Ligation Independent Cloning: Efficient Directional Cloning of PCR Products

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Ligation independent cloning (LIC) was developed for the directional cloning of PCR products without restriction enzyme digestion or ligation reactions (1, 2). LIC vectors are created by treating a linearized backbone with T4 DNA polymerase in the presence of only one dNTP. The 3' to 5' exonuclease activity of T4 DNA polymerase removes nucleotides until it encounters a residue corresponding to the single dNTP present in the reaction mix. At this point, the 5' to 3' polymerase activity of the enzyme counteracts the exonuclease activity to effectively prevent further excision. Plasmid sequences adjacent to the site of linearization are designed to produce specific non-complementary 13- and 14-base single stranded overhangs in the LIC vector. PCR products with complementary overhangs are created by building appropriate 5' extensions into the primers (see Fig. 1). 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 the appropriate dNTP to generate the specific vector-compatible overhangs. Cloning is very efficient because only the desired product is formed by annealing. The annealed 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.

Novagen has developed a series of LIC vectors in which one overhang encodes the enterokinase cleavage site. As shown in Fig.1, the following sequences must be added to the 5' end of target gene PCR primers to generate vector-compatible overhangs:

sense primer:
5' `GAC GAC GAC AAG AT`-insert specific sequence 3'

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

The sense primer encodes the last four amino acids of the enterokinase (EK) cleavage site plus the C-terminal flanking amino acid Met or Ile (ATX). Ile (ATC, ATA, ATT) at this position recreates the natural EK site found in trypsinogen. In this case, the target protein retains only one non-native amino acid at the N-terminus after EK cleavage. Met at this position allows the removal of all vector-encoded sequences.

The antisense primer may encode an in-frame stop codon or allow read through to the vector-encoded stop codon present after the C-terminal His•Tag® sequence. In the latter case, the ORF established by the insert must be maintained. For example, the first two 5' insert-specific bases of the antisense primer must represent the first two bases in the codon XXA in the sense strand.

LIC vectors with various expression options are shown in Fig. 2. All of these vectors possess the same LIC cloning site, so one LIC-prepared target gene insert can be cloned into any or all of them.

Table 1 shows the high cloning efficiency and low background obtained with Novagen's LIC vectors. When positive con-

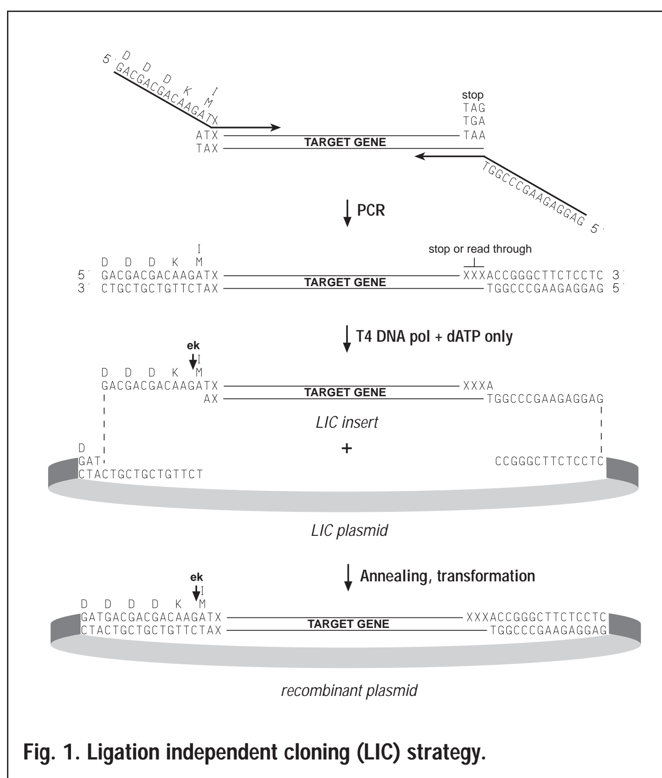

Fig. 1. Ligation independent cloning (LIC) strategy.

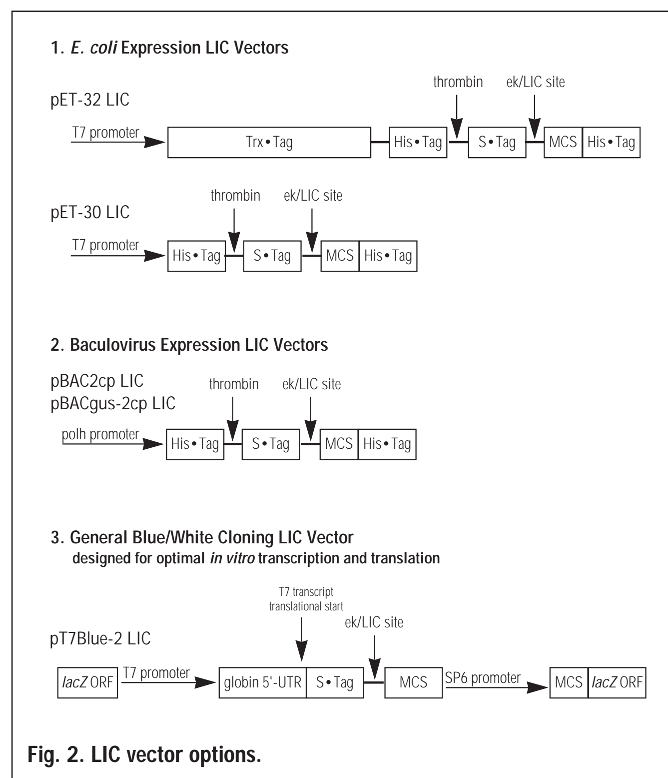

Fig. 2. LIC vector options.

LIC Vector	LIC Insert	Vector plated (ng)	Colonies	Vector Efficiency*	% Recombinants
pT7Blue-2 LIC	β-gal	1.25 ng	606	4.8×10^5	ND
pT7Blue-2 LIC	gus	1.25 ng	531	4.2×10^5	100%
pT7Blue-2 LIC	none	1.25 ng	11	background	NA
pET-32 LIC	β-gal	1.25 ng	414	3.3×10^5	100%
pET-32 LIC	gus	1.25 ng	339	2.7×10^5	ND
pET-32 LIC	none	1.25 ng	8	background	NA

* Expressed as colony forming units (cfu)/µg vector. The transformation efficiency of the competent cells was > 2×10^8 cfu/µg.
ND = not done; NA = not applicable

Table 1. LIC cloning efficiency with two different inserts.

trol inserts were cloned into pT7Blue-2LIC and pET-32 LIC, a 42 to 56-fold increase in colony forming units over background was observed.

pET LIC Vectors for Expression in *E. coli*

Two pET LIC Vectors are available, which are based on specifically constructed pET-32 LIC and pET-30 LIC plasmids. The pET-32 LIC Vector is designed for cloning and high-level expression of peptide sequences fused to the 109 amino acid (aa) (11,675 Da) thioredoxin (*trxA*, Trx•Tag™) protein. Many proteins, which are normally produced in an insoluble form in the *E. coli* cytoplasm, tend to become more soluble when fused with thioredoxin (3, 4). In addition, pET-32 LIC encodes the 6 aa His•Tag® and 15 aa S•Tag™ sequences upstream of the cloning site for simple detection and purification of target proteins. The N-terminal fusion sequences can be removed with thrombin (Trx•Tag, His•Tag) or enterokinase (all three tags). The pET-32 LIC Vector also carries the T7*lac* promoter, T7 transcription terminator, *lacI* gene, pBR322 origin of replication, f1 origin for single stranded plasmid production, and *bla* gene for ampicillin (carbenicillin) resistance.

The pET-30 LIC Vector has many of the same features as the pET-32 LIC Vector except that it lacks the thioredoxin gene sequence. In addition, the pET-30 LIC Vector carries the gene for kanamycin resistance instead of the *bla* gene for ampicillin resistance.

The pET LIC Vector Kits include the strain NovaBlue and the expression hosts BL21(DE3) and BL21(DE3)pLysS as competent cells. NovaBlue is a convenient host for initial cloning of target DNA into the pET LIC Vectors, since it is *recA*⁻ and gives high transformation efficiencies and good plasmid yields. For protein production, a recombinant plasmid is prepared from NovaBlue and transformed into expression host strains BL21(DE3) or BL21(DE3)pLysS, which are lysogenic for bacteriophage λDE3. The DE3 strains possess a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. Target protein expression is induced by the addition of IPTG to a growing culture, which allows production of T7 RNA polymerase (gene *1*) and subsequent high-level transcription of target gene sequences from the T7 promoter (5).

Expression strains derived from the thioredoxin reductase mutant AD494 (6) are available separately. AD494(DE3) hosts allow the formation of disulfide bonds in the *E. coli* cytoplasm; soluble proteins containing disulfide bonds may therefore fold properly in these strains.

pBAC LIC Vectors for Expression in Insect Cells

The pBAC™-2cp LIC and pBACgus-2cp LIC Vectors are baculovirus transfer plasmids designed for convenient and highly efficient cloning of target genes for subsequent expression in insect cells. After recombinants are established in *E. coli*, plasmids are isolated and co-transfected into insect cells with baculovirus DNA (BacVector™-1000 or -2000 Triple Cut Virus DNA) to create baculovirus recombinants. Inserts are cloned in-frame with upstream sequences encoding (in order) His•Tag, thrombin cleavage site, S•Tag, and enterokinase cleavage site for protein quantification, detection, and purification (see Fig. 3). As with the other LIC vectors, all vector-encoded amino terminal sequences can be removed by cleavage with enterokinase.

The two pBAC LIC vectors are identical except that pBACgus-2cp includes a *gus* marker gene for color identification of baculovirus recombinants.

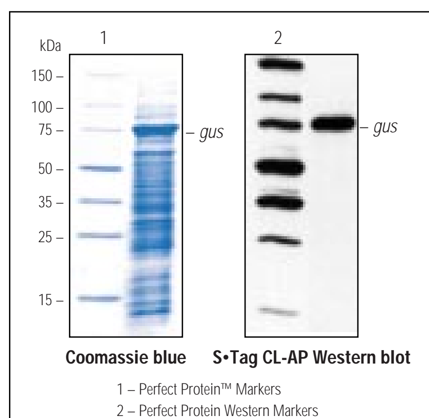

Fig. 3. Expression of β-glucuronidase (*gus*) from a BacVector-1000/pBAC-2cp LIC recombinant.

β–glucuronidase expressed from a pBAC-2cp LIC recombinant was detected by SDS-PAGE and staining with Coomassie blue (left panel) and by Western blotting using S-protein AP Conjugate and CDP-Star chemiluminescent substrate.

pT7Blue-2 LIC Vector for General Cloning and Optimal Expression by *in vitro* Transcription/Translation

The pT7Blue-2 LIC Vector is a general blue/white screening vector also designed for optimal expression of cloned inserts by *in vitro* transcription and translation. LIC inserts are placed downstream of T7 promoter, *Xenopus* β-globin 5' UTR, optimal translation initiation site, and S•Tag peptide sequences. While the other LIC vectors are also compatible with transcription/translation analysis using several strategies, the globin 5' UTR and optimal translation

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initiation site serve to enhance the produc-

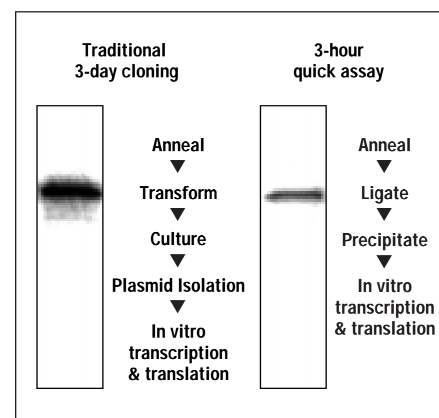

Fig. 4. *In vitro* transcription/translation analysis using pT7Blue-2 LIC.

Both autoradiographs show *in vitro* expression of ^{35}S-met labeled β-glucuronidase (*gus*). The left panel shows protein produced from plasmid DNA obtained by conventional cloning and isolation from *E. coli*. The right panel shows protein produced directly from ligated pT7Blue-2 LIC vector + insert DNA without a cloning step.

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tion of authentic proteins in a reticulocyte-based system (7, 8).

Templates for analysis can be produced by a variety of strategies. For example, following transformation of LIC insert + vector annealing reactions, bacterial colonies can be analyzed directly by colony PCR followed by transcription/translation of the PCR products. As an alternative to cloning, LIC insert + vector annealing reactions can be treated with T4 DNA ligase, precipitated, resuspended, and used directly for analysis. This procedure allows *in vitro* synthesis of the protein products of an appropriately amplified DNA sequence in about 3 hours (see Fig. 4).

Blue/white screening for recombinants with strain NovaBlue and IPTG/X-gal plates is made possible by the presence of upstream *lac* promoter and downstream *lacZ* α-peptide coding regions in pT7Blue-2. In practice, non-recombinant background is typically so low that blue/white screening is not necessary (see Table 1).

Please see page 14 for ordering information.

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