Ligation Independent Cloning (LIC) Procedure

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LIC cloning allows insertion of DNA fragments without using restriction enzymes into specific vectors containing engineered overhangs. The procedure is based on the generation of complementary single stranded DNA overhangs in vector and insert using the proofreading capability of T4 DNA polymerase. The overhang should be preceded at the 5'site by any of the 4 bases (A, C, G or T) which is not present (in a double-stranded configuration after vector cleavage) within the overhang sequence. Advantages of the method are: No restriction enzymes and ligase are required during cloning (cost effective); and (if multiple LIC vectors are available) efficient, versatile cloning into multiple vectors (e.g. with different tags, suited for different hosts, etc.) is possible. To illustrate the method, a vector from the NKI-LIC vector suite (ref) containing an N-terminal his-tag is used in the following example.

Vector preparation

- **6x-His-tag vector containing LIC sequence, KpnI restriction site and 3C protease recognition sequence**

```
-ATGGCACATCACCACCACCATCACTCCGCGGCTCTTGAGGTGCTCTTTCAGGGACCCGGGTACCCGGGCTTCTCCTCGAG-
-TACCGTGTAGTGTGTGGTAGTGAGGCGCCGAGAACTCCACGAGAAAGTCCCTGGGCCCATGGGCCCGAAGAGGAGCTC-
```

The LIC vector has been designed such that the KpnI site is flanked by the forward and reverse LIC overhangs. The forward overhang is also part of the sequence encoding the 3C protease recognition site. Note that the overhangs are preceded by a T-base at the 5'site (underlined in the sequence above). These bases serve to stall the exonuclease activity of T4 polymerase.

GGTACC – KpnI restriction site

5'-CAGGGACCCGGGT-3' = forward overhang in vector

5'-CGAGGAGAAGCCCGG-3'= reverse overhang in vector

CATCACCACCACCATCAC = 6 x his-tag

CTTGAGGTGCTCTTTCAGGGACCC = (part of) 3C protease recognition sequence

- **Vector: cleavage by KpnI**

```
-TTTCAGGGACCCGGGTAC          CCGGGCTTCTCCTCGAG-
-AAAGTCCCTGGGCC          CATGGGCCCGAAGAGGAGCTC-
```

Vector is digested by KpnI to allow the LIC overhangs to be accessible for T4 DNA polymerase exonuclease treatment.

- **Vector: T4 polymerase + dTTP incubation**

```
-TTT                                  CCGGGCTTCTCCTCGAG-
-AAAGTCCCTGGGCC                                    TC-
```

T4 DNA Polymerase will remove bases (3' to 5' direction) until it encounters a T. Note that the T-base present in the GGTACC sequence is also removed because it is a single-stranded DNA base which is also susceptible to exonuclease activity by T4 polymerase.

Design of PCR fragment

Upon designing the primers, the following LIC overhangs need to be added to the 5'sequence of the forward and reverse primers :

5'-CAGGGACCCGGT-3' = forward overhang in PCR fragment

5'-CGAGGAGAAGCCCGGTTA-3'= reverse overhang in PCR fragment ; containsTAA stop

This should result in the following PCR fragment :

```
CAGGGACCCGGTyyyyyyyyyyyyyTAACCGGGCTTCTCCTCG
GTCCCTGGGCCAXXXXXXXXXXXXXATTGGCCCGAAGAGGAGC
```

- **PCR fragment: T4 polymerase + dTAP incubation**

```
CAGGGACCCGGTyyyyyyyyyyyyyTAA
            AXXXXXXXXXXXXXATTGGCCCGAAGAGGAGC
```

Note that the PCR fragment is treated with the complementary base (dATP) compared to the base that is use in treatment of the vector (dTTP) to create the proper single-stranded DNA overhangs. The A-bases at the 5'site of the overhangs(underlined) serve to stop exonuclease processing.

- **Annealing of PCR fragment and vector**

```
-TTTCAGGGACCCGGTyyyyyyyyyyyyyTAACCGGGCTTCTCCTCGAG-
-AAAGTCCCTGGGCCAXXXXXXXXXXXXXATTGGCCCGAAGAGGAGCTC-
```

T4 polymerase-treated vector and PCR fragment are mixed, incubated at room temperature and transformed into E. coli competent cells.

- **Final construct**

```
ATGGCACATCACCACCACCATCACTCCGCGGCTCTTGAGGTGCTCTTTCAGGGACCCGGTyyyyyyyy
 M  A  H  H  H  H  H  H  S  A  A  L  E  V  L  F  Q  G  P  G  Z  Z  Z

yyyyyyyyyyyyTAACCGGGCTTCTCCTCGAG
 Z  Z  Z  Z stop
```

After 3C Protease cleavage

```
G  P  G z  z  z  z  z  z  z  z
```

The final protein (after 3C protease cleavage) contains 3 additional residues (GPG) at its N-terminus.

LIC Cloning Protocol

This protocol has been established at the Protein Facility of the Netherlands Cancer Institute (NKI) and is commonly used for cloning of DNA fragments into the NKI-LIC vectors (ref). The protocol has been generalized to be applicable for any type of LIC vector.

Vector preparation

- Digest 10 µg of LIC vector with appropriate restriction enzyme according to the manufacture protocol.

Note 1: *Typically, 2 hr incubation with 4-5 units of enzyme per µg of DNA (i.e. 40-50 units per 10 µg) in the suggested buffer should yield properly digested vector.*

- Add loading dye to the DNA sample and run the sample on a 1% agarose gel in 1x TBE buffer. Run for 30 minutes at 100kV and place the gel under a UV lamp to check for proper separation of vector and other 'contaminants' (un-cleaved vector, RNA, 'insert' DNA removed upon digestion). Digest the vector fragment from gel and purify using a commercially available gel-extraction kit. During the last step, collect your DNA in a volume of 20 to 25 µl 10 mM Tris pH 8.0.

Note 2: *Cleaved vector can also be purified using a spin column instead of isolating it from agarose gel. Howver, if the initial vector DNA is not pure, contaminants may be co-purified, which could decrease the efficiency of LIC cloning.*

- Determine the DNA concentration (in ng/µl) using a Nanodrop device or by running 2 µl of DNA next to a DNA sample of known concentration on a 1% agarose gel.
- Calculate the DNA concentration in pmol/µl using equation 1:

Equation 1: # pmol/µl = # ng/µl / (650 * fragment size (in kb)).

E.g. 200 ng/µl 5300 bp vector = 200 / (650 x 5.3) = 0.058 pmol/µl

- The concentration of the vector should ideally be around ~0.03 pmol/µl (= ~100 ng/µl). If the concentration is higher, dilute the DNA with 10 mM Tris pH 8.0 to 0.03 pmol/µl. Prepare aliquots of 3 µl vector (~0.1 pmol) into 0.2 ml PCR tubes and store the aliquots at -20 °C.

Note 3: *If the concentration is lower than 0.03 pmol/µl, adjust the volume of the aliquot such that the total amount of DNA is still 0.1 pmol/µl. The volume should however not exceed 5.5 µl (thus the minimal concentration of DNA is ~0.016 pmol/µl).*

Insert preparation

- Perform a PCR reaction to obtain your DNA insert PCR reactions. Primers should contain the proper LIC overhang sequences! PCR reactions can be performed using standard procedures for any other PCR reactions, taking into account the Tm of the primer and using a polymerase that has proofreading capability to reduce the incorporation of unspecific mutations.
- Purify the DNA fragment by gel extraction (see procedure described for vector preparation) and determine the DNA concentration using equation 1. The DNA concentration should be between 0.016 and 0.1 pmol/µl. If the concentration is higher, dilute (part of) your fragment to get within this range.

T4 polymerase exonuclease reaction

- To create the specific overhangs for vector and PCR fragments, prepare the following solutions (in 0.2 µl PCR tube):

  Note that only the DNA base which is preceding the LIC overhang sequence and stalls the exonuclease activity should be added to the reactions; in case of the NKI vectors this means **dTTP** for the vector treatment and **dATP** for treatment of the PCR fragment.

Vector	**Insert**
3 µl of cleaved vector stock (~ 0.1 pmol)	X µl purified PCR product (~0.1 pmol)
1 µl 10x buffer	1 µl 10x buffer
1 µl 25 mM **DNA base (e.g. dTTP)**	1 µl 25 mM **DNA base (e.g. dATP)**
1 µl 50 mM DTT	1 µl 50 mM DTT
1 µl 1 mg/ml BSA (=10x BSA)	1 µl 1 mg/ml BSA (=10x BSA)
0.5 µl T4 DNA polymerase (1,500 units)	0.5 µl T4 DNA polymerase (1,500 units)
2.5 µl H_2O	Y µl H_2O
Total volume: 10 µl	Total Volume: 10 µ

- Transfer the tubes to a PCR machine and run the following program:
- 25 minutes 22 °C followed by 20 min 75 °C enzyme inactivation and a final cooling down to 10 °C.

Note 4: *If the vector concentration was different, adjust the volume of vector and H_2O, such that ~0.1 pmol of vector is added.*

Note 5: *We always use the New England Biolab (NEB) buffer 2 as 10x buffer. If you don't have this solution, prepare your own 10x buffer:* *50mM NaCl*
10mM Tris-HCl
10mM MgCl2
1mM DTT
pH 7.9@25°C

Note 6: *We purchase T4 DNA polymerase from NEB, but any T4 polymerase should be fine.*

Annealing Reaction and transformation

- Mix 1.5 µl of vector (~0.015 pmol) + 3 µl of insert (~0.03 pmol) and incubate 5 min at room temperature. The remainder of T4-treated vector and fragment can be stored at -20 °C.
- Add 2 µl 50 mM EDTA and incubate for another 5 minutes at room temperature
- Transform 2.5 µl of DNA into 50 µl of E. coli competent cells (any cloning strain is fine; e.g. DH5α, HB101, Top10, XL1 Blue etc.). Incubate DNA and cells for 10 minutes on ice, heatshock for 45 seconds at 42 °C, incubate 10 minutes on ice and add 500 µl SOC or LB buffer. Incubate for 1 hr at 37 °C and plate 200 µl onto a LB-agar plate containing the respective antibiotic
- The remainder of T4-treated vector and fragment can be stored at -20 °C.

Note 7: *The number of colonies obtained after transformation depends on quality and concentration of DNA fragments and competency of E. coli cells. The number of colonies may vary between 20 to > 200.*