Manual

Jena Bioscience
Building Blocks of Life

CyClone Ligase-free Cloning Kit

Efficient plasmid construction for transformation of *Escherichia coli*

Cloning Kit

Cat. No.	Amount
PP-110S	20 reactions
PP-110L	100 reactions

For in vitro use only
Quality guaranteed for 12 months
Store at -20°C, avoid frequent thawing and freezing

CyClone Polymerase (red cap)

2.5 units/µl CyClone Polymerase in storage buffer

dNTP Mix (white cap)

10 mM each dNTP (dATP, dCTP, dGTP, dTTP)

CyClone Buffer (green cap)

10x concentrated

PCR-grade Water (white cap)

Supplements (to be provided by user)

Restriction enzymes (available from Jena Bioscience)
PCR purification kit (available from Jena Bioscience)
Gel extraction kit (available from Jena Bioscience)
Competent *E. coli* cells

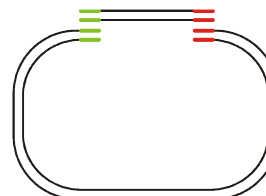
Description

CyClone Ligase-free Cloning Kit is based on a new cloning technique invented by Quan and Tian in 2009. It offers a number of advantages over conventional cloning methods. The system:

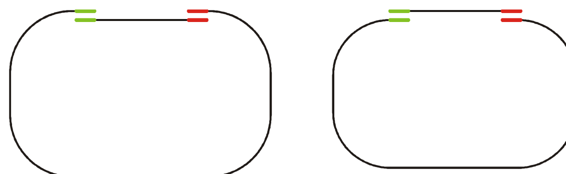
- works with any vector that can be linearized
- allows efficient cloning even into blunt end vectors
- allows directed cloning into single-cut vectors
- allows fast and easy preparation of vector and insert with no or only few purification steps
- does not require to dephosphorylate the vector
- allows the use of any restriction enzyme that linearizes the vector even if its recognition site(s) are present in the insert
- does not need a ligation step
- does not add additional sequences to the plasmid or the insert

Principle

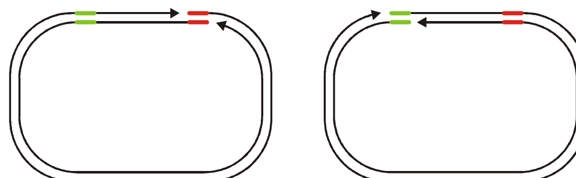
CyClone Ligase-free Cloning is based on generation of inserts with homologous ends to the linearized vector.



In a circularization reaction, vector and insert anneal due to their homologous ends.



Using a specially selected DNA polymerase, the resulting single-stranded plasmids are recircularized.



These plasmids can directly be used for transformation. They still have two nicks each, which will be repaired by *E. coli*'s endogenous DNA repair system and thus do not have to be ligated in vitro.

CyClone Ligase-free Cloning Kit

Efficient plasmid construction for transformation of *Escherichia coli*

Cloning Kit

Protocol

1 Vector preparation

Linearization with more than one enzyme will result in a higher percentage of positive clones. If the used restriction sites are not reconstructed after cloning and not present in the target fragment, the inactivation or purification steps can be omitted. Make sure the vector is linearized completely to reduce background in the transformation step (chapter 5).

1.1 With enzymes that can be heat inactivated:
- Linearize 500 ng of vector in 20 µl total reaction volume in CyClone Buffer, use at least 2 units of enzyme per µg of plasmid DNA
- Refer to Table 1 for optimal buffer concentrations for each enzyme
- Heat inactivate restriction enzymes

1.2 With enzymes that cannot be heat inactivated:
- Linearize 2 µg of vector in 40 µl total reaction volume in CyClone Buffer, use at least 2 units of enzyme per µg of plasmid DNA
- Refer to Table 1 for optimal buffer concentrations for each enzyme
- Remove enzymes with a DNA purification kit

Enzyme	CyClone Buffer	Enzyme	CyClone Buffer	Enzyme	CyClone Buffer
ApaI	1x	HpaI	1x	PvuII	1x
ApaLI	0.9x	KpnI	1x	SalI	2x
AsuII	1x	MluI	1x	ScaI	1x
BamHI	1x	MspCI	2x	SlaI	1x
BglII	1x	NaeI	1x	SmaI	1x
BseAI	1x	NcoI	1x	SnaBI	1x
BssHII	1x	NdeI	1x	SpeI	1x
CspAI	1x	NheI	1x	SphI	2x
DraI	1x	NotI	1x	SseBI	1x
EcoRI	1x	NruI	1x	SspI	1x
EcoRV	1x	PstI	1x	SstI	1x
HindIII	1x	PvuI	1x	XbaI	1x

Table 1: Optimal final CyClone buffer concentrations for individual restriction enzymes

2 Primer design

- Primers need a 5' extension with the same sequences as the ends of the linearized vector
- Melting temperature of the overlap should be in the range of 60-70 °C
- When linearizing the plasmid with enzymes that create sticky ends, use the strand with the overhang for calculations
- HPLC purification of the primers is recommended

Example: Vector was cut with NcoI and NotI. Vector is shown in lower case, primers in capitals, with the overlap in red and bold. Ns denote the primer sequence for amplification of the insert.

Forward primer:

CCTTGCCACCAGATCTGCCATGNNNNNN...

...gtgccttgccaccagatctgc
...cacggaacggtggtctagacggtac

Reverse primer:

ggccgccctcctcctcctttcttgttc...
cgggaggaggaggaaagaacaag...
...NNNNNN**CCGGCGGGAGGAGGAGGAAAGA**

3 Insert preparation

3.1 Standard preparation
- Perform PCR in 1x CyClone Buffer with 0.2 mM dNTPs and 1 µl of CyClone Polymerase per 100 µl of reaction volume and a final primer concentration of 1 µM for 25-30 cycles
- Set elongation temperature to 68 °C and elongation time to 1.5 min per 1000 bp
- Isolate fragment from agarose gel

3.2 Quick preparation

This works only if the PCR template does not have the same antibiotic resistance as the target vector, or if the PCR template is removed by Dpn I digestion.

- Perform PCR in 1x CyClone Buffer with 0.2 mM dNTP Mix and 1 µl of CyClone Polymerase per 100 µl of reaction volume and a final primer concentration of 125 nM for 25-30 cycles
- Further purification of the insert is not necessary

4 Circularization reaction

4.1 Components

Prepare the following reaction (20 µl volume):

- 100 ng of linearized vector
- 10-fold molar excess of insert
- 1x CyClone Buffer (note that fragment and/or vector may already contain buffer, add only that amount of buffer to reach 1x final concentration)
- 0.2 mM dNTP Mix

Jena Bioscience GmbH | Loebstedter Str. 71 | 07749 Jena, Germany
Phone +49-3641-6285 000 | Fax +49-3641-6285 100
www.jenabioscience.com

IFTA AG
Certified QMS and EMS according to
DIN EN ISO 9001 and DIN EN ISO 14001
Reg.-No.: ICV03597 534 and ICV03597 034 14

CyClone Ligase-free Cloning Kit

Efficient plasmid construction for transformation of *Escherichia coli*

Cloning Kit

- 0.5 units CyClone Polymerase
- Add PCR-grade Water to a final volume of 20 µl

As a control, perform the same reaction without adding any insert.

4.2 Reaction conditions

- Step 1: 30 sec 94 °C
- Step 2: 30 sec 50 °C (depends on annealing temperature of the overlaps, 50 °C will work in most cases)
- Step 3: x min 68 °C (depends on vector or fragment size, whichever is larger, set to 1.5 min / 1000 bp)
- 10 cycles

5 Transformation

- Transform competent *E. coli* cells, using your standard transformation protocol, with 5 µl of the circularization reaction or the control
- Transformation with the reaction should yield 100-1000 colonies, of which 95-99% are positive (less if the vector has not been cut efficiently)
- Transformation of the control usually gives 1-50 colonies and shows the amount of background from negative clones with non-linearized vector

References

Quan and Tian (2009) Circular polymerase extension cloning of complex gene libraries and pathways. *PLoS* One **4**:e6441