USER GUIDE

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ViraPower™ BacMam Expression System and BacMam pCMV-DEST Vector Kit

BacMam and Gateway® adapted system which enables transfection and transduction of large genes

Catalog Numbers A24223, A24227

Revision A.0

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Product information

Kit contents and storage

Types of products

This manual is supplied with the products listed below. For a list of the reagents supplied with each catalog number, see below and the next page.

Product	Quantity	Cat. No.
ViraPower™ BacMam Expression System	1 kit	A24227
BacMam pCMV-Dest Vector	1 kit	A24223

Kit components

Each catalog number contains the components listed below.

Component	A24227	A24223
BacMam pCMV-DEST Vector	✓	✓
Cellfectin® II Reagent	✓	
MAX Efficiency® DH10Bac™ Competent Cells	✓	

Shipping/storage

The ViraPower™ BacMam Expression System and BacMam pCMV-DEST Vector Kit is shipped as described below. Upon receipt, store each component as detailed below.

Box	Component	Shipping	Storage
1	BacMam pCMV-DEST Vector	Ambient	–20°C
2	Cellfectin® II Reagent	Dry ice	–20°C
3	MAX Efficiency® DH10Bac™ Competent Cells	Dry ice	–80°C

BacMam pCMV-DEST vector

The BacMam pCMV-DEST vector (20 µL) is supplied in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM NaCl at a concentration of 500 ng/µL. **Store the vector at –20°C.**

Cellfectin® II Reagent

Cellfectin® II Reagent (Box 2). **Store Box 2 at –20°C for up to 6 months.** For long-term storage, store at –80°C.

Reagent	Composition	Amount
Cellfectin® II Reagent	Proprietary	1 mL

DH10Bac™ Competent Cells

The MAX Efficiency® DH10Bac™ Competent Cells includes the following items. Transformation efficiency is ≥ 1 x 10^8 cfu/µg DNA. **Store at –80°C.**

Reagent	Composition	Amount
MAX Efficiency® DH10Bac™ Competent Cells	—	5 × 100 µL
pUC19 Control DNA	10 pg/µL in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0	50 µL

DH10Bac™ genotype

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G /pMON14272 / pMON7124

Accessory products

Additional products

The products listed in this section are available separately from Life Technologies for use with the ViraPower™ BacMam Expression System and BacMam pCMV-DEST Vector Kit. For more information, visit **www.lifetechnologies.com** or contact Technical Support (page 43).

Product	Amount	Catalog no.
GeneArt® Seamless PLUS Cloning Assembly Kit	1 kit	A14603
One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli*	20 x 50 µL	C8620-03
LR Clonase®II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
Cellfectin® Reagent	1 mL	10362-010
MAX Efficiency® DH10Bac™ Competent *E. coli*	5 x 100 µL	10361-012
Sf-900™ III SFM (1X) liquid	500 mL	12658-019
Sf9 Cells, SFM-adapted	1.5 mL, 10^7 cells/mL	11496-015
Grace's Insect Cell Culture Medium, Unsupplemented (1X), liquid	500 mL	11595-030
PureLink® Quick Plasmid Miniprep kit	50 preps	K2100-10
PureLink® HiPure Plasmid Miniprep Kit	100 preps	K2100-03
Platinum® PCR Supermix	100 reactions	12532-016
Ampicillin, sodium salt	200 mg	11593-027
Kanamycin Sulfate (100X), liquid	100 mL (10 mg/mL)	15160-054
Gentamicin, liquid	10 mL (50 mg/mL)	15750-060
Tetracycline	5 g	Q100-19
Bluo-gal	1 g	15519-028
Isopropylthio-ß-galactoside (IPTG)	1 g	15529-019
BacMam Enhancer Kit	1 kit	B10107

Dulbecco's Phosphate Buffered Saline (D-PBS) without Ca^{++}/Mg^{++}	1,000 mL	14190-136
GIBCO® Recovery™ Cell Culture Freezing Medium	50 mL	12648-010
Opti-MEM® Reduced-Serum Medium (1X), liquid (w/o phenol red)	500 mL	11058-021
Hank's Balanced Salt Solution (HBSS) (1X), liquid contains calcium and magnesium, but no phenol red	500 mL	14025-092

Entry vectors

To recombine your gene of interest into BacMam pCMV-DEST vector, you need either an entry clone containing the gene of interest or another stretch of DNA containing your gene sequence. Many entry vectors are available from Life Technologies to facilitate generation of entry clones. For more information about each vector, visit **www.lifetechnologies.com/gateway** or contact Technical Support (page 43).

CellLight® reagents

CellLight® reagents are ready-to-use, pre-packaged BacMam particles for highly efficient and targeted delivery to a range of mammalian cell types. CellLight® reagents come in a variety of colors and targets for multiplexing and co-localization studies. For more information, visit **www.lifetechnologies.com/celllight** or contact Technical Support (page 43).

Product description

Introduction

The ViraPower™ BacMam Expression System and BacMam pCMV-DEST Vector Kit allows you to construct an engineered viral genome called a bacmid using Gateway® technology, restriction enzyme cloning or seamless assembly. The bacmid DNA is then transfected into insect cells to produce BacMam reagent (baculovirus) that when transduced into mammalian cells initiates the expression of your gene of interest.

The vector uses Gateway® technology to create an expression clone by recombining an entry clone containing your gene of interest with the BacMam pCMV destination vector (BacMam pCMV-DEST). Alternatively you can utilize restriction enzyme sites or design your own seamless assembly approach to clone you gene of interest into the BacMam pCMV-DEST vector.

Advantages of BacMam technology

Using BacMam technology for gene expression in mammalian cells offers the following advantages:

- High transduction efficiency across a broad range of cell types, including primary and stem cells
- Capability of expressing large gene insert in mammalian system. Inserting more than 38Kbp had been demonstrated.
- Minimal microscopically observable cytopathic effects
- Highly reproducible and titratable expression
- Biosafety level 1 handling as the virus does not replicate in mammalian cells Note: Consult local safety regulations for details as Biosafety level 2 may apply depending on the transgene expressed.
- Allows for simultaneous delivery of multiple genes
- Compatibility with standard liquid handling procedures

Important

Before generating your own BacMam reagent, we strongly recommend determining if your cell type of choice can be transduced using BacMam technology. Visit **www.lifetechnologies.com/bacbam** to see if your cell type is compatible with BacMam technology. If your cell type is not listed, we suggest that you test your cell type for efficacy using our CellLight® products—targeted fluorescent proteins delivered using BacMam technology **www.lifetechnologies.com/celllight**.

Features of BacMam pCMV-DEST vector

BacMam pCMV-DEST vector, has the following features:

- The CMV promoter from Cytomegalovirus for high-level expression of the gene of interest in mammalian cells
- Mini-Tn*7* elements for site-specific transposition into the bacmid DNA propagated in *E. coli* (Craig, 1989; Luckow *et al.*, 1993)
- Two recombinatorial sites, *att*R1 and *att*R2, for Gateway® recombinational cloning of a gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- The *ccd*B gene located between the two *att*R sites for negative selection
- The SV40 polyadenylation signal for efficient transcription termination and polyadenylation of mRNA
- Ampicillin resistance gene for selection of transformants in *E. coli*
- Gentamicin resistance gene for selection of transformants containing recombinant bacmid DNA
- The pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*
- *WPRE or Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element* for increased duration of gene expression
- *VSV-G or Vesicular stomatitis virus G protein* enables viral delivery to mammalian cells

For more information and map of BacMam pCMV-DEST vector, see page 41.

Kit components description

The major components of the ViraPower™ BacMam Expression System and BacMam pCMV-DEST Vector Kit are described below.

- The BacMam pCMV-DEST vector into which the gene(s) of interest is cloned using Gateway® technology, restriction enzyme cloning or seamless assembly. The expression of the gene(s) of interest is controlled by the cytomegalovirus (CMV) promoter for high-level expression in mammalian cells. This expression cassette is flanked by the left and right arms of Tn*7*, and also contains a gentamicin resistance gene and an SV40 polyadenylation signal to form a mini Tn*7* (see page 42 for details).
- An important component of the kit is the DH10Bac™ *E. coli* strain that is used as the host for your BacMam pCMV-DEST vector. DH10Bac™ cells contain a baculovirus shuttle vector (bacmid) with a mini-*att*Tn*7* target site and a helper plasmid. Once the BacMam pCMV-DEST expression plasmid is transformed into DH10Bac™ cells, transposition occurs between the mini-Tn*7* element on the BacMam pCMV-DEST vector and the mini-*att*Tn*7* 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.
- The Cellfectin® II Reagent is used to transfect insect cells with the bacmid DNA.

Once you have performed the transposition reaction, you will isolate the high molecular weight recombinant bacmid DNA.

Gateway® Technology

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

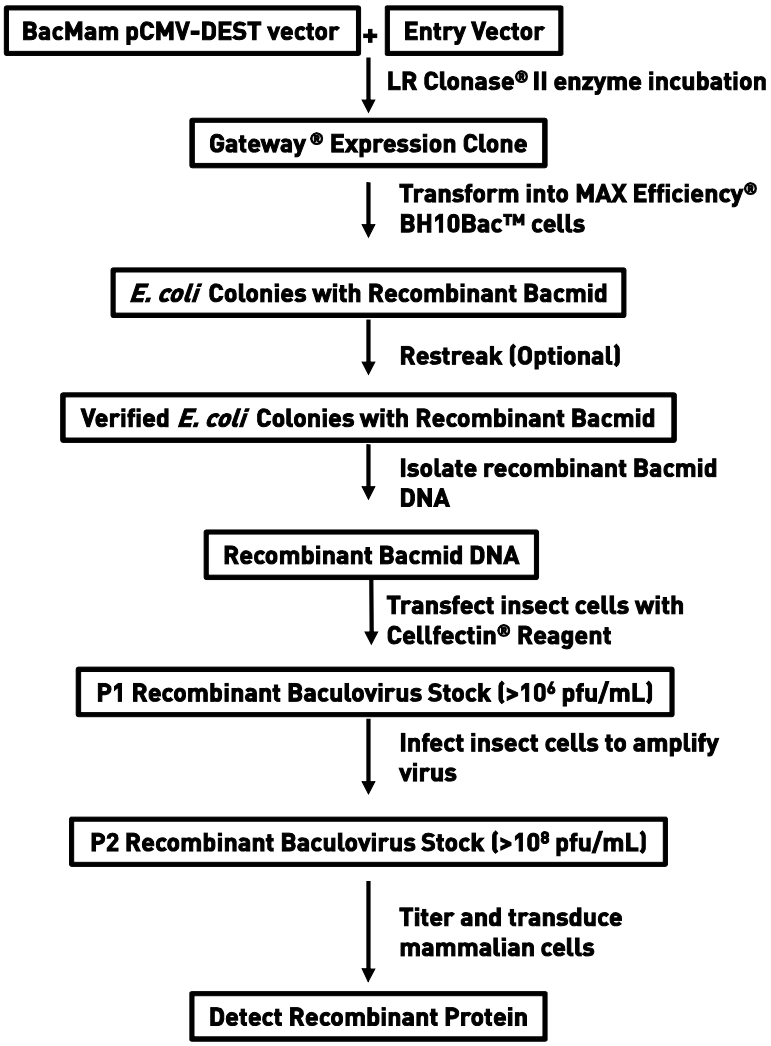
1. Clone your gene of interest into a Gateway® entry vector of choice to create an entry clone.
2. Perform an LR recombination reaction between the entry clone and BacMam pCMV-DEST.
3. Transform One Shot® Mach1™ T1^{R} Chemically Competent *E. coli* and select for an expression clone.
4. Transform DH10Bac™ *E. coli*.

For more detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual. This manual is available for downloading from **www.lifetechnologies.com/gateway** or by contacting Technical Support (page 43).

Workflow

Experimental outline

The figure below describes the steps necessary to clone and express your gene of interest using BacMam pCMV-DEST vector.



Gateway® cloning and bacmid generation

Generate an entry clone

Introduction

To recombine your gene of interest into BacMam pCMV-DEST, you need an entry clone containing the gene of interest. Many entry vectors are available from Life Technologies to facilitate generation of entry clones (see table below). For more information about each vector, visit **www.lifetechnologies.com/gateway** or contact Technical Support (page 43).

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

Once you have selected an entry vector, refer to the manual for the specific entry vector you are using for instructions to construct an entry clone. All entry vector manuals are available for downloading from **www.lifetechnologies.com** or by contacting Technical Support (page 43).

Cloning considerations

It is important to have a properly designed entry clone before recombining with the destination vector. To recombine your entry clone with BacMam pCMV-DEST vector your insert should contain an ATG start codon for proper initiation of translation and a stop codon.

Create an expression clone

Introduction

After you have generated an entry clone, perform the LR recombination reaction to transfer the gene of interest into the BacMam pCMV-DEST vector to create your expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the next section entitled **Perform LR Recombination Reaction**, pages 11–12 before beginning.

Experimental outline

To generate an expression clone:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing BacMam pCMV-DEST vector.

   Note: Both the entry clone and the destination vector should be supercoiled

2. Transform the reaction mixture into a suitable One Shot® Mach1™ T1^{R} Chemically Competent *E. coli* host (page 13).
3. Select for expression clones (page 41 for illustrations of the recombination region of expression clones in BacMam pCMV-DEST).

Perform LR recombination reaction

Introduction

Once you have produced an entry clone containing your gene of interest, you are ready to perform an LR recombination reaction between the entry clone and the BacMam pCMV-DEST vector, and to transform the reaction mixture into One Shot® Mach1™ T1^R Chemically Competent *E. coli* to select for an expression clone. It is important to have everything you need set up and ready to use to ensure that you obtain the best results. We recommend that you include a positive control (see below) and a negative control (no LR Clonase® II) in your experiment.

Positive control

The pENTR™-gus plasmid is included with Gateway LR clonase enzyme mix can be used as a positive control for LR recombination and expression. Use of the pENTR™-gus entry clone in an LR recombination reaction with any pDEST™ vector allows you to generate an expression clone containing the gene encoding β-glucuronidase (*gus*).

LR Clonase® II Enzyme Mix

LR Clonase® II enzyme mix is available from Life Technologies (page 3) to catalyze the LR recombination reaction. The LR Clonase® II Enzyme Mix combines the proprietary enzyme formulation and 5X LR Clonase® Reaction Buffer previously supplied as separate components in LR Clonase® Enzyme Mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 12 to perform the LR recombination reaction using the LR Clonase® II Enzyme Mix.

Materials needed

- Entry clone containing your gene of interest (50–150 ng/µL in TE, pH 8.0)
- BacMam pCMV-DEST vector (500 ng/µL in TE, pH 8.0)
- pENTR™-gus positive control (if desired, supplied with the LR Clonase® II Enzyme Mix, Box 2; 50 ng/µL in TE, pH 8.0)
- LR Clonase® II enzyme mix (keep at –20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Proteinase K solution (supplied with the LR Clonase® II Enzyme Mix; thaw and keep on ice until use)
- Water bath set at 37°C
- 1.5 mL microcentrifuge tubes

continued on next page

Perform LR recombination reaction, continued

LR recombination reaction

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

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

Component	Sample	Positive control
Entry clone (50–150 ng/reaction)	1–7 µL	—
BacMam pCMV-DEST vector (500 ng/ µL)	0.5 µL	0.5 µL
pENTR™ –gus (50 ng/ µL)	—	2 µL
1 X TE Buffer, pH 8.0	to 8 µL	5 µL

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

   Reminder: Immediately return the LR Clonase® II Enzyme Mix to –20°C.
5. Incubate reactions at 25°C for 1 hour.

   Note: For most applications, 1 hour yields sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation will yield more colonies.
6. Add 1 µL Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transform One Shot® Mach1™ T1^R Chemically Competent *E. coli***, page 13.

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

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

Introduction

Once you have performed the LR recombination reaction, transform One Shot® Mach1™ T1^R Chemically Competent *E. coli*.

Materials needed

- LB plates containing 100 µg/mL ampicillin (two for each transformation; warm at 37°C for 30 minutes)
- 42°C water bath
- 37°C shaking and non-shaking incubator
- One Shot® Mach1™ T1^R Chemically Competent *E. coli*
- S.O.C. Medium (supplied with the kit)

Transformation protocol

1. Thaw on ice, one vial of One Shot® Mach1™ T1^R Chemically Competent *E. coli* for each transformation.
2. Add 2 µL of the LR recombination reaction (from Step 7, previous page) into a vial of One Shot® Mach1™ T1^R Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down.**
3. Incubate on ice for 30 minutes.
4. Heat-shock the cells for 30 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice.
6. Add 250 µL of room temperature S.O.C. Medium.
7. Cap the tube tightly and shake the tube horizontally (225 rpm) at 37°C for 1 hour.
8. Plate 20 µL and 100 µL from each transformation on a prewarmed LB plates containing 100 µg/mL ampicillin and incubate overnight at 37°C. We generally plate 2 different volumes to ensure that at least 1 plate has well-spaced colonies.

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

Analyze transformants

Analyze positive clones

1. Pick 5–7 colonies (from Step 8, previous page) and culture them overnight in LB or SOC medium containing 100 µg/mL ampicillin.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink® Quick Plasmid Miniprep Kit (page 3) or equivalent.
3. Analyze the plasmids by restriction analysis or colony PCR to confirm the presence of the insert.

Confirm the expression clone

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

Sequencing

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

Long-term storage

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

To prepare a glycerol stock:

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

Prepare plasmid DNA

Prepare plasmid DNA from two separate selected expression clones using the PureLink® HiPure Plasmid Miniprep Kit (page 3) or equivalent.

Transform DH10Bac™ *E. coli*

Introduction

Once you have generated your BacMam pCMV expression construct, you are ready to transform the purified plasmid DNA into DH10Bac™ *E. coli* for transposition into the bacmid. Using blue/white selection, identify colonies containing the recombinant bacmid. MAX Efficiency® DH10Bac™ chemically competent cells are supplied with the kit, but are also available separately from Life Technologies (page 3). Guidelines and instructions to transform DH10Bac™ cells are provided in this section.

Materials needed

- Your purified BacMam pCMV expression construct (200 pg/µL in TE, pH 8.0)
- MAX Efficiency® DH10Bac™ chemically competent cells (supplied with the kit; use 1 tube of competent cells for every transformation)
- pUC19 (supplied with MAX Efficiency® DH10Bac™ *E. coli*; use as a control for transformation, if desired)
- LB agar plates containing kanamycin, gentamicin, tetracycline, Bluo-gal, and IPTG (3 plates for each transformation; use freshly prepared plates; see recommendation on the next page)
- LB agar plate containing 100 µg/ml ampicillin (for plating pUC19 transformation control)
- S.O.C. Medium (supplied with most competent cells)
- 42°C water bath
- 37°C shaking and non-shaking incubator

Prepare LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/ml Bluo-gal, and 40 µg/mL IPTG to select for DH10Bac™ transformants. See (page 3) to order antibiotics, Bluo-gal, and IPTG, and page 39 for instructions to prepare plates. If you are preparing LB plates using a pre-mixed formulation, we recommend using Luria Broth Base (Life Technologies, Catalog no. 12795-027) instead of Lennox L (LB). Using Lennox L plates will reduce the color intensity and may reduce the number of colonies obtained.

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

Prepare for transformation

For each transformation, you will need one vial of competent cells and three selective plates.

- Equilibrate a water bath to 42°C.
- Warm selective plates at 37°C for 30 minutes.
- Warm the S.O.C. Medium to room temperature.

Transform DH10Bac™ *E. coli*, continued

Transformation procedure

Follow the procedure below to transform MAX Efficiency® DH10Bac™ chemically competent cells with your expression construct. We recommend including positive control for transformation (*i.e.* pUC19) in your experiment to help you evaluate your results.

1. Thaw **on ice** one vial of MAX Efficiency® DH10Bac™ competent cells for each transformation.
2. Add the appropriate amount of plasmid DNA to the cells and mix gently. **Do not pipet up and down to mix.**
   - Your BacMam pCMV expression construct: 0.1–0.5 ng (5 µL)
   - pUC19 control: 50 pg (5 µL)
3. Incubate cells on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Immediately transfer the tubes to ice and chill for 2 minutes.
6. Add 900 µl of room temperature S.O.C. Medium.

   Note: For BacMam pCMV transformations: Shake tubes at 37°C at 225 rpm for 4 hours.

   Note: For pUC19 transformation: Shake tube at 37°C at 225 rpm for 1 hour.
7. **For each BacMam pCMV transformation:** Prepare 10-fold serial dilutions of the cells (10^{-1}, 10^{-2}, 10^{-3}) with S.O.C. Medium. Plate 20 and 150 µL of **each** dilution on an LB agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.

   For the pUC19 transformation: Dilute the cells 1:100 with S. O.C. Medium. Plate 100 µL of the dilution on an LB agar plate containing 100 µg/mL ampicillin.
8. Incubate plates for 48 hours at 37°C. Pick white colonies for analysis (see the next page for analysis recommendations).

   Note: We do not recommend picking colonies earlier than 48 hours as it may be difficult to distinguish between white and blue colonies.

Important

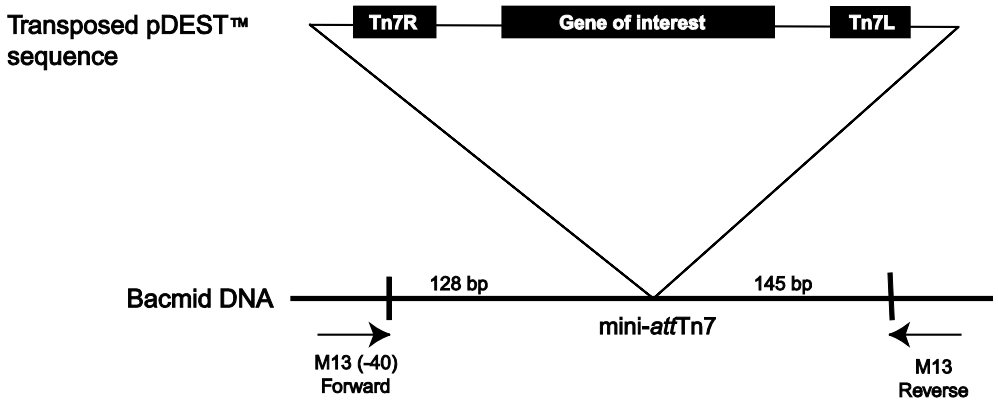
Insertions of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupt the expression of the LacZα peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies that harbor the unaltered bacmid. **Select white colonies for analysis.** True white colonies tend to be large; therefore, to avoid selecting false positives, choose the largest, most isolated white colonies. Avoid picking colonies that appear gray or are darker in the center as they can contain a mixture of cells with empty bacmid and recombinant bacmid.

Analyze recombinant bacmid DNA by PCR

Introduction

Analyze white colonies containing the recombinant bacmid DNA. Since bacmid DNA is >135 kb in size, restriction analysis is difficult to perform with DNA of this size, hence, we recommend using PCR analysis to verify the presence of your gene of interest in the recombinant bacmid. The bacmid contains M13 Forward (–40) and M13 Reverse priming sites flanking the mini-*att*Tn7 site within the *lac*Zα-complementation region to facilitate PCR analysis (see figure below). Guidelines and instructions are provided in this section to perform PCR using the M13 Forward (–40) and M13 Reverse primers.

Note: It is possible to verify successful transposition to the bacmid by using agarose gel electrophoresis to look for the presence of high molecular weight DNA. This method is less reliable than performing PCR analysis as high molecular weight DNA can be difficult to visualize.



Analyze PCR with M13 primers

To verify the presence of your gene of interest in the recombinant bacmid using PCR, you may:

- Use the M13 Forward (–40) and M13 Reverse primers
- Use a combination of the M13 Forward (–40) or M13 Reverse primers and a primer that hybridizes within your insert.

The M13 Forward (–40) and M13 Reverse primers can be purchased from Life Technologies custom primer service.

Primer	Sequence
M13 Forward (–40)	5′d[GTTTTCCCAGTCACGAC]3′
M13 Reverse	5′d[CAGGAAACAGCTATGAC]3′

DNA polymerase

You may use any DNA polymerase of your choice for PCR including Platinum® *Taq* DNA Polymerase (Life Technologies, Catalog no. 10966-018). If the expected PCR product is >4 kb, we recommend using a polymerase mixture such as Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies, Catalog no. 11304-011) for best results.

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Analyze recombinant bacmid DNA by PCR, continued

Produce the PCR product

Use the procedure below to amplify your recombinant bacmid DNA using the M13 Forward (–40) and M13 Reverse primers and Platinum® *Taq* polymerase. If you are using a combination of the M13 Forward (–40) or M13 Reverse primer and a primer specific for your gene, you will need to determine the amplification conditions to use. If you are using another polymerase, follow the manufacturer's recommendations for the polymerase you are using.

Note: Amplification conditions may need to be optimized if your insert is > 4 kb. In some cases, you may need to purify the bacmid DNA as described on page 19 and then perform the PCR.

1. For each sample, aliquot 20 µL of PCR master mix (containing DNA polymerase, salts, magnesium, and dNTPs) into a 0.5 mL microcentrifuge tube. Add 5 pmoles each of the forward and reverse PCR primer.
2. Pick 5–7 colonies and resuspend them individually in 20 µL of the PCR master mix containing primers (remember to make a patch plate to preserve the colonies for further analysis).
3. Incubate reaction for 10 minutes at 94°C to lyse cells and inactivate nucleases.
4. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	3 minutes	93°C	1X
Denaturation	45 seconds	94°C	25–35X
Annealing	45 seconds	55°C	
Extension	5 minutes	72°C	
Final Extension	7 minutes	72°C	1X

5. Remove 5–10 µL from the reaction and analyze by agarose gel electrophoresis.

What you should see

If transposition occurred and you used the M13 Forward (–40) and M13 Reverse primers for amplification, you should see a PCR product of the following size on the agarose gel:

Sample	Size of PCR Product
Bacmid alone	~300 bp
Bacmid transposed with BacMam pCMV-DEST	~2300 bp + size of your insert

If you used a combination of the M13 Forward (–40) or M13 Reverse primer and a gene-specific primer for amplification, you need to determine the expected size of your PCR product. Refer to the diagram on page 17 to help you calculate the expected size of your PCR product.

Isolate recombinant bacmid DNA

Introduction

Purify recombinant bacmid DNA from positive DH10Bac™ transformants containing your gene of interest. Purified bacmid DNA is suitable for use in PCR analysis or transfection.

Note

You may purify bacmid DNA using the procedure described below. This procedure was originally developed to isolate large plasmids (>100 kb) and has been adapted to allow isolation of bacmid DNA. You may also use the PureLink® HiPure Plasmid Purification Kit (page 3) available from Life Technologies or another method of choice to purify bacmid DNA.

Materials needed

- LB medium containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/ml tetracycline
- Solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A; filter-sterilize and store at 4°C)
- Solution II (0.2 N NaOH, 1% SDS; filter-sterilize)
- 3 M potassium acetate, pH 5.5 (autoclave and store at 4°C)
- Isopropanol
- 70% ethanol
- 1X TE Buffer, pH 8.0

Bacmid DNA isolation procedure

1. Using a sterile toothpick, inoculate a single, isolated bacterial colony in 2 mL LB medium containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline.
2. Grow culture in a 37°C shaking incubator until cells reach stationary phase, typically 2–3 days.
3. Transfer 1.5 mL of bacterial culture to a 1.5 mL microcentrifuge tube and centrifuge at 3,000 × *g* for 15 minutes to pellet cells.
4. Remove the supernatant by vacuum aspiration and resuspend the cell pellet in 0.3 ml Solution I. Gently vortex or pipet up and down to resuspend.
5. Add 0.3 mL Solution II and gently mix. Incubate at room temperature for 5 minutes.

   Note: The appearance of the suspension should change from turbid to almost translucent.
6. Slowly add 0.3 mL 3 M potassium acetate, pH 5.5, mixing gently during addition. A thick white precipitate of proteins and *E. coli* genomic DNA forms. Place the sample on ice for 10 minutes.
7. Centrifuge for 10 minutes at 14,000 × *g*.
8. Gently transfer the supernatant to a microcentrifuge tube containing 0.8 mL isopropanol. **Do not** transfer any white precipitate. Invert the tube a few times to mix and place on ice for 5–10 minutes. Proceed directly to Step 9 or store sample at –20°C overnight.

continued on next page

Isolate recombinant bacmid DNA, continued

Bacmid DNA isolation procedure, continued

Protocol continued from previous page

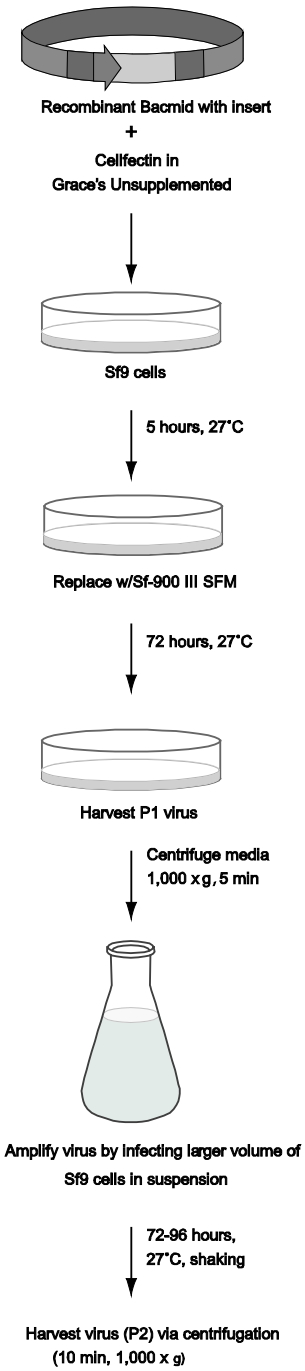
9. Centrifuge the sample for 20 minutes at 14,000 × *g* at room temperature.
10. Carefully remove the supernatant, taking care not to disturb the pellet. Add 0.5 ml 70% ethanol. Invert the tube several times to wash the pellet.
11. Centrifuge for 20 minutes at 14,000 × *g* at room temperature. Repeat Steps 10-11, if desired.
12. Remove as much of the supernatant as possible, taking care not to disturb the pellet. Air dry the pellet for 5–10 minutes at room temperature. **Do not** overdry the pellet.
13. Dissolve the DNA pellet in 40 µL 1X TE Buffer, pH 8.0. To avoid shearing, do not mechanically resuspend the DNA. Allow the solution to sit in the tube with occasional gentle tapping of the bottom of the tube.
14. Store the DNA at 4°C. We do not recommend storing the purified bacmid DNA at –20°C as repeated freezing and thawing may shear the DNA.

BacMam virus production

Experimental overview

Experimental outline

The figure below describes the steps necessary to generate your BacMam reagent from Bacmid with insert.



Culture Sf9 cells and prepare recombinant bacmid DNA

Introduction	Before you start your experiments, be sure to have cultures of Sf9 cells growing and have frozen master stocks available.
Cells for transfection	You will need Sf9 log-phase cells with >95% viability to perform a successful transfection. Refer to page 24 to determine how many cells you will need for transfection.
Cell culture instructions	Refer to the instructions supplied with Sf9 cells and media for cell culture information. You may also refer to the Insect Cell Lines manual available at **www.lifetechnologies.com** or contact Technical Support. These manuals contain information on: • Thawing frozen cells • Maintaining and passaging cells • Freezing cells • Growing cells in suspension • Scaling up cell culture
Recombinant bacmid DNA	You need to generate bacmid DNA containing your gene of interest using appropriate strategy. See protocol above.

Transfect insect cells

Introduction

Once you have confirmed that your recombinant bacmid contains the gene of interest, you are ready to transfect Sf9 insect cells to produce recombinant baculovirus. Guidelines and instructions to transfect insect cells are provided in this section.

Transfection method

We recommend using a cationic lipid such as Cellfectin® Reagent for transfection. Cellfectin® Reagent is available separately from Life Technologies (page 3).

Cellfectin® Reagent

Cellfectin® Reagent is a cationic lipid formulation in membrane-filtered water suitable for the transfection of DNA into insect cells. Cellfectin® Reagent has been found to be superior for transfection of Sf9 and other insect cells.

Sf9 insect cells

The Sf9 insect cell line is a clonal isolate derived from the parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE and is suitable for transfection and identification of recombinant plaques.

The Sf9 cell line is prepared from low passage Master Cell Bank cultures that are only 40 to 50 total passages and 10 to 20 passages serum-free. The cells are adapted to serum-free suspension growth in Sf-900™ III SFM, a serum-free medium optimized for growth of Sf9 cells.

Refer to the manual supplied with the cells for details on thawing, culturing, and passaging Sf9 cells.

Transfection media

For the highest transfection efficiency, we recommend performing the transfection in unsupplemented Grace's Insect Cell Culture Medium is available separately from Life Technologies (page 3).

Note: The Grace's Insect Cell Culture Medium **should not** contain supplements or fetal bovine serum (FBS) as the proteins in the FBS and supplements may interfere with the Cellfectin® Reagent, inhibiting the transfection.

If you are culturing Sf9 cells in Sf-900™ III SFM, you can perform the transfection in unsupplemented Grace's Medium and then easily switch back to Sf-900™ III SFM after transfection.

continued on next page

Transfect insect cells, continued

Materials needed

- Purified recombinant bacmid DNA from your construct (500 ng/µL in TE Buffer, pH 8.0)
- Sf9 cells cultured in the appropriate medium
- Cellfectin® Reagent (store at 4°C until use)
- Grace's Insect Cell Medium, Unsupplemented (**media should not contain supplements, FBS, or antibiotics**)
- 6-well tissue culture plates and other tissue culture supplies
- Sterile 1.5 mL microcentrifuge tubes
- Complete growth medium for culturing insect cells (Sf-900™ III SFM)

Calculate the number of Sf9 cells that you need for your transfection experiment and expand cells accordingly. Make sure that the cells are healthy and >97% viability before proceeding to transfection.

Transfection conditions

We generally produce baculoviral stocks in Sf9 cells using the following transfection conditions. Note that these conditions should be used as a starting point for your transfection. To obtain the highest transfection efficiency and low non-specific effects, you may optimize transfection conditions by varying DNA and Cellfectin® Reagent concentrations, and cell density.

Condition	Amount
Tissue culture plate size	6-well (35 mm) plate (one well per bacmid)
Number of Sf9 cells to transfect	8×10^5 cells
Amount of bacmid DNA	1 µg (can vary from 0.1 to 2 µg)
Amount of Cellfectin® Reagent	6 µL (can vary from 1.5 to 9 µL)

continued on next page

Transfect insect cells, continued

Transfection procedure

Use the following procedure below to transfect Sf9 cells in a **6-well format or 35 mm tissue culture dish**. If you wish to transfect cells in other tissue culture formats, you will need to determine the optimal conditions to use. Remember to use **unsupplemented** Grace's Medium that does not contain FBS or antibiotics for transfection.

1. In a 6-well or 35 mm tissue culture plate, seed 8×10^5 Sf9 cells per well in 2 ml Sf-900™ III SFM. Allow cells to attach at 27°C for at least 1 hour.
2. **For each transfection sample**, prepare bacmid DNA:Cellfectin® Reagent complexes as follows in sterile 1.5 mL microcentrifuge tubes.
   a. Dilute 1 µg of purified bacmid DNA in 100 µL unsupplemented Grace's Insect Cell Culture Medium.
   b. Mix Cellfectin® Reagent thoroughly before use by inverting the tube 5–10 times. Remove 6 µL Cellfectin® Reagent and dilute in 100 µL unsupplemented Grace's Insect Cell Culture Medium.
   c. Combine the diluted bacmid DNA with the diluted Cellfectin® Reagent (total volume is ~210 µL). Mix gently by inversion and incubate for 15–45 minutes at room temperature.
3. While DNA:lipid complexes are incubating, remove the media from the cells and wash once with 2 mL unsupplemented Grace's Insect Cell Culture Medium. Remove the wash media **immediately before** adding DNA/lipid complexes to cells.
4. Add 0.8 mL unsupplemented Grace's Insect Cell Culture Medium to each tube containing the DNA:lipid complexes. Mix gently and add the DNA:lipid complexes to cells from Step 3.
5. Incubate the cells in a 27°C incubator for 5 hours with **gentle shaking**.
6. Remove the DNA:lipid complexes and add 2 mL Sf-900™ III SFM to the cells.
7. Incubate the cells in a 27°C humidified incubator for 72 hours **without shaking**. Proceed to **Isolate P1 Viral Stock**, next page.

Isolate P1 viral stock

Introduction

Budded virus should be released into the medium 72 hours after transfection. However, if your transfection efficiency was not optimal, cells may not show all of the signs of viral infection until 4 or 5 days post-transfection. Beginning at 72 hours after transfection, you should visually inspect the cells daily for signs of infection (see below). Once the cells appear infected (*i.e.* demonstrate characteristics typical of late to very late infection), harvest the virus from the cell culture medium using the procedure below.

Characteristics of infected cells

Virally-infected insect cells typically display the following characteristics as observed from visual inspection using an inverted phase microscope with a 25–40x objective lens. The time points provided below assume that the transfection was successful (*i.e.* transfection efficiency was high).

Signs of infection	Phenotype	Description
Early (first 24 hours)	Increased cell diameter	A 25-50% increase in cell diameter may be seen.
	Increased size of cell nuclei	Nuclei may appear to "fill" the cells.
Late (24-72 hours)	Cessation of cell growth	Cells appear to stop growing when compared to a cell-only control.
	Granular appearance	Signs of viral budding; 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 P1 viral stock

1. Once the transfected cells from Step 7, previous page demonstrate signs of late stage infection (*e.g.* 72 hours post-transfection), collect the medium containing virus from each well (~1.7 mL) and transfer to sterile 15 mL tubes. Centrifuge the tubes at 1,000 × g for 5 minutes to remove cells and large debris.
2. Transfer the clarified supernatant to fresh 15 mL tubes. **This is the P1 viral stock** and is typically 1–10 × 10^6 plaque forming units (pfu)/mL.
3. Store at 4ºC, **protected from light**. See the next page for additional storage information.

continued on next page

Isolate P1 viral stock, continued

Store P1 viral stocks

Store P1 viral stocks as follows:

- Store viral stock at 4ºC, **protected from light**.
- If medium is serum-free (*e.g.* Sf-900™ III SFM), add fetal bovine serum to a final concentration of 3%. Serum proteins act as substrates for proteases.
- For long-term storage, store an aliquot of the viral stock at –80ºC for later reamplification.
- **Do not** store routinely used viral stocks at temperatures below 4ºC. Repeated freeze/thaw cycles can result in a 10- to 100-fold decrease in virus titer.

Next steps

Once you have obtained your clarified P1 baculoviral stock, you may:

- Amplify the viral stock (see the next section for details). This procedure is recommended to obtain the highest viral titers and optimal results in your expression studies.
- Determine the titer of your viral stock via a traditional viral plaque assay.

Amplify baculoviral stock

Introduction

The P1 viral stock is a small-scale, low-titer stock. We recommend using this stock to infect Sf9 cells to generate a high-titer P2 stock. The titer of the initial viral stock obtained from transfecting Sf9 cells generally ranges from 1×10^6 to 1×10^7 pfu/mL. Amplification allows production of a P2 viral stock with a titer of 1×10^8 pfu/mL or higher and is highly recommended. Guidelines and protocols are provided in this section to amplify the recombinant baculovirus to prepare a P2 viral stock.

Materials needed

- Sf9 cells cultured in the appropriate growth medium
- P1 baculoviral stock
- Any appropriate tissue culture vessel (see **Important** below)
- Tissue culture reagents
- 27°C humidified incubator

Important

To amplify your P1 viral stock, you may infect Sf9 cells growing in suspension or monolayer culture. Depending on your needs, you may amplify your P1 viral stock at any scale, but remember that you may be limited by the amount of P1 viral stock available. We generally amplify our P1 viral stock in a 50 mL suspension culture at 1×10^6 cells/mL. Calculate the number of Sf9 cells that you will need for infection and expand cells accordingly. Make sure that the cells are healthy and >95% viability before proceeding to infection.

Multiplicity of Infection (MOI)

To amplify your viral stock, infect cells at a multiplicity of infection (MOI) ranging from 0.05 to 0.1. MOI is defined as the number of virus particles per cell. Use the following formula to calculate how much viral stock to add to obtain a specific MOI:

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

Note: If you have not determined the titer of your P1 viral stock, you may assume that the titer ranges from 1×10^6 to 1×10^7 pfu/mL.

Example

If you wish to infect a 10 mL culture at 2×10^6 cells/mL using an MOI = 0.1 assuming the titer of your P1 viral stock is 5×10^6 pfu/mL.

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

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

continued on next page

Amplify baculoviral stock, continued

Amplification procedure

Follow the guidelines below to amplify your P1 viral stock in a 250 mL cell culture vessel.

1. On the day of infection, infect log phase Sf9 cells at an MOI of 0.1 by adding 1.5 ml P1 viral stock to 50 ml Sf9 cells at 1.5×10^6 cells/mL growing in Sf-900™ III SFM.
2. Incubate the cells for 72–96 hours in a 27ºC humidified incubator with **shaking**.
3. Harvest the medium containing virus by centrifuging the entire culture at 1,000 x g for 10-15 minutes to remove cells and large debris.
4. Transfer the supernatant to a fresh 50 mL tube. This is the **P2 viral stock** and is typically 1×10^8 pfu/mL or higher.
5. Store viral stock at 4ºC, **protected from light**. For long-term storage, you may store an aliquot of the P2 stock at –80ºC, protected from light. See page 27 for storage guidelines.
6. Proceed to the next section to determine the titer of your P2 viral stock.

Scale up the amplification procedure

Once you have generated a high-titer P2 baculoviral stock, you may scale-up the amplification procedure to any volume of your choice. To produce this high-titer P3 stock, scale up the amount of cells and volume of virus used appropriately, and follow the guidelines and procedure outlined in this section.

Generate high-titer stocks from frozen master stock

If you have stored your viral master stock at –80ºC, we recommend amplifying this stock to generate another high-titer stock for use in expression experiments. Viral titers generally decrease over time when virus is stored at –80ºC, and will lose 5–10 fold infectivity with each freeze/thaw cycle. Follow the guidelines and amplification procedure detailed in this section.

Determine viral titer (Optional)

Introduction

We recommend using the BaculoTiter™ Assay kit, available separately from Life Technologies, to determine the titer of your baculoviral stock. The BaculoTiter™ Assay kit provides accurate results for your baculovirus titer in two days as compared to 10-day dilution assays. For more information, refer to **www.lifetechnologies.com** or contact Technical Support page 43).

Factors affecting viral titer

A number of factors can influence viral titers including:

- **Size of your gene of interest:** Titers generally decrease as the size of the insert increases.
- **Transfection efficiency:** For the highest transfection efficiency, we recommend transfecting Sf9 cells using Cellfectin® Reagent. Prepare DNA: lipid complexes in unsupplemented Grace's Insect Cell Culture Medium (pages 23-25 for details).
- **Age of your baculoviral stock:** Viral titers may decrease with long-term storage at 4ºC or –80ºC. If your baculoviral stock has been stored for 6 months to 1 year, we recommend titering or re-titering your baculoviral stock prior to use in an expression experiment.
- **Number of freeze/thaw cycles:** If you are storing your viral stock at –80ºC, viral titers can decrease as much as 10% with each freeze/thaw cycle.
- **Improper storage of your baculoviral stock:** For routine use, baculoviral stocks should be aliquotted and stored at 4ºC, **protected from light**.

What you should see

When titering baculoviral stocks, we generally obtain titers ranging from:

- 1×10^6 to 1×10^7 pfu/mL for P1 viral stocks
- 1×10^8 to 1×10^9 pfu/mL for P2 viral stocks

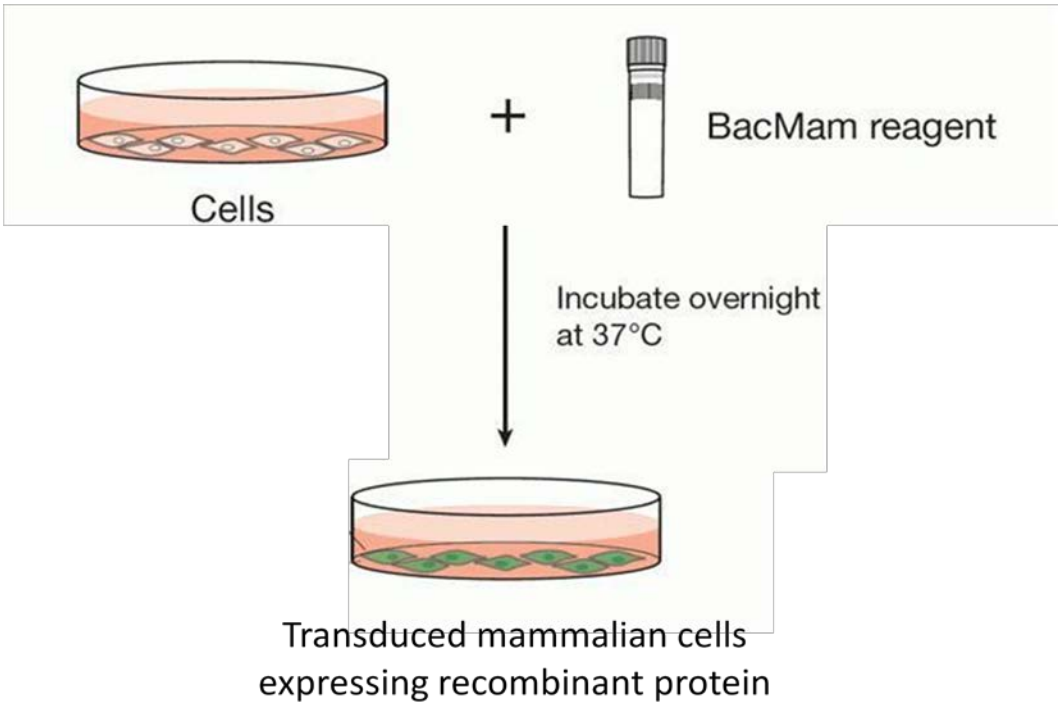
Note: If the titer of your baculoviral stock is less than 1×10^6 pfu/mL or 1×10^7 pfu/mL for a P1 or P2 viral stock, respectively, we recommend producing a new baculoviral stock. See **Troubleshooting** section for more tips and guidelines to optimize your viral yield.

Mammalian cell transduction and protein expression

Experimental overview

Experimental outline

The figure below describes the steps necessary to transduce your BacMam reagent for protein expression and detection.



Transduce with BacMam reagent

Important

Cell types successfully transduced using BacMam delivery technology include: CHO, COS-7, HEK 293, Hep G2, HeLa, human embryonic and mesenchymal stem cell, numerous primary human cell types (e.g., aortic smooth muscle cells and cardiomyocytes, primary human hepatocytes, keratinocytes and umbilical vein endothelial cells), Saos-2, and U-2 OS. Currently, the delivery system does not work well for hematopoietic or macrophage cells.

Before generating your own BacMam reagent, we strongly recommend determining if your cell type of choice can be transduced with the BacMam reagent. **www.lifetechnologies.com** to see if your cell type is compatible for use with BacMam technology. If your cell type is not listed, we suggest that you test your cell type for efficacy using our CellLight® products—targeted fluorescent proteins delivered using BacMam technology **www.lifetechnologies.com/celllight**.

Introduction

Instructions for transducing your mammalian cells with the BacMam reagent are described below.

Before you start your experiments, be sure to have cultures of mammalian cells growing and make sure you have titered your baculovirus stock.

Materials needed

- BacMam Reagent P2 viral stock
- Mammalian cells cultured in appropriate media
- Appropriate cell culture growth media

Before starting

- Plate ~1–4 × 10^6 mammalian cells in 2 × 100 mm culture dish for initial studies. Allow cells to adhere and grow for approximately 4–24 hours at 37ºC and 5% CO_2 before proceeding with the transduction. Optimal transduction occurs when cells are at 70–80% confluency.

Transduction procedure

Day 1: Labeling

1. Plate the cells at the desired density and allow them sufficient time to adhere. BacMam reagents work best when used on low-passage-number cells.

Note: If particle per cell (PPC) is not determined then BacMam solution can be added to the culture media using dilution method. Usually 5% solution (v/v) is enough (i.e. 5 ml of BacMam solution to 9.5 mL of culture media).

Note: If particle per cell (PPC) is known, for most cell types PPC of 30-200 is enough. This range will have to be optimized for cell type and culture conditions. For example, if user have the particle concentration of 1×10^8 per mL then to label 40,000 cells with a PPC of 30 12 μL will be needed per mL of culture media

2. Mix the BacMam viral particle solution several times by inversion to ensure a homogenous solution. Do not vortex.
3. Add the calculated volume of BacMam viral particle solution directly to the cells in complete cell medium and mix gently.
4. Return the cells to the culture incubator overnight (≥16 hours).

Day 2: Analysis

After overnight incubation (≥16 hours), most of cell types generate enough protein that protein can be detected or purified using appropriate technique, such as imunocytochemistry, western blot or immuno precipitation. If any of the fluorescent protein such as GFP or RFP had been used as tags they can be visualized also in the live cells.

Depending upon the cell type the expression of desired protein will maximize in 24–48 hours after transduction. Depending upon the stability of expressed protein, some cell types had been known to keep producing the protein after one or two weeks after transduction.

Troubleshooting

Cloning into BacMam pCMV-DEST vector

The table below lists some potential problems that you may encounter when generating your BacMam pCMV construct. Possible solutions that may help you troubleshoot your cloning are provided.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Check the antibiotic resistance marker and use the correct antibiotic to select for entry clones or expression clones.
	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
	Clonase® II enzyme mix is inactive or didn't use suggested amount of Clonase® II enzyme mix	• Test another aliquot of the Clonase® II enzyme mix. • Make sure that you store the Clonase® II enzyme mix at –20°C or –80°C. • **Do not f**reeze/thaw the Clonase® II enzyme mix more than 10 times. • Use the recommended amount of Clonase® II Enzyme Mix (page 12).
	Too much entry clone was used in an LR reaction	Use equal fmol of destination vector and entry clone.
	Large destination vector or entry clone (>10 kb)	• Incubate the LR reaction overnight. • Linearize the destination vector or the entry clone. • Relax the destination vector with topoisomerase I.
	Impurities in DNA	Purify insert DNA. Make sure to remove excess phenol, proteins, detergents, and ethanol from the DNA solution.
Few or no colonies obtained from the transformation control	Competent cells stored or handled improperly	Store competent cells at –80°C. Thaw cells on ice; use immediately after thawing; do not vortex.
	Transformation performed incorrectly	Follow the instructions described in this manual for transformation.
	Insufficient amount of *E. coli* plated	Increase the amount of *E. coli* plated.

continued on next page

Troubleshooting, continued

Cloning into the BacMam pCMV-DEST vector, continued

Problem	Reason	Solution
Few or no colonies obtained from the transformation control, continued	Problem with antibiotic	• Confirm use of the correct antibiotic; confirm antibiotic concentration. • Check that the antibiotic is not degraded (*i.e.* change in color of solution or the appearance of precipitate). Use fresh antibiotic.
	Cells not heat-shocked or incubated properly during transformation	Follow the recommended transformation procedure for the cells you are using.

Generating recombinant bacmid DNA

The table below lists some potential problems that you may encounter when generating the recombinant bacmid following transformation into DH10Bac™ *E. coli*. Possible solutions that may help you troubleshoot the transposition reaction are provided.

Problem	Reason	Solution
No blue (non-recombinant) colonies obtained (*i.e.* all colonies are white) **Note:** Although you will pick white colonies, you should expect to see some blue colonies. 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 a minimum of 4 hours before plating.
	Bluo-gal and IPTG omitted from plates	Prepare fresh selective plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.
	Too many colonies on the plate	Serially dilute the transformation mixture and plate to give well-separated colonies.
	Plates too old or stored in light	• Do not use plates that are more than 4 weeks old. • Store plates protected from light.
	Incorrect incubation period or temperature	Wait at least 48 hours before picking colonies. Incubate plates at 37°C.
All colonies are blue	BacMam pCMV-DEST DNA used for transformation was of poor quality	• Use purified plasmid DNA for transformation. • Check the quality of your plasmid DNA; make sure that the DNA is not degraded.
	Gentamicin omitted from plates	Prepare fresh selective plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL IPTG.

continued on next page

Troubleshooting, continued

Generating recombinant bacmid DNA, continued

Problem	Reason	Solution
Few colonies obtained	Used LB medium for recovery/expression period	Use S.O.C. Medium for the 4 hours 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 µg/mL. • Use dark and light backgrounds to view plates.
	Too many or too few colonies on plate	Adjust the serial dilutions of cells to obtain an optimal number of colonies.
	Incubation period 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. Using a range of 20–60 µg/mL IPTG generally gives optimal color development.

Isolating bacmid DNA

The table below lists some potential problems and possible solutions to help you troubleshoot recombinant bacmid DNA isolation.

Problem	Reason	Solution
Bacmid DNA is degraded	DNA stored improperly	• Store purified bacmid DNA in aliquots at –20°C. • Do not freeze/thaw repeatedly.
	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 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/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 choose one that contains recombinant bacmid DNA only.

Virus troubleshooting

Problem	Reason	Solution
Low yield of virus	Low transfection efficiency	• Use Cellfectin® Reagent for transfection. • Perform transfection in unsupplemented Grace's Insect Cell Culture Medium; make sure that no supplements, FBS, or antibiotics are present during transfection. • Harvest viral supernatant when signs of infection are visible (*i.e.* >96 hours post-transfection).
	Cells plated too sparsely	Plate insect cells at the recommended cell density.
	Used too much or too little Cellfectin® Reagent	Optimize the amount of Cellfectin® Reagent used.
	Time of incubation with DNA:lipid complexes too short or too long	Optimize the incubation time (*e.g.* 3 to 8 hours).
	Recombinant bacmid DNA is degraded	• Check the quality of your recombinant DNA by agarose gel electrophoresis prior to transfection.
	Bacmid DNA is not pure (*i.e.* contains recombinant bacmid and empty bacmid)	• Screen other DH10Bac™ transformants and choose one that contains only recombinant bacmid. • Perform plaque purification to isolate recombinant baculovirus.

Protein expression troubleshooting

Problem	Reason	Solution
Low protein expression	Viral stock contains a mixture of recombinant and non-recombinant baculovirus	• Perform plaque purification to isolate recombinant baculovirus.
	Baculovirus not recombinant	• Verify transposition by PCR analysis of bacmid DNA using the M13 forward (–40) and M13 reverse primers. • Re-transfect insect cells with new recombinant bacmid DNA.
	Used less virus per cell	• Optimize infection conditions by varying the dose (MOI).

	Time of cell harvest not optimal	• Perform a time course of expression to determine the optimal time to obtain maximal protein expression.
	Cell line not optimal	• Try other mammalian cell lines.
	Cell culture conditions	• If using cells with high passage number, try using cells with low passage number. • If using over-confluent cells, try cells at lower confluency (70% to 80% is generally optimal).
	Poor transduction efficiency	• Based on your results from initial transduction experiments, increase the BacMam reagent concentration or decrease the number of cells.
	Problems with fluorescent protein detection system or reagents	• Increase the labeling time or labeling reagent concentration to obtain optimal results. • Ensure the labeling reagent is active and has not lost activity upon storage. Perform experiments with fresh labeling reagent. • Use optimized filter set to visualize fluorescent proteins.

Appendix

Recipes

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. For the antibiotics below, prepare and store the stock solutions as directed:

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

*If you are using tetracycline from Life Technologies (Catalog no. Q100-19), prepare in water. If you are using the free-base form of tetracycline, prepare in 100% ethanol.

IPTG

Follow the procedure below to prepare a 200 mg/mL stock solution of IPTG.

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

Follow the guidelines below to prepare a 20 mg/mL stock solution of Bluo-gal.

- Dissolve the Bluo-gal in dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) to make a 20 mg/mL stock solution. Use a glass or polypropylene tube. **Important:** Exercise caution when working with dimethylformamide. Dispense solutions in a vented chemical hood only.
- Do not filter the stock solution.
- Store at –20°C protected from light.

continued on next page

Recipes, continued

LB (Luria-Bertani) plates

Follow the procedure below to prepare LB agar plates.

1. Prepare LB medium, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add antibiotic(s) and pour into 10 cm plates.
4. Let harden, then invert and store at 4°C, in the dark. Plates containing antibiotics are stable for up to 4 weeks.

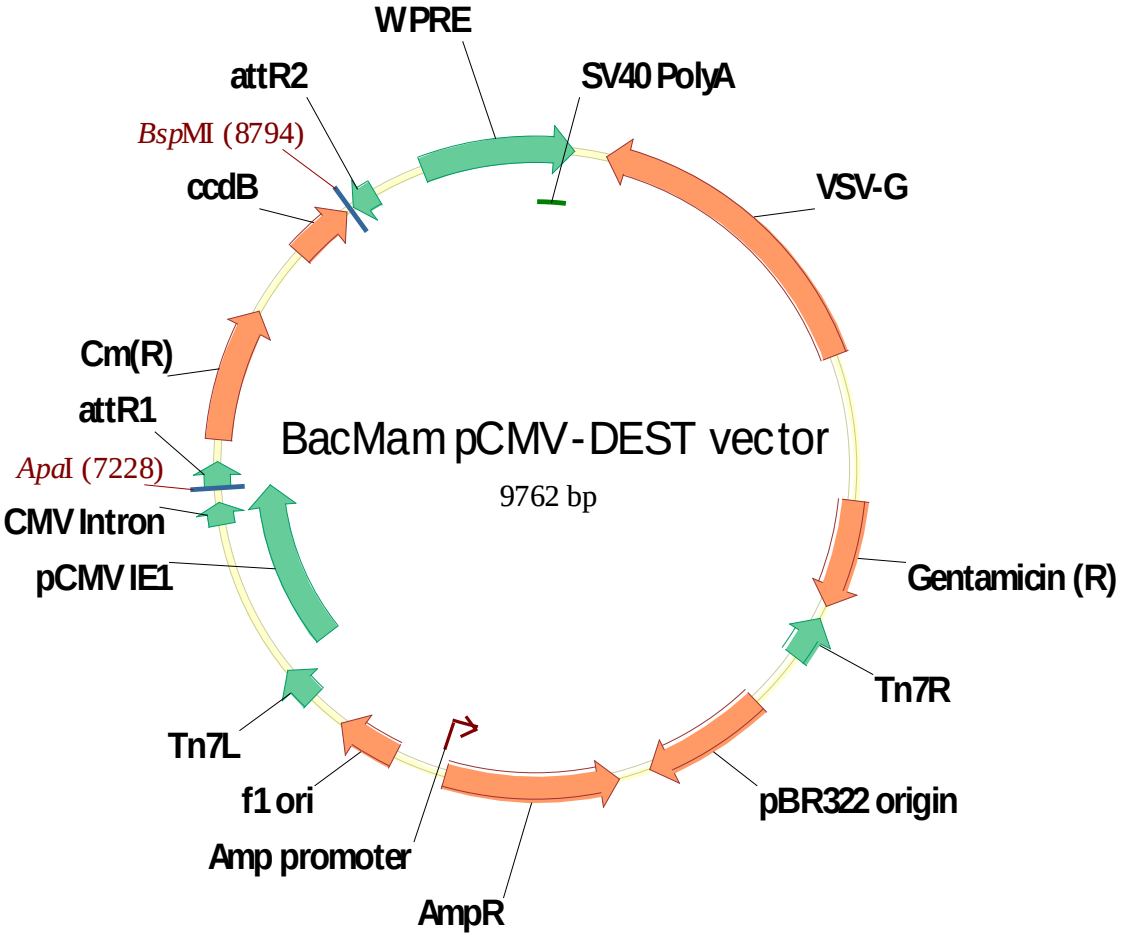
LB agar selective plates for DH10Bac™ transformation

1. Follow Steps 1-2 in the procedure above.
2. After autoclaving, cool to ~55°C, and add the following:
   - 50 µg/mL kanamycin
   - 7 µg/mL gentamicin
   - 10 µg/mL tetracycline
   - 100 µg/mL Bluo-gal
   - 40 µg/mL IPTG
3. Let harden, then invert and store at 4°C, in the dark. Tetracycline and Bluo-gal are light sensitive, so make sure that plates are stored protected from light.

Map of BacMam pCMV-DEST vector

BacMam pCMV-DEST vector map

The figure below summarizes the features of the BacMam pCMV-DEST vector (9762 bp). For a more detailed explanation of each feature, see page 42. The complete sequence of BacMam pCMV-DEST vector is available from **www.lifetechnologies.com** or from Technical Support (page 43).



SV40 PolyA 1-138
VSV-G 325-1860
Gentamicin 2582-3115
Tn7R 3182-3406
pBR322 3694-4313
AmpR 4468-5328
F1 ori 5592-5898
Tn7L 6080-6264
pCMV IE1 6284-7227
attR1 7232-7356
Chloramphenicol resistance 7465-8124
ccdB 8466-8771
attR2 8812-8936
WPRE 9195-167

Features of BacMam pCMV-DEST vector

Features

The features of BacMam pCMV-DEST vector (9762 bp), are described below.

Features	Function
CMV promoter	Allows efficient, high-level expression of your recombinant protein in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Mini-Tn7 element (Tn7R and Tn7L)	Allows site-specific transposition of your gene of interest into a bacmid propagated in *E. coli* (Craig, 1989; Luckow *et al.*, 1993).
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that allow recombinational cloning of the gene of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene	Permits counterselection of the expression clone.
*ccd*B gene	Permits negative selection.
SV40 polyadenylation sequence	Efficient transcription termination and polyadenylation of mRNA.
pUC origin	Permits high copy replication and maintenance in *E. coli*.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (*bla*)	Allows selection of transformants in *E. coli*.
Gentamicin resistance gene	Allows selection of transformants containing recombinant bacmid DNA.
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element for increased duration of gene expression
VSV-G	Vesicular stomatitis virus G protein, which enables viral delivery to mammalian cells

Documentation and support

Obtaining support

For the latest services and support information for all locations, go to:
www.lifetechnologies.com/support
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- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search though frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
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