Ek/LIC Cloning Kits

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About the Kits

Kit	Cat. No.
pET-30 Ek/LIC Vector Kit	69077-3
pET-32 Ek/LIC Vector Kit	69076-3
pET-41 Ek/LIC Vector Kit	71071-3
pET-43.1 Ek/LIC Vector Kit	71072-3
pET-44 Ek/LIC Vector Kit	71144-3
pET-46 Ek/LIC Vector Kit	71335-3
pCDF-2 Ek/LIC Vector Kit	71337-3
pRSF-2 Ek/LIC Vector Kit	71364-3
pBAC™-2cp *E. coli* Ek/LIC Vector Kit	70021-3
pBACgus-2cp *E. coli* Ek/LIC Vector Kit	70051-3
pIEx™-1 Ek/LIC Vector Kit	71237-3
pIEx-2 Ek/LIC Vector Kit	71240-3
pIEx-3 Ek/LIC Vector Kit	71245-3
pIEx-7 Ek/LIC Vector Kit	71339-3
pTriEx™-4 Ek/LIC Vector Kit	70905-3

Description

Novagen ligation-independent cloning (LIC) vectors are designed for rapid cloning and expression of genes in multiple expression systems (*in vitro*, *E. coli*, insect cell, and mammalian). Ek/LIC vectors designed for expression in *E. coli* can also be used for the coexpression of up to six target proteins. Ligation-independent cloning was developed for the directional cloning of PCR products without the need for restriction enzyme digestion or ligation reactions (1, 2). The Ek/LIC vectors are engineered to express the target protein immediately downstream of an enterokinase cleavage site so that all vector-encoded fusion sequences can be removed from the purified protein. All Ek/LIC vectors possess the same Ek/LIC cloning site so that an Ek/LIC-prepared target insert can be annealed into any or all of the Ek/LIC vectors in a 5-minute reaction.

Ek/LIC vectors are constructed by treating a linearized backbone with T4 DNA Polymerase in the presence of only one dNTP. The 3'→5' exonuclease activity of T4 DNA Polymerase removes nucleotides from one strand of each specially designed end until it encounters a residue corresponding to the dTTP present in the reaction mix. At this point the polymerase activity of the enzyme counteracts the exonuclease activity to effectively prevent further excision. This treatment produces specific non-complementary 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 page 4). The PCR product is purified to remove dNTPs (and original plasmid if it was used as template) and then treated with T4 DNA Polymerase in the presence of dATP to generate the specific vector-compatible overhangs. Cloning is very efficient because only the desired product forms during the annealing process. 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 is ready for expression in bacteria or transfection into insect or mammalian cells depending on the characteristics of the chosen vector. Coexpression of multiple target proteins 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).

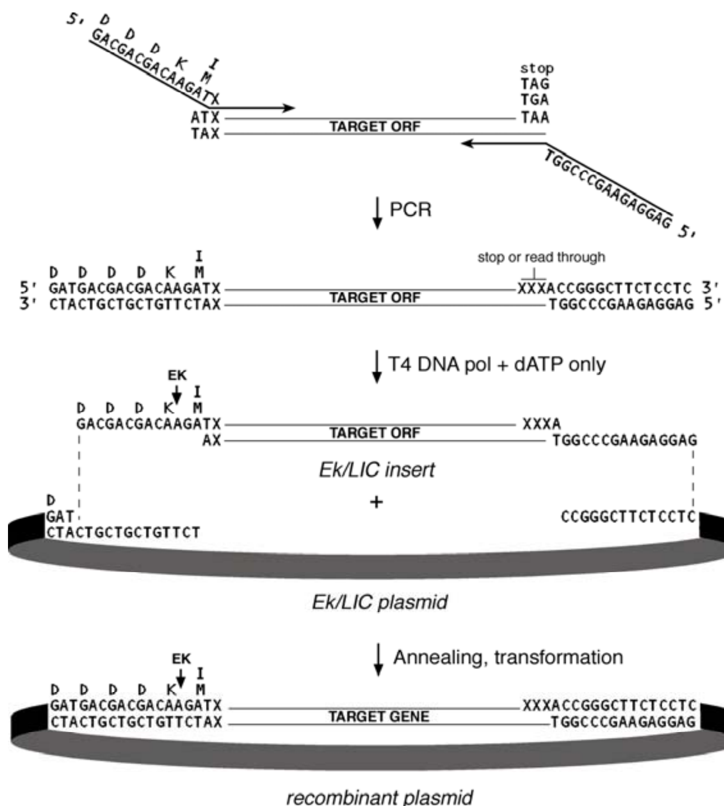


Diagram of the Ek/LIC strategy. After amplification with primers that include the indicated 5' LIC extensions, the PCR insert is treated with LIC-qualified T4 DNA Polymerase (+dATP), annealed to the Ek/LIC vector, and transformed into competent *E. coli*.

Note: *For simultaneous cloning of two inserts into the Ek/LIC vectors, 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 (ampicillin resistant)

pBAC™ *E. coli* Ek/LIC Vector Kits

1 µg	pBAC Ek/LIC Vector
8 µl	Ek/LIC GUS Control Insert <u>or</u> 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 (ampicillin resistant)

Note: *Reagents for transfection and expression in insect cells must be purchased 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 (ampicillin resistant)

Note: *Reagents for transfection and expression in insect cells must be purchased separately.*

pTriEx™-4 Ek/LIC Vector Kit

1 µg	pTriEx-4 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
2 × 0.2 ml	Origami™ B(DE3)pLacI Competent Cells
5 × 2 ml	SOC Medium
10 µl	Test Plasmid (ampicillin resistant)

Note: Reagents for transfection and expression in insect cells and mammalian cells must be purchased separately.

Storage

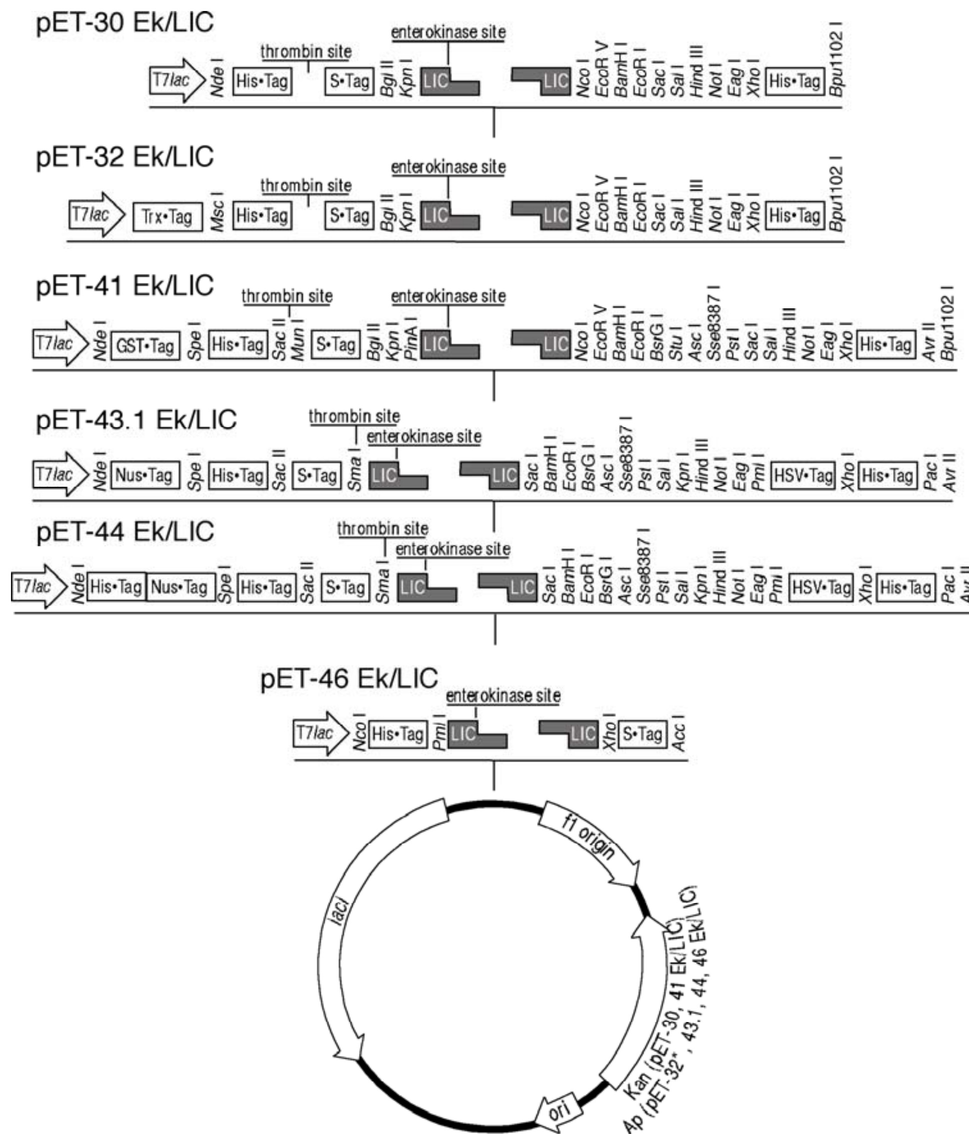
Store Competent Cells, SOC Medium, and Test Plasmid at –70°C. Store all other components at –20°C.

Ek/LIC Vectors

The full range of Ek/LIC Vectors available for cloning and expression in *E. coli*, insect, and mammalian cells is described in this section. Note that after the PCR Ek/LIC insert is prepared, it can be annealed to any of the Ek/LIC Vectors.

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

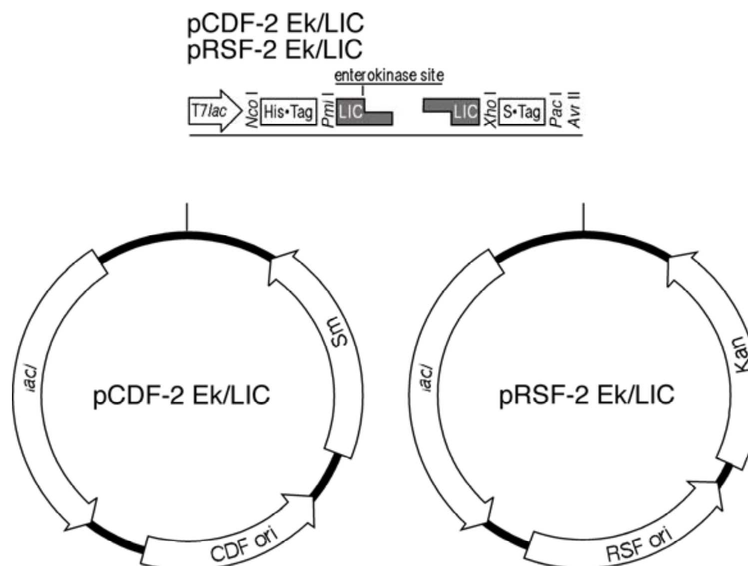
The pET Ek/LIC Vectors include an extensive range of fusion tag options for expression in *E. coli*.



* Ap gene is in opposite orientation in pET-32 Ek/LIC

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

The pRSF-2 and pCDF-2 Ek/LIC 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.



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

The pET, pRSF-2, and pCDF-2 Ek/LIC vectors are compatible with each other in the same cell and can be used for coexpression (6, 7). Single inserts 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 (See User Protocol TB384). Creating recombinants with two inserts per Ek/LIC vector allows the coexpression of up to six target proteins. Use the following tables to determine the appropriate combinations of vectors and host strain for coexpression.

Note: *The difference in target gene dosage attributed to plasmid copy number between any of the plasmids could be used to influence relative target protein expression levels according to the relative plasmid copy number where pRSF > pET> pCDF > pACYC.*

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 (Ap^R)	pRSF-2 Ek/LIC (Kn^R)	pCDF-2 Ek/LIC (Sm^RSp^R)	3–6	Group A
pET (Ap^R)	pRSF-2 Ek/LIC (Kn^R)	pCDF-2 Ek/LIC (Sm^RSp^R)	3–5	Group A
pET Ek/LIC (Ap^R)	pRSF-2 Ek/LIC (Kn^R)		2–4	Group A
pET Ek/LIC (Ap^R)	pCDF-2 Ek/LIC (Sm^RSp^R)		2–4	Group B
pRSF-2 Ek/LIC (Kn^R)	pCDF-2 Ek/LIC (Sm^RSp^R)		2–4	Group A

Ap: ampicillin/carbenicillin; Kn: kanamycin; Cm: chloramphenicol

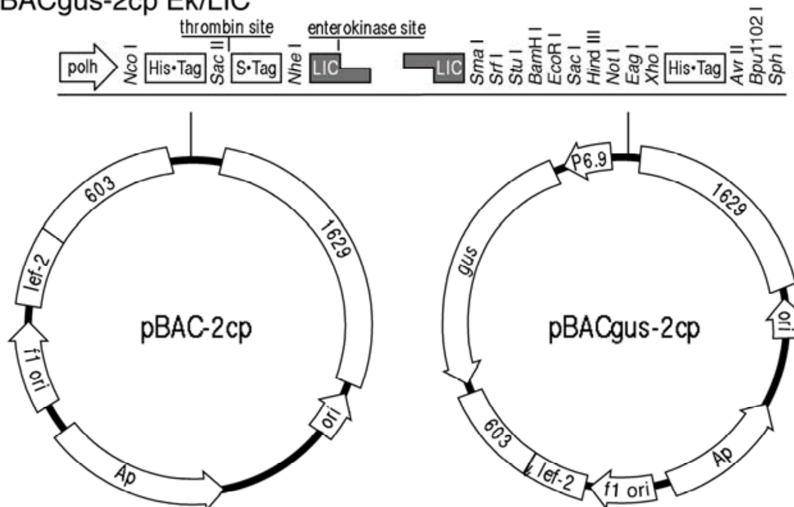
Strain Groups

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

*Selection for pCDF vectors requires the use of spectinomycin because these strains carry the *rpsL* mutation that confers streptomycin resistance.

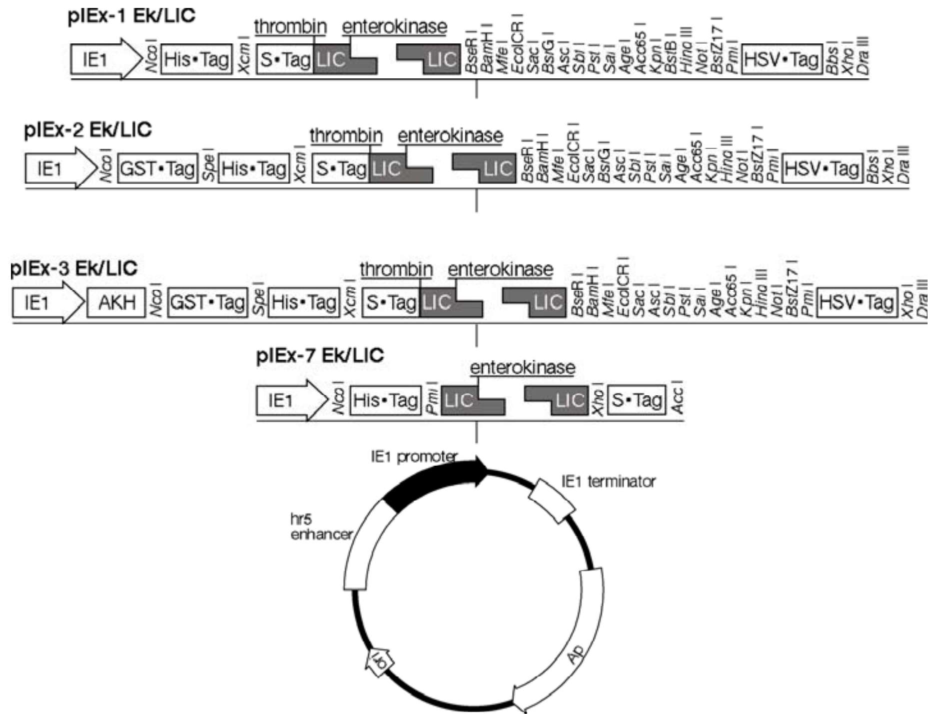
pBAC™ Ek/LIC Vectors for baculovirus-mediated expression in insect cells

The pBAC Ek/LIC Vectors are baculovirus transfer plasmids. After plasmid recombinants are established in *E. coli*, these vectors are isolated and cotransfected into insect cells with baculovirus DNA (BacVector™-1000, -2000, or -3000 Triple Cut Virus DNA) to create baculovirus recombinants.



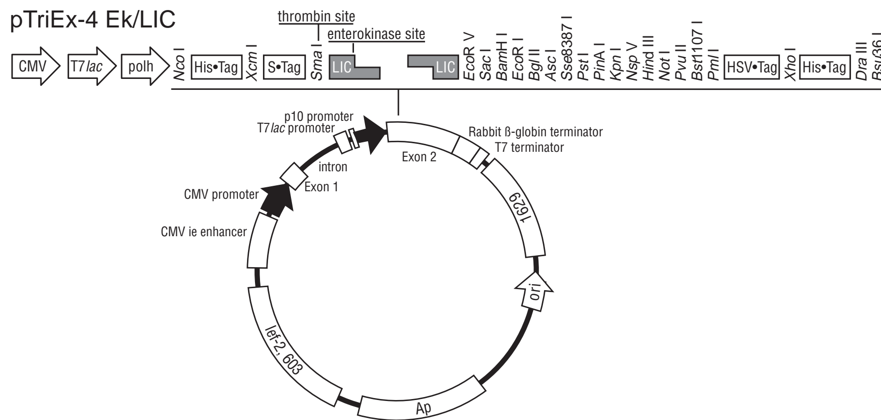
pIEx™ Ek/LIC Vectors for expression in Sf9 Insect Cells

The pIEx Ek/LIC Vectors are expression vectors designed for transfection into *Spodoptera*-derived insect cells. After recombinants are established in *E. coli*, plasmids are isolated and transfected into Sf9 or Sf21 insect cells for target protein expression. The 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, thus eliminating the need to utilize baculovirus and avoiding the cytopathic effects associated with infection.



pTriEx™-4 Ek/LIC Multisystem Vector for expression in *E.coli*, insect, and mammalian cells

The pTriEx-4 Ek/LIC Vector incorporates the CMV ie enhancer-promoter combination followed by T7*lac* and p10 promoters to direct the high-level expression of target genes in mammalian cells, *E.coli*, and baculovirus-infected insect cells.



Insert Preparation

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

Production and purification of PCR product

When amplifying the target ORF by PCR to create an Ek/LIC insert, Novagen highly recommends the use of thermostable KOD DNA polymerases (KOD HiFi DNA Polymerase, see User Protocol TB320; KOD Hot Start DNA Polymerase, see User Protocol TB341). KOD HiFi and KOD Hot Start DNA Polymerases have robust elongation rates and very low mutation frequencies (12), resulting in high yields and low error rates.

When there is a low amount of starting template, such as in reverse transcription reactions from total RNA or mRNA, or from a cDNA library, numerous template doublings are required to generate sufficient target. In these cases a low mutation frequency is especially important. In addition to using a high-fidelity DNA polymerase, such as KOD HiFi or Hot Start, the likelihood of PCR-generated mutations can also be minimized by creating a sequence-verified plasmid clone to serve as a template in subsequent amplifications. Fewer cycles are needed to generate ample material for the LIC when a high amount of this 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 represents sufficient material to perform > 75 LIC reactions.

Note: *For optimal PCR results that significantly reduce the likelihood of primer-derived mutation, we strongly recommend the use of purified primers.*

1. Amplify the desired insert sequence using appropriately designed PCR primers. The 5'-end of the primers must incorporate the following sequences (see page 3):

   sense primer: 5' GAC GAC GAC AAG AT<u>X</u>*–insert specific sequence 3'

   antisense primer: 5' GAG GAG AAG CCC GGT–insert specific sequence 3'

   *The first nucleotide of the insert-specific sequence must complete the codon AT<u>X</u>.

   Optimal PCR conditions should produce a strong DNA product of the appropriate size with minimal extraneous products when analyzed by agarose gel electrophoresis.
2. The dNTPs from the PCR product must be completely removed prior to T4 DNA Polymerase treatment. If the PCR template and the Ek/LIC vector have the same antibiotic resistance marker, the PCR product must be separated from the PCR template.
3. To avoid the generation of false positives by residual polymerase activity, inactivate the enzyme used for PCR if the insert is not gel purified. This can be accomplished by extracting the reaction with 1 volume CIAA [chloroform:isoamyl alcohol (24:1)]. Add the CIAA, vortex for 1 min and spin at 12,000 × g for 1 min. Remove and save the aqueous phase.
4. The choice of purification method will be dictated by: 1) antibiotic resistances of the template and Ek/LIC vector (same or different), and 2) the quality of the PCR product (e.g., a single amplification product versus multiple extraneous bands or primer dimmers). If extraneous bands or interfering template plasmids are present, the PCR product should be run on an agarose gel and the target band extracted using SpinPrep™ Gel Kit or a similar method. If these contaminants are not present, the agarose gel step is unnecessary and the PCR product can be purified with SpinPrep PCR Clean Up Kit, on a spin column, or by binding to a membrane or solid matrix. <u>The purified PCR product should be suspended in TlowE buffer</u> (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) with T4 DNA Polymerase treatment. Include a positive control to verify the system performance. It is recommended to include a negative control, lacking insert.

The Ek/LIC β-gal Control Insert is included with pET, pRSF, pCDF, pBAC™gus-2cp, pIEx™, and pTriEx™-4 Ek/LIC Vector Kits and requires treatment with T4 DNA Polymerase to verify system performance. It is 3085 bp long with a picomolar mass corresponding to 2 µg/pmol. Use 4 µl of the 100 ng/µl solution provided for each treatment. The pBAC-2cp Ek/LIC Vector Kit contains the Ek/LIC GUS Control Insert, which requires treatment with T4 DNA Polymerase to verify system performance. It is 1815 bp long with a picomolar mass corresponding to 1.18 µg/pmol. Use 2.4 µl of the 100 ng/µl solution provided for each treatment.

1. Assemble the following components in a sterile 1.5-ml microcentrifuge tube kept 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). (number bp in insert × 650 = pg/pmol)
2 µl	10X T4 DNA Polymerase Buffer
2 µl	25 mM dATP
1 µl	100 mM DTT
0.4 µl	2.5 U/µl T4 DNA Polymerase (LIC-qualified; 0.5 unit per 0.1 pmol PCR product)
y µl	Nuclease-free Water
20 µl	Total volume (Final concentration of insert is 0.01 pmol/µl)

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

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

Annealing the Vector and Ek/LIC Insert

The following describes the protocol for annealing of a single insert into the Ek/LIC Vectors. For annealing of two inserts using the LIC Duet™ Adaptor Kits see User Protocol TB384.

1. 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

2. Mix by stirring with the pipet tip and incubate at 22°C for 5 min.

Notes:

a) Greater volumes of the treated insert may be used; however, the concentration of vector in the reaction will decrease and subsequently fewer nanograms will be plated if a constant amount of annealing reaction is used for transformation.

b) Annealing is complete within 5 min of incubation; reactions can be incubated up to 1 h with equivalent results.

Transformation

NovaBlue GigaSingles™ Competent Cells (Cat. No. 71127) are provided in the Ek/LIC Vector Kits and should be used for initial cloning with all Ek/LIC Vectors. NovaBlue is a convenient host for initial cloning of target DNA into the Ek/LIC Vector due to its high transformation efficiency and the high yields and excellent plasmid DNA that results from *rec*A *endA* mutations. Single-use NovaBlue GigaSingles Competent Cells are provided in 50-µl aliquots. The pET, pCDF-2, pRSF-2, and pTriEx™-4 Ek/LIC Vector Kits also receive expression host strains in standard, 0.2 ml aliquots. The standard transformation reaction calls for 20 µl of cells, so each tube contains enough cells for 10 transformations. The following protocol is appropriate for transformations using either GigaSingles or Standard Competent Cells with noted difference for the steps effected.

Note: *Upon receipt of competent cells from Novagen, 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 cells, flick the tube 1–3 times. NEVER vortex competent cells.*

1. Remove the appropriate number of competent cell tubes from the freezer (include on extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is immersed in ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells and gently flick the cells 1–2 times to evenly resuspend the cells.
3. *GigaSingles Kits:* If a Test Plasmid sample is included , proceed to Step 4, if not go directly to Step 5.

   Standard Kits: 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.
4. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Gently flick the tube to mix and return to the ice.
5. Add 1 µl of the annealing reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return to the ice. Repeat for additional samples.
6. Incubate on ice for 5 min.
7. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.
8. Place the tubes on ice for 2 min.
9. *GigaSingles Kits:* Add **250 µl** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.

   Standard Kits: Add **80 µl** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.
10. Incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective medium.
11. 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, 30 µg/ml kanamycin for the kan resistance marker, 50 µg/ml of streptomycin and/or spectinomycin for the *aadA* gene, and 34 µg/ml chloramphenicol for the *cat* gene). 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 of the NovaBlue transformation mix or 10 µl of any strain with a 2×10^6 efficiency in a

"pool" of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampicillin resistance gene, *bla*).

12. Set plates on the bench for several minutes to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

Colony Screening

If the cloning was successful, there are usually many more colonies produced from annealing in the presence of the insert than with the negative control. Colonies can be screened for inserts without the need for plasmid minipreps by colony PCR using Novagen vector-specific primers followed by agarose gel analysis of the DNA products. Because Ek/LIC is directional, vector-specific primers can be used at both ends. However, one vector-specific primer also can be used in combination with an appropriate insert-specific primer (assuming compatible annealing conditions).

In addition, it is possible to quickly assess the ability of an individual clone to express a target protein *in vitro* by amplifying a colony with appropriate primers and using the DNA product as template for *in vitro* transcription and translation using Novagen Single Tube Protein® System 3 (STP3®) T7, or EcoPro™ T7 System. Note that the EcoPro T7 System is compatible only with vectors encoding an *E. coli* ribosome binding site (e.g., pET, pCDF, pRSF, or pTriEx™ Vectors). Upstream primers that provide a "spacer" region before the T7 promoter allow efficient transcription of the PCR product with T7 RNA Polymerase. This method allows rapid testing of clones for potential mutations, such as those that insert a stop codon (nonsense mutations).

Appropriate 5' primers for *in vitro* transcription and translation analysis and other primers useful for PCR colony screening are listed in the table below.

Note: *The pIEx™ Vectors do not contain a T7 promoter and are not appropriate templates for* in vitro *transcription/translation analysis without the addition of the necessary promoter and spacer regions in the 5' primer sequence (See Technical Bulletin 206).*

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	69214-3
ACYCDuetUP1 Primer	pCDF-2, pRSF-2	71178-3
T7/polh Primer	pBAC™-2cp, pBACgus-2cp	See note
TriEx™UP Primer	pTriEx-4	70846-3
Upstream primers for colony screening only		
T7 Promoter Primer	pET-30, pET-32, pET-46, pTriEx-4	69348-3
IE1 Promoter Primer	pIEx-1, pIEx-7	69103-3
S•Tag™ 18mer Primer	pET-30, pET-32, pET-43.1, pET-44, pIEx-1, pIEx-2, pIEx-3, pTriEx-4	70828-3
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, 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	70847-3
IE1 terminator Primer	pIEx-1, pIEx-2, pIEx-3, pIEx-7	71247-3

Note: Novagen does not currently offer these specified 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. Sequence details also can be found at www.novagen.com.

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Colony PCR for transcription/translation analysis

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 collect as many cells as possible. If a copy of the colony is desired, touch the pipet tip to a plate before transferring the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a 0.5-ml tube containing 50 µl sterile water. Vortex to disperse the cells.
3. Place the tube in boiling water or a heat block at 99°C for 5 min to lyse the cells and denature DNases.
4. Centrifuge at 12,000 × g for 1 min to remove cell debris.
5. Transfer 10 µl of the supernatant to a fresh 0.5-ml tube for PCR. Place on ice until use.
6. Make a master reaction mix on ice using the following amounts per reaction. To account for pipetting losses, it is convenient to 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	Nova*Taq*™ DNA Polymerase(1.25 U)
40 µl	total volume

Note: *If using the Nova*Taq *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, if necessary centrifuge briefly . Add 40 µl of the master mix to each sample, mix gently, overlay 2 drops mineral oil, cap the tubes and place 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 warmed to 80°C before the addition of the master mix. Alternatively, use Nova*Taq *Hot Start DNA Polymerase. For greatest accuracy, specificity, and yield of long complex targets use KOD HiFi, KOD Hot Start, and KOD XL DNA Polymerases, respectively.*

8. Process in the 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
Final extension 5 min at 72°C

9. To remove the oil overlay and inactivate the polymerase, add 100 µl chloroform, mix 30 sec, and centrifuge for 1 min. The top aqueous phase (which may appear cloudy) contains the DNA products. Transfer the aqueous phase to a fresh tube. If desired, remove a 5–10 µl sample for gel analysis. Store the remainder at –20°C.
10. If an appropriate PCR primer combination was used, 2 µl of the PCR product can be added directly to a Single Tube Protein® System 3, T7 reaction for protein synthesis.
11. Prior to use in EcoPro™ T7 reactions, PCR products should be precipitated to remove salts. To precipitate a 50-µl PCR reaction, add 5.2 µl 3 M sodium acetate 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 the pellet to remove residual ethanol and resuspend in 50 µl deionized water. The addition of Pellet Paint® Co-Precipitant to the DNA facilitates recovery in

the precipitation step without affecting performance in the EcoPro reaction. Use 2–4 µl in the EcoPro T7 reaction.

Note: The expected size of the PCR product produced from the Ek/LIC β-gal Control Insert is approximately 3 kbp, and approximately 2 kbp for the Ek/LIC gus Control Insert (the specific size will vary with the vector and primer combination).

Plasmid purification

After positive clones are identified, plasmid DNA can be isolated for transformation into expression hosts, restriction mapping, and sequence analysis. Plasmid DNA from candidate recombinants may also be evaluated using *in vitro* transcription/translation analysis. It is important that the template be RNase-free for *in vitro* transcription and translation. When isolating pET, and pCDF plasmids with Mobius™ or UltraMobius™ Kits use the low-copy number protocol provided. For pTriEx™, pIEx™, pBAC™ and pRSF plasmids, use the high-copy number provided. Plasmid DNA isolated with Mobius or UltraMobius Kits is essentially RNase-free. However, plasmids DNA isolated with SpinPrep™ Plasmid Kits or kits from other manufacturers may require an additional phenol:CIAA extraction to eliminate RNases. A satisfactory procedure is to add TE to 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 the final aqueous phase to a fresh tube and add 0.1 vol 3 M Na acetate and 2 vol 100% ethanol. Mix and place at –20°C for 30 min, spin 5 min at 12,000 × g, remove the supernatant, and rinse the pellet with 70% ethanol. Dry and resuspend the 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 recovery of the DNA (the –20°C incubation can be eliminated if using Pellet Paint Co-Precipitant).

Plasmid Preparation Kit	Scale	DNA Yield	Cat. No.	Size
Mobius™ 1000 Plasmid Kit	100-ml (high-copy) 250-ml to 1.5 -L (low-copy)	> 1 mg (high-copy) 200 µg–1 mg (low- copy)	70854-3 70853-3 70853-4	2 rxn* 10 rxn* 25 rxn*
UltraMobius™ 1000 Plasmid Kit	100-ml (high-copy) 250-ml to 1.5 -L (low-copy)	> 1 mg (high-copy) 200 µg–1 mg (low-copy)	70907-3 70906-3 70906-4	2 rxn* 10 rxn* 25 rxn*
Mobius 500 pET Plasmid Kit	500-ml culture	500 µg (low-copy)	70969-3	10 rxn
Mobius 200 Plasmid Kit	35-ml culture (high-copy or low-copy)	> 200 µg (high-copy) > 30 µg (low-copy)	70970-3	25 rxn
UltraMobius 200 Plasmid Kit	35-ml culture (high-copy or low-copy)	> 200 µg (high-copy) > 30 µg (low-copy)	71090-3	25 rxn
SpinPrep™ Plasmid Kit	1- to 3-ml culture	5–10 µg (high-copy) 0.25–1 µg (low-copy)	70957-3 70851-3	20 rxn 100 rxn

*The kit sizes described are for the 100 ml (high-copy) or 250 ml (low-copy) preparations. Additional buffers are required for > 250 ml (low-copy) scale (User Protocol TB279).

Protein Expression, Detection, Purification, and Quantification

Detailed protocols for expression, purification, and quantification are found in other Novagen technical bulletins (see the table on page 14). All the technical bulletins are available on the Novagen website at www.novagen.com.

After a recombinant has been established in NovaBlue cells, T7 promoter-based plasmids may be induced for protein expression using either of the following methods. T7 RNA polymerase may be delivered to the NovaBlue cells harboring recombinants derived form pET, pCDF, pRSF, or pTriEx vectors by infecting the cultures with bacteriophage CE6. Recombinant plasmids may also be isolated from the NovaBlue cells and transformed into BL21(DE3) or BL21(DE3)pLysS competent cells (provided in the pET, pCDF, and pRSF Ek/LIC Vector Kits) or Origami™ B(DE3)pLacI competent cells (provided in the pTriEx Ek/LIC Vector Kit) which are all lysogenic for bacteriophage λDE3. The DE3 strains possess a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter.

Important: pTriEx™-4 recombinants must be transformed into (DE3)pLacI hosts. pTriEx-4 is a high copy, T7*lac* promoter-based vector 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, transform pTriEx-4 recombinants into DE3 expression strain containing pLacI.

In addition to the expression strains provided in the vector kits, Novagen offers an extensive selection of other λDE3 lysogenic hosts for expression. Expression strains with thioredoxin reductase (*trxB)* and glutathione reductase *(gor*) mutations, including Origami™, Origami B, and Rosetta-gami™, Rosetta-gami B strains (13–15), are available separately. The *trxB/gor* hosts allow the formation of disulfide bonds in the cytoplasm; soluble proteins containing disulfide bonds may therefore fold properly in these strains. When using these strains for production of disulfide-bond containing proteins, folding may be enhanced by shaking the induced culture at 4°C for several hours prior to harvest (J. Beckwith, personal communication). The *trxB/gor* hosts are compatible with ampicillin-resistant plasmids pET-32, pET-43.1, pET-44 and pET-46 Ek/LIC Vectors and with the streptomycin/spectinomycin-resistant plasmid pCDF-2. The Rosetta™, RosettaBlue™, and Rosetta-gami strains are designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* (15–18). Expression of these proteins can be dramatically increased when the level of rare tRNAs is increased within the host (18). These strains supply tRNAs under the control of their native promoters for the codons AUA, AGG, AGA, CUA, CCC and GGA on a compatible chloramphenicol-resistant plasmid (15).

Topic	Technical Bulletin No./Description and Features
Protein Expression	
E. coli protein expression	TB055, TB007, pET and pTriEx expression and target protein verification protocols
Baculovirus construction and insect cell expression	TB216, pBAC, and pTriEx transfection and protein expression protocols
Insect Cell Transfection	TB356, TB359, pIEx transfection and expression protocols
Mammalian transfection and expression	TB250, pTriEx transient and stable cell line transfection and protein expression protocols
Detection	
GST•Tag™ Monoclonal Antibody	TB325, 236, IF, IP, QA, WB
His•Tag® Monoclonal Antibody	TB283, IF, IP, WB
HSV•Tag® Monoclonal Antibody	TB067, WB
Nus•Tag™ Monoclonal Antibody	TB328, WB
S•Tag™ detection	TB082, 164, 145, 143, 097, 136, WB, IF
T7•Tag® Monoclonal Antibody	TB015, 106, 212, 213, 112, 137, IF, IP, WB
IF: immunofluorescence, IP: immunoprecipitation, QA: quantitative assay, WB: Western blotting	
Quantification	
GST•Tag Assay Kit	TB236, colorimetric assay, Limit 8 pmol
S•Tag Rapid Assay Kit	TB082, Limit 20 fmol
FRETWorks™ S•Tag Assay Kit	TB251, fluorescent assay, Limit < 1 fmol
Protein Extraction	
BugBuster® Protein Extraction Reagent	TB245, Extract proteins from *E. coli* cultures without sonication. Tris and primary amine-free formulations are available
CytoBuster™ Protein Extraction Reagent	TB306, Extract proteins from insect and mammalian cell cultures
Protein Purification	
GST•Tag purification	TB235, Magnetic and non-magnetic agarose are available.
His•Tag purification	TB054, 273, Magnetic IDA agarose or Ni-NTA and IDA agarose formats. Bulk resin or prepacked cartridge and columns are available.
S•Tag purification	TB087, 160, S-protein agarose
T7•Tag purification	TB125, T7•Tag Antibody Agarose

High-throughput Protein Extraction and Purification	
PopCulture® Reagent	TB323, Extract proteins from *E. coli* without centrifugation
Insect PopCulture Reagent	TB344, Extraction proteins from insect cells without centrifugation
RoboPop™ Purification	TB327, TB346 Extract proteins from *E. coli* cells without centrifugation in a 96-well format. His•Tag® and GST•Tag™ fusion protein purification
Insect RoboPop Purification	TB368, Extract proteins from insect cells without centrifugation. His•Tag fusion protein purification in a 96-well format

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Academic and Non-profit Laboratory Assurance Letter

The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patent applications assigned to Brookhaven Science Associates (BSA). This technology, including bacteria, phages and plasmids that carry the gene for T7 RNA polymerase, is made available on the following conditions:

1. The T7 expression system is to be used for noncommercial research purposes only. A license is required for any commercial use, including use of the T7 system for research purposes or for production purposes by any commercial entity. Information about commercial licenses may be obtained from the Patent Office, Brookhaven National Laboratory, Upton, New York, 11973, Telephone: (631) 344-7134. Contact: Christine Brakel.
2. No materials that contain the cloned gene for T7 RNA polymerase may be distributed further to third parties outside of your laboratory, unless the recipient receives a copy of this assurance letter and agrees to be bound by its terms. This limitation applies to any of the following materials that are included in this kit and to any derivatives you may make of them:

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™(DE3)
E. coli HMS174(DE3)pLysS	*E. coli* Rosetta-gami(DE3)pLysS
E. coli HMS174(DE3)pLysE	*E. coli* Rosetta-gami(DE3)pLacI
E. coli NovaBlue(DE3)	*E. coli* Rosetta-gami 2(DE3)
E. coli Origami™(DE3)	*E. coli* Rosetta-gami 2(DE3)pLysS
E. coli Origami(DE3)pLysS	*E. coli* Rosetta-gami 2(DE3)pLacI
E. coli Origami(DE3)pLacI	*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

3. 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.

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Bacterial Strain Non-distribution Agreement

By purchase of the Origami™, Origami 2, Origami B, Rosetta™, Rosetta 2, RosettaBlue™, Rosetta-gami™, Rosetta-gami 2, or Rosetta-gami B host strains and acceptance of the following terms, Novagen grants a limited license to use the Origami, Origami 2, Origami B, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B host strains for the cloning and expression of genes. The intent of this license is not to limit the research use of these materials, but to protect against unauthorized commercial distribution of the strains by third parties.

1. The Origami, Origami 2, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B host strains or any derivative therefrom is not to be offered for resale or distributed outside your laboratory.
2. Gene clones and libraries in the Origami, Origami 2, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B host strains may be distributed for research purposes only, provided that the recipient acknowledge the foregoing condition.
3. DE3 lysogens of host strains Origami, Origami 2, Rosetta, Rosetta 2, RosettaBlue, Rosetta-gami, Rosetta-gami 2, or Rosetta-gami B are covered by U.S. Patent No. 4,952,496. Commercial customers must obtain a research license agreement from Brookhaven Science Associates before purchase.

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