Ek/LIC Cloning Kits

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About the Kits

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Description

Novagen ligation-independent cloning (LIC) vectors facilitate rapid cloning and gene expression in multiple expression systems (*in vitro*, *E. coli*, insect cell, and mammalian). Ligation-independent cloning enables directional cloning of PCR products without the need for restriction enzyme digestion or ligation reactions (1, 2). An enterokinase cleavage site is present immediately prior to the target protein, so that all vector-encoded fusion sequences can be removed following protein purification. All Ek/LIC vectors possess the same Ek/LIC cloning site; thus, the same Ek/LIC-prepared target insert can be annealed into any of the Ek/LIC vectors.

The LIC method uses the 3'→5' exonuclease activity of T4 DNA Polymerase to create specific 13- or 14-base single-stranded overhangs in the Ek/LIC vector. PCR products with complementary overhangs are created by building appropriate 5' extensions into the primers (see p 4). Purified PCR product is treated with LIC-qualified T4 DNA Polymerase in the presence of dATP to generate specific vector-compatible overhangs. Cloning is very efficient, as primarily the desired product is formed by annealing. The annealed Ek/LIC vector and insert are transformed into competent *E. coli* cells. Covalent bond formation at the vector-insert junctions occurs within the cell to yield circular plasmid. After verification, the construct may be used for bacterial expression or transfection into insect or mammalian cells, depending on vector characteristics. Ek/LIC vectors designed for *E. coli* expression can also be used for coexpression of up to six target proteins. Coexpression in *E. coli* can be achieved by cloning single inserts into compatible Ek/LIC expression vectors and/or by simultaneously cloning two LIC inserts into an Ek/LIC vector with the aid of LIC Duet™ Adaptor Kits (see User Protocol TB384).

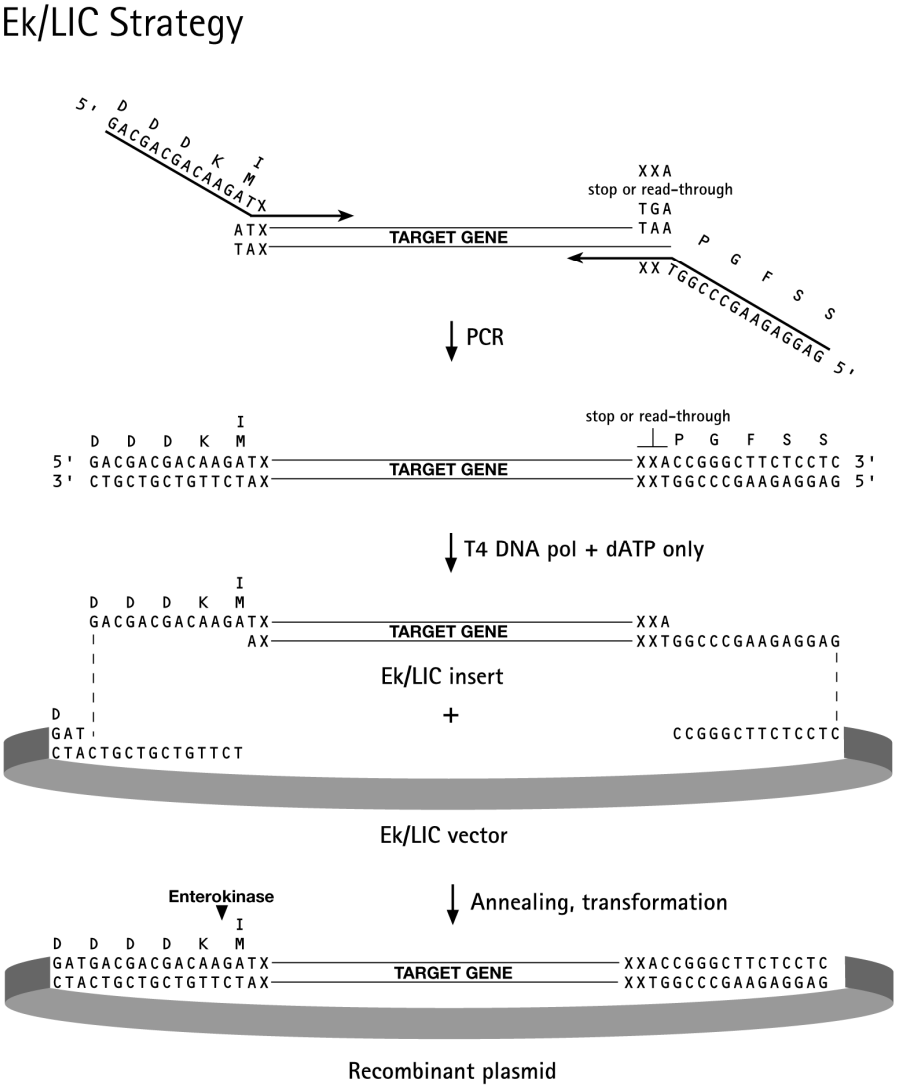

Figure 1. Diagram of the Ek/LIC strategy. After amplification with primers that include the indicated 5' LIC extensions, the PCR insert is treated with LIC-qualified T4 DNA Polymerase (+dATP), annealed to the Ek/LIC vector, and the resultant nicked, circular plasmid is transformed into competent *E. coli*.

Note: For simultaneous cloning of two inserts into the Ek/LIC vectors LIC Duet™ Adaptors may be used (see User Protocol TB384).

Components

pET, pCDF, and pRSF Ek/LIC Vector Kits

1 µg	pET, pCDF, or pRSF Ek/LIC Vector
8 µl	Ek/LIC β-gal Control Insert
25 U	T4 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
22 × 50 µl	NovaBlue GigaSingles™ Competent Cells
0.2 ml	BL21(DE3) Competent Cells
0.2 ml	BL21(DE3)pLysS Competent Cells
5 × 2 ml	SOC medium
10 µl	Test Plasmid, 0.2 ng/µl in TB buffer (ampicillin resistant)

pBAC™ and pBACgus™-2cp Ek/LIC Vector Kits

1 µg	pBAC™ Ek/LIC Vector
8 µl	Ek/LIC GUS Control Insert or Ek/LIC β-gal Control Insert
25 U	T4 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
22 × 50 µl	NovaBlue GigaSingles™ Competent Cells
5 × 2 ml	SOC Medium
10 µl	Test Plasmid, 0.2 ng/µl in TB buffer (ampicillin resistant)

Note: Reagents for transfection and expression in insect cells are sold separately.

pIEx™ Ek/LIC Vector Kits

1 µg	pIEx™ Ek/LIC Vector
8 µl	Ek/LIC β-gal Control Insert
25 U	T4 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
22 × 50 µl	NovaBlue GigaSingles™ Competent Cells
5 × 2 ml	SOC Medium
10 µl	Test Plasmid, 0.2 ng/µl in TB buffer (ampicillin resistant)

Note: Reagents for transfection and expression in insect cells are sold separately.

pIEx/Bac™ Ek/LIC Vector Kits

1 µg	pIEx/Bac™ Ek/LIC Vector
8 µl	Ek/LIC β-gal Control Insert
25 U	T4 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
22 × 50 µl	NovaBlue GigaSingles™ Competent Cells
5 × 2 ml	SOC Medium
10 µl	Test Plasmid, 0.2 ng/µl in TB buffer (ampicillin resistant)

Note: Reagents for transfection and expression in insect cells are sold separately.

pTriEx™ Ek/LIC Vector Kits

1 µg	pTriEx™ Ek/LIC Vector
8 µl	Ek/LIC β-gal Control Insert
25 U	T4 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
22 × 50 µl	NovaBlue GigaSingles™ Competent Cells
0.2 ml	Origami™ B(DE3)pLacI Competent Cells
5 × 2 ml	SOC Medium
10 µl	Test Plasmid, 0.2 ng/µl in TB buffer (ampicillin resistant)

Note: Reagents for transfection and expression in insect cells and mammalian cells are sold separately.

Storage

Store Competent Cells, SOC Medium, and Test Plasmid at –70°C. Store Ek/LIC vectors at –20°C or –70°C. Store all other components at –20°C.

Ek/LIC Vectors

This section describes the range of Ek/LIC Vectors available for cloning and expression in *E. coli*, insect, and mammalian cells. PCR-prepared Ek/LIC insert(s) can be annealed to any of the Ek/LIC Vectors.

pET Ek/LIC Vectors for expression in *E. coli*

The pET Ek/LIC Vectors for protein expression in *E. coli* feature a T7*lac* promoter and an extensive choice of fusion tags (see table below). These vectors have a ColE1-derived replicon. pET-30 Ek/LIC and pET-41 Ek/LIC bear a kanamycin resistance marker. Other pET Ek/LIC vectors bear an ampicillin/carbenicillin resistance marker.

	Fusion tags							Protease Cleavage Site	Vector Map
Vector	GST•Tag	Nus•Tag	Strep•Tag II	Trx•Tag	His•Tag	S•Tag	HSV•Tag		
pET-30 Ek/LIC					N,C	N		Tb, Ek	TB162
pET-32 Ek/LIC				N	N, C	N		Tb, Ek	TB161
pET-41 Ek/LIC	N				N, C	N		Tb, Ek	TB317
pET-43.1 Ek/LIC		N			N, C	N	C	Tb, Ek	TB318
pET-44 Ek/LIC		N			N (2x), C	C	C	Tb, Ek	TB339
pET-46 Ek/LIC					N	C		Ek	TB397
pET-51 Ek/LIC			N		C			Ek	TB437

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

pRSF-2 and pCDF-2 Vectors for expression in *E. coli*

The pRSF-2 and pCDF-2 Ek/LIC Vectors carry a T7*lac* promoter for expression in *E. coli.* pRSF-2 contains the RSF1030 replicon (3, 4) and a kanamycin resistance marker. pCDF-2 contains the CloDF13 replicon (5) and a streptomycin/spectinomycin resistance marker. See vector characteristics in table below.

Vector	Fusion Tags		Protease Cleavage Sites	Vector Map
	His•Tag®	S•Tag™		
pCDF-2 Ek/LIC	N	C	Ek	TB394
pRSF-2 Ek/LIC	N	C	Ek	TB395

N: N-terminal; C: C-terminal; Ek: enterokinase

Using pET, pCDF and pRSF Ek/LIC Vectors for coexpression in *E. coli*

The pCDF-2 and pRSF-2 Ek/LIC Vectors differ in replicons and drug resistance markers. Since different plasmids can be maintained in the same *E. coli* cell if they have different replicons (6) and selectable markers, the pCDF-2 and pRSF-2 Ek/LIC Vectors are suitable for coexpression of proteins in combination with each other or with many pET vectors. A single insert can be cloned into each of these vectors to allow coexpression of up to three target proteins. Alternatively, two inserts can be simultaneously cloned into each vector using the LIC Duet™ Adaptor cloning strategy (7) (See User Protocol TB384). Creating recombinants with two inserts per Ek/LIC vector allows coexpression of up to six target proteins. Use the following tables to determine appropriate combinations of vectors and host strain for coexpression.

Note: Difference in copy number between plasmids used for coexpression may influence relative target protein expression. Relative plasmid copy number is pRSF > pET> pCDF.

Vector and host strain compatibility

Compatible Vector Combinations			Number of coexpressed target proteins	Compatible expression host strains*
Vector 1	Vector 2	Vector 3		
pET Ek/LIC (Amp^R)	pRSF-2 Ek/LIC (Kan^R)	pCDF-2 Ek/LIC (Sm^R)	3–6	Group A
pET (Amp^R)	pRSF-2 Ek/LIC (Kan^R)	pCDF-2 Ek/LIC (Sm^R)	3–5	Group A
pET Ek/LIC (Amp^R)	pRSF-2 Ek/LIC (Kan^R)		2–4	Group A
pET Ek/LIC (Amp^R)	pCDF-2 Ek/LIC (Sm^R)		2–4	Group B
pRSF-2 Ek/LIC (Kan^R)	pCDF-2 Ek/LIC (Sm^R)		2–4	Group A
pET Ek/LIC (Kan^R)	pCDF-2 Ek/LIC (Sm^R)		2–4	Group A
pET (Kan^R)	pCDF-2 Ek/LIC (Sm^R)		2–4	Group A

Amp: ampicillin or carbenicillin, 50 µg/ml; Kan: kanamycin, 30 µg/ml; Sm: streptomycin/spectinomycin, 50 µg/ml

* See page 8 for Host Strain Groups table.

Strain Groups	
Group A	**Group B**
B834(DE3)	B834(DE3)
B834(DE3)pLysS	B834(DE3)pLysS
BL21(DE3)	BL21(DE3)
BL21(DE3)pLysS	BL21(DE3)pLysS
BLR(DE3)	BLR(DE3)
BLR(DE3)pLysS	BLR(DE3)pLysS
HMS174(DE3)	HMS174(DE3)
HMS174(DE3)pLysS	HMS174(DE3)pLysS
NovaBlue(DE3)	NovaBlue(DE3)
NovaBlue(DE3)pLysS	NovaBlue(DE3)pLysS
Origami™ 2(DE3)*	Origami 2(DE3)*
Origami 2 (DE3)pLysS*	Origami 2(DE3)pLysS*
Rosetta™(DE3)	Origami B(DE3)
Rosetta™(DE3)pLysS	Origami B(DE3)pLysS
Rosetta™ 2(DE3)	Rosetta™(DE3)
Rosetta™ 2(DE3)pLysS	Rosetta™(DE3)pLysS
RosettaBlue™(DE3)	Rosetta™ 2(DE3)
RosettaBlue™(DE3)pLysS	Rosetta™ 2(DE3)pLysS
Rosetta-gami™ 2(DE3)*	RosettaBlue™(DE3)
Rosetta-gami™ 2(DE3)pLysS*	RosettaBlue™(DE3)pLysS
Tuner™(DE3)	Rosetta-gami™ 2(DE3)*
Tuner™(DE3)pLysS	Rosetta-gami™ 2(DE3)pLysS*
Tuner™(DE3)	Rosetta-gami™ B(DE3)
Tuner™(DE3)pLysS	Rosetta-gami™ B(DE3)pLysS

*These *E. coli* strains carry a mutation in *rpsL*, resulting in ribosomal protein resistance to streptomycin. pCDF vectors bear the *aadA* gene, which confers resistance to both streptomycin and spectinomycin. Thus, when transforming *rpsL* strains with pCDF vectors, spectinomycin must be used for transformation selection. (It is not necessary to include streptomycin during growth to maintain *rpsL* strain genotype.)

pBAC™ Ek/LIC Vectors for baculovirus-mediated expression in insect cells

The pBAC™ Ek/LIC Vectors are baculovirus transfer plasmids and carry a polh promoter. To create baculovirus recombinants, first establish plasmid recombinants in *E. coli*. Then isolate and cotransfect plasmids with baculovirus DNA (BacVector®-1000, -2000, or -3000 Triple Cut Virus DNA or BacMagic™ DNA) into insect cells.

Vector	Fusion Tags		Protease Cleavage Sites	Vector Map
	His•Tag™	S•Tag™		
pBAC™-2cp Ek/LIC	N, C	C	Tb, Ek	TB128
pBACgus™-2cp Ek/LIC	N, C	C	Tb, Ek	TB141

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

pIEx™ Ek/LIC Vectors for expression in Insect Cells

The pIEx™ Ek/LIC Vectors are expression vectors for transfection into *Spodoptera*-derived insect cells. After establishing recombinants in *E. coli*, isolate plasmids and transfect into Sf9 or Sf21 insect cells for transient target protein expression. Vectors employ an optimal combination of AcNPV baculovirus-derived transcription elements, the hr5 enhancer and the IE1 immediate early promoter (8-11). This promoter/enhancer combination recruits endogenous insect cell transcription machinery for rapid, high-yield protein expression without the time consuming process of creating recombinant baculovirus or cytotoxic effects associated with infection.

Vector	Signal Sequence	Fusion Tags					Protease Cleavage Sites	Vector Map
		GST•Tag™	Strep•Tag™ II	His•Tag™	S•Tag™	HSV•Tag™		
pIEx™-1 Ek/LIC				N	N	C	Tb, Ek	TB349
pIEx™-2 Ek/LIC		N		N	N	C	Tb, Ek	TB348
pIEx™-3 Ek/LIC	N	N		N	N	C	Tb, Ek	TB351
pIEx™-7 Ek/LIC				N	C		Ek	TB399
pIEx™-8 Ek/LIC			N	C			Ek	TB439
pIEx™-10 Ek/LIC	N		N	C			Ek	TB441

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

pIEx/Bac™ Ek/LIC Vector for expression in insect cells

The pIEx/Bac™ Ek/LIC Vector is a dual purpose expression vector for transfection into *Spodoptera*-derived insect cells and as a transfer vector for generating recombinant baculovirus. After establishing recombinants in *E. coli*, isolate plasmids and transfect into Sf9 or Sf21 insect cells for rapid target protein expression and screening. Purified plasmid may also be used to create baculovirus recombinants by cotransfecting with BacMagic™ DNA or BacVector® Triple Cut Virus DNA (see User Protocols TB459 or TB216, respectively). The vector employs an optimal combination of AcNPV baculovirus-derived transcription elements (hr5 enhancer and IE1 immediate early promoter) (8-11) to drive transcription in transient transfection mode. This promoter/enhancer combination recruits endogenous insect cell transcription machinery. Therefore, expression levels of multiple constructs can be analyzed rapidly, prior to committing resources to generation of baculovirus stocks. The vector also features the AcNPV-derived p10 promoter, which is strongly active in late/very late stages of baculovirus infection. Therefore, when used in the baculovirus mode, this unique vector directs expression of target protein at all stages of the baculovirus infection process - thereby allowing optimal harvest point determination. If desired, vectors can also be used for generation of drug-resistant stable cell lines by cotransfection with the plasmid pIE1-Neo (see User Protocol TB176).

Vector	Fusion Tags			Protease Cleavage Sites	Vector Map
	Strep•Tag™ II	GST•Tag™	His•Tag™		
pIEx/Bac™-1 Ek/LIC	N		C	Ek	TB468
pIEx/Bac™-4 Ek/LIC		N	C	Ek	TB492

N: N-terminal; C: C-terminal; Ek: enterokinase

pTriEx™ Ek/LIC Multisystem Vector for expression in *E.coli*, insect, and mammalian cells

The pTriEx™ Ek/LIC Vector contains the CMV-IE enhancer-promoter combination followed by T7*lac* and p10 promoters to direct high-level expression of target genes in mammalian cells, *E.coli*, or baculovirus-infected insect cells. For the baculovirus application, the pTriEx™ vector functions as a transfer vector. Recombinant pTriEx™ constructs have the necessary baculovirus-derived flanking sequences for recombination with BacVector® Triple Cut Virus DNA or BacMagic™ DNA (see User Protocol TB216 or TB459, respectively), yielding recombinant baculovirus.

Vector	Signal Sequence	Fusion Tags				Protease Cleavage Sites	Vector Map
		His•Tag™	Strep•Tag™ II	S•Tag™	HSV•Tag™		
pTriEx™-4 Ek/LIC		N, C		N	C	Tb, Ek	TB282
pTriEx™-5 Ek/LIC		C	N			Ek	TB442
pTriEx™-7 Ek/LIC	N	C	N			Ek	TB444

N: N-terminal; C: C-terminal; Tb: Thrombin; Ek: enterokinase

Insert Preparation

The following protocols are specific for cloning a single insert into an Ek/LIC vector. For simultaneous cloning of two inserts, see User Protocol TB384 (LIC Duet™ Adaptor Kits).

Amplification and purification of target insert

When PCR-amplifying inserts, we strongly recommend using KOD DNA Polymerase or KOD Hot Start DNA Polymerase (see User Protocol TB320 or TB341, respectively). These polymerases have robust elongation rates and very low mutation frequencies, resulting in high yields and few errors (12).

When starting template is limiting (such as in reverse transcription reactions from total RNA or mRNA, or cDNA library templates), high fidelity is especially important. In addition to using a high-fidelity DNA polymerase, such as KOD or KOD Hot Start, the likelihood of PCR-generated mutations can be further reduced by creating a sequence-verified plasmid clone to serve as a template in subsequent amplifications. Fewer cycles are needed to generate sufficient material for LIC cloning when a high amount of verified template (50–250 ng plasmid) is used for PCR. Only 0.02 pmol target (13 ng of a 1,000 bp insert) is required per LIC reaction. Therefore, as little as 1 µg amplified target is sufficient to perform > 75 LIC reactions.

Note: Use HPLC-purified primers for optimal PCR results and to greatly decrease the possibility of primer-derived mutations.

1. Amplify the desired insert sequence using appropriately designed PCR primers. Because the system is ligation independent, 5’ phosphorylation of the primers is not necessary. Primer 5'-ends must incorporate the following sequences (see page 4):

   sense primer: `5' GAC GAC GAC AAG ATX*`–insert-specific sequence 3'
   antisense primer: `5' GA GGA GAA GCC CGG TXX**`–insert-specific sequence 3'

   *The first nucleotide of the insert-specific sequence on the sense primer must complete the codon ATX resulting in Met (X=G) or Ile (X=A, C or T).
   **If a C-terminal tag is not desired, include an in-frame stop codon in the insert-specific sequence. (see page 4). For example, complete the TXX anticodon on the antisense primer as TTA. On the coding strand this is TAA, a translational stop codon.
   e.g. antisense primer with stop codon: 5’ GA GGA GAA GCC CGG TTA–insert specific sequence 3’

2. The PCR product must be purified to remove dNTPs completely, to inactivate the polymerase, and to remove contaminating DNA. If extraneous products are present in the PCR reaction or if the template plasmid and the EK/LIC vector have the same antibiotic resistance marker, run the PCR product on an agarose gel. Then, excise and extract the target band using a SpinPrep™ Gel Kit (see User Protocol TB285), D-Tube™ Dialyzer with Electroelution Assessory Kit (see User Protocol TB422), or a similar method.

If spurious products are not present, the agarose gel purification step is unnecessary. In this case, one of the following methods can be used to remove dNTPs and residual enzyme:

- Purify the PCR product on a spin column or other solid support (e.g., SpinPrep™ PCR Clean-Up Kit, Cat. No. 70976).
- Inactivate the enzyme and remove dNTPs by CIAA extraction and isopropanol precipitation. Extract the reaction with 1 volume CIAA [chloroform:isoamyl alcohol (24:1)]. Vortex for 1 min and spin at 12,000 × *g* for 1 min. Remove and save aqueous phase. Add 0.1 volume 3 M sodium acetate, pH 5.2 and 1 volume isopropanol. Vortex. Incubate at room temperature for 5 min. Centrifuge at 14,000 × *g* for 5 min. Remove supernatant and rinse pellet with 70% ethanol. Allow pellet to air-dry. Resuspend purified PCR product in TlowE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

T4 DNA Polymerase treatment of target insert

Generate compatible overhangs on the insert(s) by treating purified PCR product with T4 DNA Polymerase. To verify system performance, include a positive control reaction with the supplied positive control insert. Also include a negative control, omitting insert.

Control inserts require treatment with T4 DNA Polymerase to generate compatible overhangs. The Ek/LIC β-gal Control Insert included with pET, pRSF, pCDF, pBAC™, pIEx™, pIEx/Bac™ and pTriEx™ Ek/LIC Vector Kits is 3085 bp long (2 µg/pmol). For each treatment, use 4 µl of the 100 ng/µl solution provided. The pBAC™-2cp Ek/LIC Vector Kit contains the Ek/LIC GUS Control Insert, which is 1815 bp long (1.18 µg/pmol). For each treatment, use 2.4 µl of the 100 ng/µl solution provided.

1. Assemble the following components in a sterile 1.5-ml microcentrifuge tube on ice:

x µl	0.2 pmol purified PCR product in up to 14.6 µl TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) (Note: number bp in insert × 650 = pg/pmol)
2 µl	10X T4 DNA Polymerase Buffer
2 µl	25 mM dATP
1 µl	100 mM DTT
y µl	Nuclease-free Water
0.4 µl	2.5 U/µl T4 DNA Polymerase (LIC-qualified; 0.5 unit per 0.1 pmol PCR product)
20 µl	Total volume (Final concentration of insert is 0.01 pmol/µl)

2. Start reaction by adding enzyme. Stir with pipet tip to mix. Incubate at 22°C for 30 min.
3. Inactivate enzyme by incubating at 75°C for 20 min.
4. Prepared insert can be annealed to any of the Ek/LIC vectors. Store prepared Ek/LIC insert at –20°C. Inserts stored for several months have been used successfully for cloning.

   Note: The T4 DNA Polymerase in Novagen Ek/LIC kits is specifically qualified for ligation-independent cloning. Use of unqualified T4 DNA Polymerase may cause variable cloning efficiency.

Annealing the Vector and Ek/LIC Insert

Use the following protocol to anneal an insert into an Ek/LIC Vector. Perform a negative control (lacking insert) and a positive control (T4 DNA polymerase-treated control insert), as described above.

For each insert, assemble the following components in a sterile 1.5-ml microcentrifuge tube:

1 µl	Ek/LIC Vector
2 µl	T4 DNA Polymerase treated Ek/LIC insert (0.02 pmol)
	Incubate at 22°C for 5 min, then add:
1 µl	25 mM EDTA
4 µl	Total volume

Mix by stirring with pipet tip. Incubate at 22°C for 5 min.

Note: reater volumes of treated insert may be used; however, vector concentration will be decreased. Compensate by using a larger volume of annealing reaction for transformation (see page 12).

Annealing is complete within 5 min of incubation. Reactions can be incubated up to 1 h with no negative effects.

Transformation

NovaBlue GigaSingles™ Competent Cells (Cat. No. 71127) are provided in Ek/LIC Vector Kits and should be used for initial cloning with all Ek/LIC Vectors. NovaBlue is a convenient cloning host because the *recA endA* mutations facilitate high transformation efficiency and high yields of excellent plasmid DNA. NovaBlue GigaSingles™ Competent Cells are provided in 50-µl single-use aliquots. The pET, pCDF-2, pRSF-2, and pTriEx™ Ek/LIC Vector Kits also contain competent cells of expression host strains in 0.2 ml aliquots (10 transformations). The following protocol is for transformations using either GigaSingles™ or Standard (0.2 ml) Competent Cells.

Note: Upon receipt of competent cells, verify that cells are frozen and dry ice is present in the shipping container. Immediately store competent cells at –70°C or below. Do not allow cells to thaw prior to use. To prevent cells from warming, handle only the very top of the tube and the tube cap. Keep cells on ice while in use unless otherwise noted.

1. Remove appropriate number of competent cell tubes from freezer. (Include an extra sample for Test Plasmid positive control.) Immediately place tubes on ice, immersing all but the cap. Allow cells to thaw on ice for 2–5 min.
2. Visually examine cells for thawing and gently flick tube 1–2 times to resuspend cells evenly. Never vortex competent cells.
3. *GigaSingles™ Kits:*
   If a Test Plasmid sample is included, proceed to Step 4. If not, go directly to Step 5.

   Standard Kits:
   Pre-chill required number of 1.5-ml snap-cap polypropylene tubes on ice. Pipet 20 µl cell aliquots into each pre-chilled tube.
4. **(Optional)** To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to a tube containing cells. Gently flick tube to mix. Return to ice.
5. Add 1 µl annealing reaction directly to cells. Stir gently to mix. Return to ice.
6. Incubate on ice for 5 min.
7. Heat tubes for exactly 30 s in a 42°C water bath. Do not shake.
8. Place tubes on ice for 2 min.
9. *GigaSingles™ Kits:*
   Add **250 µl** room temperature SOC medium to each tube. Keep tubes on ice while handling.

   Standard Kits:
   Add **80 µl** room temperature SOC medium to each tube. Keep tubes on ice while handling.
10. Incubate at 37°C with shaking (250 rpm) for 60 min prior to plating on selective medium.
11. To select transformants, plate a portion of the transformation on medium containing antibiotic for the plasmid-encoded drug resistance (50 µg/ml carbenicillin or ampicillin for Amp resistance marker, 30 µg/ml kanamycin for Kan resistance marker, or 50 µg/ml of streptomycin or spectinomycin for Strep resistance marker). Additional antibiotics may be necessary to ensure maintenance of host-specific feature(s). Appropriate plating volume depends on annealing and transformation efficiencies (see Certificate of Analysis for competent cell transformation efficiency). For NovaBlue recombinants, expect 10^5–10^7 transformants per µg plasmid. When plating less than 25 µl, first pipet a pool of SOC onto the plate, and then pipet the transformation into the SOC. For Test Plasmid, plate 5 µl NovaBlue transformation mix in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin.
12. Set plates on the bench for several minutes to allow excess liquid to be absorbed. Invert plates and incubate overnight at 37°C.

Colony Screening

If cloning is successful, there are usually many more colonies resulting from annealing reactions including insert than in the negative control. Colonies can be screened for inserts by colony PCR using Novagen vector-specific primers, followed by agarose gel electrophoresis. Because Ek/LIC is directional, appropriate vector-specific primers can be used at both ends. A vector-specific primer can also be used in combination with an appropriate insert-specific primer.

Alternatively, *in vitro* transcription/translation can be used to assess clones quickly. PCR-amplify insert DNA from a colony using appropriate primers, and use this product as template for *in vitro* transcription and translation. Novagen Single Tube Protein® System 3 (STP3®) T7 (Cat. No. 70192) or EcoPro™ T7 System (Cat. No. 71231) are recommended. Refer to User Protocols TB206 and TB278, respectively. *In vitro* transcription/translation analysis allows rapid testing of clones for potential mutations, such as those inserting a stop codon (nonsense mutation).

Appropriate primers for *in vitro* transcription and translation analysis or for colony PCR are listed in the table below.

Note: The pIEx™, pIEx/Bac™, and pBAC™ Vectors do not contain a T7 promoter and are not appropriate templates for in vitro *transcription/translation analysis.*

Product	Applicable Vector(s)	Cat. No.
Upstream primers for colony screening plus *in vitro* transcription and translation		
pET Upstream Primer	pET-32, pET-30, pET-41, pET-43.1, pET-44, pET-46, pET-51	69214-3
ACYCDuetUP1 Primer	pCDF-2, pRSF-2	71178-3
T7/polh Primer	pBAC™-2cp, pBACgus™-2cp	See note
TriEx™UP Primer	pTriEx™-4, pTriEx™-5, pTriEx™-7	70846-3
Upstream primers for colony screening only		
T7 Promoter Primer	pET-30, pET-32, pET-46, pET-51, pTriEx™-4, pTriEx™-5, pTriEx™-7	69348-3
IE1 Promoter Primer	pIEx™-1, pIEx™-7, pIEx™-8, pIEx™-10, pIEx/Bac™-1, pIEx/Bac™-4	69103-3
S•Tag™ 18mer Primer	pET-30, pET-32, pET-43.1, pET-44, pIEx™-1, pIEx™-2, pIEx™-3, pTriEx™-4	See note
Nus•Tag™ Primer	pET-43.1, pET-44	See note
S•Tag BAC Primer	pBAC™-2cp, pBACgus™-2cp,	See note
Blue-2S•Tag Primer	pET-30, pET-32,	See note
Downstream primers		
T7 terminator Primer	pET-30, pET-32, pET-34, pET-41, pET-46, pET-51, pCDF-2, pRSF-2	69337-3
ColiDOWN Primer	pET-43.1, pET-44	See note
1629DWN Primer	pBAC™-2cp, pBACgus™-2cp,	See note
TriExDOWN	pTriEx™-4, pTriEx™-5, pTriEx™-7	70847-3
IE1 terminator Primer	pIEx™-1, pIEx™-2, pIEx™-3, pIEx™-7, pIEx™-8, pIEx™-10, pIEx/Bac™-1, pIEx/Bac™-4	71247-3

Note: Novagen does not currently offer these primers for sale; however each primer has been tested for PCR and sequencing applications. The sequence and binding location for these primers is indicated on the respective vector map.

Colony PCR for recombinant verification and transcription/translation analysis

1. Pick a colony from an agar plate using a sterile pipet tip or toothpick. Choose colonies that are at least 1 mm in diameter. Try to collect as many cells as possible. Touch the pipet tip to a plate (to cultivate a reference colony) before transferring the bulk of the cells to a tube, as described in the next step.
2. Transfer bacteria from the pipet tip or toothpick to a 0.5 ml tube containing 50 µl sterile water. Vortex to disperse cells.
3. Place tube in boiling water or a heat block at 99°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 PCR tube. Place on ice until use.
6. Make a master reaction mix on ice using the following volumes per reaction. To account for pipeting loss, multiply the amounts by X.5, where X is the number of reactions.

   Per reaction:

31.5 µl	PCR-grade Water
1 µl	dNTPs (10 mM each dATP, dCTP, dGTP, and 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	Novataq™ DNA Polymerase(1.25 U)
40 µl	total volume

   Note: If using the Novataq™ Buffer without $MgCl_2$, add $MgCl_2$ to a final concentration of 1.5–2.5 mM and decrease the volume of water added to compensate.

7. Mix gently and centrifuge briefly. Add 40 µl master mix to each sample and mix gently. Cap tubes and place samples in a thermal cycler.

   Optional: Use a hot start procedure (warm cell lysate samples to 80°C before adding master mix). Alternatively, use Novataq™ Hot Start DNA Polymerase. For greatest accuracy, specificity, or yield of long, complex targets use KOD HiFi, KOD Hot Start, or KOD XL DNA Polymerases, respectively.

8. Process in thermal cycler for 35 cycles, as follows:

Denature	1 min at 94°C
Anneal	1 min at the proper annealing temperature (usually 55°C for vector primers)
Extend	2 min at 72°C

   Repeat for 35 cycles, and conduct final extension for 5 min at 72°C.

9. Remove 10–25 µl, add 1/10 volume 10X loading dye, and load on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. Include at least one lane of Perfect DNA™ Markers as a size standard. A strong band should be present, with size corresponding to the distance between (and including) the primers.

   Note: The expected product size of the Ek/LIC β-gal Control Insert is ~3 kb (~2 kb for the Ek/LIC GUS Control Insert). Specific size depends upon vector and primer combination.

   Recombinants identified as having appropriately-sized insert can be assessed for ability to express target protein using either the STP3® T7 or the EcoPro™ T7 *in vitro* transcription/translation systems (see page 13):

   - Add 2 µl PCR product directly to a STP3® T7 reaction.
   - Prior to use in EcoPro™ T7 reactions, precipitate PCR products to remove salts. To precipitate a 50 µl PCR reaction, add 5 µl 3 M sodium acetate pH 5.2 and 115 µl 95% ethanol. Vortex briefly and spin at 14,000 × *g* for 5 min. Wash the pellet briefly with 70% ethanol, followed by 100% ethanol. Dry pellet to remove residual ethanol. Resuspend in 50 µl deionized water. Pellet Paint® Co-Precipitant (Cat. No. 69049, see User Protocol TB146) facilitates recovery during precipitation, without affecting performance in the EcoPro™ T7 reaction. Use 2–4 µl per EcoPro™ T7 reaction.

Plasmid purification

After identifying positive clones, isolate plasmid DNA for restriction mapping, sequence analysis, and transformation into expression hosts. Plasmid DNA from candidate recombinants may also be evaluated using *in vitro* transcription/translation analysis. It is critical that template for *in vitro* transcription and translation be RNase-free.

When isolating pET and pCDF plasmids, use the low-copy number method described in the User Protocol for each kit. For pTriEx™, pIEx™, pBAC™, pIEx/Bac™ and pRSF plasmids, use the high-copy number protocol method. Isolated plasmid DNA may require an additional phenol:CIAA extraction to eliminate RNases. To do this, add TE to a final volume of 100 µl and then extract successively with 1 vol TE-buffered phenol, 1 vol phenol:CIAA (1:1; CIAA is chloroform:isoamyl alcohol, 24:1), and 1 vol CIAA. Transfer final aqueous phase to a fresh tube and add 0.1 vol 3 M sodium acetate pH 5.2 and 2 vol 100% ethanol. Mix and place at –20°C for 30 min. Centrifuge for 5 min at 12,000 × *g*, remove supernatant, and rinse pellet with 70% ethanol. Dry and resuspend DNA in 30 µl TE. If desired, 2 µl Pellet Paint® or Pellet Paint® NF Co-precipitant can be added with the TE buffer before extraction to facilitate DNA recovery. (The –20°C incubation can be eliminated if using Pellet Paint® Co-Precipitant).

Protein Expression, Detection, Purification, and Quantification

After a recombinant construct has been established in NovaBlue cells and its sequence confirmed, the construct can be used for protein expression. Detailed protocols for protein expression, purification, and quantification are found in other Novagen User Protocols. All User Protocols are available at www.merck4biosciences.com.

E. Coli Expression

T7 promoter based-plasmids may be induced for protein expression with T7 polymerase using either of two methods:

1. Recombinant plasmid is isolated from NovaBlue cells and transformed into Novagen *E. coli* DE3 expression host strains which are lysogenic for bacteriophage λDE3. BL21(DE3) and BL21(DE3)pLysS competent cells are provided in the pET, pCDF, and pRSF Ek/LIC Vector Kits. Origami™ B(DE3)pLacI competent cells are provided in the pTriEx™ Ek/LIC Vector Kit. In addition to the expression strains provided in the vector kits, Novagen offers an extensive selection of other λDE3 lysogenic expression hosts. See User Protocol TB009 for more information on available strains.
   Important: *pTriEx™ recombinants must be transformed into (DE3)pLacI hosts (see User Protocol TB250 for more information).*
2. T7 RNA polymerase is delivered to the NovaBlue cells harboring pET or pTriEx™ recombinants by infecting the cultures with bacteriophage CE6 (see User Protocol TB007).

Insect Cell Expression

pBAC™, pIEx™, pIEx/Bac™ and pTriEx™ vectors may be introduced into insect cells for transient expression and/or recombinant baculovirus production using Insect GeneJuice® Tranfection Reagent (User Protocol TB359). See respective User Protocols for information regarding transient protein expression in insect cells using pIEx™ vectors (TB356) or pIEx/Bac™ vectors (TB474). For information regarding recombinant baculovirus production with pBAC™, pIEx/Bac™, or pTriEx™ vectors, see User Protocols for the BacMagic™ DNA Kit (TB459) or BacVector® Transfection Kits (TB216).

Mammalian Cell Expression

pTriEx™ constructs may be introduced into mammalian cells using GeneJuice® Transfection Reagent (User Protocol TB289) or by classical transfection methods. For protein expression protocols using pTriEx™ in mammalian cells, see User Protocol TB250

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The initial purchaser may refuse to accept the above conditions by returning the kit unopened and the enclosed materials unused. By accepting or using the kit or the enclosed materials, you agree to be bound by the foregoing conditions.