

LINKeed. Rapid DNA Ligation Kit

DESCRIPTION

Two crucial procedures in cloning are the ligation of the foreign DNAs to the vector DNA and the transformation of bacteria using those ligated DNA constructs (the recombinant molecules). Ligation is accomplished using the enzyme DNA ligase (usually from the bacteriophage T4). It requires ATP and magnesium ions to catalyze the reaction of a 3'-OH and a 5'-P on double-stranded DNA to form a phosphodiester bond. The DNA ends can be cohesive ends, such as those formed between molecules that have been digested with the same restriction endonuclease, or they can be blunt ends. Ligation between cohesive-ended molecules is much more efficient than ligation between blunt-ended molecules. Because of this, when ligating blunt-ended molecules, the DNA concentration must be higher than when ligating cohesive-ended molecules. The rates of blunt-end and cohesive-end ligation of DNA by T4 DNA ligase are increased by orders of magnitude in the presence of Polyethylene glycol (PEG) [Pheiffer, B.H., Zimmerman, S.B., Nucleic Acids Res., 11, 7853-7871, 1983]. The LINKeed. Rapid DNA Ligation Kit is based on this PEG-assisted rapid ligation of DNA and enables sticky- and blunt-ended DNA ligation in only 10 min. 5×Rapid Ligation Buffer (RLB) supplied in this kit is specially designed for the efficient rapid ligation of DNA. For an unknown reason, while ligation efficiency appears to increase for high PEG concentration, the transformation efficiency for molecules ligated in this condition decreases. Therefore, appropriate PEG concentration should be selected. At the PEG concentration utilized in the LINKeed. Rapid DNA Ligation Kit, ligation and transformation are both increased significantly.

STORAGE

All components should be stored at -20°C. Do not substitute the reaction buffer supplied in this kit with other one.

CONTENTS

For research use only. The LINKeed. Rapid DNA Ligation Kit contains sufficient reagents to perform approximately 30 × 20 µl DNA ligation reactions.

Materials Provided	Size (30 reactions)
T4 DNA ligase	30 µl
5 × Rapid Ligation Buffer (5x RLB)	200 µl

CHARACTERISTICS

Sufficient ligation efficiency can be archived at room temperature (20-25°C) for 10-30 min.

STORAGE BUFFER

10 mM	Tris-HCl (pH 7.5)
50 mM	KCl
1 mM	Dithiothreitol
50%	Glycerol

QUALITY CONTROL

T4 DNA ligase supplied in this kit is free of detectable exo- or endonuclease activities. Each lot is functionally tested in the ligation reaction. Additionally, each lot is analyzed by SDS polyacrylamide gel electrophoresis for the presence of detectable contaminating proteins (less than 5%).

• Exonuclease assay:

After incubation of a 50 µl reaction containing T4 DNA ligase with 1 µg of sonicated [³H]-labeled *E.coli* DNA (10⁵ cpm/µg) for 4 hours at 37°C. The DNA is precipitated with trichloroacetic acid and the radioactivity of the supernatant is evaluated. Exonuclease activity is expressed as a percent of total DNA radioactivity released into the acid soluble fraction. The exonuclease activity was determined to < 0.1% radioactivity. The limit of detectability of this assay is approximately 0.05%.

• Endonuclease assay:

Incubation of a 50 µl reaction containing T4 DNA ligase with 1 µg of supercoiled plasmid DNA for 4 hours at 37°C resulted in < 0.1% conversion to nicked or linear plasmid DNA as determined by agarose gel electrophoresis.

PROTOCOL

A. Cloning of DNA fragments into plasmid vectors

We recommend to use a 1:1 or 1:3 molar ratio of vector:insert DNA. These ratios will vary with the types of vectors, for example, cDNA and genomic DNA cloning vectors. To calculate the required amount of insert DNA, the following formula can be used.

$$\frac{(\text{ng of vector}) \times (\text{kbp size of insert})}{\text{kbp size of vector}} \times \text{molar ratio of } \frac{\text{Insert}}{\text{vector}} = \text{ng of insert}$$

[Example]

How much 0.5 kbp insert DNA should be added to a ligation in which 100 ng of 6 kbp vector will be used? The desired vector:insert ratio will be 1:3.

$$\frac{(100\text{ng vector}) \times (0.5\text{kbp size of insert})}{6\text{kbp size of vector}} \times \frac{3}{1} = 25 \text{ ng}$$

1. Prepare the following reaction in a sterile microcentrifuge tube:

Vector DNA	100 ng
Insert DNA	25 ng
5 × RLB	4 µl
T4 DNA ligase	1 µl
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Nuclease-free water to final volume	20 µl

2. Incubate the reaction at room temperature for 10 min.
3. Competent *E. coli* was transformed with the ligation mixture.
(Competent cell efficiency: > 1 × 10⁷)

B. Recircularization of linear DNA

The amount of DNA is important in recircularization. For the enhanced recircularization, lower DNA concentration is helpful compared to the cloning of DNA fragment into plasmid vectors because low DNA concentration makes more intramolecular ligation (recircularization) and suppress intermolecular ligation. Intramolecular ligation is Concentration-independent.

[Example]

1. Prepare the following reaction in a sterile microcentrifuge tube:

Vector DNA	20~30 ng
5 × RLB	4 μl
T4 DNA ligase	1 μl
Nuclease-free water to final volume	20 μl

2. Incubate the reaction at room temperature for 10 min.
3. Competent *E. coli* was transformed with the ligation mixture.
(Competent cell efficiency: $> 1 \times 10^7$)

C. Adaptor ligation

Adaptor ligation conditions are basically the same as for cloning of DNA fragments into plasmid vectors (8 bases or longer). However, if the adaptor is shorter than 8 bases or if GC-contents are low in adaptors, the ligation reaction should be carried out at 16°C for 30minutes to 2 hours. We recommended vector/linker molar ratio is:

- Dephosphorylated vector: Phosphorylated linker $> 1:100$

[Example]

1. Prepare the following reaction in a sterile microcentrifuge tube:

DNA fragment	100 ng
Adaptor	25 ng
5 × RLB	4μl
T4 DNA ligase	1 μl
Nuclease-free water to final volume	20 μl

2. Incubate the reaction at 16°C for 1 hour.
3. Competent *E. coli* was transformed with the ligation mixture.
(Competent cell efficiency: $>1 \times 10^7$)

or

In the case of adaptor ligation to termini, inactivate T4 DNA ligase by heating at 70°C for 10 min and appropriate purification is performed for user's purpose.

TROUBLESHOOTING GUIDE

There are many problems with ligation reaction itself and with the competent cells, the selection medium, the restriction endonuclease digestion of the vector, and the phosphatase treatment of the vector. Because transformation of some competent cells is inhibited by components of the ligation reaction, the reaction should be diluted fivefold before being used for transformation. Some possible causes of unsuccessful ligation are listed next along with suggested solutions.

Causes of problem	Suggested solution
Inhibitors of DNA ligase are present in the DNA	Purify the DNA with phenol extraction and ethanol precipitation. Contaminants from fragments eluted from agarose gels can often inhibit ligase. Use of spermidine (at 3-5 mM) can alleviate some of this inhibition.
DNA ligase is inactive	Use fresh T4 DNA ligase
ATP in the reaction buffer has degraded	Use 5× Rapid Ligation Buffer that is < 24 months old and store the buffer at -20 °C.
Restriction endonucleases are present, causing redigestion of ligated products	Remove the restriction endonucleases by phenol extraction and ethanol precipitation. Or heat-inactivation may be helpful.
DNA is degraded by nonspecific endonucleases contaminating the reaction mixture	Use fresh components and autoclaved molecular biological grade H ₂ O.

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