Xa/LIC Cloning Kits

Table of Contents

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About the Kits

pET-30 Xa/LIC Vector Kit	70073-3
pET-32 Xa/LIC Vector Kit	70072-3

Description

Novagen® ligation-independent cloning (LIC) vectors provide for rapid cloning and gene expression in *E. coli*. The LIC method facilitates the directional cloning of PCR products without restriction enzyme digestion or ligation reactions (1, 2). The Xa/LIC vectors are engineered to express a fusion protein containing a Factor Xa cleavage site positioned so that all vector encoded sequences can be cleaved from the target protein by Factor Xa digestion of the purified fusion protein. Both Xa/LIC vectors possess the same Xa/LIC cloning site so that a Xa/LIC-prepared target insert can be annealed with either of the Xa/LIC vectors in a 5-minute reaction. Xa/LIC vector DNA is supplied as ready-to-use linear double-stranded DNA with single stranded overhangs for annealing to target DNA.

The LIC method uses the 3'→5' exonuclease activity of T4 DNA Polymerase to create specific 12- or 15-base single-stranded overhangs in the target DNA. PCR products with ends complementary to the vector LIC cloning sites are created by including appropriate 5' sequences in the primers (see below). The purified PCR product is treated with T4 DNA Polymerase in the presence of dGTP to generate the specific vector- compatible overhangs. Cloning is very efficient because the desired product is the predominant molecule formed by annealing. The annealed Xa/LIC vector plus insert is transformed into competent *E. coli* cells. Covalent bond formation at the vector-insert junctions occurs within the cell to yield circular plasmid. DNA is isolated and the plasmid construct is verified for structure, and then transformed into appropriate *E. coli* hosts for protein expression.

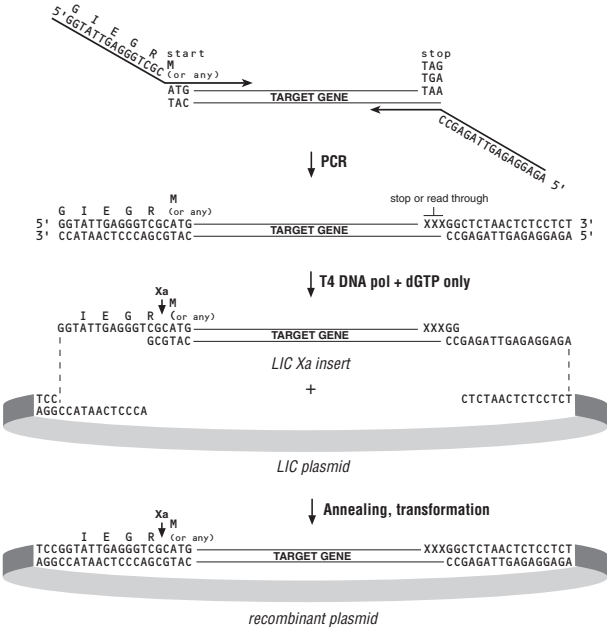


Figure 1. Diagram of the Xa/LIC strategy.

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

Components

1 µg	pET Xa/LIC Vector
8 µl	Xa/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 dGTP
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
2 ng	Test Plasmid, 0.2 ng/µl (Amp^R)

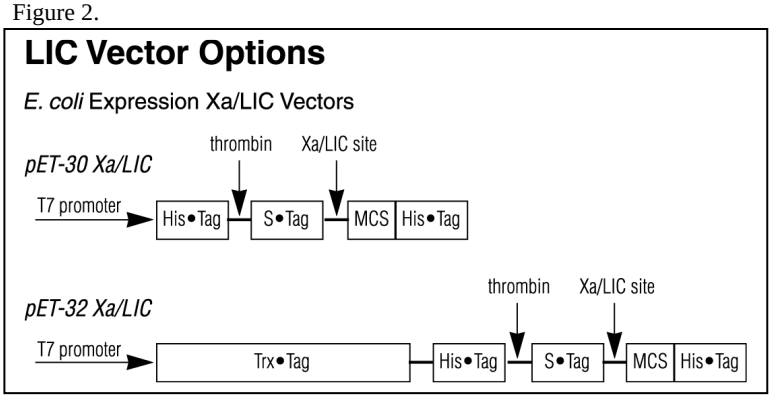
Storage

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

Xa/LIC Vectors

The pET Xa/LIC Vectors combine the advantages of the strong T7 promoter and expression control elements of the pET vector series with the convenience of LIC cloning. Vectors feature a T7*lac* promoter and a choice of fusion tags (diagramed below). These vectors have a ColE1-derived replicon. pET-30 Xa/LIC has a kanamycin resistance marker. pET-32 Xa/LIC has an ampicillin/carbenicillin resistance marker.

Figure 2.



Insert Preparation

Production 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 (3).

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 2):

   sense primer: `5' GGT ATT GAG GGT CGC` – insert-specific sequence 3'
   antisense primer: `5' AGA GGA GAG TTA GAG CCX`*–insert-specific sequence 3'

   *If a C-terminal tag is not desired, include an in-frame stop codon in the insert-specific sequence. (see page 2). If C-terminal tags are desired, design the insert specific sequence so as to maintain the reading frame indicated in the antisense primer.
2. The PCR product must be purified to remove dNTPs, 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 Xa/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.

The Xa/LIC β-gal Control Insert is included with the vector kits and requires treatment with T4 DNA Polymerase to generate compatible overhangs. It is 3085 bp long (2 µg/pmol). For each treatment, use 4 µl of the 100 ng/µl solution provided.

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). (Note: number bp in insert × 650 = pg/pmol)
2 µl	10X T4 DNA Polymerase Buffer
2 µl	25 mM dGTP
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 the 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 Xa/LIC vectors. Store the prepared Xa/LIC insert at –20°C. Inserts have been stored for several months and used successfully for cloning.

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

Annealing the Vector and Xa/LIC Insert

Use the following protocol to anneal an insert into an Xa/LIC Vector. Perform a negative control (lacking insert) and a positive control (Control Insert after T4 DNA polymerase treatment, as above).

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

1 µl	Xa/LIC Vector
2 µl	T4 DNA Polymerase treated Xa/LIC insert (0.02 pmol)

2. Incubate at 22°C for 5 min, then add:

1 µl	25 mM EDTA
4 µl	Total volume

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

Note: Greater 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 6).

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 Xa/LIC Vector Kits and should be used for initial cloning with Xa/LIC Vectors. NovaBlue is a convenient host for initial cloning of target DNA because it has high transformation efficiency and gives high yields of excellent quality plasmid DNA. The Xa/LIC Vector Kits contain NovaBlue GigaSingles™ Competent Cells in 50 µl single-use aliquots and expression host strains in standard, 0.2 ml aliquots. The following protocol is for transformations of NovaBlue GigaSingles™ Competent Cells with DNA from annealing reactions or transformation of expression host with plasmid DNA of the isolated recombinant constructs of Xa/LIC vectors.

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 to determine transformation efficiency.) 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:* Proceed to Step 3.	*Standard Kits:* For each transformation, pre-chill 1.5 ml snap-cap polypropylene tube on ice. Pipet 20 µl thawed competent cells into each pre-chilled tube.

4. Add DNA
   1 µl annealing reaction directly to NovaBlue GigaSingles™ cells.
   or 1 µl (1 – 10 ng) purified recombinant plasmid to expression host competent cells
   or 1 µl (0.2 ng) Test Plasmid to tube of competent cells.
5. 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. Also include any additional antibiotics necessary to maintain host-specific features. Use 50 µg/ml carbenicillin or ampicillin for Amp resistance marker or 30 µg/ml kanamycin for Kan resistance marker. BL21(DE3)pLysS requires 34 µg/ml chloramphenicol to maintain the plysS plasmid. Appropriate plating volume depends on annealing and transformation efficiencies (see Certificate of Analysis for competent cell transformation efficiency). For NovaBlue expect 10^5–10^7 transformants per µg annealed vector DNA. For Test Plasmid, plate 5 µl NovaBlue transformation mix, For transformation of expression host with a purified recombinant plasmid, plate 5–20 µl. When plating less than 25 µl, first pipet a pool of SOC onto the plate, and then pipet the transformation into the SOC. Spread.
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

Many more colonies in transformation with annealing reactions (vector plus insert) than with the negative control (vector alone) usually indicates successful cloning. Colonies can be screened for inserts by colony PCR using vector-specific primers, followed by agarose gel electrophoresis. Because Xa/LIC is directional, vector-specific primers can be used at both ends. Alternatively, a vector-specific primer can be used in combination with an appropriate insert-specific primer.

Appropriate primers for colony PCR of clones in the Xa/LIC vectors followed by in vitro transcription translation are the pET Upstream Primer (Cat. No. 69214-3) and the T7 Terminator Primer (Cat. No. 69337-3).

For colony PCR (not followed by in vitro transcription/translation) use either the T7 Promoter Primer (Cat. No. 69348-3) or the pET Upstream Primer and the T7 Terminator Primer.

Colony PCR

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. Spin at 12,000 x *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 NovaTaq™ Buffer without $MgCl_2$, add $MgCl_2$ to a final concentration of 1.5–2.5 mM, decreasing the volume of water.

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

   Note: As an optional step, a hot start procedure can be used in which the samples are warmed to 80°C before the addition of the master mix. Alternatively, use NovaTaq™ Hot Start DNA Polymerase. For greater accuracy, specificity, and yield of long complex targets use KOD, KOD Hot Start, or 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, 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 size of PCR product produced from the Xa/LIC β-gal Control Insert is ~ 3 kbp. The specific size depends on the vector and primer combination.

Plasmid purification

After identifying colonies containing positive clones, isolate plasmid DNA for restriction mapping, sequence analysis, and transformation into expression hosts. Plasmid DNA of 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 plasmids, use a method for low-copy number plasmids. 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 plasmid has been isolated from NovaBlue and its structure verified, use the DNA to transform an expression host. BL21(DE3) or BL21(DE3)pLysS competent cells (provided in the pET Xa/LIC Vector Kits) are both lysogenic for bacteriophage λDE3. The DE3 strains possess a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. After plasmids are established in a λDE3 lysogen, expression of the target ORF can be induced by growing cultures in Overnight Express™ Autoinduction Medium, or by adding IPTG to cultures in conventional medium. Overnight Express Autoinduction Medium directs high-density cell growth in the absence of induction followed by autoinduction and continued cell growth during an overnight incubation (See User Protocol TB383.)

For induction of protein expression in conventional medium, add IPTG to final concentration of 0.4–1 mM when the bacterial cell culture reaches an OD_{600} of 0.6. Continue incubation for 3 hr before harvesting cells for protein analysis. For information regarding induction optimization, protein detection, and quantification, or for cell extract preparation refer to the pET System Manual (See User Protocol TB055).

Detailed protocols for protein purification, and quantification are found in other Novagen Technical Bulletins. All Technical Bulletins are available http://www.merck4biosciences.com.

In addition to the expression strains provided in the vector kits, EMD Chemicals Inc. offers an extensive selection of other λDE3 lysogenic hosts for expression. These include host strains that enhance disulfide bonds formation and strains that supplement tRNAs for codons rarely used in *E. coli*. See User Protocol TB009 for more information on the competent cells available.

Alternatively, the T7 promoter-based pET plasmids may be induced for protein expression in non-DE3 lysogens by infection with Bacteriophage CE6. See User Protocol TB007.

References

1. Aslanidis, C. and de Jong, P. J. (1990) *Nucl. Acids Res.* **18**, 6069–6074.
2. Haun, R. S., Serventi, I. M., and Moss, J. (1992) *Biotechniques* **13**, 515–518.
3. Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., Oka, M., and Imanaka, T. (1997) *Appl. Environ. Microbiol.* **63**, 4504–4510.

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