Cat. # 6024

For Research Use

TaKaRa

TaKaRa DNA Ligation Kit LONG

Product Manual

v201707Da

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I. Description

TaKaRa DNA Ligation Kit LONG enables the efficient cloning of longer DNA fragments. It is a powerful tool for cloning DNA over 10 kb in length, and also for the construction of BAC libraries. This kit is especially well suited to the following applications:

- Plasmid construction with a long DNA fragment
- BAC library construction
- Long cDNA cloning

Stellar™ Competent Cells (Cat. #636763/636766) and Stellar Electrocompetent Cells (Cat. #636765) are recommended for transformation. These cells have advantages that make them well suited for constructing clones with longer insert DNA or cDNA.

II. Components (for 50 reactions*)

DNA Ligase <LONG>	50 µl
10X LONG Ligation Buffer	300 µl
Control Vector (pUC118/*Hin*d III/BAP)	10 µl
Control Insert DNA/*Hin*d III (18 kb)	30 µl
dH_2O	1 ml x 2

* For cohesive-end ligation, this kit is designed for 50 reactions with a 50-µl reaction volume.
For blunt-end ligation, this kit is designed for 10 reactions with a 50-µl reaction volume.

III. Storage

-20℃

IV. Protocol

[1] Standard protocol for vector ligation of cohesive-end DNA

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

Reagent	Volume
Vector DNA*1	X μl (25 - 50 ng)
Insert DNA*1	Y μl
10X LONG Ligation Buffer	5 μl
dH_2O	Z μl
Total	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 with 4 - 10 μl of the ligation reaction mixture. When more than 10 μl of the ligation reaction mixture is used for transformation, or transformation is performed via electroporation, the ligated DNA should be ethanol precipitated and dissolved in an appropriate buffer prior to use.*2

*1 Use vector and insert DNA dissolved in TE buffer, etc.
The recommended concentration of vector DNA (2 - 10 kb) in the reaction is 0.5 - 1.0 ng/μl. A 1:1 ratio of vector:insert DNA will likely provide successful results. However, if there is an issue with efficiency, try performing the reaction at a molar ratio of vector : insert = 2:1 - 10:1.
Refer to "V. Notes 1, 3".

*2 Refer to "V. Notes 5, 6".

[2] Standard protocol for vector ligation of blunt-end DNA

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

Reagent	Volume
Vector DNA*1	X μl (50 - 100 ng)
Insert DNA*1	Y μl
10X LONG Ligation Buffer	5 μl
dH_2O	Z μl
Total	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 15 hours at 16℃.
5. Transform 100 μl of *E. coli* competent cells with 4 - 10 μl of the ligation reaction mixture. When more than 10 μl of the ligation reaction mixture must be used for transformation, or transformation is performed via electroporation, the ligated DNA should be ethanol precipitated and dissolved in an appropriate buffer prior to use.*2

*1 Use vector and insert DNA dissolved in TE buffer, etc.
The recommended concentration of vector DNA (2 - 10 kb) in the reaction is 1 - 2 ng/μl. A 1:1 ratio of vector:insert DNA will likely provide successful results. However, if there is an issue with efficiency, try performing the reaction at a molar ratio of vector : insert = 1:2 - 10:1.
Refer to "V. Notes 1, 3".

*2 Refer to "V. Notes 5, 6".

[3] Control reaction

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

Reagent	Volume
Control Vector (pUC118/*Hin*d III/BAP) (25 ng/μl)	1 μl
Control Insert DNA/*Hin*d III (18 kb) (25 ng/μl)	3 μl
10X LONG Ligation Buffer	5 μl
dH_2O	40 μl
Total	49 μl

2. Heat the reaction mixture (without enzyme) at for 3 minutes 65℃. Immediately cool on ice for 3 minutes.
3. Add 1 μl of DNA Ligase <LONG>.
4. Incubate for 3 - 15 hours at 16℃.
5. Transform 100 μl of the *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 (Stellar, DH5α, JM109, and HST02) with a transformation efficiency of 1.0×10^8 cfu/μg, over 1×10^6 white colonies can be obtained.

V. Notes

1. Preparation of vector DNA and long insert DNA

- Minimize exposure of the cloning vector and insert DNA to UV irradiation during purification via gel electrophoresis, as this can lead to a decrease in cloning efficiency.
- For higher cloning efficiency, perform a cohesive-end ligation, as it is 10 - 100 times more effective than blunt-end ligation.

2. Cloning of PCR products

- We do not recommend the TA-cloning of large PCR products that have a 3′ A-overhang. If cloning this type of PCR amplicon, blunt the fragment's ends with T4 DNA polymerase and clone into a linearized vector with blunt ends.
- PCR products may include some non-specific amplification products, even when only a single band is observed in gel electrophoresis. To effectively clone long PCR products, we recommend that the fragment of interest is first purified via gel electrophoresis.
- PCR products amplified with a high-fidelity DNA polymerase (e.g., PrimeSTAR® HS DNA Polymerase, etc.) have non-phosphorylated blunt ends due to the 3′→5′ exonuclease activity of the DNA polymerase. Therefore, a phosphate group must be added to the 5′ end of the primers used in the PCR reaction, or the amplification products themselves must be phosphorylated with T4 polynucleotide kinase before the ligation reaction.

3. Insert DNA/vector DNA ratio

When insert DNA is low as compared with vector DNA at a 1:1 molar ratio, efficiency of the ligation reaction will decrease. On the other hand, plasmids containing multiple inserts tend to appear when the amount of insert DNA is high. Optimization of vector:insert ratio and concentration are important factors for successful ligation. Utilization of a higher concentration of vector DNA results in an increase in the total number of recombinants, but a decrease in cloning efficiency.

Cohesive ends
For vectors between 2 and 10 kb, the optimum concentration of vector DNA is 0.5 to 1 ng/μl in the reaction solution. Optimal reaction conditions use a vector: insert molar ratio of 2:1 - 10:1.

Blunt ends
For vectors between 2 and 10 kb, the optimum concentration of vector DNA is 1 to 2 ng/μl in the reaction solution. Optimal reaction conditions use a vector: insert molar ratio of 1:2 - 10:1.

4. Reaction time

Perform cohesive-end ligation for 3 - 15 hours at 16℃ (Figure 1). Blunt-end ligation proceeds more slowly, and should therefore be incubated for at least 15 hours at 16℃ (Figure 2).

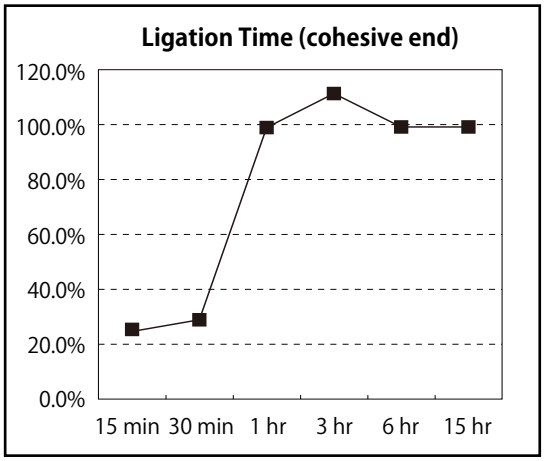

Figure 1. Reaction time of cohesive-end ligation

An 18-kb DNA fragment digested with *Hin*d III was ligated into a 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℃. The number of colonies on the plate were counted and the ratios of colony number at each time point are shown as 100% of the colony number at 15 hr.

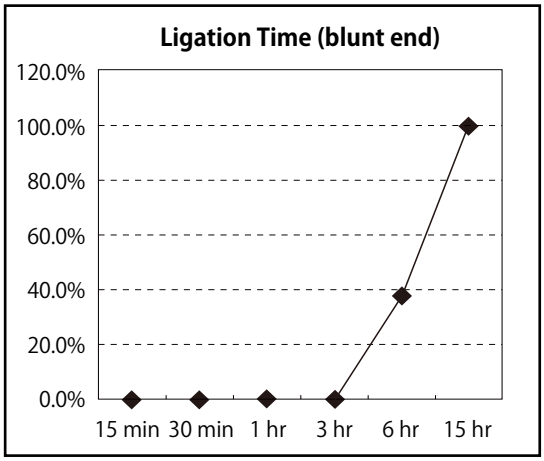

Figure 2. Reaction time of blunt-end ligation

An 18-kb DNA fragment digested with *Sma* I was ligated into a 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℃. The number of colonies on the plate were counted and the ratios of colony numbers at each time are shown as 100% of the colony number at 15 hr.

5. Transformation via electroporation

- We confirmed that a ligated plasmid less than 20 kb could be transformed into chemically competent *E. coli* cells. Electroporation can increase transformation efficiency and be a powerful method for constructing large recombinant DNA (more than 20 kb) or libraries.
- Ligation reaction mixtures cannot be used directly for electroporation. Before electroporating, the reaction buffer must be replaced with H_2O or TE buffer by ethanol precipitation or dialysis, etc. We recommend the Drop Dialysis method (refer to Section 6) from among the various available dialysis methods. Do not use a phenol/chloroform extraction, as this can cause a decrease in transformation efficiency.
- *E. coli* has mechanisms for the restriction of foreign DNA. These mechanisms are the *Eco*K I restriction system (encoded by *hsdRMS*) and the methylation-requiring restriction system (encoded by *mcrA*, *mcrB*, *mcrC*, *hsdRMS*, *mcr*, and *mrr*). These systems create significant problems in the cloning of foreign DNA, especially for longer fragments, and result in substantially reduced recovery of desired clones. We recommend using an *E. coli* strain that has mutations in these systems (e.g., HST08 Premium) for cloning large DNA fragments.

  E. coli HST08 Premium Competent Cells (Cat. #9128)
  E. coli HST08 Premium Electro-Cells (Cat. #9028)

  [Genotype of HST 08 Premium]
  F^-, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, φ80d*lacZ*Δ*M15*, Δ (*lacZYA-argF*) U169, Δ (*mrr-hsdRMS-mcrBC*), Δ*mcrA*, λ^-

6. Procedure for the 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 (120 mm diameter) on ice.
(2) Float a 0.025-μm Type-VS Millipore membrane on the 1/10 TE buffer.
(3) Carefully drop the ligation reaction onto the center of the filter using a wide-bore pipette tip.
(4) Cover the petri dish and dialyze for over 3 hours on ice. Carefully stir the 1/10 TE buffer every 30 minutes - 1 hour.
(5) Carefully recover the dialyzed DNA using a wide-bore pipette tip and place in a microcentrifuge tube.
(6) Transform electrocompetent *E. coli* cells with 1 to 10 μl of the recoverd sample.

VI. Reference

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

VII. Related Products

Stellar™ Competent Cells (Cat. #636763/636766)
Stellar™ Electrocompetent Cells (Cat. #636765)
TaKaRa DNA Ligation <Mighty Mix> (Cat. #6023)
T4 Polynucleotide Kinase (Cat. #2021S/A/B)
T4 DNA Polymerase (Cat. #2040A/B)