TaKaRa DNA Ligation Kit LONG

Cat.# 6024
v.0704

TaKaRa

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I. Description:

Takara Ligation Kit LONG provides the reagents optimized for the construction of recombinant DNA with long DNA fragment. It is powerful tool for the cloning of over 10 kbp DNA, especially for the construction of BAC library. Takara Ligation Kit LONG is suitable for applications described below:

- Vector construction with long size DNA fragment
- BAC library construction
- Long size cDNA cloning

II. Kit Component (For 50 reactions*):

DNA Ligase <LONG>	50 μl
10 × LONG Ligation Buffer	300 μl
Control Insert DNA/*Hin*d III(18 kb)	50 μl
Control Vector (pUC118/Hind III/BAP)	10 μl
dH_2O	1 ml

* For cohesive-end ligation, it is designed for 50 reactions.
For blunt-end ligation, it is designed for 10 reactions.

III. Storage:

Store at − 20℃

IV. Procedure:

(1) Standard protocol for vector ligation of cohesive-end DNA

1. Assemble the reaction mixture described below in a microcentrifuge tube or PCR tube at room temperature.

[reaction mixture]		
vector DNA*[1]	X μl	(25 – 50 ng)
insert DNA*[1]	Y μl	
10 × LONG Ligation Buffer	5 μl	
dH_2O	Z μl	
	49 μl	

2. Heat the reaction mixture (without enzyme) for 3 minutes at 65 ℃ and immediately cool on ice.
3. Add 1 μl of DNA Ligase <LONG> to the reaction mixture on ice.
4. Incubate the reaction for 3 - 15 hours at 16 ℃ .
5. Transform 100 μl of *E.coli* competent cells directly with 4 – 10 μl of the ligation reaction mixture. If more than 10 μl of the ligation reaction mixture must be used for transformation, then the ligated DNA should instead be ethanol precipitated and dissolved in an appropriate buffer prior to use.*[2]

*1 Dissolve DNA in an appropriate buffer such as TE buffer. Refer to V. Note 1,3.
*2 Refer to V. Note 5, 6.

(2) Standard protocol for vector ligation of blunt-end DNA

1. Assemble the reaction mixture described below in a microcentrifuge tube or PCR tube at room temperature.

[reaction mixture]		
vector DNA*1	X μl	(50 – 100ng)
insert DNA*1	Y μl	
10 × LONG Ligation Buffer	5 μl	
dH_2O	Z μl	
	45 μl	

2. Heat the reaction mixture (without enzyme) for 3 minutes at 65 ℃ and immediately cool on ice.
3. Add 5 μl of DNA Ligase <LONG> to the reaction mixture on ice.
4. Incubate for 3 – 15 hours at 16 ℃ .
5. Transform 100 μl of *E.coli* competent cells directly with 4 – 10 μl of the ligation reaction mixture. If more than 10 μl of the ligation reaction mixture must be used for transformation, then the ligated DNA should instead be ethanol precipitated and dissolved in an appropriate buffer prior to use.*2

*1 Dissolve DNA in an appropriate buffer such as TE buffer. Refer to V. Note 1, 3.
*2 Refer to V. Note 5, 6.

(3) Control Reaction

1. Assemble the reaction mixture described below in a microcentrifuge tube or PCR tube at room temperature to prepare a reaction mixture described below:

[reaction mixture]	
Control Vector (pUC118/*Hin*d III/BAP) (25 ng/ μl)	1 μl
Control Insert DNA/*Hin*d III (18 kbp) (25 ng/ μl)	3 μl
10 × LONG Ligation Buffer	5 μl
dH_2O	40 μl
	49 μl

2. Heat the reaction mixture (without enzyme) at for 3 minutes 65℃ . Immediately cool on ice.
3. Add 1 μl of DNA Ligase <LONG> to the reaction mixture on ice.
4. Incubate for 3 – 15 hours at 16 ℃ .
5. Transform *E.coli* competent cells using 4 μl of the ligation reaction mixture and spread the transformed *E.coli* cells on LB-Amp plates containing X-Gal and IPTG. When using *E.coli* competent cells (DH5a, JM109 and HST02) possessing 1.0 x 10^8 cfu/ μg of the transforming efficiency, over 1 x 10^6 white colonies can be obtained.

V. Note:

1. Preparation of vector DNA and long size insert DNA
   - Minimize exposure of cloning vector and insert DNA to UV irradiation which can cause a decrease in the cloning efficiency.
   - To prevent unwanted shearing of the large DNA fragments that will be cloned, use wide-bore pipette tips when transferring the DNA.
   - If you need higher cloning efficiency, perform a ligation reaction using DNA fragments with cohesive-ends because cohesive-end ligation is 10 - 100 times as effective as blunt-end ligation.

2. Cloning of PCR product
   - We cannot recommend the TA-cloning of large PCR products containing 3′ A-overhang. If you need to clone such PCR products, blunt the fragment′ s ends with T4 DNA polymerase and clone the blunted PCR product into a blunted vector.
   - PCR products may include some non-specific amplification products, even though you observe only a single band on the electrophoresis gel corresponding to your product. Therefore, for effective cloning of large PCR products, we recommend that you gel purify your fragment of interest.
   - PCR products amplified with high fidelity DNA polymerase (PrimeSTAR® HS DNA Polymerase etc.) have non-phosphorlyrated blunt ends due to the 3′ → 5′ exonuclease activity of the DNA polymerase. When using high fidelity DNA polymerase, phosphate group must be added to the 5′ end of primers used in PCR reaction or the amplification products themselves must be phosphorylated with T4 polynucleotide kinase.

3. Ratio of Insert DNA/Vector DNA

In general, a lower ratio of vector/insert DNA will decrease a efficiency of ligation reaction, whereas a higher ratio of vector/insert DNA will favor formation of multiple insert clone (chimera).Optimization of vector/insert ratio and concentration are important factor for success of ligation. Utilization of higher concentration of vector DNA results in increasing in the total number of recombinants, but decreasing in the cloning efficiency. The concentration of insert DNA depends on its size relative to the size of vector

Cohesive end

For 2 kbp to10 kbp vector, the optimum concentration of vector DNA is 0.5 ng to 1 ng/ μl in reaction solution. Find a optimum condition with molor ratio of vector : insert =2:1 ～ 10:1.
It is recommended to use same amount (ng) of Insert DNA and vector DNA.
The molar ratio of vector increases by the size of insert compared with the usual ligation condition.

Blunt end

For 2 kbp to10 kbp vector, the optimum concentration of vector DNA is 1 ng to 2 ng/ μl in reaction solution. Find a optimize condition with molor ratio of vector : insert =1:2 ～ 10:1.
It is recommended to use same amount (ng) of Insert DNA and vector DNA.
The molar ratio of vector increases by the size of insert compared with the usual ligation condition.

4. Reaction time

Perform cohesive-end ligation for 3 – 15 hours at 16 ℃ (Figure 1). Blunt-end ligation proceeds more slowly, therefore incubate reaction mixtures at lease for 15 hours at 16 ℃ (Figure 2).

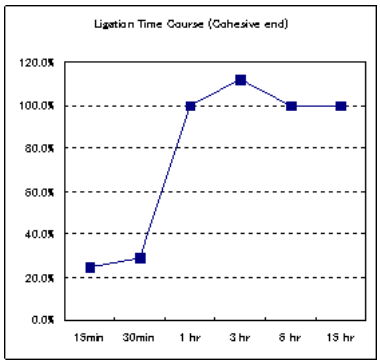

Figure 1. Time course of cohesive-end ligation

18 kbp DNA fragment digested with *Hin*d III were ligated into the pUC118/*Hin*d III/BAP vector by following the standard protocol. Ligation products were transformed into chemically competent cells and grown overnight on LB-amp plates at 37℃ .

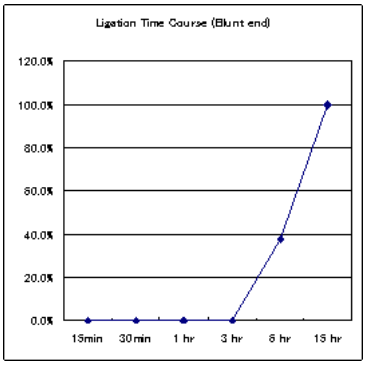

Figure 2. Time Course of blunt-end ligation.

18 kbp DNA fragments digested with *Sma* I were ligated into the pUC118/*Hin*c II/BAP vector by following the standard protocol. Ligation products were transformed into chemically competent cells and grown overnight on LB-amp plates at 37℃ .

5. Transformation with electroporation method

- We confirmed that about less than 20 kbp of ligation products could be transformed using a chemically competent *E.coli* competent cells. Electroporation method can increase transformation efficiency and be powerful method for constructing a large size recombinant DNA (more than 20 kbp) and a library.

- Ligation reaction mixture can not be used directly for electroporation. Before using the reaction products for electroporation, reaction buffer must be replaced to H_2O or TE using an ethanol precipitation or a dialysis etc. We recommend Drop Dialysis method (refer to 6) among various dialysis methods. Do not use a phenol/chloroform isolation to prevent the disruption of large size ligation products.
- *E.coli* has mechanism for restriction of foreign DNA. This mechanism is known as *Eco*K I restriction system encoded by hsdRMS and the metylation-requiring restrictionsystem encoded by mcrA, mcrB, mcrC, mcr and mrr. These systems has significant problem in cloning of foreign DNA, especially long size fragments, resulting in substantially reduced recovery of desired clones. We recommend using an *E.coli* strain that has mutation of these systems like HST02 for cloning of large size DNA fragments.

E. coli HST02 Competent Cells (TaKaRa Cat.# 9127)
E. coli HST02 Electro Competent Cells (TaKaRa Cat.# 9026)
Genotype of HST0 2: F' (*laclq Δ lacZM15 proAB*) *endA1 gyrA96 thi supE44 relA1 traD36 Δ (lac-proAB) e14 (mcrA) Δ deoR recA Δ (mrr-mcrBC hsdRMS)*

6. Procedure of Drop Dialysis method (buffer replacement for electropolation)[1)]
   1) Pour 25 ml of 1/10 TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), into a petri dish (90 mm diameter) on ice.
   2) Float a 0.025 μ m Type-VS Millipore membrane on the 1/10 TE buffer.
   3) The Ligation solution drop carefully onto the center of the filter using a wide bore pipette tip.
   4) Cover the petri dish and dialyze for 4 hours on ice. Carefully stir the 1/10 TE buffer every 1 hour.
   5) Carefully recover the dialyzed DNA using a wide bore pipette tip and place in a microcentrifuge tube.
   6) Transform competent *E.coli* Electro cells with 1 to 10 μ l of the recoverd sample.

VI. Reference:

1) Osoegawa, K. *et al*., (1998) *Genomics* **52**, 1.

VII. Related products:

E. coli HST02 Competent Cells (TaKaRa Cat.# 9127)
E. coli HST02 Electro Competent Cells (TaKaRa Cat.# 9026)
TaKaRa DNA Ligation <Mighty Mix> (TaKaRa Cat.# 6023)
TaKaRa BKL Kit (TaKaRa Cat.# 6126)
T4 Polynucleotide Kinase (TaKaRa Cat.# 2021A/B)
T4 DNA Polymerase (TaKaRa Cat.# 2040A/B)