1 with an MOI of 5. Infect Spinner #2 with an MOI of 10 (to calculate the amount of virus to add, see **Multiplicity of Infection,** page 34).

   Example: A 100 mL spinner flask contains 50 mL of cell culture at a density of 2×10^6 cells/mL. To infect at an MOI of 5 (5 virions/cell in culture), you will need (5 virions/cell) (2×10^6 cells/mL) (50 mL) = 5×10^8 virions.
   If your high-titer stock has been titered at 1×10^8 virions/mL, then you need 5 mL of your stock to yield an MOI of 5.
4. Incubate spinners at 27°C with a spin rate of 80 to 90 rpm.
5. Remove 1 mL aliquots of cells every 24 hours over a period of 5 days and transfer each sample to a microcentrifuge tube.
6. Pellet cells at $800 \times g$ for 10 minutes at 4°C. It is best to keep samples at 4°C or on ice to prevent proteolysis.
7. Transfer supernatant to a new tube. Label each tube.
8. Store the cell pellet and the supernatant samples at –70°C. Storage at –70°C will reduce proteolysis of the recombinant protein. You will lyse the cell pellet later when you have collected all time points.
9. Proceed to **Processing of Time Point Samples**, page 40.

Continued on next page

Time Course for Expression of Recombinant Protein, continued

Seeding Densities and Volumes for Infections

The table below gives approximate seeding densities and volumes for typical vessel sizes. Infection at these densities in the minimal volumes listed will yield optimal infection.

Minimal Volumes: The total volumes used are lower than those used in general cell culture and maintenance. This is done so that the virus added is kept concentrated and can infect cells more readily.

Cell Density: Cell density in adherent culture is approximately 50% confluent to allow maximal cell surface area for contact with virus and subsequent infection.

MOI: Use an MOI of 5–10 for a time course of protein expression or a large-scale protein preparation (see **Multiplicity of Infection,** page 34).

Amount of Virus to Add: The amount of virus to add will depend on MOI.

Type of Vessel	Cell Density	Final Volume (culture medium + added virus)
96-well plate	2.0×10^4 cells/well	100 µL
24-well plate	5.0×10^5 cells/well	500 µL
12-well plate	7.0×10^5 cells/well	750 µL
6-well plate	1.0×10^6 cells/well	1 mL
60 mm^2 plate	2.5×10^6 cells/plate	3 mL
25 cm^2 flask	3.0×10^6 cells/flask	5 mL
75 cm^2 flask	5.0×10^6 cells/flask	10 mL
150 cm^2 flask	1.8×10^7 cells/flask	15–20 mL
spinners (all)	$2.0–2.5 \times 10^6$ cells/mL	no more than half of the total volume of the flask

Processing Time Point Samples

Introduction

If you are using the protocols on pages 35–38, you should now have a total of twenty time point samples, with two samples for each of the ten time points you have harvested (five for each MOI). Set aside the supernatant samples on ice until you are ready to proceed to SDS-PAGE and/or western blot analysis. The following table summarizes the labeled samples you will have at this point.

MOI	Time Point	Supernatant	Cell Pellet
5	T=24 T=48 T=72 T=96 T=120	5S24 5S48 5S72 5S96 5S120	5P24 5P48 5P72 5P96 5P120
10	T=24 T=48 T=72 T=96 T=120	10S24 10S48 10S72 10S96 10S120	10P24 10P48 10P72 10P96 10P120
0	Uninfected (24–48 hours.)	control (S)	control (P)
5–10	Wild-type Infected 24–48 hours.	WT(S)	WT(P)

Important

If you are expressing a secreted protein (e.g., using pMelBac or a native signal sequence), you will need to analyze the **supernatant** for the presence of **secreted, recombinant protein** and the **cell pellet** for the presence of **unprocessed recombinant protein**. You will need to make a lysate for your cell pellet samples. When compared to the supernatant sample, the lysate sample will provide information on whether or not the recombinant protein is being secreted, and will give you an idea of how much protein has been secreted versus how much protein is still intracellular at different time points. This will help you to optimize your time points for maximal protein levels.

Materials Required

You will need the following reagents and equipment:

- Phosphate-buffered saline (see page vi for ordering information)
- Tris-buffered saline (TBS, see **Recipes**, page 62)
- Protease inhibitors (see next page)
- 18-gauge needle and 3 cc syringe
- *Optional*: Triton X-100 or Nonidet P-40 (NP-40)
- *Optional*: Sonicator with microtip

Continued on next page

Protease Inhibitors

We recommend that you add one or more protease inhibitors to each of the lysis buffers that are described in the protocols below. The following table summarizes recommended protease inhibitors, their method of action and working concentrations.

Protease Inhibitor	Method of Action	Stock Solution	Working Concentration
PMSF	Serine protease inhibitor	10 mg/mL in isopropanol	100 µg/mL
Leupeptin	Serine and thiol protease inhibitor	50 µg/mL in deionized water	0.5 µg/mL
Aprotinin	Serine protease inhibitor	50 µg/mL in deionized water	0.5 µg/mL
Pepstatin A	Acid protease inhibitor	100 µg/mL in methanol	1 µg/mL

You can store all of the above protease inhibitor solutions at –20°C except for PMSF. Store PMSF at room temperature in isopropanol. PMSF is not stable in aqueous solution and therefore must be added to the lysis buffer just before use.

PMSF (phenylmethylsulfonylfluoride) is very harmful if inhaled, swallowed, or contacted by the skin. Wear protective clothing and gloves when handling.

Continued on next page

Processing Time Course Samples, continued

Detergent Lysis

Detergent lysis is a quick and efficient way to lyse cells and extract intracellular protein. The protocol below uses Triton X-100, but you may also NP40. If you do not want to use detergent to lyse your cell samples (e.g., sensitivity of your protein to detergent), see protocols for sonication and/or freeze-thawing, pages 43–44.

1. Place all cell pellets from the time course on ice. Be sure to include the control sample.
2. Make up 2–5 mL of lysis buffer (0.1% Triton X-100 in PBS or TBS). You will be using 100 µL for each 10^6 cells.
3. Add each of the protease inhibitors (Leupeptin, Aprotinin and Pepstatin A) at the working concentrations described on the previous page. You should perform this step on ice. **Add PMSF just after adding the lysis buffer to the cell pellet (Step 5).**
4. Add lysis buffer to each cell pellet. You will be adding 100 µL for each 10^6 cells in the pellet.

   Adherent Cell Procedure: If you followed the adherent cell procedure (page 37), use 100 µL for each cell pellet.

   Suspension Cell Procedure: If you followed the suspension cell procedure (page 38), use 200 µL for each cell pellet.
5. Add PMSF to each sample to a final concentration of 100 µg/mL.
6. Vortex each cell sample to break up the cell pellet and begin lysis.
7. **Lysis/Incubation:** Incubate all samples on ice for 30–45 minutes, vortex at 10 minute intervals to assist lysis.
8. Once all samples have been lysed, pellet cellular debris at 1,000 × *g* for 10 minutes at 4°C.

   Check for lysis efficiency: To check for cell lysis, take a 10 µL sample, add 1 µL of Trypan Blue and load onto a hemacytometer. See **Growth and Maintenance of Insect Cell Lines** for protocols. All cells should stain blue when lysis is complete.
9. Transfer supernatant (lysate) to a new tube. Keep on ice. Proceed to **Analyzing Time Point Samples,** page 45.

   Note: We recommend that you save the pellet from the lysate (the insoluble portion) as it may be useful for analysis if you cannot detect proteins in the lysate or the supernatant.

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Lysis by Sonication

Sonication is an alternate method of lysis for those who do not wish to use detergent. You will need access to a sonicator with a microtip. Be sure to follow the manufacturer's recommendations for sonication of eukaryotic cells. If you do not have access to a sonicator and do not wish to use detergent lysis, proceed to the protocol for **Freeze-Thawing**, page 44.

1. Place all cell pellets from the time course on ice. Be sure to include the control sample.
2. Make up 2–5 mL of PBS or TBS. Add each of the protease inhibitors (Leupeptin, Aprotinin and Pepstatin A) at the working concentrations described on page 41. You should perform this procedure on ice. **Add the PMSF at Step 4.**
3. Add buffer with protease inhibitors to each cell pellet. You will be adding 100 µL for each 10^6 cells in the pellet. Try not to disturb the pellet; sonication of a pellet gives a more effective burst at the onset of lysis.

   Adherent Cell Procedure: If you followed the adherent cell procedure (page 37) use 100 µL for each cell pellet.

   Suspension Cell Procedure: If you followed the suspension cell procedure (page 38) use 200 µL for each cell pellet.
4. Add PMSF to each sample at 100 µg/mL.
5. Sonicate each cell sample to break up the cell pellet and begin lysis. Keep all samples on ice while sonicating.
6. Incubate all samples on ice for 30–45 minutes, sonicate at intervals to assist lysis.
7. Once all samples have been lysed, pellet cellular debris at 1,000 × *g* for 10 minutes at 4°C.

   Check for lysis efficiency: To check for cell lysis, take a 10 µL sample, add Trypan Blue and load into a hemocytometer. See **Growth and Maintenance of Insect Cell Lines** manual for protocols. All cells should stain blue when lysis is complete. If all cells do not stain blue, sonicate the cells at intervals until they are all blue.
8. Transfer supernatant (lysate) to a new tube and place on ice. Proceed to **Analyzing Time Point Samples,** page 45.

   Note: We recommend that you save the pellet from the lysate (the insoluble portion) as it may be useful for analysis if you cannot detect proteins in the lysate or the supernatant.

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Lysis by Freeze-Thawing

Alternating cycles of freezing and thawing, combined with shearing through an 18-gauge needle, is an effective method for lysing cells. It avoids the use of detergents, when undesirable, and does not require special equipment.

1. Place all cell pellets from the time course on ice. Be sure to include the control sample.
2. Make up a freezing bath of isopropanol (or ethanol) and dry ice. Equilibrate a water bath to 37°C.
3. Make up 2–5 mL of PBS (see page vi for ordering information) or TBS (see page 62 for recipe). Add each of the protease inhibitors (Leupeptin, Aprotinin and Pepstatin A) at the working concentrations described on page 41. You should perform this procedure on ice. **Do not add PMSF until just after freeze-thawing.**
4. Add buffer with protease inhibitors to each cell pellet. You will be adding 100 µL for each 10^6 cells in the pellet.

   Adherent Cell Procedure: If you followed the adherent cell procedure (page 37), use 100 µL for each cell pellet.

   Suspension Cell Procedure: If you followed the suspension cell procedure (page 38), use 200 µL for each cell pellet.
5. Vortex each cell sample to break up the cell pellet and begin lysis. Keep all samples on ice.
6. Incubate all samples in freezing bath until just frozen (2 minutes).
7. Transfer to 37°C water bath until just thawed (2 minutes).
8. Repeat Steps 5 and 6 until you have repeated at least 5 cycles.
9. Transfer all samples to ice. **Add PMSF to a final concentration of 100 µg/mL.**
10. Using an 18-gauge needle and a 3 cc syringe, shear each sample by passing through the needle 3–4 times. Keep the samples on ice.
11. Once all samples have been lysed, pellet cellular debris at 1,000 × *g* for 10 minutes at 4°C.

    Check for lysis efficiency: To check for cell lysis, take a 10 µL sample, add the Trypan Blue and load onto a hemocytometer. See **Growth and Maintenance of Insect Cell Lines** manual for protocols. All cells should stain blue when lysis is complete. If all cells do not stain blue, repeat shearing (Step 10) until they do.
12. Transfer supernatant (lysate) to a new tube and place on ice. Proceed to **Analyzing Time Point Samples,** page 45.

    Note: We recommend that you save the pellet from the lysate (the insoluble portion) as it may be useful for analysis if you cannot detect proteins in the lysate or the supernatant.

Identification of Recombinant Protein

Introduction

There are many different methods that you can use to identify your recombinant protein expressed using the baculovirus expression system. Many of them require a monoclonal or polyclonal antibody for detection, but there are alternative protocols that you can use which do not require an antibody. This section provides information to help you identify your recombinant protein. You can find detailed protocols in **Protocols to Evaluate Expression,** beginning on page 51.

Analyzing Time Point Samples

The next step after lysing your cell pellets from all of your time point samples is to analyze for your protein by either SDS-PAGE or western blot (see protocols on pages 51 and 52, respectively) to determine the optimal time point. Once you determine the optimal time point for expression, you may use the MOI and cell line you have tested to proceed with **Large-Scale Expression** (page 49), or you may wish to try optimization using other MOIs or cell lines (see **Optimizing Protein Expression Levels,** page 47).

Analyzing Secreted Proteins: In addition to analyzing the supernatant, we recommend that you analyze the lysate as well to determine if all of your protein is being secreted. This can assist you in optimizing your time course for expression.

Analyzing Intracellular Proteins: We recommend that, in addition to the cell lysate, you also analyze the supernatant to determine if you have lost any protein due to cell lysis during infection. This can assist you in optimizing your time course for expression.

Detecting Recombinant Protein

Use the table below to determine the best method to detect your recombinant protein.

Do you have an antibody to the recombinant protein?	Use these methods:
If you **do** have an antibody:	1. Western Blot (page 52). Do this first. 2. Immunofluorescence (page 57). If neither of these works for you, proceed to methods for those who do not have an antibody.
If you **do not** have an antibody:	1. Metabolic labeling (page 59). 2. Northern blot (*i.e.*, Cytoplasmic RNA Purification and RNA dot blots, page 55).

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Identification of Recombinant Protein, continued

If You Have an Antibody

If you have a monoclonal or a polyclonal antibody to your protein, we recommend that you perform western blot analysis. If you are using a construct that was derived from pBlueBacHis2 (or pBlueBacHis), you can use the Anti-Xpress™ Antibodies (see page vi) which recognizes the Xpress™ leader peptide with the sequence Asp-Leu-Tyr-Asp-AspAsp-Asp-Lys.

Antibody detection methods will help to determine when and where your protein is being expressed during the time course. It is often very difficult to assay for intracellular proteins using only SDS-PAGE analysis because of the high background of cellular proteins found in insect cell lysates. We also recommend a control of uninfected cells or wild-type infected cells as background can be a problem even with the use of monoclonal antibodies. You may also use immunofluorescence (page 57).

If You Do Not Have an Antibody

If you do not have an antibody to your recombinant protein, and are having difficulty identifying your protein over background using SDS-PAGE (page 51), you may use metabolic labeling (page 59) or Northern blot analysis (page 55).

Glycosylation

When expressing and purifying a glycosylated protein in a heterologous expression system, it is desirable to quickly determine whether the protein is glycosylated properly. Recently, some protocols for carbohydrate analysis of proteins have been published to allow the molecular biologist to characterize glycosylated proteins of interest (Ausubel *et al.*, 1994). Further information about glycosylation in eukaryotes is available in a recent review (Varki and Freeze, 1994).

Other AcMNPV Genes and ORFs

Other native AcMNPV proteins may be prominent at the time point that you have selected for expression. You may wish to know more about what other proteins are expressed during infection in addition to your own. For more information on other AcMNPV genes and their protein sizes and locations in the genome, see O'Reilly *et al.*, 1992.

Optimizing Protein Expression Levels

Introduction

Once you have successfully identified your recombinant protein, you can optimize expression levels. This is usually done by manipulating the MOI (multiplicity of infection), the time of harvest and/or the cell line used. The following section will give you some guidelines on how to optimize expression levels.

Trying Different Cell Lines

One of the ways to optimize protein expression is to carry out a time course in more than one cell line (e.g., you might try both Sf9 cells and High Five™ cells). This will determine if the protein is expressed more efficiently in one cell line versus the other. The High Five™ cell line has been shown to express proteins at higher levels than the Sf9 cell line, with secreted proteins showing the most dramatic increase (see below for more information on High Five™ cells). A time course must be performed for each cell line and each MOI that is tested in that cell line (see **Time Course for Expression of Recombinant Protein,** page 34).

High Five™ Cells

High Five™ cells (see page v for ordering information) are particularly well suited for expression of secreted recombinant proteins. This cell line (BT1-TN-5B1-4) was originally developed by the Boyce Thompson Institute, Ithaca, NY and originated from the egg cells of the cabbage looper, *Trichoplusia ni*, the native host of AcMNPV (Davis *et al.*, 1992). This cell line has the following characteristics:

- Grows well in monolayer and doubles in less than 24 hours for ease of use
- Adaptable to suspension culture and serum-free medium for high-level protein expression and purification
- Provides 5–10 fold higher secreted expression than Sf9 cells (Davis *et al.*, 1993)

For more information about High Five™ cells or a protocol for adaptation to suspension culture, call Technical Support (see page 76).

Trying Different MOIs

Multiplicity of infection can greatly affect your expression results. It has a direct influence on the kinetics of expression and the percentage of cells that are infected at time zero. It is recommended that several MOIs in the range of 5–10 be tested prior to large scale expression. A time course must be performed for each MOI that is tested (see **Time Course for Production of Recombinant Protein**, page 34).

If you have performed the time course using an MOI of 5 and an MOI of 10, you may want to try an intermediate MOI to optimize infection kinetics.

For example: If an MOI of 5 gives you protein over a wide range of times, but an MOI of 10 lyses all infected cells before sufficient protein can accumulate, you might want to try an MOI of 6 and/or an MOI of 8. The objective in trying different MOIs is to find the MOI which yields the highest protein levels and the least loss due to lysis and proteolysis.

Continued on next page

Optimization of Protein Expression Levels, continued

Optimizing Time Points

Once you have determined the general time frame during which your protein is expressed (e.g., between 48 and 72 hours), you may try several intermediate time points between these two times (e.g., 52, 60, 68 hours). This will enable you to maximize the amount of protein you can harvest before proteolysis occurs. Simply repeat the **Time Course for Production of Recombinant Protein**, page 34, using your selected intermediate time points.

Note: This must be repeated for each cell line and for each MOI that you wish to test. Change only a single, given variable in any one flask.

Using Suspension Culture vs. Adherent Culture

The use of suspension culture (spinner or shake flask) versus adherent culture can increase the cell density per mL of culture, and therefore can potentially increase the relative yield of protein per mL of culture. Protocols for suspension and adherent culture can be found in **Time Course for Production of Recombinant Protein,** pages 37–38.

Large-Scale Expression of Recombinant Protein

Introduction

Now that you have successfully optimized expression levels, you may wish to proceed to large-scale expression of your recombinant protein. For some researchers, this may mean moving to suspension culture for the first time or moving up to larger vessels (1 liter or more). Others may wish to go larger still and utilize airlift bioreactors and/or fermenters. This section will summarize the requirements and options that exist for large-scale expression of protein using the baculovirus expression system.

Large-Scale Expression in Spinner Flasks

If you are moving into suspension culture or scaling up your suspension culture up to 1 liter spinner flasks, see the sections on Suspension Cell Culture in the **Growth and Maintenance of Insect Cell Lines** manual. This manual provides information on how to adapt Sf9, Sf21, or High Five™ insect cell lines to suspension culture as well as protocols for maintaining and scaling-up suspension cultures.

To scale-up to 1 liter spinner flasks (500 mL total culture volume), we recommend the following:

- Generate a large-scale, high-titer stock of the desired recombinant virus (see page 29). This will allow you to infect many large-scale cultures and ensure consistency in protein expression.
- Start with 100 or 250 mL spinner flasks (50–125 mL of insect cell culture) and scale up to 1 liter spinners with 500 mL of cell suspension.
- Seed cultures at 1×10^6 cells/mL and subculture cell suspension when the density reaches $2.0–2.5 \times 10^6$ cells/mL.
- Check cell viability daily to ensure the culture is >95% viable.
- Add Pluronic F-68 to a final concentration of 0.1% in your spinner culture. This protects the cells from shearing forces and allows you to increase the impellar speed to 120 rpm for larger cultures. Increasing the impeller speed increases aeration of the culture for better growth.

Continued on next page

Large-Scale Expression of Recombinant Protein, continued

Large-Scale Expression Options

The following table summarizes other methods, requirements, benefits and references for scale-up production of recombinant protein using the baculovirus expression system.

Method	Requirements	Benefits	References
Stirred Bioreactor	For a 5-liter Bioreactor: • 5-liter bioreactor • Sterilized tubing • Microbial air filters • High purity nitrogen, oxygen, and air. • pH, dissolved oxygen and temperature probes. • External dissolved oxygen controller. • External pH controller. • Peristaltic pump for acid/base lines. • Linear recorder to monitor dissolved oxygen and pH control. • Laminar flow hood in close proximity to the bioreactor.	• Addresses increased oxygen needs of large-scale culture. • Controlled growth and optimization of variables in the culture. • Increased cell densities. • Elevated protein production. • Reproducible results for batch production of protein.	(Tom *et al.*, 1995) (Murhammer and Goochee, 1988) (Maiorella *et al.*, 1988) (O'Reilly *et al.*, 1992)
Airlift Fermentor	For a 5-liter Airlift Fermentor: • 5-liter airlift fermentor system • Dissolved oxygen control module. • Dissolved oxygen electrode. • Microbial air filters. • 1/4" stainless-steel tubing. • Silicone tubing. • Circulating water bath.	• Addresses increased oxygen needs of large-scale culture. • Control over environmental variables in the culture. • Increased cell densities. • Elevated protein production.	(Maiorella *et al.*, 1988) (Murhammer and Goochee, 1988) (Onken and Weiland, 1983) (O'Reilly *et al.*, 1992)
Insect Larvae	• *Trichoplusia ni* larvae and facilities to grow larvae	• More accurate posttranslational modification of recombinant protein – does not rely on one cell type only. • Higher levels than in cell culture. • Inexpensive.	(Medin *et al.*, 1990) (Wood *et al.*, 1993)

Protocols to Evaluate Expression

SDS-PAGE Analysis of Recombinant Protein

Introduction

This section provides references and suggestions for analyzing protein expression by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). SDS-PAGE will allow you to analyze the solubility, purity, and yield of the fusion protein. We recommend that you use 10% Tricine gels to analyze cell lysates if the fusion protein is less than 20 kD. This gel system resolves differences between low molecular weight proteins.

Types of SDS-PAGE Gels

There are many types of SDS-PAGE gels. A wide range of pre-cast Novex® NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Life Technologies. The NuPAGE® Gel System avoids the protein modifications associated with LaemmLi-type SDS-PAGE, ensuring optimal separation for protein analysis. For more information about the pre-cast polyacrylamide gels available from Life Technologies, visit our website (www.lifetechnologies.com) or contact Technical Support (see page 76).

General Procedure for Sample Preparation

1. Before lysing the cells and preparing your samples, assemble the SDS-PAGE gel or use a pre-cast polyacrylamide gel.
2. Lyse the cells (see **Processing of Time Point Samples,** page 40). Take 10 µL aliquots from supernatant and mix with 10 µL of 2X SDS-PAGE sample buffer (see page vi). Do the same for each cell lysate sample. Be sure to also do the same for the controls (see **Controls,** page 36).
3. Boil the samples 5 minutes and load all 20 µL onto the SDS-PAGE gel. If aggregates form after boiling, remake the sample and load without boiling.
4. Electrophorese the gel and process according to your protocol of choice.
5. Analyze for the presence of the desired protein, extent of processing or solubility, purity, or yield.

Western Blot (Immunoblotting)

Introduction

This procedure can be used for detection of recombinant protein expression particularly when levels of expression are low. The table below outlines the basic steps of a western blot. You may also sue the iBlot® Dry Blotting System available separately from Life Technologies. The iBlot® device is a self contained unit that uses disposable blotting stacks with integrated nitrocellulose and PVDF membranes, and allows blotting of proteins from polyacrylamide gels efficiently and reliably in less than seven minutes without the need for additional buffers or an external power supply. For more information on the iBlot® Dry Blotting System refer to our website (**www.lifetechnologies.com**) or contact Technical Support (page 76).

Step	Action
1	Run an SDS polyacrylamide gel of the purified or partially purified protein or cell lysate with appropriate controls.
2	Transfer the proteins electrophoretically to a nitrocellulose or PVDF membrane.
3	Probe the blot with antibody to your recombinant protein.
4	Incubate the blot with an IgG secondary antibody conjugated to a color development enzyme such as alkaline phosphatase or horseradish peroxidase (HRP).
5	Add the color development substrate and watch for the appearance of colored band on the nitrocellulose or PVDF membrane. You may also use chemiluminescence to detect the band representing your protein.

Solutions Required

You may use alkaline phosphatase conjugated to the appropriate IgG antibody to detect binding of your antibody to the recombinant protein. Other detection methods are also suitable to detect your protein. Note that alkaline phosphatase is more sensitive than HRP. The following materials and solutions are needed for immunoblotting:

- Tris-Buffered Saline (TBS: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5)
- Tris-Buffered Saline + Tween 20 (TBST: TBS plus 0.05% Tween-20, w/v)
- Bovine Serum Albumin (BSA), powdered
- Blocking buffer (TBS + 3% BSA, w/v)
- Dilution buffer (TBST + 1% BSA, w/v)
- Secondary Antibody: IgG (whole molecule) Alkaline Phosphatase
- Enzyme Substrates for alkaline phosphatase: Bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). Prepare 10 mL of a 50 mg/mL stock solution of each substrate. Use 100% dimethylformamide (DMF) to dissolve BCIP and 70% DMF to dissolve NBT. We recommend the ready-to-use Novex® AP Chromogenic Substrate (BCIP/NBT), available separately from Life Technologies, as a convenient alternative (see page vi).
- Alkaline phosphatase buffer (100 mM diethanolamine, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5)

Continued on next page

Immunoblotting Protocol

Prepare an SDS polyacrylamide gel (either Tris/Glycine or Tris/Tricine) designed to resolve your recombinant protein. Prepare your samples for electrophoresis. For information about SDS-polyacrylamide gel electrophoresis, see (Ausubel *et al.*, 1994).

1. Load your samples and electrophorese your SDS polyacrylamide gel.
2. Transfer proteins to nitrocellulose electrophoretically. We use 25 mM Tris, 192 mM glycine, 20% v/v methanol pH 8.3 as a transfer buffer.
3. Run at 100V, 150 mA (100V, 240 mA at the finish) for 1 hour. Be sure to have a cooling system in place and operational with these electrophoretic settings. You may also transfer overnight at 30V, 40 mA (30V, 90 mA at the finish).
4. Remove nitrocellulose and incubate in 10 mL blocking buffer. Gently agitate using a rocker platform for 1 hour at room temperature.
5. Wash nitrocellulose in 20 mL TBST for 5 minutes with gentle agitation. Repeat wash once more.
6. Transfer membrane to a tray containing the antibody to your recombinant protein. Dilute the antibody into the appropriate amount of dilution buffer. Incubate with gentle agitation for 1–2 hours.

   Note: You may prefer overnight incubation, since longer incubations usually increase sensitivity of detection. In our hands, 1 hour incubation is usually sufficient for detection.
7. Transfer membrane to a tray containing 20 mL TBST and wash for 5 minutes with gentle agitation. Repeat wash once more.
8. Transfer membrane to a tray containing the secondary antibody. Dilute the secondary antibody according to the manufacturer's recommendation into dilution buffer. Incubate with gentle agitation for 1 hour.
9. Wash two times in TBST as described in Step 7.

Continued on next page

Detection Reaction

The protocol below was developed using alkaline phosphatase-conjugated secondary antibody. If using a different system, be sure to follow the manufacturer's instructions.

1. Transfer blot from Step 9, previous page, to a tray containing TBS and wash for 5 minutes to remove detergent.
2. Prepare fresh substrate solution immediately before use.

   Note: For alkaline phosphatase conjugated antibody, add 66 µL of the NBT stock to 10 mL alkaline phosphatase buffer and mix well. Then add 33 µL of the BCIP stock and mix thoroughly. Use within 1 hour.
3. Rinse the blot twice with alkaline phosphatase buffer, then add 10 mL of the substrate solution. Incubate with gentle agitation at room temperature and watch for color development.

   Note: When detecting higher concentrations of protein, the purple signal should develop within 10 minutes. Lower concentrations will take longer to develop but should be visible within 30 minutes. Color development will continue for up to 4 hours; however, high backgrounds will occur with longer incubation times.
4. Stop the color development by washing the membrane in distilled water for 10 minutes. Change the water at least once during the 10 minute incubation.
5. Air-dry membrane on filter paper.

Cytoplasmic RNA Purification and RNA Dot Blots

Introduction

If the level of protein expression is not as high as expected, compare the steady-state level of polyhedrin mRNA produced by wild-type virus with the recombinant mRNA. You can accomplish this by using a probe specific for the polyhedrin promoter and the foreign gene mRNA (O'Reilly and Summers, 1988b). If the steady state mRNA levels are similar, the low level of protein expression may be due to post-transcriptional factors and processes such as the efficiency of ribosomal binding, co- and posttranslational processing, protein stability, etc.

Growth of Virus

You must evaluate two isolates (*i.e.*, two high-titer stocks amplified from two separate plaques) as two independent stocks of virus are necessary to conclude that any variation in the levels of RNA (or protein) are due to the viral construct, and not due to variation in MOI or many other factors which might affect the end result. Include duplicate controls of wild-type virus and/or uninfected cells.

Note: The following is an example of a time course that you can use. However, you may also use other flask sizes, including spinners (for seeding densities and volumes, see page 39). Infections in spinner culture eliminate the need for separate flasks for each time point as you can simply remove a sample from the spinner at each desired time point. MOIs other than 10 may also be used (see **Multiplicity of Infection**, page 34).

1. Seed 25 cm^2 tissue culture flasks with 6×10^6 cells per flask.

   Note: You will require 2 flasks per virus per 24-hour time period plus duplicate wild-type and mock-infected controls. For example, for a 72-hour experiment with 2 virus types, you would seed 12 flasks for the viruses, and 4 flasks for controls.
2. Infect the flasks at an MOI of 10 with the desired virus.
3. Incubate at 27°C, harvesting one set of duplicates at 24-hour intervals (see below).

Harvesting and Extracting RNA

At each time point, you will need to harvest the cells and extract the RNA. **Be sure to use RNase-free reagents and equipment.**

1. To harvest cells:

   Adherent: Slough cells from the monolayer, remove cell suspension and transfer to a sterile 15 mL conical centrifuge tube.

   Suspension: Pipette 2 to 3 mL of cell culture from the spinner flask and transfer to a sterile 15 mL conical centrifuge tube.
2. Centrifuge the samples at 1,000 rpm for 12 minutes.
3. Discard the supernatant and resuspend each pellet in 500 µL of (4°C) Grace's insect medium and transfer to an RNase-free, sterile microcentrifuge tube.
4. Centrifuge these samples at 1,000 rpm for 15 seconds and discard the supernatant.
5. Resuspend the pellet in 45 µL of cold, RNase-free 1X TE buffer (10 mM Tris, 1.0 mM EDTA) and place on ice.

Procedure continued on next page

Continued on next page

Cytoplasmic RNA Purification and RNA Dot Blots, continued

Harvesting and Extracting RNA, continued

Procedure continued from previous page

6. Lyse cells by adding 5 µL of RNase-free 5% NP-40 detergent. Mix by vortexing. Allow to incubate on ice for 5 minutes and vortex again.
7. Add an additional 5 µL of NP-40 and mix.
8. Centrifuge for 2–5 minutes at 1,000 rpm to sediment the nuclei. A pellet should be visible.
9. Transfer 50 µL of the supernatant to a new microcentrifuge tube containing 30 µL of 20X SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) and 20 µL of formaldehyde.
10. Mix and incubate at 65°C for 15 minutes then freeze this cytoplasmic fraction at –70°C until ready for use.

Dot Blot Hybridization of RNA

1. Dilute 10 µL of the cytoplasmic fraction into 190 µL of 15X SSC (sterile, RNase-free 2.25 M NaCl, 225 mM sodium citrate, pH 7.0) in a round bottom 96-well microtiter plate.
2. Make 1:5 dilutions by taking 40 µL from the above dilution and mixing with 160 µL of 15X SSC. Similarly, make 1:25 dilutions by mixing 40 µL of the 1:5 dilution with 160 mL of 15X SSC.
3. Assemble the dot blot apparatus and prepare membrane as recommended by manufacturer. You should equilibrate the membrane with 15X SSC before blotting.
4. Apply 75 µL aliquot to each well. You should apply duplicates for each of the dilutions described above including the control RNAs.
5. Process the membrane according to the manufacturer's instructions in preparation for nucleic acid probing.
6. Hybridize using appropriate probes.

Indirect Immunofluorescence of Recombinants

Introduction

Indirect immunofluorescence analysis of insect cells infected with a recombinant virus may be used to evaluate protein expression. Immunofluorescence microscopy is particularly useful for studying proteins that localize, for example, to the cell nucleus or are expressed in the cytoplasm or on the plasma membrane. You can optimize the following protocol like the time course for protein expression (page 47). For more information regarding the factors affecting optimization, see pages 47–48.

Preparing Coverslips

1. Soak coverslips (Bellco #1943-00015) overnight in 70% ethanol + 1% HCl. Rinse 8 times with water, then sterilize by baking overnight.
2. Using aseptic technique, transfer one coverslip to each well of a 12-well plate.
3. Treat the coverslips for $\geq$ 5 minutes with a drop of Poly-D-Lysine (Sigma #P7280; 200 µg/mL in water).
4. Rinse the coverslips in the wells with water.

Attaching Cells to Coverslip

1. Seed 7×10^5 cells per well in 750 µL complete TNM-FH per well. Let cells attach for 1 hour.
2. Add enough high-titer virus stock to yield an MOI of 5–10 (for more information on MOI, see page 34).
3. Allow infections to proceed for 48 hours (other time points may also be assessed, see page 48).
4. Remove the medium and rinse each well with 1 mL of PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9).
5. Meanwhile, prepare an incubation chamber by lining the bottom and top of a plastic tip box with 3 MM filter paper. Keep the filter paper wet with distilled water throughout the incubation steps. Place a piece of parafilm cut to size on the bottom filter paper.

Fixing Cells

1. For each coverslip, place one 100 µL drop of 2% paraformaldehyde (prepared in PHEM buffer and heated with stirring in a hood to ~90°C for 30–45 minutes) on the parafilm.
2. Place the coverslip **cell side down** on the drop, close the incubation chamber, and allow fixing for 10–15 minutes.
3. Float the coverslip by carefully squirting 200 µL PHEM buffer underneath it.
4. Transfer the coverslip, cell side down, to a 100 µL drop of 0.1% Triton® X-100 prepared in PHEM buffer.
5. Solubilize for 10 minutes in the incubation chamber.
6. Float the coverslip as in Step 3, and rinse by placing on a 200 µL drop of PBS. Repeat the rinse.

Continued on next page

Indirect Immunofluorescence of Recombinants, continued

Adding Antibody

1. Transfer the coverslips from Step 6, previous page, cell side down, to a drop containing the primary antibody diluted in PHEM buffer plus 1% normal goat serum.
2. Incubate for 60 minutes in the incubation chamber.
3. Float the coverslip and rinse by placing on a 200 µL drop of PBS. Repeat the rinse twice.
4. Place the coverslip on a 50 µL drop of fluorescein isothiocyanate-conjugated goat anti-rabbit/mouse diluted appropriately in PHEM buffer containing 1% normal goat serum.
5. If desired, stain cell nuclei by transferring coverslips, cell side down, to a 10 µL drop of 4´,6-diamidino-2-phenylindole (DAPI, Sigma; 1 µg/mL in water).
6. Float coverslips and transfer to rack (Thomas #8542-E40). Rinse 3 times for 5 minutes in beaker containing PBS. After last wash, dab the edge of the coverslip onto a tissue to remove most of the PBS.
7. Place coverslip, cell side down, on 5 µL drop of mountant on a microscope slide.

   Note: To prepare the mountant, dissolve 100 mg p-phenylenediamine in PBS, adjust pH to 9.0 and the volume to 10 mL, and add 90 mL glycerol. Store the mountant in the dark at –20°C.
8. When viewing the slides, remember that the nuclei of infected cells are very large, and may "fill" the cell. Staining the cells with DAPI (Step 5) aids in distinguishing the nuclei from the cytoplasm in infected cells.

Metabolic Labeling of Recombinant Protein

Introduction

The following protocol will enable you to identify proteins that are not expressed at sufficient levels to be visualized by Coomassie staining using SDS-PAGE analysis. This protocol is also useful in cases where an antibody to the protein is not available, or where the polyclonal antibody used produces too much background to successfully visualize the recombinant protein.

Choice of Isotope

In some cases, a protein may have low methionine content. In these cases, it is necessary to label with ^{35}S-cysteine to incorporate sufficient label for autoradiography.

Materials Needed

- 6-well plate
- Log-phase insect cells (Sf9, Sf21 or High Five™ cells)
- Grace's Insect medium (methionine-deficient) or EX-CELL 400 (methionine-deficient)
- ^{35}S-methionine and/or ^{35}S-cysteine (depending on the amino acid content of your protein)

Protocol

The following protocol is a time course for production of metabolically labeled protein and may need to be optimized as with the **Time Course for Production of Recombinant Protein,** page 34. See this section for guidelines on optimization. You must perform a separate assay for each cell line and for each MOI that you wish to test.

1. Seed each well in a 6-well plate with 10^6 cells. Let the cells attach for 30 minutes.
2. Label one of each of the wells as follows: T=24, T=48, T=72, T=96, T=120. These represent the times of harvest for each well. You should label the sixth well "control" to use with uninfected cells as a background control.
3. Remove all medium and replace with 1 mL of complete TNM-FH (or EX-CELL 400 for High Five™ cells).
4. Infect with enough of your high titer stock to yield an MOI of 5.
5. Incubate at 27°C. Parafilm the edges or incubate in a plastic bag with a moist paper towel enclosed to prevent evaporation.

   You must perform Steps 6–13 for each time point beginning 6–8 hours prior to lysis.
6. At 6–8 hours prior to the time point to be taken (e.g., at 18 hours post-infection for the T=24 well), remove the medium from the well and add 1 mL of methionine deficient Grace's medium (supplemented with 1–2% serum). Take care **not** to dislodge the cells from each well.
7. Incubate the plate at 27°C for one hour to starve them of methionine. This will enhance the uptake of the labeled methionine.

Procedure continued on next page

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Metabolic Labeling of Recombinant Protein, continued

Protocol, continued

Procedure continued from previous page

8. After the 1 hour incubation, add 100 Ci of ^{35}S-methionine or a combination of ^{35}S-methionine and ^{35}S-cysteine to each well.
9. Incubate at 27°C for 4–6 hours to allow incorporation of the label.
10. Scrape the cells from the well and transfer the entire solution (approximately 1–1.5 mL) from each well to a microcentrifuge tube.
11. Pellet cells at 800 × *g* for 10 minutes at 4°C. It is best to keep samples at 4°C or on ice to minimize proteolysis.
12. Transfer supernatant to a new tube, label these with the MOI, "S" for supernatant, and a number for the well from which you took the sample (e.g., "5S1" would be MOI = 5, supernatant, well #1). Do the same for the pellet samples (e.g., "5P1" would be the pellet from the same sample).
13. Wash the cell pellet gently with 2 mL PBS to remove excess label.
14. Store the washed cell pellet and the supernatant at –70°C. The cell pellet will be lysed later when all time points have been collected.
15. To lyse cell pellets, proceed to **Processing Time Point Samples,** page 40.

Autoradiography

Following lysis and SDS-PAGE analysis, you must dry the gel, expose the film with it overnight at –70°C, and then process for development.

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 using Grace's Insect Medium, Supplemented from GIBCO® (see page vi), add 55 mL of FBS. Mix well.
2. Add Gentamicin (see page vi) to 10 µg/mL final concentration.
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.

PEG/NaCl Solution

20% Polyethylene glycol 8000
1 M NaCl

1. For 100 mL, mix 20 g PEG 8000 and 5.84 g NaCl in 100 mL water.
2. Autoclave 20 minutes at 15 lbs/sq. in.
3. While the solution is still warm (~55°C), swirl carefully to mix thoroughly.

Agarose-TNM-FH Solution

In a sterile 100 mL bottle, dilute 25 mL of melted 2.5% agarose 1:1 with 25 mL of Grace's Insect Medium, 2X supplemented with FBS. Mix well and place the bottle in 47°C water bath until use.

X-gal, Blu-gal, or Bluo-gal Solution

50 mg/mL X-gal, Blu-gal or Bluo-gal

1. Dissolve 100 mg of X-gal, Blu-gal, or Bluo-gal (see page vi) in 2 mL dimethylformamide (DMF).
2. Store in an amber bottle at –20°C.

Continued on next page

Tris-Buffered Saline

20 mM Tris-HCl
500 mM NaCl
pH 7.5

1. Dissolve the following in 800 mL deionized water:
   2.42 g Tris base
   29.2 g NaCl
2. Adjust pH to 7.5 with concentrated HCl.
3. Bring the volume to 1 liter.
4. Autoclave for 20 minutes on liquid cycle to sterilize.
5. Store at 4°C or room temperature.

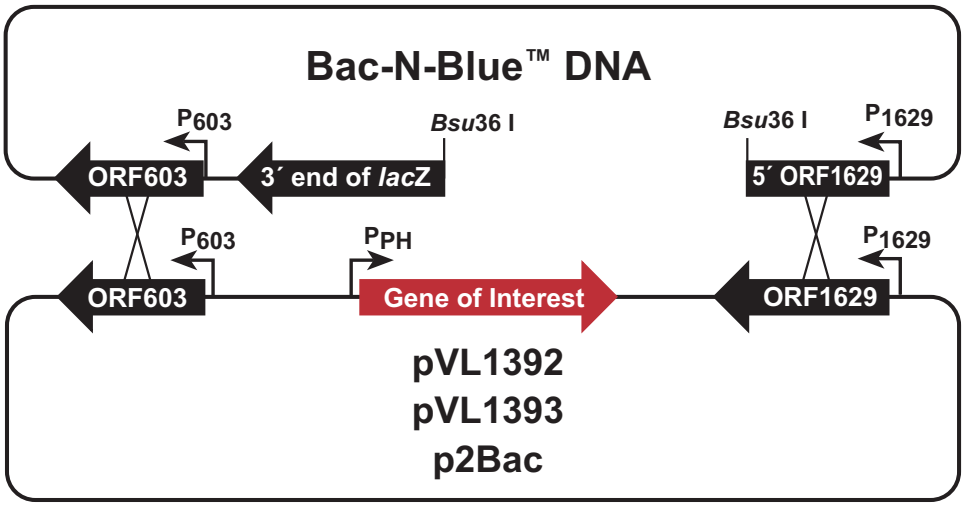
Recombination between Bac-N-Blue™ DNA and Transfer Vector

Recombination Events between Bac-N-Blue™ DNA and Specific Transfer Vectors

Recombination between Bac-N-Blue™ DNA and the transfer vector occurs at two sites: the ORF1629 sequences and either the ORF603 or *lac*Z sequences. If recombination occurs at the *lac*Z sequences, active β-galactosidase is produced, creating blue, recombinant plaques on medium containing X-gal or Bluo-gal. Bac-N-Blue™ DNA works with all our pBlueBac vectors and any polyhedrin promoter-based transfer vectors containing sequences from ORF603 and ORF1629.

Note: pVL1392, pVL1393, pBlueBacIII, pBlueBac4, pBlueBac4.5, pBlueBacHis A, B, and C, pBlueBacHis2 A, B, and C, pAc360, p2Bac vectors are no longer available from Life Technologies.

A. For vectors that do not contain *lac*Z sequences (*i.e.*, pVL1392, pVL1393, p2Bac), recombination occurs at ORF603 and ORF1629. Recombinant plaques (occ⁻) do not produce polyhedra and will appear dull and flat in appearance when compared to occ⁺ plaques that are shiny and crystalline in appearance.



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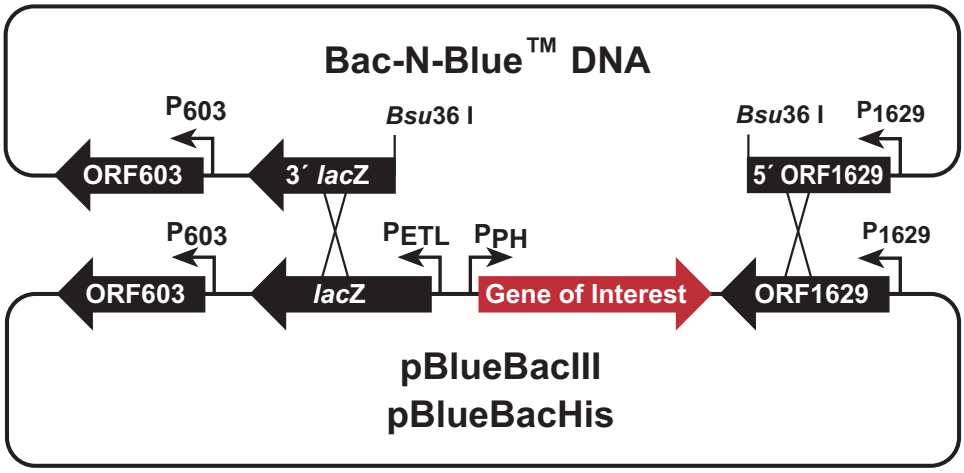
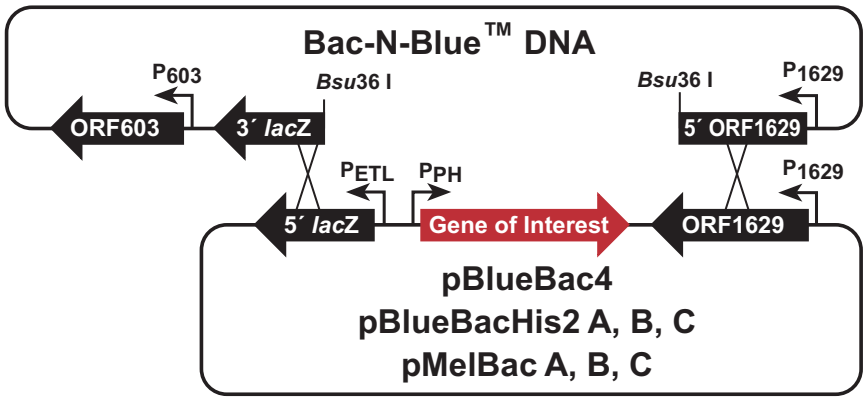
Recombination between Bac-N-Blue™ DNA and Transfer Vector, continued

Recombination Events between Bac-N-Blue™ DNA and Specific Transfer Vectors

B. For vectors that contain the complete *lac*Z gene under control of a baculovirus promoter (pBlueBacIII, pBlueBacHis), recombination can occur between the ORF1629 sequences and either the *lac*Z sequences or the ORF603 sequences, resulting in the formation of blue, recombinant plaques when plated on medium containing X-gal or Bluo-gal.



C. For vectors that contain only a 5´ portion of the *lac*Z and the ETL promoter (pBlueBac4.5, pBlueBacHis2, pMelBac A, B, and C), recombination occurs between the *lac*Z and ORF1629 sequences, forming blue, recombinant plaques on medium containing X-gal or Bluo-gal.



Additional Protocols

Trypan Blue Exclusion Assay

1. Mix 0.1 mL of Trypan Blue solution (see page vi) with 1 mL of cells and examine under a microscope at low magnification.
2. Dead cells will take up Trypan Blue while live cells will exclude it. Count live cells versus dead cells. Cell viability should be at least 98% for healthy log-phase cultures.

Purifying Budded Virus

For most purposes, it is not necessary to purify budded virus. You can store viral stocks as medium supernatants for extended periods of time at 4°C protected from light. However, there may be times when it is necessary to concentrate or purify budded (recombinant) virus. See Lee and Miller, 1978 for a protocol to purify budded virus.

Purifying DNA from Infected Cells

If you wish to purify total DNA from infected cells for Southern blot analysis, see Summers and Smith, 1985. You may also purify viral DNA by the above method **Purification of Budded Virus** and then proceed with cesium chloride/ethidium bromide density-gradient centrifugation (Ausubel, *et al.*, 1994).

Purifying DNA Polyhedral Occlusion Bodies

If you wish to purify wild-type AcMNPV DNA from infected cells, see (Miller and Dawes, 1978).

Baculovirus Biology and Life Cycle

Baculoviruses: A Brief Overview

Baculovirus Hosts: The baculoviruses *(Baculoviradae)* are a family of double-stranded DNA viruses that are found primarily in insects. They are not known to infect any nonarthropod hosts.

Baculovirus Family: The family can be divided into two subfamilies; occluded baculoviruses *(Eubaculovirinae)* and nonoccluded baculoviruses *(Nudibaculovirinae).*

Most Commonly Used: The two *Eubaculovirinae* most commonly used in expression work are *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV). Both contain circular genomes of approximately 130 kb.

AcMNPV vs. BmNPV: The basic technology for AcMNPV and BmNPV based systems are very similar, but AcMNPV has several advantages for cell culture applications. BmNPV is optimal for expression in larvae. The differences between these two baculovirus systems are summarized in the following table.

Why larvae?: Expression in larvae may provide other types of posttranslational modification. This is due to expression by many cell types rather than a single cell type (as in cell culture applications).

AcMNPV	BmNPV
Cell lines supporting replication double every 18–24 hours.	Cell lines supporting replication double every 4–5 days.
Broad range of transfer vectors.	Limited transfer vectors available.
Variety of promoters active at different times during infection.	May only express a single gene or a polyhedrin fusion under the polyhedrin promoter.
Can be used for expression in larvae, size of larvae is less preferable than BmNPV. Larvae size: 300–500 mg Yields: 50 µL of hemolymph per insect.	Preferred system for expression in larvae due to larger size of larvae. Larvae size: 5 g Yields: 0.5 mL of hemolymph per insect.

AcMNPV: *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is the prototype virus of the *Baculoviradae* family. AcMNPV is most commonly used in insect cell culture applications due to the superior cell lines and transfer vectors that are available for production of recombinant virus and protein. The Bac-N-Blue™ Baculovirus Expression System utilizes the AcMNPV system.

Continued on next page

Baculovirus Biology and Life Cycle, continued

Baculovirus Structure

Genome Size: The baculovirus genome is double-stranded and circular, averaging 80–200 kb. The genome of AcMNPV is 130 kb.

Viral Capsid Size: The name *baculo*virus refers to the rod shaped capsids of the viral particles. Baculovirus capsids range in size from 40–50 nm in diameter and from 200–400 nm in length (Harrap, 1972b).

Note: Capsids can extend in length to accommodate larger genomes (e.g., insertion of a recombinant gene).

Viral Capsid Structure: Inside the capsid, the baculovirus genome is condensed into a nucleoprotein structure called the *core.* This DNA *core* combined with the viral *capsid* constitute the *nucleocapsid.*

Nucleocapsid Formation: During AcMNPV infection, two forms of viral progeny are produced: budded virus particles, and occluded virus particles. They differ in the way each type of *nucleocapsid* is processed after it is made in the nucleus of infected cells.

1. **Budded virus** is processed by migrating through the plasma membrane, creating a loose membraned capsid.
2. **Occluded virus** is processed by acquiring a membrane through the nuclear membrane, creating a snug membraned capsid. Occluded virus is embedded in proteinaceous viral inclusions called polyhedra (Rohrmann, 1986). Occluded virus serves its primary function during the baculovirus life cycle in nature (see page 69).

Polyhedral Occlusion Bodies (PIBs): Occluded virus formed in the nucleus is embedded in a crystalline protein matrix. This matrix protein is known as **polyhedrin.** The polyhedrin matrix with multiple occluded virions embedded in it is referred to as a polyhedral occlusion body or PIB. PIBs have a carbohydrate rich external surface coat known as the *calyx* that is thought to contribute to its stability in nature (see page 69).

Wild-Type AcMNPV Virus: Polyhedral occlusion bodies can be seen in the tissue culture infection of wild type AcMNPV virus. The crystalline nature of the polyhedrin matrix gives the PIBs a refractive nature under a dissection microscope. Both occluded virus and budded virus are found in wild-type AcMNPV infected cells.

Recombinant AcMNPV Virus: Recombinant AcMNPV viruses lack the polyhedrin gene, do not display PIBs, and do not appear refractive. Budded virus is the only type of virus found in recombinant AcMNPV infected cells.

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Baculovirus Biology and Life Cycle, continued

Baculovirus Life Cycle in Nature

Occluded virus is responsible for horizontal transmission among susceptible insects, while the budded virus is responsible for secondary and cell-to-cell infection in cultured cells or in the insect host. Multiple occluded virions embedded in polyhedrin protein are released into the environment when the insect dies. The polyhedrin gene and the viral occlusions are not essential for virus infection or replication in tissue culture. The table and figure below depict the unique biphasic life cycle of a typical baculovirus.

Stage	Description
1	A susceptible caterpillar ingests the viral occlusions from a food source.
2	The occlusions dissociate in the gut of the susceptible caterpillar to release the infectious virus particles.
3	Virions invade the cells lining the gut, penetrate to the nucleus, and uncoat.
4	Viral DNA replication is detected by 6 hours.
5	By 10–12 hours post-infection, virus buds from the surface to infect other cells and tissues.
6	Late in infection (18–24 hours post-infection) the viral encoded polyhedrin protein assembles in the nucleus of the infected cell and virus particles become embedded in the proteinaceous occlusions.
7	Budded virus levels reach a maximum (1×10^8 pfu/mL) between 36 and 48 hours post-infection.
8	The viral occlusions accumulate to large numbers and the cells lyse.
9	The caterpillar stops feeding and undergoes rapid physical changes resulting in liquefaction. The larval disintegration releases the viral occlusions to the environment for consumption by the next caterpillar. The cycle repeats.

For a detailed review of the baculovirus life cycle and viral structure, see King and Possee, 1992, O'Reilly, *et al.*, 1992, or Richardson, 1995.

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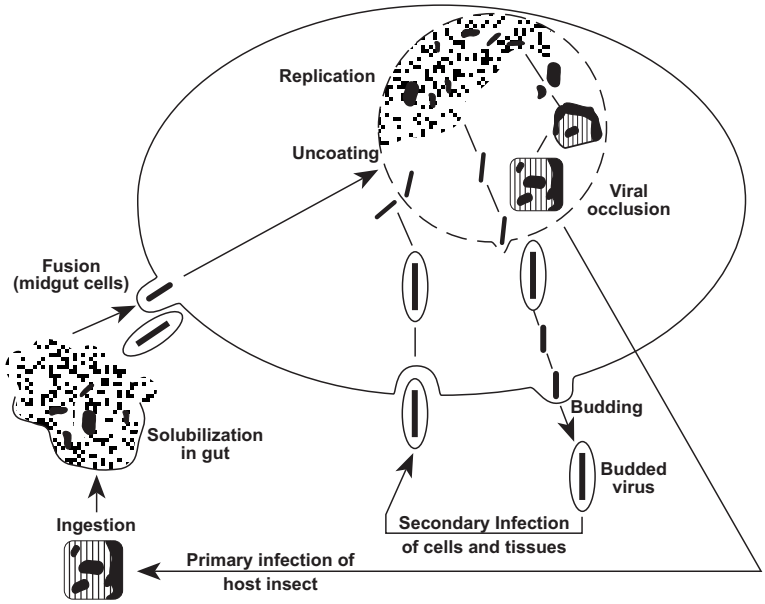
Baculovirus Biology and Life Cycle, continued

Diagram of Baculovirus Life Cycle

Figure adapted from "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", M. D. Summers and G. E. Smith, Texas Agricultural Experiment Station Bulletin No. 1555. and used with permission.



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Baculovirus Biology and Life Cycle, continued

Native Role of Viral Occlusions

Horizontal Transmission: Viral occlusions are an important part of the native virus life cycle, providing the means for horizontal transmission of the virus. When infected larvae die, millions of polyhedra are left in the decomposing tissue.

Protection: Viral occlusions protect the embedded virus particles from inactivation by environmental factors that would otherwise rapidly inactivate budded virus.

Dissemination in the Gut: When larvae feed on contaminated plants, they ingest the polyhedra. The occlusions dissolve in the alkaline environment of the caterpillar gut, releasing virus which invade and replicate in the cells of the midgut tissue. Secondary infection spreads to other caterpillar tissues via the budded form of the virus.

Baculovirus Life Cycle - In Cell Culture

In cell culture, the infection cycle occurs in three phases: early, late, and very late (O'Reilly, *et al.*, 1992).

Stage of Infection	Hours Post-infection	What is Occurring?	Visible Signs
Early	0–6	• Cells are being reprogrammed for viral replication. • Cytoskeletal rearrangements, host chromatin is dispersing in the nucleus.	• Nuclear Swelling.
Late	6–24	• Production of budded virus. • Extensive viral replication.	• Nuclear Swelling. • Viral budding (granular appearance of the cell).
Very Late	20–24	• Production of *occluded virus*. • Production of budded virus is greatly reduced or terminated. • Nucleus fills up with PIBs (in wild-type infection).	• PIBs in nucleus. • Refractive appearance of wild-type infected cells under the dissection microscope.
p10	72 +	• Fibrous material accumulates in the nucleus, most of it is *p10* (see page 66). • Cells are no longer synthesizing proteins and are lysing.	• Lysis. • Debris/cell death.

Gene Regulation

The Polyhedrin Gene

Sequence: The polyhedrin gene of AcMNPV has been mapped and sequenced. For a complete sequence of the polyhedrin gene, see O'Reilly, *et al*, 1992.

Function: The polyhedrin gene codes for a protein which serves as a matrix for imbedded nucleocapsids, found in polyhedral occlusion bodies. These viral occlusions are responsible for the transmission of the virus (insect to insect) in nature. The polyhedrin protein is not necessary for cell to cell transmission (e.g., tissue culture applications).

Very Late Gene: The polyhedrin gene is expressed during the very late phase of infection, beginning at about 20–24 hours post-infection.

Expression Levels: In infected *Spodoptera frugiperda* cell cultures, polyhedrin (29 kDa) accumulates to very high levels, typically 0.5 to 1 g/mL. This accounts for more than 50% of the total Coomassie stained protein in the cell when detected on SDS-polyacrylamide gels.

Nonessential for Replication: This gene has been shown to be nonessential for infection or replication of the virus in tissue culture (Smith *et al.*, 1983).

Occ$^-$ vs. Occ$^+$: Deletions or insertions inactivate the polyhedrin gene and result in the production of an occlusion-negative virus. An occlusion-negative virus will form occlusion-negative plaques (occ$^-$) that are distinctly different from those of a wild-type, occlusion-positive (occ$^+$) virus. These distinctive plaque morphologies provide a visual method to screen for recombinant viruses in which the wild-type AcMNPV polyhedrin gene has been replaced with a hybrid gene of choice. This screening process can be very difficult at first, but is very simple when using a blue-screening transfer vector such as pBlueBac4.5 or pBlueBacHis2. Both transfer vectors will produce a recombinant virus that is occ$^-$ and produce blue plaques that are easily visualized.

Ideal for Expression: The nonessential nature, the high levels of expression of the polyhedrin gene, and the ease with which recombinant occ$^-$ viruses can be detected makes the polyhedrin promoter particularly suitable for recombinant protein expression.

The p10 Gene

Sequence: The p10 gene of AcMNPV has been mapped and sequenced (O'Reilly, *et al.*, 1992).

Function: While occlusion bodies are being formed in the nucleus, a large amount of fibrous material accumulates in the nucleus and cytoplasm. Most of this fibrous material is made up of a 10 kD protein, p10 (VanDerWilk *et al.*, 1987). The p10 protein is thought to play a lytic role as deletion of the p10 gene has been shown to delay lysis up to two weeks post-infection (Williams *et al.*, 1989).

Very Late Gene: The p10 gene, like the polyhedrin gene, is expressed during the very late phase of infection, beginning at about 20–24 hours post-infection.

Nonessential for Replication: This gene has been shown to be nonessential for infection or replication of the virus in tissue culture (Kuzio *et al.*, 1984; Williams *et al.*, 1989).

Ideal for Expression: The nonessential nature and high levels of expression of the p10 gene make the p10 promoter particularly suitable for recombinant protein expression.

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Polyhedrin and p10 Promoter Regulation

Leader Sequences: Polyhedrin transcription is activated by the binding of factors to sequences found in the untranslated leader region of the (Gross and Rohrmann, 1992; Rasmussen and Rohrmann, 1993). These sequences are responsible for the very high levels of expression that occur at very late times in infection. (Ooi and Miller, 1990). Although the p10 promoter is less characterized than the polyhedrin promoter, it is thought to be regulated in a similar way (Ooi *et al.*, 1989; Weyer and Possee, 1989).

TAAG: TAAG sequences are rare in the AcMNPV genome and are found mainly at late or very late transcriptional start sites (e.g., p10 and polyhedrin). They are the primary determinant of late and very late promoter activity (Possee and Howard, 1987; Rohrmann, 1986; Thiem and Miller, 1989; Wilson *et al.*, 1987).

Dependent Process: Late and very late transcription is dependent on viral DNA replication and on early viral gene expression (Huh and Weaver, 1990; Rice and Miller, 1986; Thiem and Miller, 1989; Wilson *et al.*, 1987).

Moving Promoters: The late and very late promoters are compact and remain functional even when moved to different locations in the genome (O'Reilly, *et al.*, 1992).

Polyhedrin Regulation: Several AcMNPV genes are known to be involved in expression of the polyhedrin promoter (Li and Fields, 1993; Morris and Miller, 1994; Passarelli and Miller, 1993a). The Polyhedrin Promoter-Binding Protein (PPBP) is one of the factors known to directly interact with the polyhedrin promoter (Mukherjee *et al.*, 1995).

Transcriptional Termination

Polyadenylation Signal: This standard polyadenylation signal A_2UA_3 appears in the downstream ORF 1629, approximately 20 nucleotides upstream of the transcript cleavage site in polyhedrin (Possee *et al.*, 1991). This signal is maintained in all of Life Technologies' transfer vectors, so addition of a polyadenylation site is not necessary.

Polyhedrin Transcripts: Polyhedrin is transcribed into at least three sense mRNAs during infection (Friesen and Miller, 1985; Ooi and Miller, 1990). Little is known about transcription termination in the native polyhedrin gene.

Addition of Polyadenylation Signals: Addition of polyadenylation signals when using the transfer plasmids is not recommended as little is known about how these might affect mRNA levels or stability.

AU-Rich Residues: AU-rich sequences in the 3´ untranslated regions of mRNAs have been shown to cause mRNA instability (Yost *et al.*, 1990). However, removal of these residues should be addressed only after preliminary expression work with the unmodified gene has been conducted.

Translation

For information regarding recommendations for designing your heterologous gene for optimal translation in the baculovirus expression system, see the manual supplied with your transfer vector.

Processing of Recombinant Protein

Posttranslational Modification in Baculovirus

The following table briefly summarizes the types of modifications that have been evidenced using the baculovirus expression system. The proteins listed represent a small sample of the several hundred foreign genes that have been expressed utilizing the Baculovirus Expression System.

Type of Processing	Sub-Types	Protein	References
Proteolytic Processing	Signal cleavage.	Human tissue plasminogen activator	(Jarvis and Summers, 1989)
	Proprotein processing.	Influenza virus hemagglutinin Fowl plaque virus hemagglutinin	(Kuroda *et al.*, 1989; Kuroda *et al.*, 1991)
N-terminal blocking	Acetylalanine at the N-terminus	Human aldose reductase	(Nishimura *et al.*, 1991)
Phosphorylation	Serine/threonine phosphorylation Tyrosine phosphorylation	SV40 large T antigen Tyrosine kinase -pp60$^{c\text{-}src}$	(Hoss *et al.*, 1990) (Lanford *et al.*, 1989) (Piwnica-Worms *et al.*, 1990)
Glycosylation	N-glycosylation	Herpes simplex virus glycoprotein D Human transferrin receptor Human insulin receptor	(Krishna *et al.*, 1989) (Domingo and Trowbridge, 1988) (Herrera *et al.*, 1988) (Paul *et al.*, 1990)
	O-glycosylation	Pseudorabies virus glycoprotein Human B-choriogonadotropin	(Thomsen *et al.*, 1990) (Chen *et al.*, 1991b)
Lipid Modification	Fatty acid acylation	SV40 large T antigen Human transferrin receptor	(Lanford, 1988)) (Murphy *et al.*, 1988) (Domingo and Trowbridge, 1988)
	Polyisoprenylation	H-ras, Rap1A	(Buss *et al.*, 1991)
	Myristolation	Picornavirus capsid protein Hepatitis B virus surface antigen	(Belsham, 1991) (Lanford *et al.*, 1989)

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Processing of Recombinant Protein, continued

Posttranslational Modification in Baculovirus, continued

Type of Processing	Sub-Types	Protein	References
α-amidation		*M. sexta* diuretic hormone Frog peptidylglycine monooxygenase	(Maeda, 1989) (Suzuki *et al.*, 1990)
Subcellular localization		Rat glucocorticoid receptor	(Alnemri *et al.*, 1991)
Folding	Disulfide bond formation	Immunoglobulin heterodimers	(Hasemann and Capra, 1990)
	Dimerization Oligomerization	Human interleukin-5	(Ingley *et al.*, 1991)
Gene splicing		Silk moth chromosomal genes SV-40 small T antigen	(Iatrou and Meidinger, 1989) (Jeang *et al.*, 1987)

Assays for Posttranslational Modification

There are many types of assays that can be performed to determine the type and extent of posttranslational modification that is occurring during expression. These include assays for glycosylation, phosphorylation and fatty acid acylation.

Modification	Types of Assays
Glycosylation	Labeling with radioactive sugars. Cleavage with glycosidases. Inhibitors of glycosylation.
Phosphorylation	Labeling with ^{32}P in a time course prior to SDS-PAGE analysis.
Acylation	Labeling with ^{3}H-myrystic acid or palmytic acid prior to SDS-PAGE analysis.
Proteolytic Processing	Pulse-chase labeling prior to SDS-PAGE analysis.

Kits are often commercially available for the above mentioned assays. For detailed information and sample protocols, see O'Reilly, *et al.*, 1992.

Proteins Expressed Using the Baculovirus Expression System

Types of Proteins Expressed Using Baculovirus

The following table summarizes some of the types of proteins that have been expressed in the Baculovirus Expression System. One or two examples of each type are given for reference.

Protein Type	Examples	References
Viral Proteins	Avian leukemia virus gp-85 Hepatitis B surface antigen Human influenza virus hemagglutinin	(Noteborn *et al.*, 1990) (Kang *et al.*, 1987) (Takehara *et al.*, 1988) (Possee, 1986) (Kuroda *et al.*, 1989; Kuroda *et al.*, 1991)
Receptor Protein (Membrane-bound Proteins)	Human CD4 receptor Human EGF receptor Human insulin receptor	(Webb and Summers, 1990) (Greenfield *et al.*, 1988) (Herrera *et al.*, 1988) (Paul *et al.*, 1990)
Nuclear Proteins	Xeroderma pigmentosum group B ERCC3 (DNA helicase activity)	(Ma *et al.*, 1994)
Secreted Proteins	Human haptoglobin Human immunoglobulin heavy chain (γ-1) Human immunoglobulin light chain (91A3)	(Heinderyckx *et al.*, 1989) (Hasemann and Capra, 1990)
Envelope Proteins	HIV type 1 gp120	(Cochran *et al.*, 1987) (Richardson *et al.*, 1988)
Fungal Proteins	*Neurospora crassa* qa-1f activator protein	(Baum *et al.*, 1987)
Plant Proteins	*Phaseolus vulgaris* phaseolin *Carica papaya* papain	(Bustos *et al.*, 1988) (Menard *et al.*, 1990)
Protozoan Proteins	*Plasmodium falciparum* circumsporozoite protein	(Dontafraid *et al.*, 1988) (Good *et al.*, 1990)

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