pTriEx™ System Manual

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About the Kits

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Description

The pTriEx System enables high-level, transient target gene expression in three systems: mammalian cells, baculovirus-infected insect cells, and *E. coli* cells. Multi-system expression is possible due to the unique design the pTriEx DNA.

The pTriEx DNA contains a mammalian promoter (Chicken β-actin, or CMVie), insect promoter (p10), and *E. coli* promoter (T7*lac*). Translation initiation signals include a ribosome binding site (rbs) for bacterial expression followed by an optimal Kozak consensus sequence for mammalian cell expression. The promoter and cloning regions are flanked on each side by segments of baculovirus genomic DNA that facilitate the generation of baculovirus recombinants at the viral *polh* locus. The pTriEx DNA contains a comprehensive cloning region, N-terminal fusion tags, signal sequences, protease cleavage sites, and optional C-terminal fusion tags.

pTriEx-1.1, pTriEx-2, pTriEx-3, and pTriEx-4 are available as stable expression vectors. These vectors are designed to efficiently select mammalian cell lines expressing target genes. Stable selection is achieved by translation of both the target gene and a downstream selective marker gene, specifically hygromycin (Hygro) or neomycin (Neo). Selection ensures that all drug-resistant cells also produce the target protein.

pTriEx-4, pTriEx-5, pTriEx-6, and pTriEx-7 are available in Ek/LIC or 3C/LIC Vector Kits. For more information on the ligation-independent cloning (LIC) method, please see User Protocol TB163.

Components

pTriEx™ DNA

- 20 µg pTriEx DNA (uncut)

pTriEx™ Hygro or Neo DNA

- 20 µg pTriEx Hygro or Neo DNA (uncut)
- 2 µg pTriEx Hygro or Neo β-gal Transfection Control

pTriEx Ek/LIC or 3C/LIC Vector Kit

- 1 µg pTriEx LIC Vector (linearized)
- 8 µl LIC β-gal Control Insert
- 25 U 4 DNA Polymerase, LIC-qualified
- 50 µl 10X T4 DNA Polymerase Buffer
- 100 µl 100 mM DTT
- 40 µl 25 mM dATP
- 50 µl 25 mM EDTA
- 1.5 ml Nuclease-free Water
- 0.2 ml Origami™ B(DE3)pLacI Competent Cells
- 11 × 50 µl NovaBlue GigaSingles™ Competent Cells
- 2 × 2 ml SOC Medium
- 10 µl Test Plasmid (Amp^R)

Storage

Store pTriEx DNA at –20°C. Store Competent Cells, SOC and Test plasmid at –70°C. Store all other components at –20°C. The pTriEx LIC Vectors can also be stored at –70°C.

pTriEx Vectors

The table below highlights the unique features of each pTriEx vector. Additional information including vector maps and sequences can be found online at http://www.merck4biosciences.com.

Vector	Promoters	N-terminal Tags	C-terminal Tags (optional)	Protease Cleavage Sites	Stable Vector	LIC	Vector Map
pTriEx-1.1	T7*lac*, p10, β-actin	None	HSV•Tag® His•Tag®	None	Hygro Neo	No	TB280 TB294 (Hygro) TB293 (Neo)
pTriEx-2	T7*lac*, p10, β-actin	His•Tag S•Tag™	HSV•Tag His•Tag	Thrombin rEK	Hygro Neo	No	TB277 TB296 (Hygro) TB295 (Neo)
pTriEx-3	T7*lac*, p10, CMVie	None	HSV•Tag His•Tag	None	Hygro Neo	No	TB281 TB298 (Hygro) TB297 (Neo)
pTriEx-4	T7*lac*, p10, CMVie	His•Tag S•Tag	HSV•Tag His•Tag	Thrombin rEK	Hygro Neo	Yes	TB282 TB300 (Hygro) TB299 (Neo) TB419 (Ek/LIC)
pTriEx-5	T7*lac*, p10, CMVie	Strep•Tag® II	His•Tag	rEK	No	Yes	TB434 TB442 (Ek/LIC)
pTriEx-6	T7*lac*, p10, CMVie	Strep•Tag II	His•Tag	3C Thrombin	No	Yes	TB435 TB443 (3C/LIC)
pTriEx-7	T7*lac*, p10, CMVie	Mouse IgM Strep•Tag II	His•Tag	rEK	No	Yes	TB436 TB444 (Ek/LIC)

Cloning Target Genes into pTriEx™ Vectors

Note: For pTriEx Ek/LIC and 3C/LIC Vector Kits, follow User Protocols TB163 or TB453, respectively.

Vector preparation

Use the restriction enzyme manufacturer recommended buffer, and incubation conditions for digestion. Many enzymes can be digested at the same time in the same buffer.

Note that each enzyme digests with different efficiencies, especially when two sites are close together. In general, enzymes with compatible buffers and whose sites are more than 10 bp apart can be used together in the same reaction. If one enzyme is a poor cutter, if buffers are incompatible, or if sites are separated by 10 bp or less, then digestions should be performed sequentially. That is, the first digestion should be performed with the enzyme that is the poorest cutter and the second enzyme added after digestion has been verified by running a sample of the reaction on an agarose gel.

Note that some restriction enzymes may display "star activity," a less stringent sequence dependence that results in altered specificity. Conditions that can lead to star activity include high glycerol concentration (> 5%), high pH, and low ionic strength.

If cloning into a single site, dephosphorylate the vector after restriction digestion to decrease the background of non-recombinants resulting from self-ligation of the vector. Molecular biology grade calf intestinal or shrimp alkaline phosphatase should be used according to manufacturer instructions.

It is also useful to dephosphorylate vectors cut with two enzymes, especially when the sites are close together or if one of the enzymes is a poor cutter. This decreases non-recombinant background caused by incomplete digestion with one of the enzymes, which is undetectable by gel analysis.

Following digestion, it is worthwhile to gel-purify the vector before insert ligation to remove residual nicked and supercoiled plasmid, which transform very efficiently relative to the desired ligation products. This step is optional, but usually reduces the effort required to screen for the correct construction. The SpinPrep™ Gel DNA Kit (Cat. No. 70958) is ideal for rapid isolation of DNA fragments from agarose gel slices.

Insert preparation

Preparing inserts by restriction digestion followed by gel purification is usually straightforward. Note that when subcloning into pTriEx vectors from vectors with the same selective marker (even with PCR as discussed below), it is necessary to gel-purify the fragment of interest to remove the original plasmid, which will transform very efficiently. As little as 10 pg of contaminating supercoiled plasmid (i.e., less DNA than can be visualized on an agarose gel) can typically result in many more colonies containing the original plasmid than the desired pTriEx subclone.

PCR can be used to isolate and/or modify target genes for expression in pTriEx plasmids. With this approach, it is possible to design primers that will (1) isolate the translated portion of a cDNA sequence, (2) add convenient restriction enzyme sites or LIC overhangs, and (3) place the coding region in the proper reading frame. In general, primers should contain a minimum of 15 (preferably 18–21) nucleotides complementary to the sequence of interest with a GC content of approximately 50%, and restriction sites should be flanked by 3–10 "spacer" nucleotides (depending on the enzyme) at the 5' end to allow for efficient digestion.

One risk in using PCR for insert preparation is the potential to introduce mutations. PCR error rate can be minimized in several ways:

- Use an enzyme with high fidelity, such as KOD DNA polymerase, Hot Start DNA polymerase, or XL DNA Polymerase (Cat Nos. 71085, 71086, or 71087).
- Limit the number of PCR cycles.
- Increase the concentration of target DNA.
- Increase the primer concentration.

Ligation

For maximum ligation efficiency, the pTriEx™ vector and insert should be free of phenol, ethanol, salts, protein, and detergents. Dissolve the vector and insert in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or in water.

Note: EMD Chemicals Inc. offers a Clonables™ 2X Ligation Premix (Cat. No. 70573). This enables ligation of any DNA (sticky or blunt ends) and plating for recombinants in less than 1 h [inNovations 9] with NovaBlue Singles™ Competent Cells. See User Protocol TB233 for information on Clonables.

Vector and insert concentrations can be determined by A_{260} readings (where 1 A_{260} unit = 50 µg/ml DNA), or estimated by agarose gel electrophoresis of a DNA sample along side known amounts of DNA of similar sizes in adjacent wells. Dilute solutions of DNA can be concentrated easily and with high recovery using Pellet Paint® Co-Precipitant (Cat. No. 69049). Pellet Paint is compatible with ligation and transformation.

1. For a standard reaction using DNA fragments with 2–4 base sticky ends, use 50–100 ng (0.02–0.03 pmol) pTriEx vector with an equimolar to 5-fold excess of insert (i.e., 50 ng of a 500 bp fragment) in a volume of 20 µl. Higher molar ratios of insert:vector may be used for blunt ends; however, the frequency of recombinants with multiple inserts will increase.
2. Assemble the following components in a 1.5 ml tube (these components are available separately in the DNA Ligation Kit, Cat. No. 69838) or use the Clonables 2X Ligation Premix (Cat. No. 70573). Add ligase last.

2 µl	10X Ligase Buffer (200 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$, 250 µg/ml acetylated BSA)
2 µl	100 mM DTT
1 µl	10 mM ATP
2 µl	50 ng/µl prepared pTriEx vector
1 µl	T4 DNA ligase, diluted (with ligase dilution buffer) 0.2–0.4 Weiss units/µl
x µl	Prepared target gene insert
y µl	Nuclease-free water to volume
20 µl	Total volume

3. Gently mix by stirring with a pipet tip. Incubate at 16°C for 2 h to overnight. Also set up positive and negative control reactions (see below).

   Note: For blunt ends, use 10X more ligase (i.e., undiluted enzyme), reduce the ATP concentration to 0.1 mM, and incubate for 6–16 h at 16°C or 2 h at room temperature.

For a negative control, assemble a parallel reaction containing the same amount of prepared vector. Omit the target insert; substitute volume with water or TE buffer bringing the reaction volume to 10 µl. This reaction will help determine non-recombinant background (i.e., residual circular plasmid, re-circularized vector).

For a positive control, include the β-gal Control Insert, provided in the pTriEx LIC Vector Kits.
The LIC β-gal Control Insert when annealed with the pTriEx LIC Vector and transformed into NovaBlue Singles Competent Cells according to standard protocols will produce greater than 10^4 cfu/µg vector.

Transformation

The NovaBlue strain should be used as the initial cloning host for pTriEx vectors as it is a convenient host for initial cloning of target DNA into pTriEx vectors due to its high transformation efficiency, and the high yields and excellent plasmid DNA that result from *recA endA* mutations. NovaBlue strains contain no source of T7 RNA polymerase, making it ideal for the establishment of recombinant plasmids under nonexpression conditions. Note that pTriEx vectors do not enable "blue/white" screening of recombinants by *lacZ* α complementation.

NovaBlue Singles™ Competent Cells (Cat. No. 70181) are provided in the pTriEx™ LIC Vector Kits. These single-use NovaBlue Singles Competent Cells are provided in 50 µl aliquots. NovaBlue Competent Cells are also available in standard kits, provided in 0.2 ml aliquots (Cat. No. 69825). The standard transformation reaction uses 20 µl, so each tube contains enough cells for 10 transformations. The following protocol is appropriate for transformations using either Singles or standard competent cells.

Note: Upon receipt of Novagen® competent cells, verify that the cells are frozen and that dry ice is present in the shipping container. Immediately place the competent cells at –70°C or below. For optimal results, do not allow the cells to thaw at any time prior to use. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible. To mix, flick the tube 1–3 times. NEVER vortex competent cells.

1. Remove the appropriate number of NovaBlue competent cells from freezer (include one extra sample for Test Plasmid positive control, if desired). Immediately place tubes on ice, so that all but the cap is immersed in ice. Allow cells to thaw on ice for 2-5 min.
2. Visually examine the cells to see that they have thawed and gently finger-flick the tube 1–2 times to evenly resuspend the cells. The cells are then ready to remove an aliquot (*Standard*), or for the addition of the DNA (*Singles*™).

Singles:	*Standard:*
If a Test Plasmid sample is included, go to Step 4. If not, go directly to Step 5.	Place the required number of 1.5-ml snap-cap polypropylene tubes on ice to pre-chill. Pipet 20 µl aliquots of cells into the pre-chilled tubes.

(Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.

3. Add 1 µl of a ligation reaction directly to cells. Stir gently to mix and return tube to the ice. Repeat for additional samples.
4. Place tubes on ice for 5 min.
5. Heat tubes for exactly 30 s in a 42°C water bath; do not shake.

   Note: This "heat shock" step is most easily accomplished if the tubes are in a floating rack whereby the lower halves of the tubes are exposed. Place the rack in the water bath so that the lower half of the tubes are submerged for 30 s, and then replace the rack on ice.

6. Place tubes on ice for 2 min.

Singles:	*Standard:*
Add **250 µl** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.	Add **80 µl** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.

Incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective medium.

Selection for transformants is accomplished by plating on medium containing antibiotic for the plasmid encoded drug resistance (50 µg/ml carbenicillin or ampicillin for the Amp resistance marker). Additional host-specific antibiotics may also be appropriate to ensure maintenance of the host-encoded feature(s).
When plating less than 25 µl, first pipet a "pool" of SOC onto the plate and then pipet the cells into the SOC. The appropriate amount of transformation mixture to plate varies with the efficiencies of the annealing process and of the competent cells (see certificate of analysis for efficiency). For recombinants in NovaBlue, expect 10^5–10^7 transformants/µg plasmid, depending on the particular insert and the ligation efficiency. For the Test Plasmid, plate only 5 µl NovaBlue transformation mix in a "pool" of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the amp-resistance gene, *bla*).

7. Set plates on the bench for several minutes to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C (preferably 15–18 h).

   Note: When subcloning is successful, there are usually many more colonies produced from ligation in the presence of the insert than with the negative control. However, the cloning can be successful even though the number of colonies on the two plates is roughly equivalent.

Analysis of transformants

There are several methods for analysis of transformants, including colony PCR, plasmid preparation and restriction analysis, sequencing, and *in vitro* transcription and translation. One colony PCR protocol is described below.

For plasmid DNA preparation, standard methods typically produce plasmid DNA suitable for *E. coli* transformation and routine molecular biology manipulations, including sequencing. However, when isolating plasmid for baculovirus or mammalian cell transfections, more rigorous methods are recommended as contaminants can interfere with transfection. Use only high-quality transfection DNA. If necessary, prepare DNA on CsCl gradients using standard methods.

Detailed protocols for sequencing with double stranded and single stranded templates are available from manufacturers of sequencing kits.

Colony PCR

Before growing colonies for plasmid isolation, the presence of the appropriate insert as well as its orientation can be determined using direct colony PCR. This additional step may be particularly helpful if a "dirty" (many extraneous bands), unpurified PCR product was cloned.

To determine insert orientation and size, 5 pmol (1 µl) of a 5' vector-specific PCR primer is used with 5 pmol of a 3' insert-specific primer, if available. In a second reaction, the 5' insert-specific primer and 3' vector-specific primer is used. The TriEx™UP Primer (Cat. No. 70846-3) and T7 Promoter Primer (Cat. No. 69348-3) are both suitable as 5' vector-specific primers for all pTriEx vectors. The TriExDOWN Primer (Cat. No. 70847-3) is suitable as 3' vector-specific primer for all pTriEx vectors. Alternatively, just the vector-specific primers can be used in one reaction if insert orientation information is not desired.

Note: PCR products from any pTriEx Neo or pTriEx Hygro vector using the TriExDOWN primer will contain the CITE-sequence and drug resistance gene.

1. Pick a colony from an agar plate using a 200 µl pipet tip or sterile toothpick. Choose colonies that are at least 1 mm in diameter and try to get as many cells as possible. If a copy of the colony is desired, touch pipet tip to a fresh plate before transferring.
2. Transfer bacteria to a 0.5 ml tube containing 50 µl of sterile water. Vortex to disperse cells.
3. Place tube in boiling water or a heat block at 100°C for 5 min to lyse cells and denature DNases.
4. Centrifuge at 12,000 × g for 1 min to remove cell debris.
5. Transfer 10 µl supernatant to a fresh 0.5 ml tube for PCR. Leave on ice until use.
6. Assemble the following components for colony PCR.

   Per reaction:

   - 31.75 µl Nuclease-free water
   - 1 µl dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
   - 1 µl upstream Primer, 5 pmol/µl
   - 1 µl downstream primer, 5 pmol/µl
   - 5 µl 10X Nova*Taq*™ Buffer with $MgCl_2$
   - 0.25 µl (1.25 U) Nova*Taq* DNA Polymerase
   - 40 µl Total volume

   To account for pipetting losses, it is convenient to multiply the amounts by X.5, where X is the number of reactions.

   Note: For greatest specificity, and yield of long complex targets during PCR use KOD Hot Start and KOD XL DNA Polymerases respectively, with their buffers and cycling conditions (see User Protocols TB341, TB342). KOD polymerases are not available for sale in Japan through EMD Chemicals Inc.

7. Add 40 µl of the master mix to each 10 µl sample, mix gently, add 2 drops of mineral oil, cap tubes, and put the samples in a thermal cycler.

   Note: As an optional step, a hot start procedure can be used in which the cell lysate samples are prewarmed to 80°C before the addition of the master mix.

8. Process in the thermal cycler for 35 cycles, as follows:
   - 1 min at 94°C
   - 1 min at the proper annealing temperature (usually 55°C for vector primers)
   - 2 min at 72°C
   - 6 min final extension at 72°C
9. To remove the oil overlay and inactivate the polymerase, add 100 µl of chloroform, mix 30 s, and centrifuge 1 min. The top aqueous phase (which may appear cloudy) contains the DNA.
10. Add 5 µl 10X loading dye to the top aqueous phase.
11. Load 10–25 µl per lane on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Include at least one lane of Perfect DNA™ Markers. A strong band should appear that has a size corresponding to the total number of bases between and including the primers.

Protein Expression in *E. coli*

After a pTriEx™ recombinant has been established in NovaBlue cells, the plasmid may be induced for protein expression using either of the following two methods.

Bacteriophage CE6 infection

T7 RNA polymerase may be delivered to the NovaBlue cells harboring recombinants derived form pTriEx vectors by infecting the cultures with bacteriophage CE6. The λp_L and λp_I promoters control the expression of T7 RNA polymerase in this phage. Therefore, T7 RNA polymerase is immediately produced in host cells upon CE6 infection. This in turn results in transcription of the target gene from the T7*lac* promoter present in the pTriEx plasmids. T7 RNA polymerase transcribes target DNA so actively that normal CE6 phage development cannot proceed. No T7 RNA polymerase is present in the cell before infection, so it should be possible to express any target DNA that can be cloned under control of a T7 promoter in this way. Bacteriophage CE6 is available separately and is described in User Protocol TB007.

Induction in (DE3)pLacI expression hosts

Recombinant plasmids may also be isolated from NovaBlue cells and transformed into (DE3)pLacI expression hosts which are lysogenic for bacteriophage λDE3. Strains containing (DE3)pLacI must be used as the expression host for pTriEx vectors. This is because (DE3)pLacI strains possess a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter and pTriEx is a high copy plasmid that does not encode lac repressor (*lacI* gene). To insure that the level of *lac* repressor is sufficient to repress both the T7*lac* promoter that controls target ORF expression and the lacUV5 promoter that regulates T7 RNA polymerase expression, it is critical to transform (DE3)pLacI expression strains. EMD Chemicals Inc. offers a number of (DE3)pLacI expression strains (see table below). For details on each of the strains listed below, see User Protocol TB009.

pTriEx™ Expression Strains	Cat. No.	Antibiotic Resistance
Origami™ 2(DE3)pLacI Competent Cells	71347	12.5 µg/ml Tet, 34 µg/ml Cam
Origami B(DE3)pLacI Competent Cells	70838	15 µg/ml Kan, 12.5 µg/ml Tet, 34 µg/ml Cam
Rosetta™(DE3)pLacI Competent Cells	70920	34 µg/ml Cam
Rosetta™ 2(DE3)pLacI Competent Cells	71404	34 µg/ml Cam
Rosetta-gami™ 2(DE3)pLacI Competent Cells	71353	12.5 µg/ml Tet, 34 µg/ml Cam
Rosetta-gami B(DE3)pLacI Competent Cells	71138	15 µg/ml Kan, 12.5 µg/ml Tet, 34 µg/ml Cam
RosettaBlue™ (DE3)pLacI Competent Cells	71060	12.5 µg/ml Tet, 34 µg/ml Cam
Tuner™ (DE3)pLacI Competent Cells	70625	34 µg/ml Cam

Kan = kanamycin, Tet = tetracycline, Cam = chloramphenicol

Transformation

Novagen® (DE3)pLacI competent cells are provided in standard, 0.2 ml aliquots. The standard transformation calls for 20 µl of cells, so each tube contains enough for 10 transformations. Follow the *Standard* transformation protocol (see p 7), substituting the appropriate (DE3)pLacI host for NovaBlue. Note that selection for transformants is accomplished by plating on medium containing antibiotic for the plasmid encoded drug resistance (50 µg/ml carbenicillin or ampicillin for the Amp resistance marker), as well as any host-specific antibiotics (see table above, or User Protocol TB009) to ensure maintenance of the host-encoded feature(s).

Induction

Following transformation, preliminary analysis of expression levels, cellular localization, and solubility of the target protein should be performed. For this purpose, pick a single colony from a freshly streaked plate and inoculate 50 ml LB containing the appropriate antibiotic(s) for the plasmid and host strain in a 250 ml Erlenmeyer flask. For good aeration, add medium up to only 20% of the total flask volume.

1. Prepare starter culture of the pTriEx™ recombinant in a (DE3)pLacI host as follows: inoculate 3 ml appropriate media + 1% glucose + antibiotics in culture tube with a single colony from a plate, or sterile loop of cells from a glycerol stock.

   Note: Plasmids containing toxic genes may be destabilized in (DE3)pLacI hosts due to cAMP stimulation of T7 RNA polymerase [Grossman et al., 1998]. This can be effectively delayed by including 1% glucose in the culture medium.

2. Incubate at 37°C with shaking at 250 rpm to an OD_{600} of approximately 0.5. Add the entire 3 ml culture to 100 ml medium containing 1% glucose and antibiotic(s).
3. Shake the 100 ml culture at the desired temperature until the OD_{600} is approximately 0.5–1.0 (e.g., 2–3 h in LB broth, 37°C). Monitor the OD_{600} during growth by removing aliquots aseptically.
4. Just prior to induction, split the 100 ml culture into 2 × 50 ml cultures. Add IPTG to one of the 50 ml cultures and use the other culture as an uninduced control. For full induction, use 500 µl sterile 100 mM IPTG for a final concentration of 1 mM. For lower levels of induction using *lacY* I mutant strains (i.e., Tuner™, Rosetta-gami™ B, and Origami™ B), vary the range of IPTG. Using 0.25 µM to 300 µM IPTG should yield target protein expression that ranges from barely detectable to nearly fully induced at 3 h at 37°C.
5. Incubate both cultures with shaking at the desired temperature for the appropriate amount of time, typically 2-4 h when performing a standard 37°C induction.

   Note: Overnight Express™ Autoinduction Systems are designed for high-level protein expression with IPTG-inducible bacterial expression systems including pTriEx without the need to monitor cell growth. See User Protocol TB383 for more information on these systems.

6. For target protein verification protocols, see User Protocol TB055 (Section V).

Protein Expression in Insect Cells

The pTriEx vectors contain the baculovirus p10 promoter for protein expression in infected insect cells. The p10 promoter is highly active during the very late phase of baculovirus infection, thus it is necessary to prepare a recombinant baculovirus, using the pTriEx plasmid as a transfer vector. For baculovirus construction protocols, see User Protocol TB216. Substitute the pTriEx plasmid DNA for pBAC™ plasmid DNA in this protocol.

pTriEx vectors are compatible with the Novagen® Direct Plaquing transfection protocol (see User Protocol TB216) in which the transfection mixture (virus DNA, pTriEx plasmid DNA, and Insect GeneJuice® Transfection Reagent) is added directly to a prepared Sf9 insect cell monolayer, followed by an overlay of the Novagen® pre-qualified BacPlaque™ Agarose in BacVector® Insect Cell Medium. This procedure allows recombinant plaques to be visualized 3–4 days after preparing the transfection mixture, and provides the advantage of ensuring that each plaque represents an independent recombinant baculovirus. The traditional Liquid Overlay transfection protocol should also be used in parallel with the Direct Plaquing transfection protocol (see User Protocol TB216).

After preparing a stock of the TriEx recombinant virus, maximal protein expression is achieved by infection of TriEx Sf9 Cells (Cat. No. 71023) which are optimized for vigorous cell growth and protein yield in serum-free TriEx Insect Cell Medium (Cat. No. 71022). TriEx Sf9 Cells also yield high titers of recombinant baculoviruses when used for amplifying viral stocks.

If using stable pTriEx Hygro or Neo Vectors, the β-gal Transfection Control plasmid should be used as a positive control to verify the generation of recombinant virus plaques using the transfection protocols. This plasmid provides the ability

to visualize control recombinants by staining plaques with the colorimetric substrate X-Gluc, which indicates β-glucuronidase (gus) activity.

It is also recommended that first time users include a negative control, e.g., a parallel transfection reaction containing only Triple Cut virus DNA and no transfer plasmid. This control will reveal any background plaques from residual uncut viral DNA (typically < 5% of the plaque number obtained using the β-gal Transfection Control plasmid).

For target protein verification protocols, see User Protocol TB055 (Section V).

Protein Expression in Mammalian Cells

In mammalian cells, target protein expression is mediated by the constitutive chicken β-actin or CMVie promoter. The pTriEx™ construct may be introduced into mammalian cells using GeneJuice® Transfection Reagent (Cat. No. 70967).

For pTriEx transfection protocols in mammalian cell lines, and subsequent transient protein expression protocols, see User Protocol TB289. For stable protein expression protocols, see the section below. For target protein verification protocols, see User Protocol TB055 (Section V).

For stable expression, mammalian cells transfected with pTriEx bicistronic constructs can be selected using neomycin or hygromycin. Neomycin (Neo) is an aminoglycoside that is similar in structure to G418, gentamycin, and kanamycin. In mammalian cells, neomycin blocks protein synthesis by binding to the small subunit of ribosomes. Expression of the bacterial aminoglycoside phosphotransferase gene (APH) in mammalian cells detoxifies neomycin by phosphorylation, thus conferring resistance to neomycin. Hygromycin (Hygro) is an aminoglycoside antibiotic that inhibits the growth of prokaryotic and eukaryotic microorganisms and eukaryotic cells by disrupting translocation on the 70S ribosome and promoting mistranslation of mRNA. Expression of the bacterial hygromycin phosphotransferase gene (HPH) in mammalian cells detoxifies hygromycin by phosphorylation, thus, conferring resistance to hygromycin.

Neomycin (Cat. No. 480100) and Hygromycin (Cat. No. 400049) are available as solutions that can be used directly in cell culture. When using powdered forms, reconstitute as follows:

- Calculate the concentration based on the stated amount of active drug as shown on the label.
- Dissolve antibiotic in a buffered solution (i.e., 100 mM HEPES, pH7.3) or serum-free growth medium (i.e., DMEM).
- Filter to sterilize. Store sterile solution at –20°C.

Note: As different cell lines possess various levels of natural resistance to neomycin and hygromycin, it is highly recommended that mammalian cell lines be tested for antibiotic sensitivity. A general protocol is provided below.

Determination of antibiotic sensitivity

Note: It is important to determine the minimum concentration of neomycin or hygromycin required to kill off non-transfected cells (i.e., the kill curve). The following protocol describes a simple procedure to test a range of concentrations of antibiotic, to determine the minimum concentration needed:

1. Plate cells at 25% confluency in all 6 wells of a 6-well plate. Incubate cells overnight.
2. The next day, aspirate off growth medium and replace with freshly prepared growth medium containing the following concentrations of Neo or Hygro: 0, 100, 250, 500, 750, and 1000 µg/ml.
3. Every 3–4 days, aspirate the old medium and replace with freshly prepared growth medium, containing the respective antibiotic concentrations. Observe the percentage of surviving cells.
4. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of untransfected host cells in 10–12 days from the start of antibiotic selection.

Selection of stable cells

The CITE sequence in the pTriEx bicistronic expression vectors is an attenuated version which results in reduced translation of the selectable marker. The weakened translation efficiency of the attenuated CITE favors the preferential selection of cells expressing high levels messenger RNA and thus high levels of target protein. This selective pressure will maintain the long-term stable expression of the target gene.

Unless there is a special need for clonal cell lines, a mixed population of drug-resistant cells can be used directly for experiments. To generate clonal cell lines, it is necessary to dilute the resistant cells and plate for single cells in 96 well dishes.

1. Follow the recommended transfection protocols for adherent cells as outlined in User Protocol TB289. Then, incubate the cells for 2 days at 37°C (5% CO_2).
2. Follow the antibiotic sensitivity procedure above, replacing the growth medium every 3-4 days. The growth medium shall contain the desired concentrations of selective antibiotic (either the full strength selective concentration determined above or the 50% reduced concentration).
3. Split surviving cells to fresh T-75 flasks as needed to avoid overgrowth.
4. Continue to passage cells in the presence of antibiotic for two additional passages. At this point the antibiotic-resistant cells are considered to be stable polyclonal cells and may be used for further experimentation.

   Note:During the stable cell selection process, mammalian cell lines may require initial antibiotic concentrations at 50% of the minimal concentration (as determined from the kill curve) for the first week followed by a change to the full-strength optimal selection concentration. For example, minimum neomycin and hygromycin concentrations of 1000 and 800 µg/ml, respectively, were determined for the BHK cell line using the antibiotic sensitivity procedure above. After performing the antibiotic sensitivity procedure, we recommend selecting BHK cells in neomycin or hygromycin at 500 and 400 µg/ml, respectively, for the first week followed by a switch to 1000 and 800 µg/ml for neomycin and hygromycin, respectively, for the following two weeks.

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E. coli B834(DE3)	*E. coli* Rosetta(DE3)pLysS
E. coli B834(DE3)pLysS	*E. coli* Rosetta(DE3)pLacI
E. coli BL21(DE3)	*E. coli* Rosetta 2(DE3)
E. coli BL21(DE3)pLysS	*E. coli* Rosetta 2(DE3)pLysS
E. coli BL21(DE3)pLysE	*E. coli* Rosetta 2(DE3)pLacI
E. coli BL26(DE3)pLysE	*E. coli* RosettaBlue™(DE3)
E. coli BLR(DE3)	*E. coli* RosettaBlue(DE3)pLysS
E. coli BLR(DE3)pLysS	*E. coli* RosettaBlue(DE3)pLacI
E. coli HMS174(DE3)	*E. coli* Rosetta-gami™ 2(DE3)
E. coli HMS174(DE3)pLysS	*E. coli* Rosetta-gami 2(DE3)pLysS
E. coli HMS174(DE3)pLysE	*E. coli* Rosetta-gami 2(DE3)pLacI
E. coli NovaBlue(DE3)	*E. coli* Rosetta-gami B(DE3)
E. coli Origami™ 2(DE3)	*E. coli* Rosetta-gami B(DE3)pLysS
E. coli Origami 2(DE3)pLysS	*E. coli* Rosetta-gami B(DE3)pLacI
E. coli Origami 2(DE3)pLacI	*E. coli* Tuner™(DE3)
E. coli Origami B(DE3)	*E. coli* Tuner(DE3)pLysS
E. coli Origami B(DE3)pLysS	*E. coli* Tuner(DE3)pLacI
E. coli Origami B(DE3)pLacI	Bacteriophage λCE6
E. coli Rosetta™(DE3)	Bacteriophage λDE3

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