

Bac-to-Bac™ TOPO™ Expression System

USER GUIDE

An efficient cloning and site-specific transposition system to generate recombinant baculovirus for high-level protein expression

Catalog Numbers A11100, A11101

Publication Number MAN0000699

Revision A.0



Manufacturer: Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0000699

Revision	Date	Description
A.0	28 June 2018	Rebrand
A	15 December 2008	New document

Important Licensing Information: This product may be covered by one or more Limited Use Label Licenses. By use of this product, you accept the terms and conditions of all applicable Limited Use Label Licenses.

TRADEMARKS: All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.

©2018 Thermo Fisher Scientific Inc. All rights reserved.

Contents

■	Product information	7
	Product description	7
	Kit contents and storage	7
	Types of products	7
	Shipping and storage	7
	Bac-to-Bac™ TOPO™ Cloning Kits	8
	One Shot™ Mach1™ T1 ^R Chemically Competent <i>E.coli</i>	9
	MAX Efficiency™ DH10Bac™ Competent <i>E. coli</i>	9
	ExpiFectamine™ Sf Transfection Reagent	9
	The Bac-to-Bac™ TOPO™ Expression System and Bac-to-Bac™ HBM TOPO™ Secreted Expression System	10
	System components	10
	pFastBac™ TOPO™ vector	10
	DH10Bac™ <i>E. coli</i>	11
	Baculovirus shuttle vector	11
	ExpiFectamine™ Sf Transfection Reagent	11
	Helper plasmid	11
	ExpiFectamine™ Sf Transfection Reagent	11
	Diagram of the Bac-to-Bac™ System	12
	Workflow	13
■	Methods	14
	Procedural guidelines	14
	Culture insect cells	14
	Introduction	14
	Serum-free medium	14
	Insect cell culture reference guide	15
	General cell culture guidelines	15
	Cells for transfection	15
	Generate recombinant pFastBac™ donor plasmid	16
	Introduction	16
	pFastBac™ TOPO™ vectors	16
	Generate recombinant bacmid DNA: transform DH10Bac™ <i>E. coli</i>	16
	Introduction	16
	Positive control	16
	Required materials	17

Prepare for transformation	17
Transform DH10Bac™ <i>E. coli</i>	17
Verify the phenotype	19
Isolate recombinant bacmid DNA	19
Introduction	19
Before you begin	20
Equilibrate the column	20
Prepare the cell lysate	20
Bind and wash the DNA	20
Elute and precipitate DNA	21
Analyze recombinant bacmid DNA by PCR	22
Introduction	22
Analyze by PCR with pUC/M13 primers	22
DNA polymerase	22
Generate the PCR product	23
What you should see	24
Produce recombinant baculovirus: transfect insect cells	24
Introduction	24
Plasmid preparation	24
ExpiFectamine™ Sf Transfection Reagent	24
Insect cell lines	24
Media for transfection	25
Positive control	25
Required materials	25
Transfection conditions	25
Important guidelines for transfection	26
Transfect cells cultured in supplemented Grace's Medium	26
Transfect cells cultured in serum-free medium	27
Isolate P0 virus stock	27
Introduction	27
Characteristics of infected cells	28
Prepare the P0 virus stock	28
Store the virus stocks	28
Virus stock applications	29
Amplify baculovirus stock	29
Introduction	29
Required materials	29
Multiplicity of infection (MOI)	29
Important considerations	30
Amplify P0 virus stock in a 1-L flask	30
Scale up the amplification procedure	30
Generate high-titer stocks from frozen master stock	30

Perform a plaque assay	31
Introduction	31
Experimental outline	31
Factors affecting virus titer	31
Required materials	31
Prepare the plaquing medium	32
Perform plaque assay	33
Neutral Red staining	34
Prepare Neutral Red agarose overlay (for use on Day 4)	34
Prepare Neutral Red stain (for use on Day 7–10 prior to counting plaques)	34
Calculate the titer	35
What you should see	35
Plaque purification	35
Express recombinant protein	36
Introduction	36
Recommendation when using pFastBac™ /HBM TOPO™ vector	36
Positive control	36
Guidelines for expression	37
Calculate virus volumes	37
Optimize expression	37
Harvest baculovirus infected insect cells	38
Analyze recombinant protein	38
Introduction	38
Protease inhibitors	39
Prepare cell lysates	39
Detect recombinant protein	39
Assay for β -glucuronidase	40
Purify recombinant protein	40
Remove the 6x His tag using AcTEV™ Protease	40
■ APPENDIX A Troubleshooting	41
Recombinant bacmid DNA generation	41
Bacmid DNA isolation	42
Insect cells transfection	42
Protein expression	43
■ APPENDIX B Supporting protocols	45
Antibiotic stock solutions	45
IPTG (200 mg/mL) stock solution	45
Bluo-gal (20 mg/mL) stock solution	45
LB (Luria-Bertani) Medium	46
LB (Luria-Bertani) Plates	46

■	APPENDIX C Bacmid DNA Isolation Using PureLink™ HiPure Maxiprep Kit	47
	Introduction	47
	Grow bacmid DNA stock	47
	Before Starting	48
	Equilibrate the column	48
	Prepare the cell lysate	48
	Bind and wash the DNA	49
	Elute and precipitate the DNA	49
■	APPENDIX D Ordering information	51
	Additional products	51
	Insect cell culture products	52
	Recombinant fusion protein detection	53
	Recombinant fusion proteins purification	53
	Nalgene flasks and tissue culture plates	53
■	APPENDIX E Safety	55
	Chemical safety	56
	Biological hazard safety	57
■	APPENDIX F Documentation and support	58
	Customer and technical support	58
	Limited product warranty	58



Product information

IMPORTANT! Before using this product, read and understand the information in the “Safety” appendix in this document.

Product description

The Bac-to-Bac™ TOPO™ Expression System and Bac-to-Bac™ HBM TOPO™ Secreted Expression System enable rapid and efficient generation of recombinant baculovirus by combining the ease of TOPO™ cloning with the efficiency of the Bac-to-Bac™ System. Both systems take advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating recombinant bacmid DNA. The Bac-to-Bac™ HBM TOPO™ Secreted Expression System, in particular, is designed to allow for the secreted expression of your protein of interest.

Kit contents and storage

Types of products

Product	Amount	Cat. No.
Bac-to-Bac™ C-His TOPO™ Expression System	1 kit	A11100
Bac-to-Bac™ N-His TOPO™ Expression System	1 kit	A11101
Bac-to-Bac™ HBM TOPO™ Secreted Expression System	1 kit	A11339

Shipping and storage

The Bac-to-Bac™ C-His, N-His, or HBM TOPO™ Expression System is shipped in four boxes. On receipt, store each box as described. All reagents are guaranteed for at least six months when stored properly.



Bac-to-Bac™ TOPO™ Cloning Kits

Box 1			
Reagent	Composition	Amount	Storage
One of the following vectors: <ul style="list-style-type: none"> pFastBac™/NT-TOPO™ vector (Cat. No. A11101) pFastBac™/CT-TOPO™ vector (Cat. No. A11100) pFastBac™/HBM TOPO™ vector (Cat. No. A11339) 	<ul style="list-style-type: none"> 20 mL at 10 ng/μL in 50% glycerol 50 mM Tris-HCl pH 7.4 (at 25°C) 1 mM EDTA 2 mM DTT 0.1% Triton X-100 100 μg/mL BSA 30 μM bromophenol blue 	20 μL	2 to 8°C
10X PCR Buffer	<ul style="list-style-type: none"> 100 mM Tris-HCl pH 8.3 (at 42°C) 500 mM KCl 25 mM MgCl₂ 0.01% gelatin 	100 μL	-20°C
dNTP Mix	12.5 mM each of dATP, dCTP, dGTP, and dTTP; neutralized at pH 8.0 in water	10 μL	
Salt Solution	1.2 M NaCl, 0.06 M MgCl ₂	50 μL	
Sterile Water	—	1 mL	
Control PCR template	50 ng/μL in TE buffer ^[1] , pH 8.0	10 μL	
Control PCR primers	100 ng/μL each in TE buffer, pH 8.0	10 μL	
Polyhedrin forward sequencing primer	100 ng/μL in TE buffer, pH 8.0	20 μL	
SV40 polyA reverse sequencing primer	100 ng/μL in TE buffer, pH 8.0	20 μL	
pFastBac™ 1-Gus control plasmid	0.2 ng/μL in TE buffer, pH 8.0	20 μL	

^[1] TE buffer, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0



One Shot™ Mach1™ T1^R Chemically Competent *E. coli*

Box 2			
Reagent	Composition	Amount	Storage
One Shot™ Mach1™ T1 ^R Chemically Competent <i>E. coli</i>	—	20 × 50 µL	-80°C
SOC Medium (can be stored at room temperature or 4°C)	<ul style="list-style-type: none"> • 2% tryptone • 0.5% yeast extract • 10 mM NaCl • 2.5 mM KCl • 10 mM MgCl₂ • 10 mM MgSO₄ • 20 mM glucose 	6 mL	
pUC19 Control DNA	10 pg/mL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL	

One Shot™ Mach1™ T1 ^R Chemically Competent <i>E. coli</i>	
Genotype	F ⁻ ϕ80(<i>lacZ</i>)ΔM15 Δ <i>lacX74</i> <i>hsdR</i> (r _K -m _K ⁺) Δ <i>recA</i> 1398 <i>endA1 tonA</i>
Transformation efficiency	≥ 1 × 10 ⁹ cfu/µg of DNA

MAX Efficiency™ DH10Bac™ Competent *E. coli*

Box 3			
Reagent	Composition	Amount	Storage
MAX Efficiency™ DH10Bac™ Competent <i>E. coli</i>	—	4 kits (5 × 100 µL per kit)	-80°C
pUC19 Control DNA	0.01 µg/mL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	100 µL	

MAX Efficiency™ DH10Bac™ Competent <i>E. coli</i>	
Genotype	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) ϕ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galJ galK λ⁻rpsL nupG/bMON14272/pMON7124</i>
Transformation efficiency	1 × 10 ⁸ cfu/µg of DNA

ExpiFectamine™ Sf Transfection Reagent

Box 4		
Reagent	Amount	Storage
ExpiFectamine™ Sf Transfection Reagent	1 mL	4°C (do not freeze)



The Bac-to-Bac™ TOPO™ Expression System and Bac-to-Bac™ HBM TOPO™ Secreted Expression System

System components

The Bac-to-Bac™ TOPO™ Expression System and Bac-to-Bac™ HBM TOPO™ Secreted Expression System provide a rapid and highly effective method to generate recombinant baculoviruses by combining the ease of blunt-end TOPO™ cloning with the efficiency of site-specific transposition technology of the Bac-to-Bac™ System.

The major components of the Bac-to-Bac™ Baculovirus Expression System include:

- A choice of pFastBac™/CT-TOPO™, pFastBac™/NT-TOPO™, or pFastBac™/HBM TOPO™ donor plasmids that allow rapid generation of an expression construct containing the gene of interest under the control of a baculovirus-specific strong polyhedrin (PH) promoter.
- The pFastBac™/HBM TOPO™ donor plasmid, a component of the Bac-to-Bac™ HBM TOPO™ Secreted Expression System, allows you to efficiently generate recombinant baculovirus for secreted expression of your protein of interest.
- One Shot™ Mach1™ T1^R Chemically Competent *E.coli* that enable same-day isolation of recombinant pFastBac™ expression construct from the transformation mix.
- An *E. coli* host strain, DH10Bac™, that contains a baculovirus shuttle vector (bacmid) and a helper plasmid, and allows generation of a recombinant bacmid following transposition of the pFastBac™ expression construct.
- ExpiFectamine™ Sf Transfection Reagent for fast and efficient transfection of insect cells to generate recombinant baculovirus.
- A control expression plasmid containing the Gus gene that allows production of a recombinant baculovirus which, when used to infect insect cells, expresses the *Arabidopsis thaliana* β-glucuronidase.

pFastBac™ TOPO™ vector

The first major component of the System is a pFastBac™ TOPO™ vector into which your gene of interest will be cloned.

After you amplify your gene of interest using a proofreading polymerase, you clone it into the pFastBac™ TOPO™ vector as a blunt-end PCR product, and transform One Shot™ Mach1™ T1^R Chemically Competent *E.coli*. You then select and analyze transformants for the correct insertion of your blunt-end PCR products, and use the recombinant vector as a "donor plasmid" to generate a recombinant baculovirus.

Expression of the gene of interest is controlled by the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (P_H) promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7, and contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn7.

The pFastBac™/HBM-TOPO™ vector contains the N-terminal Honey Bee Mellitin (HBM) secretion signal coding sequence on the plasmid and facilitates the secretion of the cloned gene product into the extracellular medium. The C-terminal polyhistidine tag allows easy purification of the secreted protein.



DH10Bac™ *E. coli*

The second major component of the System is the DH10Bac™ *E. coli* strain that is used as the host for your pFastBac™ construct containing your gene of interest. DH10Bac™ cells contain a bacmid with a mini-*att*Tn7 target site and a helper plasmid.

Once the pFastBac™ expression plasmid (the "donor plasmid") is transformed into DH10Bac™ cells, transposition occurs between the mini-Tn7 element on the pFastBac™ vector and the mini-*att*Tn7 target site on the bacmid to generate a recombinant bacmid. This transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid.

Once you have performed the transposition reaction, you will isolate the high molecular weight recombinant bacmid DNA and transfect the bacmid DNA into insect cells using the ExpiFectamine™ Sf Transfection Reagent to generate a recombinant baculovirus that can be used for preliminary expression experiments. After the baculovirus stock is amplified and titered, the high-titer stock can be used to infect insect cells for large-scale expression of the recombinant protein of interest.

Baculovirus shuttle vector

The baculovirus shuttle vector (bacmid), bMON14272 (136 kb), present in DH10Bac™ *E. coli* contains:

- A low-copy number mini-F replicon.
- Kanamycin resistance marker.
- A segment of DNA encoding the *lacZ* alpha peptide gene from a pUC-based cloning vector into which the attachment site for the bacterial transposon, Tn7 (mini-*att*Tn7) has been inserted. Insertion of the mini-*att*Tn7 attachment site does not disrupt the reading frame of the *lacZ* alpha peptide gene.

The bacmid propagates in DH10Bac™ *E. coli* as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* gene deletion present on the chromosome to form colonies that are blue (lac+) in the presence of a chromogenic substrate such as Blueo-gal or X-gal and the inducer, IPTG.

Recombinant bacmids are generated by transposing a mini-Tn7 element from a pFastBac™ donor plasmid to the mini-*att*Tn7 attachment site on the bacmid. The Tn7 transposition functions are provided by a helper plasmid.

ExpiFectamine™ Sf Transfection Reagent

ExpiFectamine™ Sf Transfection Reagent is a next-generation proprietary cationic lipid formulation that offers high transfection efficiency and protein expression levels in adherent or suspension Sf9 and Sf21 cell cultures. This versatile reagent exhibit low toxicity and enables transfection of insect cells using fast and simple optimized protocols to streamline the workflow.

Helper plasmid

DH10Bac™ *E. coli* also contain the helper plasmid, pMON7124 (13.2 kb), which encodes the transposase and confers resistance to tetracycline. The helper plasmid provides the Tn7 transposition function *in trans*.

ExpiFectamine™ Sf Transfection Reagent

ExpiFectamine™ Sf Transfection Reagent is a next-generation proprietary cationic lipid transfection reagent for efficient transfection of plasmid DNA and baculovirus production in insect cells. ExpiFectamine™ Sf is a component of the ExpiSf™ Expression System (Cat. No. A38841, A39112, or A39111). To learn more about this reagent and the ExpiSf™ Expression System for superior protein yields, see thermofisher.com/expisf.



Diagram of the Bac-to-Bac™ System

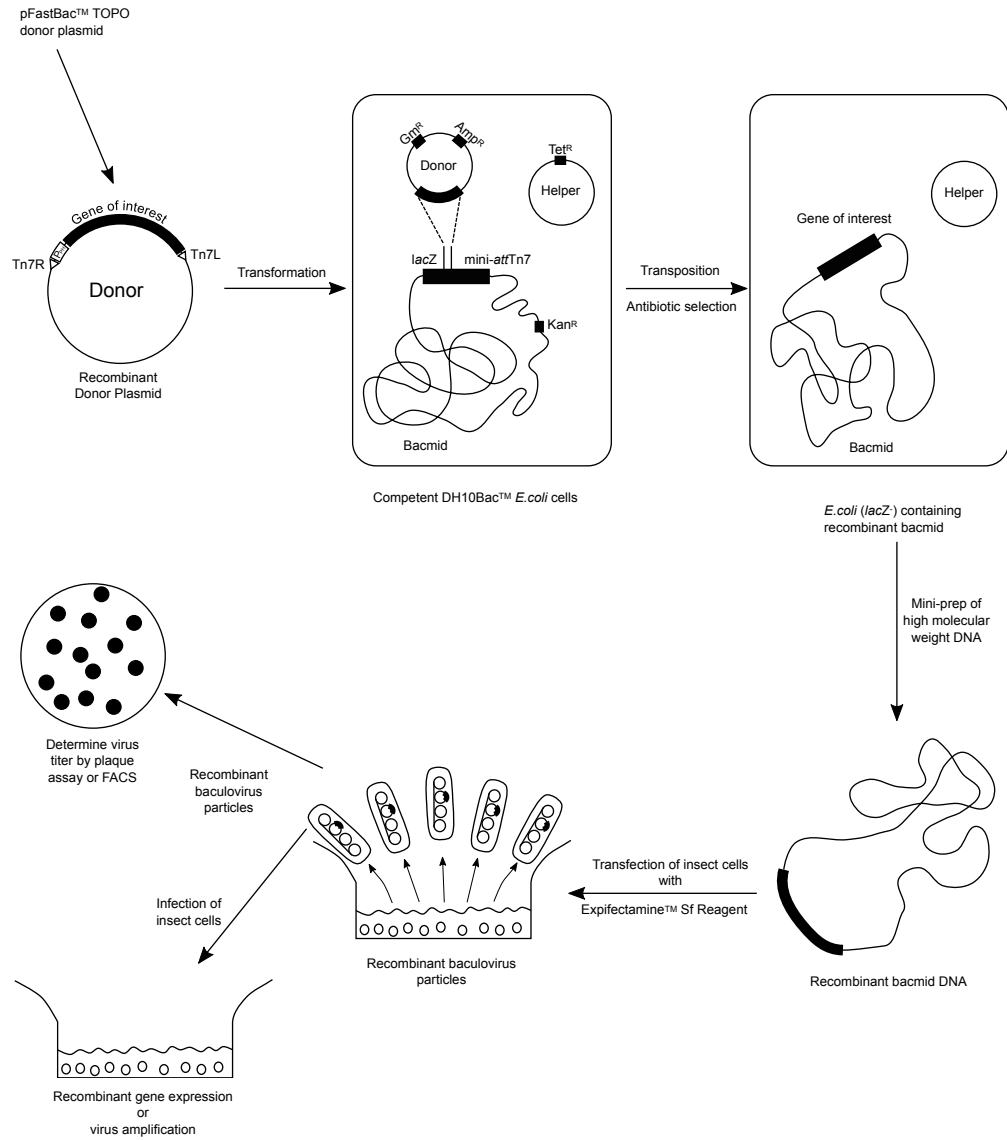


Figure 1 Generation of recombinant baculovirus and the expression of your gene of interest using the Bac-to-Bac™ TOPO™ Expression System or Bac-to-Bac™ HBM TOPO™ Secreted Expression System



Workflow

Generate recombinant pFastBac™ donor plasmid



Generate recombinant bacmid DNA: transform DH10Bac™ E. coli



Isolate recombinant bacmid DNA



Analyze recombinant bacmid DNA by PCR



Produce recombinant baculovirus: transfect insect cells



Isolate P0 virus stock



Amplify baculovirus stock (Optional)



Titer virus stock (plaque assay, FACS, or equivalent)

Perform a plaque assay



Express recombinant protein



Analyze recombinant protein



Methods

Procedural guidelines

The Bac-to-Bac™ TOPO™ Expression System and Bac-to-Bac™ HBM TOPO™ Secreted Expression System are designed to help you create a recombinant baculovirus for high-level expression of your gene of interest in insect cells. Although the systems have been designed to help you easily produce recombinant baculovirus and express your protein of interest, use of either system is geared towards those users who are familiar with baculovirus biology and insect cell culture. We highly recommend that users possess a working knowledge of virus and tissue culture techniques.

For detailed instructions for cloning your gene of interest into the pFastBac™ TOPO™ vector of choice, refer to *Bac-to-Bac™ TOPO™ Cloning Kit User Guide* (Pub. No. MAN0000698).

Culture insect cells

Introduction

We recommend using *Spodoptera frugiperda* Sf9 or Sf21 insect cells as the host for recombinant baculovirus production. You can also use the high-density, suspension ExpiSf9™ cell line, a component of the ExpiSf™ Expression System, to produce your recombinant baculovirus. For additional information on baculovirus generation and protein expression using the ExpiSf™ Expression System, go to thermofisher.com/expisf. Before you start your transfection and expression experiments, ensure to have cultures of Sf9 or Sf21 cells growing and have frozen master stocks available. Sf9 or Sf21 cells and cell culture reagents are available separately.

Note: High Five™ cells and Mimic™ Sf9 insect cells are appropriate for expression only.

Serum-free medium

Insect cells may be cultured under serum-free conditions. We recommend using Sf-900™ II SFM or Sf-900™ III SFM. If you are using the ExpiSf™ Expression System, use ExpiSf™ CD Medium for cell culture.

Both Sf-900™ II SFM and Sf-900™ III SFM are protein-free media optimized for the growth and maintenance of Sf9 and Sf21 cells, as well as for the large-scale production of recombinant proteins expressed using the Bac-to-Bac™ System.

ExpiSf™ CD Medium is an innovative chemically-defined, yeastolate-free, animal origin-free, serum-free, protein-free formulation that has been optimized for the growth and maintenance of high-density suspension ExpiSf9™ cells, and for large-scale production of recombinant proteins expressed using the Bac-to-Bac™ System. For more information, see thermofisher.com/expisf.



Insect cell culture reference guide

For guidelines and detailed information on insect cell culture, such as in the following list, refer to the *Guide to Baculovirus Expression Vector System (BEVS) and Insect Cell Culture Techniques* at thermofisher.com.

- Maintaining and passaging insect cells in adherent and suspension culture
- Freezing cells
- Using serum-free medium (includes protocols to adapt cells to serum-free medium)
- Scaling up cell culture

General cell culture guidelines

Insect cells are sensitive to environmental factors. In addition to chemical and nutritional culture factors, physical factors can also affect insect cell growth. Therefore optimization is required to maximize cell growth. Consider the following when culturing insect cells:

- **Temperature:** The optimal range to grow and infect cultured insect cells is 27°C to 28°C.
- **pH:** A range of 6.1 to 6.4 works well for most culture systems. Sf-900™ II SFM and Sf-900™ III SFM maintain a pH in this range under conditions of normal air and open-capped culture systems.
- **Osmolality:** The optimal osmolality of medium for use with lepidopteran cell lines is 345 to 380 mOsm/kg.
- **Aeration:** Insect cells require passive oxygen diffusion for optimal growth and recombinant protein expression. Active or controlled oxygenated systems require dissolved oxygen at 10% to 50% of air saturation.
- **Shear forces:** Suspension culture generates mechanical shear forces. Growing insect cells in serum-containing media (10% to 20% FBS) generally provides adequate protection from cellular shear forces. If you are growing insect cells in serum-free conditions other than Sf-900™ II SFM or Sf-900™ III SFM, supplementation with a shear force protectant such as Pluronic™ F-68 can be required.

Cells for transfection

You need log-phase Sf9 or Sf21 cells with >95% viability to perform a successful transfection. To determine how many cells you need for transfection, see “Transfection conditions” on page 25.



Generate recombinant pFastBac™ donor plasmid

Introduction

To generate a recombinant plasmid containing your gene of interest for use in the Bac-to-Bac™ TOPO™ Expression System or Bac-to-Bac™ HBM TOPO™ Secreted Expression System perform the following steps:

1. Generate a blunt-end PCR product containing your gene of interest with a thermostable proofreading DNA polymerase such as the Platinum™ Pfx DNA Polymerase or the AccuPrime™ Pfx DNA Polymerase.
2. TOPO™ clone your blunt-end PCR product into the pFastBac™/CT-TOPO™, pFastBac™/NT-TOPO™, pFastBac™/HBM-TOPO™ vector and use the reaction to transform One Shot™ Mach1™ T1^R Chemically Competent *E.coli*. **Do not transform the ligation reaction into DH10Bac™ *E. coli*.**
3. Pick colonies, isolate plasmid DNA, and screen for insert directionality by sequencing expression clones with the primers provided in the kit.

For detailed instructions, refer to the *Bac-to-Bac™ TOPO™ Cloning Kit User Guide* (Pub. No. MAN0000698).

IMPORTANT! When generating the recombinant plasmid containing your gene of interest for use in the Bac-to-Bac™ TOPO™ Expression System or Bac-to-Bac™ HBM TOPO™ Secreted Expression System, you **must** follow certain design parameters for your PCR insert and the recommendations for the transformation procedure outlined in the *Bac-to-Bac™ TOPO™ Cloning Kit User Guide* (Pub. No. MAN0000698). To ensure proper expression of your recombinant protein, it is imperative that you read the sections on generating the blunt-end PCR product, blunt-end TOPO™ cloning, transformation of One Shot™ Mach1™ T1^R Chemically Competent *E.coli*, and analysis of transformants in the *Bac-to-Bac™ TOPO™ Cloning Kit User Guide* before you begin.

pFastBac™ TOPO™ vectors

For the features and vector maps of pFastBac™/CT-TOPO™, pFastBac™/NT-TOPO™, and pFastBac™/HBM-TOPO™ refer to the *Bac-to-Bac™ TOPO™ Cloning Kit User Guide* (Pub. No. MAN0000698). See thermofisher.com.

Generate recombinant bacmid DNA: transform DH10Bac™ *E. coli*

Introduction

The pFastBac™ construct containing your gene of interest in the correct orientation is used to transform purified plasmid DNA into MAX Efficiency™ DH10Bac™ Competent Cells for transposition into the bacmid. Then, blue/white selection identifies colonies containing the recombinant bacmid.

Guidelines and instructions to transform DH10Bac™ *E. coli* using the pFastBac™ construct are provided in this section.

Positive control

The pFastBac™ 1-Gus Control Vector is supplied for use as a positive transfection and expression control. We recommend including this control vector in your DH10Bac™ transformation experiments.

For a map and a description of the features of the control plasmid, refer to the *Bac-to-Bac™ TOPO™ Cloning Kit User Guide* (Pub. No. MAN0000698).



Required materials

- Your purified pFastBac™ construct (200 pg/mL in TE, pH 8.0)
- Positive expression control plasmid (as a transposition control)
- MAX Efficiency™ DH10Bac™ Competent Cells (use 1 tube per transformation)
- pUC19 (as a transformation control)
- LB agar plates containing kanamycin, gentamicin, tetracycline, Bluo-gal, and IPTG (3 freshly prepared plates per transformation, see “Recommendation” on page 17 and Appendix B, “Supporting protocols”)
- LB agar plate containing 100 mg/mL ampicillin (for pUC19 transformation control)
- SOC Medium
- 15-mL round-bottom polypropylene tubes
- 42°C water bath
- 37°C shaking and non-shaking incubator

Recommendation

Prepare LB agar plates containing:

- 50 µg/mL kanamycin
- 7 µg/mL gentamicin
- 10 µg/mL tetracycline
- 100 µg/mL Bluo-gal
- 40 µg/mL IPTG

Note: Use Bluo-gal instead of X-gal for blue/white selection as Bluo-gal generally produces a darker blue color than X-gal.

To order antibiotics, Bluo-gal, and IPTG, see Appendix D, “Ordering information”, and for instructions to prepare plates, see “LB (Luria-Bertani) Plates” on page 46. We recommend using Luria Broth Base (see “LB (Luria-Bertani) Medium” on page 46) instead of Lennox L (LB) as the color intensity and number of colonies that are obtained on Lennox L plates is reduced.

Prepare for transformation

Each transformation requires one vial of DH10Bac™ competent cells and three selective plates.

- Equilibrate a water bath to 42°C.
- Warm selective plates at 37°C for 30 minutes.
- Warm the SOC Medium to room temperature.
- Pre-chill one 15-mL round-bottom polypropylene tube for each transformation.

Transform DH10Bac™ *E. coli*

1. Thaw on ice one vial of MAX Efficiency™ DH10Bac™ Competent Cells for each transformation.
2. For each transformation, gently mix, then transfer 100 µL of the DH10Bac™ cells into a pre-chilled, 15-mL round-bottom polypropylene tube.



3. Add the plasmid DNA to the cells, according to the following table, then mix gently.

Note: Do not pipet up and down to mix.

Table 1

Plasmid DNA	Amount to add
Your recombinant pFastBac™ construct	1 ng (5 µL)
pFastBac™ control plasmid	1 ng
pUC19 control	50 pg (5 µL)

4. Incubate the cells on ice for 30 minutes.
5. Heat-shock the cells for 45 seconds at 42°C without shaking.
6. Immediately transfer the tubes to ice and chill for 2 minutes.
7. Add 900 µL of room temperature SOC Medium.
8. Transform cells according to the following table.

To transform	Description
pFastBac™	Shake tubes at 37°C at 225 rpm for 4 hours
pUC19	Shake tube at 37°C at 225 rpm for 1 hour

9. Prepare dilutions according to the following table.

For plasmid	Description
pFastBac™	Prepare 10-fold serial (10^{-1} to 10^{-3}) dilutions of the cells with SOC Medium, then plate 100 µL of each dilution on an LB agar plate containing: <ul style="list-style-type: none"> • 50 µg/mL kanamycin • 7 µg/mL gentamicin • 10 µg/mL tetracycline • 100 µg/mL Bluo-gal • 40 µg/mL IPTG
pUC19	Dilute the cells 1:100 with SOC Medium, then plate 100 µL of the dilution on an LB agar plate containing 100 µg/mL ampicillin.

10. Incubate plates for 24–48 hours at 37°C.

IMPORTANT! Insertion of the mini-Tn7 into the mini-attTn7 attachment site on the bacmid disrupts the expression of the LacZ peptide. Therefore, colonies containing the recombinant bacmid are white. Blue colonies contain unaltered bacmid.



11. Pick white colonies for analysis.

Note: True white colonies tend to be large, therefore avoid false positives by selecting the largest, most isolated white colonies. Avoid picking colonies that appear gray or that are darker in the center as they can contain a mixture of cells with empty bacmid and recombinant bacmid.

Verify the phenotype

1. Pick up to 10 white colonies, then restreak them on fresh LB agar plates containing:
 - 50 µg/mL kanamycin
 - 7 µg/mL gentamicin
 - 10 µg/mL tetracycline
 - 100 µg/mL Bluo-gal
 - 40 µg/mL IPTG

2. Incubate the plates overnight at 37°C.

3. Pick a white colony, then inoculate a liquid culture that contains:
 - 50 µg/mL kanamycin
 - 7 µg/mL gentamicin
 - 10 µg/mL tetracycline

4. Isolate recombinant bacmid DNA, see “Isolate recombinant bacmid DNA” on page 19.

Note: For increased recombinant bacmid yield, use the procedure for the PureLink™ HiPure Plasmid Maxiprep Kit, see Appendix C, “Bacmid DNA Isolation Using PureLink™ HiPure Maxiprep Kit”.

Note: Bacmid DNA must be clean and free from phenol and sodium chloride as contaminants can kill insect cells, and salt interferes with lipid complexing, which decreases the transfection efficiency.

5. Analyze the recombinant bacmid DNA by PCR to verify successful transposition to the bacmid, see “Analyze recombinant bacmid DNA by PCR” on page 22.

Isolate recombinant bacmid DNA

Introduction

The PureLink™ HiPure Plasmid DNA Miniprep Kit allows you to purify high-quality bacmid DNA from DH10Bac™ *E. coli* (see Appendix D, “Ordering information”). The isolated bacmid DNA is appropriate for use in insect cell transfections.

Note: We do not recommend the PureLink™ HiPure Precipitator Module or the PureLink™ HiPure Plasmid Filter Mini/Midi/Maxiprep Kits for isolating bacmid DNA.

Note: When using the ExpiSf™ Expression System, we recommend isolating bacmid DNA using the PureLink™ HiPure Plasmid Maxiprep Kit (Cat. No. K2100-06). This will ensure there is enough, high-quality bacmid DNA for baculovirus generation using suspension-based transfection. Follow instructions in *PureLink™ HiPure Plasmid Purification Kits User Guide*, (Pub. No. MAN0000486) using the MaxiPrep low-copy plasmid instructions.

**Before you begin**

- Prepare LB medium containing:
 - 50 µg/mL kanamycin
 - 7 µg/mL gentamicin
 - 10 µg/mL tetracycline

Inoculate a single white bacterial colony into 2 mL of this LB medium, then incubate the culture at 37°C in a shaking water bath at 250 rpm overnight.

- Verify that RNase A is added to the Resuspension Buffer (R3).
- Ensure the Lysis Buffer (L7) contains no precipitates.

Equilibrate the column

1. Place the PureLink™ HiPure Mini column on the PureLink™ Nucleic Acid Purification Rack.
2. Add 2 mL of Equilibration Buffer (EQ1) to the column.
3. Allow the solution in the column to drain by gravity flow.

Prepare the cell lysate

1. Harvest 1.5 mL of bacterial cells by centrifuging at $9,000 \times g$ for 15 minutes, then remove all the medium.
2. Add 0.4 mL Resuspension Buffer (R3) containing RNase A to the pellet, then resuspend the cells until homogeneous.
3. Transfer the cell suspension to a centrifuge tube.
4. Add 0.4 mL Lysis Buffer (L7), then mix gently by inverting the capped tube five times.
Do not vortex.
5. Incubate at room temperature for 5 minutes.
6. Add 0.4 mL Precipitation Buffer (N3), then mix immediately by inverting the capped tube until the mixture is homogeneous.
Do not vortex.
7. Centrifuge the mixture at $>15,000 \times g$ at room temperature for 10 minutes.

Note: If the pellet does not adhere to the bottom of the tube, incubate the tube at room temperature for 5 minutes to allow the separation of the lysate and gelatinous pellet. Pipette the clear lysate into a sterile tube and centrifuge at $>15,000 \times g$ for 5 minutes at room temperature to remove any remaining cellular debris.

Bind and wash the DNA

1. Load the supernatant from step 7 onto the equilibrated column.
2. Allow the solution in the column to drain by gravity flow.
3. Wash the column twice with 2.5 mL of Wash Buffer (W8).
4. Allow the solution in the column to drain by gravity flow after each wash.
Discard the flow-through.



Elute and precipitate DNA

1. Place a sterile centrifuge tube (elution tube) under the column.
2. Add 0.9 mL of Elution Buffer (E4) to the column to elute DNA.
Note: Allow the solution to drain by gravity flow. Do not force out any remaining solution.
3. The elution tube contains the purified DNA.
Discard the column.
4. Add 0.63 mL of isopropanol to the elution tube.
5. Mix, then place on ice for 10 minutes.
6. Centrifuge the mixture at $>15,000 \times g$ at 4°C for 20 minutes.
7. Carefully remove, then discard the supernatant.
8. Resuspend the DNA pellet in 1 mL of 70% ethanol.
9. Centrifuge at $>15,000 \times g$ at 4°C for 5 minutes.
10. Carefully remove, then discard the supernatant.
11. Air-dry the pellet for 10 minutes.
12. Resuspend the DNA pellet in 40 μL TE Buffer.
Note: Allow the pellet to dissolve for at least 10 minutes on ice. To avoid shearing the DNA, pipette only 1–2 times to resuspend.
13. Store the bacmid DNA at 4°C .

IMPORTANT! Aliquot bacmid DNA in TE buffer pH 8.0, into separate tubes and store at -20°C (not in a frost-free freezer). Avoid multiple freeze/thaw cycles as it decreases the transfection efficiency. Bacmid DNA can also be stored for up to 2 weeks at 4°C in TE Buffer pH 8.0.

Prepare glycerol stocks of DH10Bac™ *E. coli* containing the bacmid DNA from mid-logarithmic phase culture grown from white colonies picked during the blue-white screening, and store at -80°C for future bacmid DNA isolation.

Note: For increased recombinant bacmid yield, use the PureLink™ HiPure Plasmid Maxiprep Kit, see Appendix C, “Bacmid DNA Isolation Using PureLink™ HiPure Maxiprep Kit”. The PureLink™ HiPure Plasmid Prep Kits allow purification of all types and sizes of plasmid DNA and are ideally suited for bacmid purification (see Appendix D, “Ordering information”).

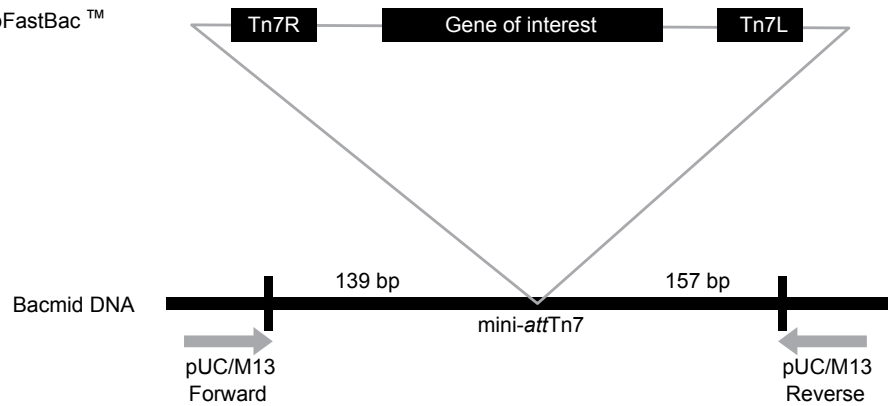


Analyze recombinant bacmid DNA by PCR

Introduction

Recombinant bacmid DNA is greater than 135 kb in size. As restriction analysis is difficult to perform with DNA of this size, use PCR analysis to verify the presence of your gene of interest in the recombinant bacmid. Use the pUC/M13 Forward and Reverse primers that hybridize to sites flanking the mini-*att*Tn7 site within the *lacZ*-complementation region to facilitate PCR analysis.

Transposed pFastBac™ sequence



Guidelines and instructions are provided in this section to perform PCR using the pUC/M13 Forward and Reverse primers.

Analyze by PCR with pUC/M13 primers

To verify the presence of the gene of interest in the recombinant bacmid using PCR, either:

- Use the pUC/M13 Forward and Reverse primers.
- Use a combination of the pUC/M13 Forward or Reverse primer and a primer that hybridizes within the insert.

pUC/M13 Forward and Reverse primers, see the following table, are not provided.

Primer	Sequence
pUC/M13 Forward	5'-CCCAGTCACGACGTTGATAAAACG-3'
pUC/M13 Reverse	5'-AGCGGATAACAATTTACACACAGG-3'

DNA polymerase

Use any DNA polymerase for PCR, including Platinum™ *Taq* DNA Polymerase. If the expected PCR product is > 4 kb, we recommend using a polymerase mixture such as Platinum™ *Taq* DNA Polymerase High Fidelity for best results. See Appendix D, "Ordering information".



Generate the PCR product

Use the procedure below to amplify your recombinant bacmid DNA using the pUC/M13 Forward and Reverse primers and Platinum™ *Taq* polymerase. If you are using a combination of the pUC/M13 Forward or Reverse primers and a primer specific for your gene, determine the amplification conditions to use. If you are using another polymerase, follow the manufacturer's recommendations for the polymerase you are using.

Note: Optimize amplification conditions when the insert is > 4 kb.

1. For each sample, set up the following 50- μ L PCR reaction in a 0.5-mL microcentrifuge tube:

Component	Amount
Recombinant bacmid DNA (100 ng)	1 μ L
10X PCR Buffer (appropriate for enzyme)	5 μ L
10 mM dNTP Mix	1 μ L
50 mM MgCl ₂	1.5 μ L
PCR Primers (1.25 μ L each 10 μ M stock)	2.5 μ L
Sterile Water	38.5 μ L
Platinum™ <i>Taq</i> polymerase (5 units/ μ L)	0.5 μ L
Total	50 μL

2. Overlay with 50 μ L (1 drop) of mineral or silicone oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	3 minutes	94°C	1X
Denaturation	45 seconds	94°C	25–35X
Annealing	45 seconds	55°C	1X
Extension	5 minutes	72°C	
Final extension	7 minutes	72°C	

4. Remove 5–10 μ L from the reaction, then analyze by agarose gel electrophoresis.

**What you should see**

Successful transposition using the pUC/M13 Forward and Reverse primers for amplification results in PCR product sizes on agarose gel according to the following table.

Bacmid transposed with	Size of PCR Product
Bacmid alone	~300 bp
pFastBac™/CT-TOPO™	~2420 bp + size of your insert
pFastBac™/NT-TOPO™	~2440 bp + size of your insert
pFastBac™/HBM-TOPO™	~2500 bp + size of your insert
pFastBac™ 1-Gus	~4200 bp

If you have used a combination of the pUC/M13 Forward and Reverse primers and a gene-specific primer for amplification, determine the expected size of your PCR product.

Produce recombinant baculovirus: transfect insect cells

Introduction

After confirmation that the recombinant bacmid contains the gene of interest, transfect insect cells to produce recombinant baculovirus. Guidelines and instructions to transfect Sf9 and Sf21 insect cells are provided in this section.

Note: For instructions to generate recombinant baculovirus using ExpiSf9™ cells as part of the ExpiSf™ Expression System workflow, refer to the *ExpiSf™ Expression System User Guide* (Pub. No. MAN0017532) at thermofisher.com/expisf.

Plasmid preparation

You may use any method to prepare purified recombinant bacmid DNA for transfection. Bacmid DNA must be clean and free from phenol and sodium chloride as contaminants may kill the cells, and salt will interfere with lipid complexing and decrease transfection efficiency. We recommend isolating bacmid DNA using the PureLink™ HiPure Plasmid DNA Miniprep Kit following the procedure that is provided, see Appendix C, “Bacmid DNA Isolation Using PureLink™ HiPure Maxiprep Kit”.

ExpiFectamine™ Sf Transfection Reagent

ExpiFectamine™ Sf Transfection Reagent is a next-generation proprietary cationic lipid formulation that offers high transfection efficiency and protein expression levels in adherent or suspension Sf9 and Sf21 cell cultures. This versatile reagent exhibit low toxicity and enables transfection of insect cells using fast and simple optimized protocols to streamline the workflow.

Insect cell lines

We recommend using Sf9 or Sf21 cells for transfection and identification of recombinant plaques. High Five™ and Mimic™ Sf9 cells are not recommended because they generally transfect less efficiently. However, after you have generated your baculovirus stock, you can use High Five™ or Mimic™ Sf9 cells for expression studies.



Media for transfection

The ExpiFectamine™ Sf Transfection Reagent:

- allows for high transfection efficiency directly into growth medium in both adherent and suspension cell culture formats
- ensures no need to replace the culture medium prior to transfection
- has low toxicity that ensures no need to replace the medium after transfection

Positive control

Include the recombinant bacmid from the pFastBac™ 1-Gus control plasmid as a positive control in your transfection and expression experiments. In this bacmid, the gene encoding β -glucuronidase (Gus) is expressed under the control of the strong polyhedrin (P_H) promoter. Assay expression of β -glucuronidase after transfection.

Required materials

- Purified recombinant bacmid DNA generated from the pFastBac™ construct (500 ng/ μ L in TE Buffer, pH 8.0)
- (Optional) Purified recombinant bacmid DNA generated from the pFastBac™ control construct (500 ng/ μ L in TE Buffer, pH 8.0)
- Sf9 or Sf21 cells cultured in the appropriate medium
- ExpiFectamine™ Sf Transfection Reagent (store at 4°C until use)
- Opti-MEM™ I Reduced Serum Medium
- 6-well tissue culture plates and other tissue culture supplies
- 1.5-mL sterile microcentrifuge tubes
- Complete growth medium for culturing insect cells, such as:
 - Sf-900™ II SFM
 - Sf-900™ III SFM
 - TNM-FH
 - Grace's Supplemented Insect Cell Culture Medium

Transfection conditions

1. Calculate the number of Sf9 or Sf21 cells required for your transfection experiment, then expand cells accordingly.
Before transfection, ensure cells are healthy, with greater than 95% viability, and are growing in the logarithmic phase with a density of $1.5\text{--}2.5 \times 10^6$ cells/mL.
2. Produce baculovirus stocks in Sf9 or Sf21 cells according to the following table of transfection conditions.

Condition	Amount per well
Tissue culture plate size	6-well (35 mm) plate (one well/bacmid)
Number of Sf9 or Sf21 cells to transfect	1×10^6 cells
Medium volume	3 mL
Bacmid DNA	1 μ g (can vary from 1 to 2 μ g)
ExpiFectamine™ Sf Transfection Reagent	10 μ L (can vary from 5 to 10 μ L)

Note: Optimize transfection efficiency by varying cell density, and DNA and ExpiFectamine™ Sf Transfection Reagent concentrations.



Important guidelines for transfection

- ExpiFectamine™ Sf Transfection Reagent does not require removal of the culture medium or washing of the cells. It is not necessary to replace the medium after transfection.
- The DNA-lipid complex formation time is shorter (~5 minutes) when using ExpiFectamine™ Sf Transfection Reagent as compared to Cellfectin™ II Reagent.
- Do not add antibiotics during transfection as this causes cell death or interferes with transfection.

Transfect cells cultured in supplemented Grace's Medium

Use the following protocol to transfect cells in a 6-well format. All amounts and volumes are given on a per well basis.

1. Verify that the Sf9 or Sf21 cells are in the log phase ($1.5\text{--}2.5 \times 10^6$ cells/mL) with greater than 95% viability.
2. Select the appropriate procedure according to the following table.

Culture conditions	Procedure
For cells without antibiotics and a density within $1.5\text{--}2.5 \times 10^6$ cells/mL	<ol style="list-style-type: none"> 1. Detach the cells, then seed 8×10^5 cells per well in 2 mL unsupplemented (without antibiotics and serum) Grace's Insect medium. Note: Do not change medium or wash the cells as the medium carried over will enhance the transfection efficiency. 2. Allow cells to attach for 15 minutes at room temperature in the hood.
For cells containing antibiotics or the cell density is not within $1.5\text{--}2.5 \times 10^6$ cells/mL	<ol style="list-style-type: none"> 1. Prepare 10 mL Plating Medium by mixing 1.5 mL Supplemented (10% FBS) Grace's Insect Medium (without antibiotics) and 8.5 mL Grace's Insect Medium, Unsupplemented (without FBS and antibiotics). 2. Detach the cells, then seed 8×10^5 cells per well. 3. Allow cells to attach for 15 minutes at room temperature in the hood. 4. Replace the medium with 2.5 mL Plating Medium.

3. For each transfection sample, dilute 10 μ L ExpiFectamine™ Sf Transfection Reagent in 250 μ L Opti-MEM™ I Reduced Serum Medium.
4. Mix by inverting 5–10 times (do not vortex), then incubate for 5 minutes at room temperature.
5. Add 1 μ g undiluted bacmid DNA per sample.
Note: It is not necessary to pre-dilute bacmid DNA prior to addition.
6. Mix by inverting 5–10 times (do not vortex), then incubate for 5 minutes at room temperature.
Note: Do not incubate the mixture for longer than 20 minutes as this decreases transfection efficiency.



7. Add the entire DNA-lipid mixture dropwise onto the cells.
8. Incubate the cells at 27°C for 72–96 hours or until visible signs of virus infection.

Transfect cells cultured in serum-free medium

Use the following protocol to transfect cells in a 6-well format. All amounts and volumes are given on a per well basis.

1. Verify that the Sf9 or Sf21 cells are in the log phase ($2-4 \times 10^6$ cells/mL) with greater than 95% viability.
2. Detach the cells, then seed 1×10^6 cells per well in 3 mL culture medium. Allow cells to attach for 30–60 minutes at room temperature in the hood or by returning the plate to the incubator.
3. For each transfection sample, dilute 10 μ L ExpiFectamine™ Sf Transfection Reagent in 250 μ L Opti-MEM™ I Reduced Serum Medium.
4. Mix by inverting 5–10 times (do not vortex), then incubate for 5 minutes at room temperature.
5. Add 1 μ g undiluted bacmid DNA per sample.
Note: It is not necessary to pre-dilute bacmid DNA prior to addition.
6. Mix by inverting 5–10 times (do not vortex), then incubate for 5 minutes at room temperature.
Note: Do not incubate the mixture for longer than 20 minutes as this decreases transfection efficiency.
7. Add the entire DNA-lipid mixture dropwise onto the cells.
8. Incubate the cells at 27°C for 72–96 hours or until visible signs of virus infection.

Isolate P0 virus stock

Introduction

Budded virus is released into the medium ~72–96 hours after transfection. However, if transfection efficiency is suboptimal, the cells might not show all the signs of virus infection until 5 days posttransfection. Starting at 72 hours after transfection, visually inspect the cells daily for signs of infection. After the cells appear infected (that is, display characteristics typical of late to very late infection), harvest the virus from the cell culture medium, as described in this section.



Characteristics of infected cells

Table 2 Characteristics displayed by baculovirus infected insect cells under an inverted phase microscope (250-400X magnification)

Signs of infection	Phenotype	Description
Early (first 24 hours)	Increased cell diameter	A 25–50% increase in cell diameter can be seen.
	Increased size of cell nuclei	Nuclei can appear to “fill” the cells.
Late (24–72 hours)	Cessation of cell growth	Cells appear to stop growing when compared to a cell-only control.
	Granular appearance	Signs of virus budding such as vesicular appearance to cells.
	Detachment	Cells release from the plate or flask.
Very Late (>72 hours)	Cell lysis	Cells appear lysed, and show signs of clearing in the monolayer.

Prepare the P0 virus stock

1. When the transfected cells show signs of late stage infection (~72–96 hours posttransfection), transfer the medium containing the virus from each well (~2 mL) to sterile 15-mL snap-cap tubes.
2. Centrifuge the tubes at $500 \times g$ for 5 minutes to remove cells and large debris.
3. Transfer this clarified supernatant to fresh 15-mL snap-cap tubes.
Store this P0 virus stock at 4°C, protected from light. See “Store the virus stocks” on page 28 (below).

Note: To concentrate the virus stock and obtain a higher titer, filter the virus supernatant through a 0.2 µm, low protein binding filter after the low-speed centrifugation step.

Store the virus stocks

- Store virus stock at 4°C, protected from light.
- For long-term storage, store an aliquot of the virus stock at –80°C for later reamplification.
- Do not store routinely used virus stocks at temperatures below 4°C. Avoid repeated freeze/thaw cycles as they can result in a 10- to 100-fold decrease in virus titer.



Virus stock applications

Clarified P0 baculoviral stock can be used to:

- Amplify the virus stock (see “Amplify baculovirus stock” on page 29). This procedure is recommended to obtain the highest virus titers and optimal results in expression studies.
- Determine the titer of the virus stock (see “Perform a plaque assay” on page 31)
- Plaque purify recombinant baculovirus (see “Perform a plaque assay” on page 31)
- Infect Sf9 or Sf21 cells for preliminary expression experiments

Note: The amount of virus stock is limited and expression conditions may not be reproducible (such as, MOI is unknown if the titer has not been determined).

Amplify baculovirus stock

Introduction

The P0 virus stock is a small-scale, low-titer stock that can be used to infect cells to generate a high-titer P1 stock. The titer of the initial virus stock obtained from transfecting Sf9 or Sf21 cells generally ranges from 1×10^6 to 1×10^7 plaque forming units (pfu)/mL. Amplification allows production of a P1 virus stock with a titer ranging from 1×10^7 to 1×10^8 pfu/mL, and is recommended.

Guidelines and protocols are provided in this section to amplify the recombinant baculovirus to prepare a P1 virus stock.

Required materials

- Sf9 or Sf21 cells (in monolayer or suspension) cultured in the appropriate growth medium
- P0 baculovirus stock
- Any appropriate tissue culture container

Note: We recommend amplification in a 10 mL to 200 mL suspension culture at 2×10^6 cells/mL.

- Tissue culture reagents
- 27°C humidified incubator

Multiplicity of infection (MOI)

Calculate how much virus stock to add to obtain a specific multiplicity of infection (MOI), according to the following formula.

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

Note: We recommend an MOI of 0.05 to 0.1 pfu/cell. If the titer of the P0 virus stock has not been determined, assume that the titer ranges from 1×10^6 to 1×10^7 pfu/mL.

EXAMPLE: To infect a 10-mL culture at 2×10^6 cells/mL using an MOI of 0.1 with a P0 virus stock titer of 5×10^6 pfu/mL.

$$\text{Inoculum required (mL)} = \frac{(0.1 \text{ pfu/cell}) \times (2 \times 10^7 \text{ cells})}{5 \times 10^6 \text{ pfu/mL}}$$

$$\text{Inoculum required (mL)} = 0.4 \text{ mL}$$



Important considerations

For successful amplification of baculovirus, pay attention to several key points:

- Use Sf9 or Sf21 cells that are in excellent health, low passage (5–20), log-phase growth, and have >95% viability.
- Use a low MOI between 0.05–0.1 as higher MOI reduces baculovirus quality.
- Harvest the virus when 60–80% of cells are dead.
- Do not amplify baculovirus indefinitely, as baculovirus acquire deleterious mutations with each passage. Usually, P3 is the highest usable passage.

Amplify P0 virus stock in a 1-L flask

1. On the day of infection, prepare an Sf9 or Sf21 cell suspension by seeding cells at 2×10^6 cells/mL in 200 mL of culture medium.
2. Add the appropriate amount of P0 virus stock to the flask.
3. Incubate the cells for 72–96 hours in a humidified incubator at 27°C on an orbital shaker, according to the following table.

Shaking orbit	Speed (rpm)
19-mm	125 ±5
25-mm	125 ±5
50-mm	95 ±5

4. Transfer the medium containing virus from the flask to sterile ≥50-mL tubes, then centrifuge the tubes at $500 \times g$ for 5 minutes to clarify the baculovirus stock.

Note: Determine optimal harvest times for each baculovirus construct. Culture viability decreases over time as cells lyse.

5. Transfer the supernatant to fresh collecting tubes.
This is the P1 virus stock.
6. Store at 4°C, protected from light. For long-term storage, aliquot the P1 stock and store at –80°C, protected from light. For storage guidelines see “Store the virus stocks” on page 28.

Scale up the amplification procedure

After generating a high-titer P1 baculovirus stock, you can scale up the amplification procedure to any volume of your choice. To produce this high-titer P2 stock, scale up the number of cells and volume of virus that is used appropriately, and follow the guidelines and procedure that is outlined in “Amplify baculovirus stock” on page 29.

Generate high-titer stocks from frozen master stock

We recommend amplifying virus master stock that is stored at –80°C to generate another high-titer stock for use in expression experiments. Virus titer generally decreases over time when virus is stored at –80°C. Follow the guidelines and amplification procedure that is detailed in “Amplify baculovirus stock” on page 29.



Perform a plaque assay

Introduction

We recommend that you perform a plaque assay to determine the titer of your virus stock. You can also perform a plaque assay to purify a single virus clone. In this procedure, you infect cells with dilutions of virus stock and identify focal points of infection (plaques) on an agarose overlay. You can also titer your virus stock by the endpoint dilution method.

Experimental outline

To determine the titer of a baculovirus stock.

Plate Sf9 or Sf21 cells in 6-well plates



Prepare 10-fold serial dilutions of baculovirus stock



Add the different dilutions of baculovirus to cells, then incubate for 1 hour



Remove the virus, then overlay the cell monolayer with Plaquing Medium



Incubate the cells for 7–10 days, then stain and count the number of plaques in each dilution

Factors affecting virus titer

- **The size of the gene of interest:** Titer generally decreases as the size of the insert increases.
- **Transfection efficiency:** For the highest transfection efficiency, we recommend transfecting Sf9 or Sf21 cells using ExpiFectamine™ Sf Transfection Reagent. Prepare DNA: lipid complexes in Opti-MEM™ I Reduced Serum Medium.
- **The age of the baculovirus stock:** Virus titer can decrease with long-term storage at 4°C or –80°C. Titer baculovirus stock that has been stored for ≥6 months before use in an expression experiment.
- **Freeze/thaw cycles:** Virus titer can decrease as much as 10% with each freeze/thaw cycle.
- **Improper storage of your baculovirus stock:** For routine use, aliquot baculovirus stock and store at 4°C, protected from light.

Required materials

- Clarified baculovirus stock (store at 4°C until use)
- Sf9 or Sf21 cells cultured in the appropriate medium (30 mL of log-phase cells at 5×10^5 cells/mL for each baculovirus stock to be titered)
- Sf-900™ II SFM, Sf-900™ III SFM or other appropriate complete growth medium
- Sf-900™ Medium (1.3X) (100 mL) or other appropriate plaquing medium
- 4% agarose gel (specifically formulated for optimal insect cell growth)
- Sterile, cell-culture grade distilled water



- 100 mL sterile glass bottle
- 6-well tissue-culture plates (2 plates for each virus stock to be titered)
- Sterile hood
- Water baths at 40°C and 70°C
- (Optional) Microwave oven
- 27°C humidified incubator
- Neutral red (Sigma-Aldrich™ Cat. No. N7005)

Note: When culturing Sf9 or Sf21 cells in serum-supplemented media, such as complete TNM-FH, have the following reagents on hand:

- Grace's Insect Cell Culture Medium, Supplemented
- Grace's Insect Cell Culture Medium (2X)
- Fetal Bovine Serum (FBS), qualified, heat-inactivated

Prepare the plaquing medium

Plaquing medium consists of a mixture of culture medium and agarose, and is used to immobilize the infected cells for the plaque assay. Prepare Plaquing Medium immediately before use by the following procedure.

Note: If you are culturing Sf9 cells in Sf-900™ II SFM or Sf-900™ III SFM, prepare Sf-900 Plaquing Medium. If you are culturing cells in TNM-FH, prepare Grace's Plaquing Medium.

1. Melt the 4% agarose gel by placing the bottle in a 70°C water bath for 20 to 30 minutes or by heating the agarose in a microwave oven.
2. While the 4% agarose gel is melting, place the following in the 40°C water bath:
 - Empty, sterile 100-mL bottle
 - Sf-900 Medium (1.3X) or Grace's Insect Cell Culture Medium (2X), as appropriate
3. When the 4% agarose gel has liquefied, move the agarose gel, medium, and empty 100-mL bottle to a sterile hood.
4. Prepare the Plaquing Medium according to the following table.

Medium	Procedure
Sf-900 Plaquing Medium	In the empty 100-mL bottle, combine 30 mL of Sf-900 Medium (1.3X) with 10 mL of the melted 4% agarose gel, then mix gently.
Grace's Plaquing Medium	<ol style="list-style-type: none"> 1. Add 20 mL of heat-inactivated FBS to the 100-mL bottle of Grace's Insect Medium (2X), then mix. 2. In the empty 100-mL bottle, combine 25 mL of Grace's Insect Medium (2X, with serum added) with 12.5 mL of cell-culture grade, sterile, distilled water and 12.5 mL of the melted 4% agarose gel, then mix gently.

Note: Work quickly so that the agarose does not harden.

5. Return the bottle of Plaquing Medium to the 40°C water bath until use.



Perform plaque assay

Use this procedure to determine the titer of pFastBac™ baculovirus stock, including control vector stocks. The amounts provided are to titer one baculovirus stock (two 6-well plates per virus stock).

Note: Include a negative control (no virus) in your experiment.

1. On the day of infection, harvest Sf9 or Sf21 cells, then prepare a 30 mL cell suspension at 5×10^5 cells/mL in Sf-900™ II SFM (or other complete growth medium).
2. Aliquot 2 mL of cell suspension into each well of two 6-well plates.
3. Incubate the cells, covered, at room temperature for 1 hour, to allow the cells to settle to the bottom of the plate.
4. After the 1 hour incubation, ensure, by observation, that the cells are attached and at 50% confluence.
5. Prepare an 8-log serial dilution (10^{-1} to 10^{-8}) of the clarified baculovirus stock.

Medium	Description
Sf-900™ II SFM	In 12 mL disposable tubes, sequentially dilute 0.5 mL of the baculovirus stock (or previous dilution) in 4.5 mL of medium.
Grace's Insect Cell Culture Medium, Supplemented, without FBS	

Note: This results in 8 tubes of diluted virus stock (10^{-1} to 10^{-8}). Only dilutions 10^{-4} to 10^{-8} are to be used.

6. Move the 6-well plates of cells and the tubes of diluted virus to a sterile hood.
7. Label the plates, in columns of 2 (a sample well plus a duplicate well) as follows: no virus (negative control), 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} .
8. Remove the medium from each well, then immediately add 1 mL of the appropriate inoculum (virus dilution). For the negative control, add the appropriate medium without virus.
9. Incubate cells with virus for 1 hour at room temperature.
10. After the 1 hour incubation, move the bottle of plaquing medium from the 40°C water bath and the 6-well plates of infected cells to a sterile hood.
11. Sequentially, going from the highest dilution (10^{-8}) to the lowest dilution (10^{-4}), remove the inoculum from the wells, then add 2 mL of plaquing medium.

Note: Work quickly to avoid desiccation of the cell monolayer.

12. Allow the agarose overlay to harden for 1 hour at room temperature before moving the plates.



13. Incubate the cells in a 27°C humidified incubator for 7–10 days until plaques are visible and ready to count.
 - To stain the plaques to facilitate counting, see “Neutral Red staining” on page 34
 - To calculate the titer, see “Calculate the titer” on page 35

Neutral Red staining

Staining with Neutral Red improves the visualization of plaques. Crystalline Blue and other plaque staining dyes containing organic solvents are not recommended because they kill the host cells. Stain the plaques by either of the following ways:

- Prepare an agarose solution containing Neutral Red and overlay this solution on the plates 4 days post-infection. Count the plaques 7–10 days post-infection.
- Prepare a Neutral Red solution and add to the plates for 1–2 hours just prior to counting plaques (7–10 days post-infection).

IMPORTANT! For plaque purification, do not stain with Neutral Red as it is a known mutagen that can alter the recombinant virus.

Prepare Neutral Red agarose overlay (for use on Day 4)

1. Prepare a 1 mg/mL Neutral Red solution in Sf-900™ II SFM (or other appropriate complete growth medium), then filter-sterilize it.
2. In a 50-mL tube, combine 16.5 mL of Sf-900™ II SFM with 1.5 mL of Neutral Red solution (1 mg/mL), then place in a 40°C water bath.
3. Microwave the 4% agarose gel until melted, then place in a 40°C water bath for 5 minutes.
4. Move the 50 mL tube of Neutral Red solution and the 4% agarose gel to a sterile hood.
5. Prepare the Neutral Red overlay by adding 6 mL of 4% agarose gel to the Neutral Red solution.
6. Add 1 mL of the Neutral Red overlay to each well.
7. When the agarose has hardened, return the plates to a 27°C humidified incubator until the plaques are ready to count.

Note: Plaques will appear as clear spots on a red monolayer.

Prepare Neutral Red stain (for use on Day 7–10 prior to counting plaques)

1. Prepare a 1 mg/mL Neutral Red solution in cell-culture grade, distilled water.
2. Add 0.5 mL of Neutral Red solution to each well, then incubate for 1–2 hours at room temperature.
3. Gently remove the excess stain with a pipet or blotter, then count the plaques.

Note: Plaques will appear as clear spots on a red monolayer.



Calculate the titer

Count the number of plaques present in each dilution, then use the following formula to calculate the titer (plaque forming units (pfu)/mL) of the virus stock.

Note: The optimal range to count is 3 to 20 plaques per well of a 6-well plate.

$$\text{Titer (pfu/well)} = \text{number of plaques} \times \text{dilution factor} \times \frac{1}{\text{mL of inoculum/well}}$$

Example: Add 1 mL of inoculum and observe 20 plaques in the well containing the 10^{-6} virus dilution. Using the formula, the titer of this virus stock is:

$$\text{Titer (pfu/mL)} = 20 \text{ plaques} \times 10^6 \times \frac{1}{1 \text{ mL of inoculum/well}}$$

$$\text{Titer (pfu/mL)} = 2 \times 10^7 \text{ pfu/mL}$$

What you should see

The expected titer for pFastBac™ baculovirus stock ranges from:

- 1×10^6 to 1×10^7 pfu/mL for P1 virus stock
- 1×10^7 to 1×10^8 pfu/mL for P2 virus stock

Note: If the baculovirus stock titer is less than 1×10^6 or 1×10^7 pfu/mL for a P1 or P2 virus stock, respectively, produce a new baculovirus stock.

For tips and guidelines to optimize the virus yield, see “Factors affecting virus titer” on page 31, and the troubleshooting section “Low yield of virus” on page 42.

Plaque purification

Prepare virus stock from a single virus clone by plaque purifying the baculovirus.

Required materials

- Plate containing well-spaced virus plaques
 - Log phase Sf9 or Sf21 cells at greater than 95% viability
 - Sterile Pasteur pipette
1. On the day of infection, harvest Sf9 or Sf21 cells, then prepare a 30 mL cell suspension at 5×10^5 cells/mL in Sf-900™ II SFM (or other complete growth medium).
 2. Aliquot 2 mL of cell suspension into each well of two 6-well plates.
 3. Incubate the cells, covered, at room temperature for 1 hour, to allow the cells to settle to the bottom of the plate.
 4. After the 1 hour incubation, ensure, by observation, that the cells are attached and at 50% confluence.
 5. Using a sterile Pasteur pipette, carefully pick a clear plaque, then transfer the agarose plug (containing virus) to a 1.5 mL microcentrifuge tube containing 500 μ L of complete growth medium.
 6. Mix well by vortexing, then add 100 μ L of the agarose plug solution to each well.
 7. Incubate the cells in a 27°C humidified incubator for 72 hours.



8. Collect the medium containing virus from each well (~2 mL), then transfer to sterile 15-mL snap-cap tubes.
9. Centrifuge the tubes at $500 \times g$ for 5 minutes to remove cells and large debris.
10. Transfer the clarified supernatant to fresh 15-mL snap-cap tubes. This is the plaque-purified virus stock.
11. Proceed to “Amplify baculovirus stock” on page 29.

Express recombinant protein

Introduction

The high titer (1×10^8 pfu/mL) pFastBac™ baculovirus stock can be used to infect insect cells and assay for expression of the recombinant protein. Guidelines for infection and expression are provided in this section.

Note: The ExpiSf™ Expression System enables high-density infection of suspension ExpiSf9™ cells to generate superior protein yields in a chemically defined, yeastolate-free growth medium. For further information, go to thermofisher.com/expisf.

Recommendation when using pFastBac™/HBM TOPO™ vector

When using the Bac-to-Bac™ HBM TOPO™ Secreted Expression System, we recommend using High Five™ cells adapted to suspension culture in serum-free medium for expression of your secreted proteins.

High Five™ cells

High Five™ cells (see Appendix D, “Ordering information”) are well suited for expression of secreted recombinant proteins. The BT1-TN-5B1-4 cell line originates from egg cells of *Trichoplusia ni* (cabbage looper ovary), the native host of AcMNPV. This cell line has the following characteristics:

- Grows well in monolayer and doubles in less than 24 hours
- 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

For more information on High Five™ cells or a protocol for adaptation to suspension culture, refer to the *Growth and maintenance of insect cell lines User Guide* (Pub. No. MAN0000030).

Positive control

Include recombinant baculovirus that are generated from pFastBac™ control plasmid for use as an expression control. After cells have been infected with pFastBac™ control virus, the gene encoding Gus and or CAT will be constitutively expressed and can be easily assayed (see “Assay for β -glucuronidase” on page 40).



Guidelines for expression

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

- **Cell line:** Depending on the application and gene of interest, use any insect cell line including Sf9, Sf21, High Five™, or Mimic™ Sf9 for expression. Grow the cells either in adherent or suspension culture using your culture container of choice.
Note: Improve expression of a secreted protein by using High Five™ cells.
- **Culture Conditions:** Culture cells in serum-free conditions using Sf-900™ II SFM, Sf-900™ III SFM, or Express Five™ SFM as appropriate. Some applications and proteins of interest require the culture to be supplemented with 0.1% to 0.5% FBS or BSA post-infection, to protect the recombinant protein from proteolysis. Protein-based protease inhibitors are generally less expensive and more effective than many synthetic protease inhibitors.
- **Infection Conditions:** Infect cultures when cells are in the mid-logarithmic phase of growth at a density of 1×10^6 to 2×10^6 cells/mL. Ensure that the culture is not rate-limited by nutritional (amino acid or carbohydrate utilization) or environmental factors (pH, dissolved O₂, or temperature) during infection.
- **MOI:** Optimal MOI varies between cell lines and the relative infection kinetics of the virus isolate or clone used. Establish a dose for each virus, medium, reactor, and cell line employed to determine the optimal infection parameters to use for protein expression. As a starting point, infect cells using an MOI of 5 to 10 pfu/cell.
- **Time course:** Perform a time course to determine the expression kinetics for your recombinant protein as many proteins can be degraded by cellular proteases that are released in cell culture.

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

Calculate virus volumes

Calculate the volume of virus stock that is needed to achieve a given MOI using the following formula.

$$\text{Volume of virus} = \frac{\text{MOI (desired)} (\text{number of cells})}{\text{titer of viral stock}}$$

Example: To infect a spinner with 50 mL of culture at a cell density of 2×10^6 cells/mL (1×10^8 total cells) at an MOI of 5 pfu/cell using a high-titer virus stock at 1×10^8 pfu (virions)/mL, calculate as follows:

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

Optimize expression

Use these guidelines to determine the optimal conditions to use for expressing your recombinant protein of interest.

Condition	Procedure
Cell line	<ol style="list-style-type: none"> 1. Infect Sf9, Sf21, High Five™, or Mimic™ Sf9 cells at a constant MOI. 2. Assay for recombinant protein expression at different times post-infection (such as 24, 48, 72, 96 hours post-infection). 3. Select the cell line that provides the optimal level of recombinant protein expression.



Condition	Procedure
MOI	<ol style="list-style-type: none"> 1. Infect a population of cells at varying MOIs (such as 1, 2, 5, 10, or 20) and assay for protein expression. 2. Use the MOI that provides the optimal level of recombinant protein expression.
Time course	<ol style="list-style-type: none"> 1. Infect cells at a constant MOI and assay for recombinant protein expression at different time points post-infection (such as 24, 48, 72, or 96 hours). 2. Select the time point at which the recombinant protein expression is optimal.

Harvest baculovirus infected insect cells

1. Seed 6×10^5 Sf9 or Sf21 cells per well in a 24-well plate, then let the cells attach for at least 30 minutes.
2. Rinse the cells one time with fresh growth media, then replace with 300 mL of fresh media.
3. Add the pFastBac™ baculovirus stock to each well at the desired MOI. Include the appropriate controls (mock-infected (uninfected) cells, pFastBac™ positive control baculovirus, previously characterized recombinant baculovirus).
4. Incubate the cells in a 27°C humidified incubator.
5. Harvest cells (or media, if the recombinant protein is secreted) at the appropriate time (24, 48, 72, or 96 hours post-infection).
Note: If harvesting cells, remove the media, then rinse the cells one time with serum-free medium.
6. Lyse the cells with 400 µL of 1X SDS-PAGE Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS).
7. Freeze samples at -20°C or boil samples for at least 3 minutes before separating proteins by SDS-PAGE.

Analyze recombinant protein

Introduction

After harvesting baculovirus infected insect cells, analyze the expression of your protein by SDS-PAGE or western blot. For secreted proteins, determine that all the protein is being secreted by analyzing both the supernatant and the cell lysate. Analyzing cell lysates can help with optimizing the MOI and time course for expression (see “Optimize expression” on page 37). After determining the optimal experimental parameters for secreted expression, proceed with large-scale expression experiments.




Protease inhibitors

We recommend that you add one or more protease inhibitors to your lysis buffer during protein isolation.

Protease inhibitor	Method of action	Stock solution	Working concentration	Storage
PMSF	Serine protease inhibitor	10 mg/mL in isopropanol	100 µg/mL	20–25°C ^[1]
Leupeptin	Serine and thiol protease inhibitor	50 µg/mL in deionized water	0.5 µg/mL	–20°C
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	

^[1] Store in isopropanol

Note: PMSF is not stable in aqueous solution. Add it to the lysis buffer just before use.

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

Prepare cell lysates

Use any method to prepare cell lysates for analysis, including:

- Detergent lysis
- Sonication lysis
- Freeze-thaw lysis

Detect recombinant protein

Select any method of choice to detect the recombinant protein of interest, including functional analysis or western blot.

The pFastBac[™]/CT-TOPO[™] and pFastBac[™]/NT-TOPO[™] vectors allow the expression of the recombinant protein of interest as a 6xHis fusion. Similarly, The pFastBac[™]/NT-TOPO[™] vector allows the expression of your recombinant protein of interest as a C-terminal 6xHis fusion. Use the antibodies (available separately, see Appendix D, “Ordering information”) in the following table to detect the recombinant protein.

For use with	Antibody	Epitope
pFastBac [™] /CT-TOPO [™]	Anti-His (C-term) Antibody Anti-His (C-term)-HRP Antibody Anti-His (C-term)-AP Antibody	Detects the C-terminal polyhistidine (6xHis) tag: HHHHHH-COOH (requires the free carboxyl group for detection)
pFastBac [™] /HBM TOPO [™]		
pFastBac [™] /CT-TOPO [™]		
pFastBac [™] /NT-TOPO [™]	Penta-His [™] mouse IgG1 monoclonal Antibody	Detects both C- and N-terminal polyhistidine (6xHis) tag
pFastBac [™] /HBM TOPO [™]		



Note: C- or N-terminal 6xHis tags and the Tobacco Etch Virus (TEV) recognition site increase the size of your protein by at least 3 kDa on PAGE gels. The HBM secretion signal coding sequence is cleaved on secretion, and therefore does not change the size of your protein.

Assay for β -glucuronidase

- Identify blue plaques on agarose plates containing the chromogenic indicator X-glucuronide.
 - a. Mix 5 mL of a 20 mg/mL X-glucuronide solution (in DMSO or dimethylformamide) with 50 mL of cell-free medium from the infected cells.
 - b. Monitor for the development of blue color within 2 hours.
- Alternatively, histochemical detection of β -glucuronidase in infected insect cells can be performed using x-Gluc, see Appendix D, "Ordering information".

Purify recombinant protein

The presence of the C- and N-terminal 6xHis tag in the pFastBac[™]/CT-TOPO[™] or pFastBac[™]/HBM TOPO[™] and pFastBac[™]/NT-TOPO[™] vectors, respectively, allows the purification of your recombinant protein with a metal-chelating resin such as ProBond[™] and Ni-NTA. For more information, go to thermofisher.com.

Remove the 6x His tag using AcTEV[™] Protease

The pFastBac[™]/CT-TOPO[™], pFastBac[™]/NT-TOPO[™] and pFastBac[™]/HBM TOPO[™] vectors contain a Tobacco Etch Virus (TEV) recognition site that allows the removal of the 6xHis tag from your recombinant fusion protein using AcTEV[™] Protease (available separately). For more information, go to thermofisher.com.



Troubleshooting

Recombinant bacmid DNA generation

Observation	Possible cause	Recommended action	
No blue colonies Although you pick white colonies, expect to see some blue colonies (contain non-recombinant bacmids).	Insufficient time for color development	Wait at least 48 hours before identifying colony phenotypes.	
	Used X-gal instead of Bluo-gal in agar plates	Use Bluo-gal in selective plates to increase the contrast between blue and white colonies.	
	Insufficient growth after transposition	Grow transformed cells in S.O.C. Medium for at least 4 hours before plating.	
	Bluo-gal and IPTG omitted from plates	Prepare fresh selective plates containing 50 mg/mL kanamycin, 7 mg/mL gentamicin, 10 mg/mL tetracycline, 100 mg/mL Bluo-gal, and 40 mg/mL IPTG.	
	Too many colonies on the plate		Serially dilute the transformation mixture and plate to give well-separated colonies.
			Adjust the serial dilutions of cells (10^{-2} to 10^{-4}) to obtain well-spaced colonies.
	Plates too old or stored in light		Do not use plates that are more than 4 weeks old.
Store plates protected from light.			
Incubation time too short or temperature too low		Wait at least 48 hours before picking colonies. Incubate plates at 37°C.	
All colonies are blue	DNA from the pFastBac™ construct used for transformation was of poor quality	Use purified plasmid DNA for transformation.	
		Check the quality of your plasmid DNA and ensure that the DNA is not degraded.	
	Gentamicin was omitted from the plates	Prepare fresh selective plates containing 50 mg/mL kanamycin, 7 mg/mL gentamicin, 10 mg/mL tetracycline, 100 mg/mL Bluo-gal, and 40 mg/mL IPTG.	
Few colonies obtained	Used LB medium for recovery/expression	Use S.O.C. Medium for the 4-hour growth time.	
	Recovery/expression time too short	Increase the recovery time to >4 hours at 37°C or 6 hours at 30°C.	
Poor blue/white colony differentiation	Agar not at correct pH	Adjust pH of LB agar to 7.0.	
	Intensity of the blue color too weak	Use Bluo-gal, not X-gal.	
		Increase the concentration of Bluo-gal to 300 mg/mL.	
		Use dark and light backgrounds to view plates.	



Observation	Possible cause	Recommended action
Poor blue/white colony differentiation	Too many or too few colonies on plate	Adjust the serial dilutions of cells to obtain an optimal number of colonies.
	Incubation time too short or temperature too low	Do not pick colonies until 48 hours after plating.
		Incubate plates at 37°C.
IPTG concentration not optimal	Optimize the IPTG concentration. A range of 20–60 mg/mL IPTG generally gives optimal color development.	

Bacmid DNA isolation

Observation	Possible cause	Recommended action
Bacmid DNA is degraded	DNA stored improperly	Store purified bacmid DNA in aliquots at 4°C for no more than 2 weeks.
		Do not freeze/thaw the bacmid DNA.
		For long-term storage of bacmid DNA, prepare glycerol stocks of DH10Bac™ <i>E. coli</i> containing the ensured bacmid DNA.
	High molecular weight bacmid DNA handled improperly	When isolating bacmid DNA, do not vortex the DNA solution.
Do not resuspend DNA pellets mechanically. Allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube.		
Poor yield	Used incorrect antibiotic concentrations	Grow transformed DH10Bac™ cells in LB medium containing 50 mg/mL kanamycin, 7 mg/mL gentamicin, and 10 mg/mL tetracycline.
Bacmid DNA contains a mixture of recombinant bacmid and empty bacmid	Picked a colony that was gray or dark in the center	Analyze more white DH10Bac™ transformants and select one that contains recombinant bacmid DNA only.

Insect cells transfection

Observation	Possible cause	Recommended action
Low yield of virus	Low transfection efficiency	Use ExpiFectamine™ Sf Transfection Reagent for transfection.
		Ensure that no supplements, FBS, or antibiotics are present in the media during transfection.
		Harvest virus supernatant when signs of infection are visible, that is >72 hours post-transfection.



Observation	Possible cause	Recommended action	
Low yield of virus	Cells plated too sparsely	Plate insect cells at the recommended cell density.	
	Used too much or too little ExpiFectamine™ Sf Transfection Reagent or other lipid reagent	Optimize the amount of ExpiFectamine™ Sf Transfection Reagent or other lipid reagent used.	
	Time of incubation with DNA/lipid complexes too short or too long	Incubate DNA/lipid complexes for only 5 minutes before addition to cells. Incubating this mixture for longer than 20 minutes can lead to lower transfection efficiencies.	
	Recombinant bacmid DNA is degraded		Check the quality of your recombinant DNA by agarose gel electrophoresis before transfection.
			Prepare bacmid DNA using PureLink™ HiPure Plasmid Prep Kits.
			Store purified bacmid at 4°C. Do not freeze at -80°C as it decreases transfection efficiency.
	Bacmid DNA is not pure, that is it contains recombinant bacmid and empty bacmid		Screen other DH10Bac™ transformants and select one that contains only recombinant bacmid.
Perform plaque purification to isolate recombinant baculovirus.			

Protein expression

Observation	Possible cause	Recommended action	
Low protein expression	virus stock contains a mixture of recombinant and non-recombinant baculovirus	Perform plaque purification to isolate recombinant baculovirus.	
	Baculovirus not recombinant		Ensure transposition by PCR analysis of bacmid DNA using the pUC/M13 Forward and Reverse primers.
			Re-transfect insect cells with new recombinant bacmid DNA.
	Used too low or too high virus titer		Optimize infection conditions by varying the MOI.
	Time of cell harvest not optimal		Perform a time course of expression to determine the optimal time to obtain maximal protein expression.
	Cell growth conditions and medium not optimal		Optimize culture conditions according to the size of your culture container and expression conditions.
			Culture cells in Sf-900™ II SFM or Sf-900™ III SFM for optimal cell growth and protein expression.
Cell line not optimal		Try other insect cell lines.	



Observation	Possible cause	Recommended action
Low protein expression	Protein expression is not optimal.	Optimize protein expression by varying such parameters as incubation temperature and oxygenation.



Supporting protocols

Antibiotic stock solutions

Antibiotics can be ordered in either dry powdered form or as a stabilized, sterile, premixed solution. Store these solutions according to the manufacturer's recommendations. Prepare and store stock solutions of the antibiotics according to the following table.

Antibiotic	Stock Solution Concentration		Storage
Ampicillin	50 mg/mL in water	filter-sterilize	-20°C, protected from light
Kanamycin	10 mg/mL in water		
Tetracycline	10 mg/mL in 100% ethanol		
Gentamicin	7 mg/mL in water		

IPTG (200 mg/mL) stock solution

1. Dissolve 2 g of IPTG in 8 mL of sterile water.
2. Adjust the volume of the solution to 10 mL with sterile water.
3. Filter-sterilize through a 0.22-micron filter.
4. Dispense the stock solution into 1-mL aliquots.
5. Store at -20°C.

Bluo-gal (20 mg/mL) stock solution

1. Dissolve the Bluo-gal in dimethylformamide or dimethylsulfoxide (DMSO) to make a 20 mg/mL stock solution.

Note: Use a glass or polypropylene tube.

IMPORTANT! Exercise caution when working with dimethylformamide. Dispense solutions in a vented chemical hood only.



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

LB (Luria-Bertani) Medium

Composition:

- 1.0% Tryptone (casein peptone)
 - 0.5% Yeast Extract
 - 1.0% NaCl
 - pH 7.0
1. For 1 L, dissolve the following in 950 mL deionized water.
 - 10 g of tryptone
 - 5 g of yeast extract
 - 10 g of NaCl
 2. Adjust the solution to pH 7.0 with NaOH, then bring the volume up to 1 L.
 3. Autoclave on liquid cycle for 20 minutes.
 4. Allow the solution to cool to $\sim 55^{\circ}\text{C}$, then add antibiotics if needed.
 5. Store at room temperature or at 4°C .

LB (Luria-Bertani) Plates

1. Prepare LB medium as described in “LB (Luria-Bertani) Medium” on page 46, but add 15 g/L of agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. Allow the solution to cool to $\sim 55^{\circ}\text{C}$, then add antibiotics before pouring into 10 cm plates.
4. Let the solution harden, then invert and store at 4°C , in the dark.
Note: Plates containing antibiotics are stable for up to 4 weeks at 4°C .



Bacmid DNA Isolation Using PureLink™ HiPure Maxiprep Kit

Introduction

After you have transformed your pFastBac™ TOPO™ construct containing your gene of interest into DH10Bac™ *E. coli* and performed the transposition reaction, use the PureLink™ HiPure Plasmid Maxiprep Kit to purify recombinant bacmid DNA from DH10Bac™ transformant.

Bacmid DNA purified by this method is suitable for use in PCR analysis or insect cell transfections.

Note: We do not recommend the PureLink™ HiPure Precipitator Module or the PureLink™ HiPure Plasmid Filter Mini/Midi/Maxiprep Kits for isolating bacmid DNA.

For more information on PureLink™ HiPure purification products, see thermofisher.com.

Grow bacmid DNA stock

Growing bacmid DNA stock from DH10Bac™ transformants in LB medium requires three days.

1. **Day 1:** Pick a single white bacterial colony from among the DH10Bac™ transformants (see “Transform DH10Bac™ *E. coli*” on page 17) and inoculate 4 mL of LB medium containing 50 mg/mL kanamycin, 7 mg/mL gentamicin and 10 mg/mL tetracycline. Alternatively, you can thaw glycerol stocks of DH10Bac™ cells harboring your verified recombinant bacmid and use 100 mL to inoculation.
2. Incubate the culture at 37°C in a shaking water bath at 250 rpm overnight.
3. **Day 2:** Transfer the entire 4 mL of overnight culture into 50 mL of fresh LB medium with antibiotics (as above) and incubate at 37°C in a shaking water bath at 250 rpm overnight.
4. **Day 3:** Transfer the entire 50 mL of overnight culture into 500 mL of fresh LB medium with antibiotics (as above) and incubate at 37°C in a shaking water bath at 250 rpm overnight.
5. **Day 4:** proceed with the PureLink™ HiPure bacmid DNA isolation procedure.



Before Starting

Before beginning, verify that RNase A has been added to the Resuspension Buffer (R3) and that no precipitate has formed in the Lysis Buffer (L7).

Equilibrate the column

Place the PureLink™ HiPure Maxi column on the PureLink™ Nucleic Acid Purification Rack (see the manual supplied with the rack for more details). Apply 30 mL Equilibration Buffer (EQ1) to the column. Allow the solution in the column to drain by gravity flow.

Prepare the cell lysate

1. Harvest 250–500 mL of the overnight culture by centrifuging at $4,000 \times g$ for 10 minutes in a bucket.
Remove all medium.
2. Add 20 mL Resuspension Buffer (R3) with RNase A to the pellet and resuspend the cells until homogeneous.
3. Transfer cell suspension to a 50-mL centrifuge tube.
4. Add 20 mL Lysis Buffer (L7). Mix gently by inverting the capped tube five times.
Do not vortex.
5. Incubate at room temperature for 5 minutes.
Note: Do not allow lysis to proceed for more than 5 minutes.
6. Add 20 mL Precipitation Buffer (N3) and mix immediately by inverting the capped tube until the mixture is homogeneous.
Do not vortex.
7. Centrifuge the mixture at $>12,000 \times g$ at room temperature for 10 minutes.
Note: If the pellet does not adhere to the bottom of the tube, incubate the tube at room temperature for 5 minutes to allow the separation of the lysate and gelatinous pellet.
8. Pipette the clear lysate into another tube and centrifuge at $>15,000 \times g$ for 5 minutes at room temperature to remove any remaining cellular debris.



Bind and wash the DNA

1. Load the supernatant onto the equilibrated column. Allow the solution in the column to drain by gravity flow.
2. Wash the column with 60 mL Wash Buffer (W8).
3. Allow the solution in the column to drain by gravity flow after each wash. Discard the flow-through.

Elute and precipitate the DNA

1. Place a sterile 30 mL centrifuge tube (elution tube) under the column.
2. Add 15 mL Elution Buffer (E4) to the column to elute DNA. Allow the solution to drain by gravity flow. Do not force out any remaining solution. The elution tube contains the purified DNA. Discard the column.
3. Add 10.5 mL isopropanol to the elution tube. Mix well.
4. Centrifuge the mixture at $>15,000 \times g$ at 4°C for 30 minutes. Carefully remove and discard the supernatant.
5. Add 1 mL 70% ethanol to the pellet in the 30 mL elution tube, displace the pellet from the side of the tube, and transfer all the pellet fragments into a 1.5 mL microcentrifuge tube.
6. Centrifuge at $>15,000 \times g$ at 4°C for 10 minutes. Carefully remove and discard the supernatant.
7. Add another 1 mL fresh 70% ethanol to the pellet in the microcentrifuge tube, and centrifuge at $>15,000 \times g$ at 4°C for another 10 minutes (second wash). Carefully remove and discard the supernatant.
8. Air-dry the pellet at room temperature until the appearance of the pellet changes from white-opaque to translucent and crystalline.
9. Resuspend the DNA pellet in 200–500 μL TE Buffer, pH 8.0 by vortexing.
10. Measure the concentration of the purified bacmid DNA. The concentration should be in range of 150–300 ng/mL



11. Store the tube at 4°C.

IMPORTANT!

You may store your bacmid DNA at -20°C if you avoid frequent freeze/thaw cycles as it decreases the transfection efficiency.

To store your purified bacmid DNA at -20°C, aliquot into separate tubes in TE Buffer, pH 8.0 to avoid more than one freeze/thaw cycle and **do not** store in a frost-free freezer. You may also store the purified bacmid DNA for up to 2 weeks at 4°C in TE Buffer, pH 8.0.

You may prepare glycerol stocks of DH10Bac™ *E. coli* containing the bacmid DNA from mid-logarithmic phase culture grown from white colonies picked during the blue-white screening, and store at -80°C for future bacmid DNA isolation.



Ordering information

Unless otherwise indicated, all materials are available through thermofisher.com.

Additional products

Item	Amount	Source
Bac-to-Bac™ C-His TOPO™ Cloning Kit	1 kit	A11098
Bac-to-Bac™ N-His TOPO™ Cloning Kit	1 kit	A11099
Bac-to-Bac™ HBM TOPO™ Cloning Kit	1 kit	A11338
MAX Efficiency™ DH10Bac™ Competent Cells	5 × 100 µL	10361012
One Shot™ Mach1™ -T1 R Chemically Competent <i>E. coli</i>	21 × 50 µL	C8620-03
ExpiFectamine™ Sf Transfection Reagent	1 mL	A38915
Platinum™ Pfx DNA Polymerase	100 units	11708-013
AccuPrime™ Pfx DNA Polymerase	200 reactions	12344-024
Pfx50™ DNA Polymerase	100 reactions	12355-012
Platinum™ Taq DNA Polymerase High Fidelity	100 reactions	11304-011
PureLink™ PCR Purification Kit	50 preps	K3100-01
PureLink™ Quick Gel Extraction System	1 kit	K2100-12
PureLink™ HiPure Plasmid Miniprep Kit	25 preps	K2100-02
	100 preps	K2100-03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100-04
	50 preps	K2100-05
PureLink™ HiPure Plasmid Maxiprep Kit	10 preps	K2100-06
	25 preps	K2100-07
Ampicillin Sodium Salt, irradiated	200 mg	11593-027
Kanamycin Sulfate	5 g	11815-024
	25 g	11815-032
Kanamycin Sulfate (100X), liquid	100 mL	15160-054



Item	Amount	Source
Gentamicin Reagent Solution, liquid (50 mg/mL)	10 mL 10 × 10 mL	15750-060 15750-078
Bluo-gal	1 g	15519-028
Isopropylthio- b -galactoside (IPTG)	1 g	15529-019
S.O.C. Medium	10 × 10 mL	15544-034
(Miller's LB Broth Base) fi Luria Broth Base, powder	500 g	12795-027
Water, distilled (cell-culture grade)	500 mL	15230-162
4% Agarose gel (optimal for insect cell growth)	40 mL	18300-012
Fetal Bovine Serum (FBS), Qualified, Heat Activated	100 mL	16140-063
X-Gluc	0.1 g	R0851
Platinum™ SuperFi™ PCR Master Mix	100 reactions	12358010

Insect cell culture products

Item	Amount	Source
Sf9 Cells, Adapted in Sf-900™ II SFM	1.5 × 10 ⁷ cells	11496-015
Sf9 Cells, Adapted in Sf-900™ III SFM	1.5 × 10 ⁷ cells	12659017
Sf21 Cells, Adapted in Sf-900™ II SFM	1.5 × 10 ⁷ cells	11497-013
Sf21 Cells, Adapted in Sf-900™ III SFM	1.5 × 10 ⁷ cells	12682019
High Five™ Cells	3 × 10 ⁶ cells	B855-02
Mimic™ Sf9 Insect Cells	1 × 10 ⁷ cells	12552014
Sf-900™ II SFM	500 mL	10902-096
Sf-900™ III SFM	500 mL	12658-019
Express Five™ SFM	1 L	10486025
Opti-MEM™ I Reduced Serum Medium	100 mL	31985062
Grace's Insect Cell Culture Medium, Supplemented	500 mL	11605-094
Grace's Insect Cell Culture Medium (2X)	100 mL	11667-037
Penicillin-Streptomycin	100 mL	15070063
Pluronic™ F-68	100 mL	24040032



Recombinant fusion protein detection

Cloning the gene of interest in frame with the N- or C- terminal polyhistidine tag allows the recombinant fusion protein expression to be detected using an antibody to the appropriate epitope. The amount of antibody supplied is sufficient for 25 western blots.

Item	Epitope	Source
Anti-His (C-term) Antibody	Detects the C-terminal polyhistidine (6xHis) tag: HHHHHH-COOH (requires the free carboxyl group for detection)	R930-25
Anti-His (C-term)-HRP Antibody		R931-25
Anti-His (C-term)-AP Antibody		R932-25
Penta-His™ mouse IgG1 monoclonal Antibody	Detects both N- and C-terminal polyhistidine (6 x His) tag	P21315

Recombinant fusion proteins purification

Use ProBond™ resin or Ni-NTA resin to purify your recombinant fusion protein.

Item	Amount	Source
ProBond™ Nickel-chelating Resin	50 mL	R801-01
	150 mL	R801-15
ProBond™ Purification System	6 purifications	K850-01
Ni-NTA Agarose	10 mL	R901-01
	25 mL	R901-15
	100 mL	R901-10
Ni-NTA Purification System	6 purifications	K950-01
Purification Columns (10 mL polypropylene columns)	50 columns	R640-50
AcTEV™	1,000 Units	12575-015
	10,000 Units	12575-023

Nalgene flasks and tissue culture plates

Item	Volume	Amount	Source
Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile, vented	125 mL	Case of 24	4115-0125
	250 mL	Case of 12	4115-0250
	500 mL	Case of 12	4115-0500
	1000 mL	Case of 6	4115-1000



Appendix D Ordering information
Nalgene flasks and tissue culture plates

Item	Volume	Amount	Source
Nalgene™ Single-Use PETG Erlenmeyer Flasks with Plain Bottom: Sterile, vented	2000 mL	Case of 4	4115-2000
	2800 mL	Case of 4	4115-2800
6-well plate	—	75 pk	140675



Safety



WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
 - Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the “Documentation and Support” section in this document.
-

Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section in this document.
 - Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
 - Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
 - Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
 - Handle chemical wastes in a fume hood.
 - Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
 - After emptying a waste container, seal it with the cap provided.
 - Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
 - Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
 - **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.
-

Biological hazard safety



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
www.cdc.gov/biosafety/publications/bmb15/BMBL.pdf
 - World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
-



Documentation and support

Customer and technical support

Visit thermofisher.com/support for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
 - Product FAQs
 - Software, patches, and updates
 - Training for many applications and instruments
- Order and web support
- Product documentation, including:
 - User guides, manuals, and protocols
 - Certificates of Analysis
 - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.thermofisher.com/us/en/home/global/terms-and-conditions.html. If you have any questions, please contact Life Technologies at www.thermofisher.com/support.

