LIC Duet™ Adaptor Kits

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About the Kits

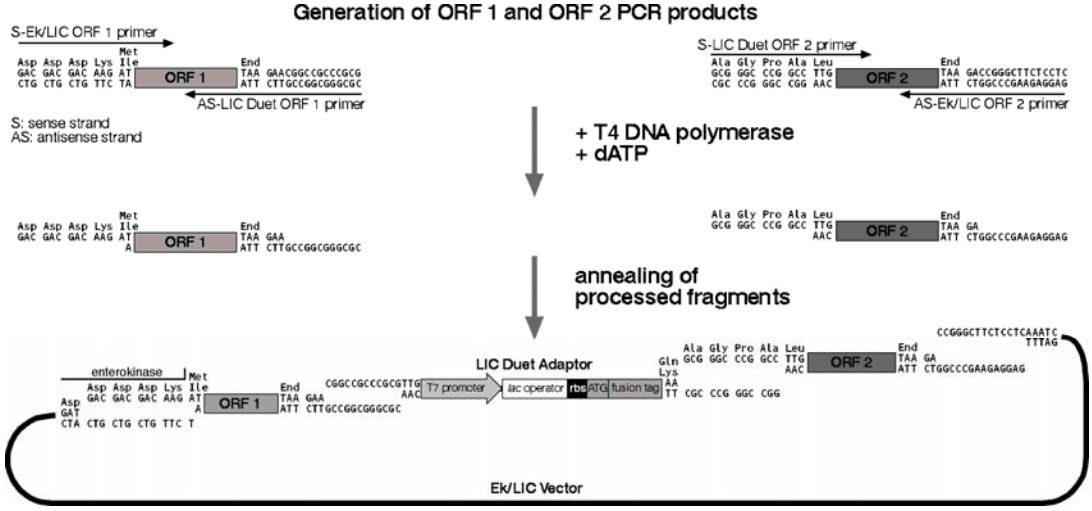
LIC Duet™ Minimal Adaptor	71362-3
LIC Duet T7•Tag® Ek Adaptor	71321-3
LIC Duet Trx•Tag™ Ek Adaptor	71322-3
LIC Duet GST•Tag™ Ek Adaptor	71323-3
LIC Duet Nus•Tag™ Ek Adaptor	71324-3

Description

LIC Duet™ Adaptors enable cloning of two open reading frames (ORFs) simultaneously into one plasmid for coexpression in bacteria (1). Ligation-independent cloning (LIC) facilitates directional cloning of PCR products without requiring restriction enzyme digestion or ligation reactions (2, 3). Properly designed inserts are cloned in-frame and downstream from vector-encoded fusion tags. All LIC Duet Adaptors include a T7*lac* promoter/operator, strong ribosome binding site (RBS), and ATG start codon. These enable efficient expression of the second, or "downstream", ORF under the same controls as the first, or "upstream", ORF when inserted into a pET, pCDF, or pRSF Ek/LIC vector. Four LIC Duet Adaptors encode a protease cleavage site in the 5'-vector-insert junction, allowing removal of all vector-encoded sequences from expressed fusion proteins. These four adaptors offer a choice of fusion tag (T7•Tag®, Trx•Tag™, GST•Tag™, or Nus•Tag™ sequence). The LIC Duet Minimal Adaptor is designed for expression of a target protein with a minimal fusion, and does not encode an enterokinase cleavage site or detectable fusion tag.

The adaptors bridge the 3'-end of the first prepared PCR product and the 5'-end of the second prepared PCR product. The LIC vector overhangs anneal with the 5'-end of the first PCR product and the 3'-end of the second PCR product. Therefore, the first target protein is fused to an N-terminal tag encoded by the LIC vector and the second target protein is fused to the N-terminal sequence encoded by the LIC Duet adaptor. Adaptors are interchangeable, since they all possess a common overhang upstream from the T7*lac* promoter and a different, yet common, overhang downstream from the Ek cleavage site (or the ATG start codon in the LIC Duet Minimal Adaptor). Given the available collection of *E. coli* Ek/LIC vectors and LIC Duet Adaptors, numerous combinations of coexpressed fusion proteins are possible (see User Protocol TB163 for available Ek/LIC Vector Kits).

Compatible target ORFs are PCR-generated using primers with specified 5'-extensions (see *Insert Preparation* on p 4). Target PCR products are purified to remove dNTPs and (if applicable) the original plasmid template. PCR products are then treated with T4 DNA Polymerase in the presence of dATP to generate overhangs compatible with Ek/LIC vector and LIC Duet Adaptor. Cloning is very efficient, as only desired product is formed by annealing. *E. coli* competent cells are transformed with an annealing reaction (pET Ek/LIC vector, target LIC insert-1, LIC Duet Adaptor and target LIC insert-2). Covalent bond formation at the vector-insert and adaptor-insert junctions occurs within the cell to yield circular plasmid. After verification, the construct may be used for bacterial expression.



Cloning strategy for LIC Duet™ Adaptors. After ORF 1 and ORF 2 are amplified with primers that include the indicated 5'-LIC extension, the PCR inserts are treated with LIC-qualified T4 DNA Polymerase (+dATP), annealed to the LIC Duet Adaptor and Ek/LIC vector, and transformed into competent *E. coli*.

Components

- 20 µl LIC Duet Adaptor (0.4 pmol at 0.02 pmol/µl)
- 4 µl LIC Duet Control Insert 1
- 8 µl LIC Duet Control Insert 2

Storage

Store all components at –20°C.

Additional available reagents

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
pET-51 Ek/LIC Vector Kit	71570-3
pRSF-2 Ek/LIC Vector Kit	71364-3
pCDF-2 Ek/LIC Vector Kit	71337-3
T4 DNA Polymerase, LIC-qualified, 250 U	70099-3

Additional user-supplied reagents

- TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0)
- 10X T4 Polymerase buffer (500 mM Tris HCl pH 8.0, 100 mM MgCl2, 0.5 mg/ml acetylated BSA; included with T4 DNA Polymerase, LIC-qualified)
- 100 mM DTT
- 25 mM dATP
- 25 mM EDTA

LIC Duet™ Adaptors

The complete nucleotide sequence for each LIC Duet™ adaptor can be found in its respective User Protocol map, as indicated in the table below. Note that after two target sequences have been amplified and treated with T4 DNA Polymerase, they can be annealed to any of the LIC Duet Adaptors for simultaneous cloning of both ORFs into any pET, pCDF, or pRSF Ek/LIC vector.

Product	Fusion tag	Tag size (aa)	Adaptor size (bp)	User Protocol
LIC Duet™ Minimal Adaptor	None*	none	138	TB389
LIC Duet T7•Tag® Ek Adaptor	T7•Tag	11	189	TB385
LIC Duet Trx•Tag™ Ek Adaptor	Trx•Tag	109	502	TB386
LIC Duet GST•Tag™ Ek Adaptor	GST•Tag	220	835	TB387
LIC Duet Nus•Tag™ Ek Adaptor	Nus•Tag	495	1,660	TB388

* LIC Duet Minimal Adaptor does not encode an EK site.

Insert Preparation

Production and purification of PCR products

When PCR-amplifying inserts, we strongly recommend using KOD HiFi 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 (4).

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 use of high-fidelity DNA polymerase such as KOD HiFi or KOD Hot Start, creating a sequence-verified plasmid to serve as a template in subsequent amplifications further minimizes the likelihood of PCR-generated mutations. 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 likelihood of primer-derived mutations.*

Amplify target ORF-1 and target ORF-2 using appropriately designed PCR primers. Primer 5' ends must incorporate the following sequences (see p 3):

a. ORF-1 primers

ORF-1 sense primer: 5' GAC GAC GAC AAG ATX[1]–insert specific sequence 3'

ORF-1 antisense primer: 5' CGC GGG CGG CCG T–insert specific sequence 3'

[1]The first nucleotide of the insert-specific sequence must complete the codon ATX.

b. ORF-2 primers

ORF-2 sense primer: 5' GCG GGC CCG GCC T[2]–insert specific sequence 3'

ORF-2 antisense primer: 5' GAG GAG AAG CCC GGT[3]– insert specific sequence 3'

[2]The first nucleotides of the insert-specific sequence must complete the codon TXX.

[3]If C-terminal tag sequences are desired, additional bases may be required in the antisense primer to ensure C-terminal sequences are in-frame. If a C-terminal tag is not desired, include a stop codon in the insert-specific sequence.

Optimal PCR conditions should produce a strong DNA band of the appropriate size and minimal extraneous products upon analysis by agarose gel electrophoresis.

Additional notes:

- All dNTPs from the PCR reaction must be completely removed prior to T4 DNA Polymerase treatment.
- If the PCR template and the Ek/LIC vector have the same antibiotic resistance, product must be purified from template.
- Choice of purification method depends on: 1) whether antibiotic resistance of the template and Ek/LIC vector are the same, and 2) quality of PCR products (e.g., single amplification product versus multiple extraneous bands or primer dimers). If extraneous bands or interfering template plasmids are present, run PCR product on an agarose gel and extract the target band using the SpinPrep™ Gel Kit (Cat. No. 70852-3) or a similar method. If contaminants are not present, the agarose gel step is unnecessary and product can be purified with SpinPrep PCR Clean-Up Kit (Cat. No. 70976-3), on a spin column, or by binding to a membrane or solid matrix.
- Purified PCR product should be eluted in TlowE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).
- To avoid generating false positives by residual polymerase activity, the polymerase used for PCR should be inactivated if insert is not gel-purified. Extract the reaction with CIAA [chloroform:isoamyl alcohol (24:1)] by adding 1 volume CIAA, vortexing for 1 min, and spinning at 12,000 × *g* for 1 min. Remove and save the aqueous (upper) phase.

T4 DNA Polymerase treatment of target inserts

T4 DNA Polymerase treatment generates compatible overhangs. Include positive control inserts to verify the system performance. Also include a negative control, omitting insert. The LIC Duet™ Adaptors already possess the desired LIC overhangs. Therefore, adaptors should be added directly to the annealing reaction described in the next section and NOT subjected to additional T4 DNA Polymerase treatment.

LIC Duet Control Inserts 1 and 2 require treatment with T4 DNA Polymerase to verify system performance. LIC Duet Control Insert 1 (0.53 µg/pmol) is 810 bp long and encodes the majority of T7 gene *10*. For each treatment, use 1.1 µl of the 100 ng/µl solution provided. LIC Duet Control Insert 2 (1.2 µg/pmol) is 1,840 bp long and encodes β-glucuronidase (*gus*). For each treatment, use 2.5 µl of the 100 ng/µl solution provided. Set up separate reactions for each experimental target insert or Control Insert (i.e., LIC Duet Control 1 insert and LIC Duet Control 2 insert are treated separately).

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 of bp in insert × 650 = pg/pmol)
2 µl	10X T4 DNA Polymerase Buffer (included with T4 DNA Polymerase and all Ek/LIC Vector Kits)
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 should be 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 target ORF-1 and target ORF-2 can be annealed to any of the LIC Duet Adaptors and any of the Ek/LIC vectors. Store prepared inserts at –20°C. Inserts that have been 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. The use of unqualified T4 DNA Polymerase may cause variability in cloning efficiency. LIC-qualified T4 polymerase is available separately (Cat. No. 70099-3).

Annealing the Vector, Adaptor, and Inserts

1. For each set of ORF-1 and target ORF-2 inserts, assemble the following components in a sterile 1.5-ml microcentrifuge tube:

1 µl	pET, pRSF, or pCDF Ek/LIC Vector
2 µl	T4 DNA Polymerase-treated target ORF-1 insert (0.02 pmol)
1 µl	LIC Duet Adaptor (0.02 pmol)
2 µl	T4 DNA Polymerase-treated target ORF-2 insert (0.02 pmol)

Incubate at 70°C for 30 sec, place at room temperature for 2 min to cool, then add:

1 µl	25 mM EDTA
7 µl	Total volume

Note: The heating step is included to optimize annealing of LIC overhangs.

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

Tips: Greater volumes of treated insert may be used; however, vector concentration will decrease. Subsequently, fewer ng will be plated if a constant volume of annealing reaction is used for transformation.

Annealing is complete within 5 min of incubation. Reactions can be incubated up to 1 h without affecting results.

Transformation

NovaBlue GigaSingles™ Competent Cells (Cat. No. 71127) are provided in Ek/LIC Vector Kits and should be used for initial cloning with all LIC Vectors. NovaBlue is a convenient host for initial cloning due to its high transformation efficiency and the high yields and excellent plasmid DNA resulting from *recA* and *endA* mutations. The pET, pRSF, and pCDF Ek/LIC Vector Kits also include expression host strains. See User Protocol TB163 for transformation protocol.

Colony Screening

If 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 by colony PCR using Novagen vector/adaptor-specific primers, followed by agarose gel analysis. Because Ek/LIC is directional, appropriate vector-specific and LIC Duet Adaptor-specific primers can be used to amplify target ORF-1 and/or target ORF-2. However, a vector-specific primer also can be used in combination with an appropriate insert-specific primer (assuming compatible annealing conditions).

In addition, *in vitro* transcription/translation can be used to assess clones quickly. Amplify insert DNA from a colony with appropriate primers and use this product as template for *in vitro* transcription and translation with Single Tube Protein® System 3 (STP3®) T7 or EcoPro™ T7 System. Upstream primers that provide a "spacer" region before the T7 promoter allow efficient transcription. This method allows rapid testing of clones for potential mutations, such as those inserting a stop codon (nonsense mutations).

Appropriate 5' primers useful for *in vitro* transcription and translation analysis or PCR colony screening are listed in the table below. See User Protocol TB163 for colony PCR protocol.

Product	Applicable Vector(s) or LIC Duet Ek Adaptors	Cat. No.
Upstream primers for *in vitro* transcription and translation or colony screening		
ACYCDuetUP1 Primer	pCDF-2, pRSF-2	71178-3
pET Upstream Primer	pET-30, pET-32, pET-41, pET-43.1, pET-44	69214-3
Downstream primers		
T7 terminator Primer	pET-30, pET-32, pET-41, pCDF-2, pRSF-2	69337-3
ColiDOWN Primer	pET-43.1, pET-44	See note
ORF-1 upstream primers for colony screening only		
S•Tag™ Primer	pET-30, pET-32, pET-41, pCDF-2, pRSF-2	69945-3
ORF-1 downstream primers		
LIC Duet ORF1DOWN	All LIC Duet Adaptors	See note
ORF-2 upstream primers for colony screening only		
LIC Duet ORF2UP Primer	Trx•Tag™, GST•Tag™, Nus•Tag™ LIC Duet Ek Adaptors	See note
T7•Tag® Primer	T7•Tag LIC Duet Ek Adaptor	See note
ORF-2 Downstream primers		
T7 terminator Primer	pET-30, pET-32, pET-41, pCDF-2, pRSF-2	69337-3
ColiDOWN Primer	pET-43.1, pET-44	See note

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 of the primers is indicated on the respective LIC Duet Adaptor Map. Sequences are also available at www.novagen.com.

Protein Expression, Detection, Purification and Quantification

Detailed protocols for protein expression, detection, purification, and quantification are found in other User Protocols (see TB163 for a listing). All user protocols are available at www.novagen.com.

After a recombinant construct has been established in NovaBlue cells, T7 promoter based-plasmids may be induced for protein expression using either of two methods. In the first possible method, recombinant plasmid is isolated from NovaBlue cells and transformed into Novagen *E. coli* DE3 expression host strains which are lysogenic for bacteriophage λDE3. DE3 strains possess a chromosomal copy of T7 RNA polymerase gene under control of the *lacUV5* promoter. See User Protocol TB009 for description of the extensive collection of λDE3 lysogenic hosts available for expression. In the second possible method, T7 RNA polymerase is delivered to the NovaBlue cells harboring pET recombinants by infecting the cultures with bacteriophage CE6 (see User Protocol TB007).

References

1. Loomis, K., Sternard, H., Rupp, S., Held, D., Yaeger, K., Novy, R. and Wong, S. (2003) *in**Nova**tions* **18**, 7–12.
2. Aslanidis, C. and de Jong, P. J. (1990) *Nucl. Acids Res.* **18**, 6069–6074.
3. Haun, R. S., Serventi, I. M. and Moss, J. (1992) *Biotechniques* **13**, 515–518.
4. Takagi, M., Nishioka, M., Kakihara, H., Kitabayashi, M., Inoue, H., Kawakami, B., Oka, M. and Imanaka, T. (1997) *Appl. Environ. Microbiol.* **63**, 4504–4510.

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 patents assigned to Brookhaven Science Associates. 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 Office of Intellectual Property and Sponsored Research, Brookhaven National Laboratory, Building 185, P.O. Box 5000, Upton, New York, 11973, Telephone: (631) 344-7134.
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 AD494(DE3)	*E. coli* Origami B(DE3)pLysS
E. coli AD494(DE3)pLysS	*E. coli* Origami B(DE3)pLacI
E. coli B834(DE3)	*E. coli* Rosetta(DE3)
E. coli B834(DE3)pLysS	*E. coli* Rosetta(DE3)pLysS
E. coli BL21(DE3)	*E. coli* Rosetta(DE3)pLacI
E. coli BL21(DE3)pLysS	*E. coli* Rosetta 2(DE3)
E. coli BL21(DE3)pLysE	*E. coli* Rosetta-gami(DE3)
E. coli BL26(DE3)pLysE	*E. coli* Rosetta-gami(DE3)pLysS
E. coli BL21*trxB* (DE3)	*E. coli* Rosetta-gami(DE3)pLacI
E. coli BL21*trxB* (DE3)pLysS	*E. coli* Rosetta-gami B(DE3)
E. coli BLR(DE3)	*E. coli* Rosetta-gami B(DE3)pLysS
E. coli BLR(DE3)pLysS	*E. coli* Rosetta-gami B(DE3)pLacI
E. coli HMS174(DE3)	*E. coli* RosettaBlue(DE3)
E. coli HMS174(DE3)pLysS	*E. coli* RosettaBlue(DE3)pLysS
E. coli HMS174(DE3)pLysE	*E. coli* RosettaBlue(DE3)pLacI
E. coli NovaBlue(DE3)	*E. coli* Tuner(DE3)
E. coli Origami(DE3)	*E. coli* Tuner(DE3)pLysS
E. coli Origami(DE3)pLacI	*E. coli* Tuner(DE3)pLacI
E. coli Origami B(DE3)	Bacteriophage λCE6
	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.