pIEx/Bac™ Vectors

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Description

The pIEx/Bac™ Vectors are dual-purpose expression vectors designed for transfection into *Spodoptera*-derived insect cells and for use as transfer vectors to generate recombinant baculovirus. After pIEx/Bac recombinants are established in *E. coli*, isolated plasmids can be transiently transfected into Sf9 or Sf21 insect cells for rapid target protein expression and screening. Purified plasmid can also be used to create baculovirus recombinants by cotransfecting with BacMagic™ DNA (see User Protocol TB459) or BacVector® Triple Cut Virus DNA (see User Protocol TB216). To generate drug-resistant stable cell lines, the vectors can be cotransfected with the plasmid pIE1-Neo (see User Protocol TB176).The pIEx/Bac vectors employ an optimal combination of AcNPV baculovirus-derived transcription elements: the hr5 enhancer and the ie1 immediate early promoter (Rodems 1993, Guarino 1994, Pullen 1995, Jarvis 1996), to drive transcription in the transient transfection mode. This promoter/enhancer combination recruits endogenous insect cell transcription machinery, allowing rapid expression analysis of multiple constructs, before committing resources to generate baculovirus stocks. A second promoter, the strong AcNPV-derived p10 promoter, is active in the late/very late stages of baculovirus infection. When used to generate a baculovirus, this unique vector directs expression of the target protein at all stages of the infection process, allowing the user to determine the optimal harvest point for any given recombinant protein.

Transfecting pIEx/Bac plasmids with Insect GeneJuice® Transfection Reagent ensures optimal transfection efficiencies and expression levels.

A pIEx/Bac Positive Expression Control DNA is included to monitor the success of transient transfection experiments. This plasmid contains a β-galactosidase insert fused to N-terminal His•Tag® and S•Tag™ coding sequences. After using the control to create a recombinant baculovirus it can also be used to monitor the success of baculovirus infection experiments.

Components

- 20 µg (40 ul) 0.5 µg/µl pIEx/Bac DNA
- 5 µg (10 ul) 0.5 µg/µl pIEx/Bac Positive ExpressionControl
- 50 µl T4 DNA Polymerase Buffer
- 25 U T4 DNA Polymerase, LIC-qualified
- 100 µl 100 mM DTT
- 40 µl 25 mM dATP
- 50 µl 25 mM EDTA
- 1.5 ml Nuclease-free Water
- 1 pkg NovaBlue GigaSingles Competent Cells
- 2 ml SOC Medium
- 10 µl Test Plasmid

Storage

Store DNA at –20°C.

Vector features

The pIEx/Bac™ vectors contain an extensive multiple cloning site (MCS) to create recombinants that express Strep•Tag® II, His•Tag®, or GST•Tag™ fusion proteins. The vectors also contain an *Nco* I site that provides the option to produce unfused target proteins. Many of the MCS restriction sites are also found in Novagen® pET, pBAC™, pIEx™, and pTriEx™ expression vectors to facilitate traditional subcloning. High-throughput (HT) compatible Ek/LIC or 3C/LIC cloning versions of the pIEx/Bac vectors are also available: pIEx/Bac-1, -4 are Ek/LIC (see User Protocol TB163) and pIEx/Bac-3, -5 are 3C/LIC (see User Protocol TB453). pIEx/Bac-1 encodes an N-terminal Strep•Tag II sequence followed by an enterokinase (Ek) cleavage site and an optional C-terminal 10x His•Tag sequence. pIEx/Bac-3 features an N-terminal 10x His•Tag coding sequence followed by a cleavage site for human rhinovirus type 14 3C (3C), the MCS, thrombin (Tb) cleavage site and an optional C-terminal Strep•Tag II coding sequence. pIEx/Bac-4 and –5 both feature N-terminal GST•Tags. pIEx/Bac-4 encodes a GST•Tag followed by an Ek site and has an optional C-terminal His•Tag sequence. pIEx/Bac-5 encodes a GST•Tag followed by a 3C site and has an optional C-Terminal Strep•Tag II sequence. Complete vector maps can be found in the User Protocols listed in the table below.

pIEx/Bac Vector Characteristics

Vector	Fusion Tags			Protease Cleavage Sites	User Protocol
	Strep•Tag II	GST•Tag	His•Tag		
pIEx/Bac-1	N		C	Ek	TB468
pIEx/Bac-3	C		N	3C, Tb	TB470
pIEx/Bac-4		N	C	Ek	TB492
pIEx/Bac-5	C	N		3C, Tb	TB493

N: N-terminal; C: C-terminal; Ek: enterokinase; 3C: HRV 3C; Tb: thrombin;

Fusion Tags

- His•Tag fusion proteins produced in insect cells can be purified using Novagen Ni-NTA His•Bind® Resins or Ni-MAC™ and Co-MAC™ Fractogel® Cartridges and can be easily detected using the His•Tag Monoclonal Antibody (Cat. No. 70796)
- The Strep•Tag II peptide is an 8 amino acid sequence that binds to Strep•Tactin® resin, an engineered streptavidin with an optimized binding site for the tag. Strep•Tag II fusion proteins can be purified using Strep•Tactin resins and detected with either the Strep•Tag II Monoclonal Antibody (Cat. No. 71590) or Strep•Tag II Antibody HRP Conjugate (Cat. No. 71591).
- GST•Tag fusion proteins produced in insect cells can be purified using GST•Bind™ Resin, GST•Bind Fractogel® Cartridges or GST•Mag™ Agarose Beads and can be easily detected using the GST•Tag Monoclonal Antibody (Cat. No. 71097). GST•Tag fusion proteins can also be quantitatively assayed with the GST•Tag Assay Kit (Cat. No. 70532).

Cloning strategies to generate target proteins without fusions

The *Nco* I restriction site (CCATGG) in the pIEx/Bac vectors can be used to clone an open reading frame (ORF) so that expression yields unfused protein (Novy 1999). Similarly, vector-encoded C-terminal fusions are prevented by including a translation stop codon in the insert.

The ATG triplet within the *Nco* I site encodes the N-terminal methionine residue of the expressed protein. Target genes or PCR-engineered inserts that contain either an *Nco* I site or sites that generate compatible overhangs (*Bsp*H I [TCATGA], *Bsp*LU11 I [ACATG], and subsets of *Afl* III [ACRYGT] and *Sty* I [CCWWGG]) at the beginning of the ORF can be cloned into the *Nco* I site. Each of these restriction sites dictates the first nucleotide of the next triplet codon, which may not generate the authentic N-terminus.

Note: Any strategy employing restriction digestion, can be complicated if there are additional recognition sites in the target gene.

If the insert contains *Bsp*H I, *Bsp*LU11 I, *Afl* III, and *Sty* I sites, it may be possible to employ an alternative strategy to generate native target protein. The table below lists several commercially available restriction enzymes that cleave "downstream" of their recognition site. Any of the restriction sites in this table can be engineered into PCR primers to generate *Nco* I-compatible overhangs. It is relatively unlikely that a given insert will contain sites for all of the enzymes listed.

Enzyme (isoschizomers)	Recognition and cleavage site	Overhangs generated
Bbs I (*Bpi* I, *Bpu*A I)	5′-GAAGAC(N)$_2$ –3′ 3′-CTTCTG(N)$_6$ -5′	GAAGACNN NNNNN CTTCTGNNNNNN N
Bsa I (*Eco* 3 1 I)	5′-GGTCTC(N)$_1$ -3′ 3′-CCAGAG(N)$_6$ -5′	GGTCTCN NNNNN CCAGAGNNNNN N
*Bsm*B I (*Esp* 3 I)	5′-CGTCTC(N)$_1$ -3′ 3′-GCAGAG(N)$_5$ -5′	CGTCTCN NNNNN GCAGAGNNNNN N
Bsp M I (*Bfu*A I)	5′-ACCTGC(N)$_4$ –3′ 3′-TGGACG(N)$_8$ -5′	ACCTGCNNNN NNNNN TGGACGNNNNNNNN N

The Ek/LIC versions of pIEx/Bac™-1, and -4 provide an alternative method for generating unfused protein. After fusion protein purification, cleavage with enterokinase (Ek) removes all N-terminal fusion sequences (see User Protocol TB163). Proteins with very minimal N-terminal fusions can be generated using the 3C/LIC versions of pIEx/Bac-3 and -5. Cleavage of the fusion protein with HRV 3C Protease leaves only 3 amino acids (gly-pro-gly) at the N-terminus. (see User Protocol TB450).

Cloning strategies to generate fusion proteins

To create N-terminal fusion proteins, use restriction enzyme sites downstream from the tag sequence and maintain the reading frame. To create fusions with a C-terminal tag, the insert must lack a stop codon and maintain the desired reading frame. Restriction enzyme-mediated cloning strategies and protocols are available in many publications describing molecular biology techniques.

Analysis of pIEx/Bac recombinants

Recombinant plasmid DNA from the *E. coli* cloning host should be assayed for the correct insert and reading frame before transfection into insect cells. Several methods are available for analysis of recombinants including colony PCR, restriction analysis, and sequencing.

Colony PCR and sequencing primers

The following table lists the appropriate primers to use for PCR and sequence analysis.

Vector	Sense Primer	Antisense Primer
pIEx/Bac-1 pIEx/Bac-3	IE1 Promoter Primer (Cat. No. 69103-3)	IE1 Terminator Primer (Cat. No. 71247-3)
pIEx/Bac-4 pIEx/Bac-5	A primer designed to the C-terminal region of the GST•Tag would be appropriate	

Plasmid DNA isolation

Standard isolation methods typically produce plasmid DNA suitable for *E. coli* transformation and routine molecular biology manipulations, including sequencing. When isolating plasmid DNA for insect cell transfection, however, more rigorous methods are often required. Plasmid DNA prepared for transfection of eukaryotic cells must not contain interfering contaminants, such as endotoxins.

Production of Recombinant Baculovirus

pIEx/Bac vectors can be used to create baculovirus recombinants by cotransfection and homologous recombination with BacMagic™ DNA (see User Protocol TB459) or BacVector® Triple Cut Virus DNA (see User Protocol TB216). Complete details for preparing insect cells and creating, amplifying, titering, and using baculovirus recombinants for target protein expression, are described in the BacMagic™ and BacVector® User Protocol TBs. For time-saving baculovirus generation we recommend BacMagic DNA.

BacMagic DNA provides positive selection of baculovirus recombinants and eliminates the time-consuming plaque purification steps necessary in the traditional method. BacMagic DNA is an AcNPV genome with a portion of the essential ORF1629 deleted and a bacterial artificial chromosome (BAC) in place of the polyhedrin (polh) coding region. This combination prevents nonrecombinant virus from replicating in insect cells, yet allows the viral DNA to be propagated as circular DNA in bacterial cells. The target coding sequence in a compatible transfer plasmid, such as pBAC™, pIEx/Bac™, or pTriEx™ vectors, is cotransfected with BacMagic DNA into insect cells. Homologous recombination within the cells restores the function of the viral ORF1629 and the target coding sequence replaces the BAC sequence. Only the recombinant baculovirus can replicate, producing a homogeneous population of recombinants.

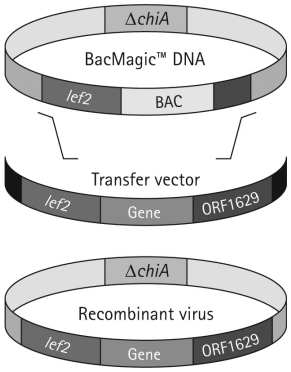

Figure 1. Constructing baculovirus recombinants using the BacMagic system

Target Protein Expression and Purification

Insect cell lines and medium

The pIEx/Bac vectors are suitable for expression in *Spodoptera*-derived insect cells, including Sf9 and Sf21 cells. Novagen Sf9 Insect Cells (Cat. No. 71104) plus BacVector Insect Cell Medium (Cat. No. 70590) are recommended for transfection of pIEx/Bac Vectors. While TriEx Sf9 Cells (Cat. No. 71023) can be used for transient or stable transfections, they may give lower transfection efficiencies than Sf9 Insect Cells. TriEx Sf9 Cells, grown in TriEx Insect Cell Medium, are recommended for superior cell growth and protein yield.

Transient Transfection

Critical factors in obtaining high expression yields during transient transfection experiments are the efficiency and cytotoxicity of the transfection reagent. Novagen® Insect GeneJuice® Transfection Reagent (Cat. No. 71259) is a proprietary liposome formulation optimized for maximal transfection efficiency of *Spodoptera frugiperda*-derived insect cells (e.g., Sf9 and Sf21). The reagent also features extremely low toxicity to the cells and can be used for both transient and stable transfections in serum-containing or serum-free medium. Insect GeneJuiceTransfection Reagent is ideal for large-scale protein expression using the pIEx/Bac Vectors.

Note: For transient transfection experiments, Insect GeneJuice Transfection Reagent is strongly recommended.

Infection with baculovirus recombinants

It is important to infect cells at a high multiplicity of infection (MOI) to ensure all cells are infected simultaneously, producing a synchronous culture. The optimal MOI is usually 5–10 pfu/cell, but should be evaluated for each particular virus. To optimize the MOI, use various MOIs to infect prepared plates or shake cultures; MOIs suggested for initial evaluation are 2, 5, and 10. Also, protein expression should be evaluated at different times after infection (24, 48, 72, and 96 h).

Target protein extraction and purification

Insect PopCulture® Reagent

Insect PopCulture® Reagent is a detergent-based lysis reagent specifically formulated for total culture extraction and affinity purification of recombinant proteins without requiring centrifugation. The improved method increases processing efficiency and target protein yields (Loomis 2002). Insect PopCulture can be used for protein extraction from insect cells in suspension or adherent cells on tissue culture plates.

Note: Novagen® Ni-NTA His•Bind® Resin, Ni-MAC™ Fractogel® and Co-Mac™ Fractogel Cartridges are compatible with purification of proteins from Insect PopCulture extracts. His•Bind Resin (IDA agarose),GST•Bind™ Resin, and GST•Bind Fractogel Cartridges are NOT compatible with purification of proteins from Insect PopCulture extracts.

CytoBuster™ Protein Extraction Reagent

CytoBuster™ Protein Extraction Reagent is a proprietary formulation of detergents optimized for efficient extraction of proteins from insect and mammalian cells. The unique composition of CytoBuster enables isolation of functionally active proteins without secondary treatment, such as sonication or freeze/thaw. CytoBuster Protein Extraction Reagent is compatible with purification of proteins using His•Bind Resin (IDA agarose), GST•Bind Resin, and GST•Bind Fractogel Cartridges and has been specifically formulated for use with Western blotting protocols, immunoprecipitation, and kinase/phosphatase assays.

Reportasol™ Extraction Buffer

Reportasol™ Extraction Buffer is designed to efficiently extract soluble reporter enzymes from mammalian and insect cells, while maintaining maximal activity. Efficient extraction requires no shaking or mixing and extracted proteins can be used in standard protein assay methods.

Insect RoboPop™ Ni-NTA His•Bind Purification Kit

Insect RoboPop™ Purification Kit is designed for HT purification of His•Tag® fusion proteins directly from transfected or infected Sf9 cultures without cell harvest, mechanical disruption, or extract clarification. The kits feature Insect PopCulture Reagent, Benzonase® Nuclease, Ni-NTA His•Bind Resin, and buffers for efficient protein extraction and affinity purification. The Insect RoboPop Purification Kit is designed to purify recombinant fusion protein from 10 ml cultures using a 2 ml well capacity filter plate. The 96 Well Filter Plate is compatible with standard filtration manifolds for manual and robotic processing. A Collection Plate and Sealer are provided for storage of the purified proteins. The RoboPop Ni-NTA His•Bind Purification Kit will purify up to 38 mg of His•Tag fusion proteins per 96 well plate (up to 0.4 mg/well). Stated yields are based on 10 ml cultures and binding capacities of the resin and can vary with expression levels for individual fusion proteins.

References

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