

Cat. # 6022

For Research Use

TAKARA

DNA Ligation Kit Ver. 2.1

Product Manual

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I. Description

The DNA Ligation Kit is a simple system that enables very rapid DNA ligation reactions. The kit allows for a small final reaction volume through the use of a single enzymatic solution containing both T4 DNA Ligase and an optimized buffer. Because of high efficiency reaction, this kit enables efficient ligation in just 3 minutes for the majority of reactions and in only 30 minutes for more challenging reactions.

Transformation efficiency for ligated circular DNA can be improved by the addition of Solution III (Transformation Enhancer) to the ligation mixture before transformation. Use of Solution III is especially recommended for ligation reactions where the amount of insert DNA is low, or when low ligation efficiency is expected. Following ligation, the reaction solution can be directly used for bacterial transformations without the need for DNA purification.

Ratios (by volume) of each solution for various types of ligations

	Ver.2.1
	volume ratio
Circular DNA • Ligation of DNA fragments with plasmid vectors • Ligation of linker DNA with plasmid vectors • Self-circularization	DNA solution : 1 Solution I : 1
Linear DNA • Linker (or Adaptor) ligation to cDNA • Ligation of DNA fragments with λ -phage vectors*	DNA solution : 1 Solution II : 1 Solution I : 2

* It is recommended to use TaKaRa DNA Ligation Kit Ver.1 (Cat. #6021) for this purpose.

II. Components

Solution I : Enzyme Solution	250 μ l x 3
Solution II : Concatenation Buffer	750 μ l
Solution III : Transformation Enhancer	200 μ l

* Sufficient components for 100 reactions when 7.5 μ l of Solution I is used per reaction.

III. Storage -20°C

If a precipitate appears in Solution III, dissolve completely by vortexing for several minutes. Solution III should be stored at room temperature once it was thawed.

IV. Precautions

1. Solutions I and II should be stored frozen at -20°C. These Solutions are not affected by freeze-thaw cycles. Solution I, which contains T4 DNA Ligase, should be thawed on ice and gently mixed before use. Solution II may be thawed and mixed at room temperature. Solution III, once thawed, should be stored at room temperature. If a precipitate appears in Solution III, dissolve by vortexing the solution before use.
2. DNA ligation mixtures can be loaded directly onto agarose gels for gel electrophoresis. Ethanol precipitation* is recommended for concentrating DNA samples. Extraction with phenol is not recommended because a white precipitate may be formed.

* Ethanol precipitation :

- 1) Add one-tenth volume of 3 M Sodium acetate (pH 5.2) (or one-twentieth volume of 5 M NaCl) and 2 - 2.5 volumes of ethanol into the ligation mixture.
- 2) Incubate at -20°C for 20 min, or at -80°C for 10 min.
- 3) Collect the DNA by centrifugation at 4°C. When a small amount of DNA is to be collected, carrier may be useful for ethanol precipitation.

V. Protocol and Examples

A. Ligation of a DNA fragment with a plasmid vector

Protocol

1. Mix linearized plasmid vector DNA and a DNA fragment in a total volume of 5 - 10 μ l. We recommend TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) for dissolving DNA. Recommended amounts of vector : fragment is 0.03 pmol : 0.03 - 0.3 pmol (0.03 pmol of pUC18 DNA (2,686 bp) corresponds to about 50 ng).
2. Add same volume of Solution I (5 - 10 μ l) as the DNA solution and mix thoroughly.
3. Incubate at 16°C for 30 minutes*¹.
4. The ligation reaction mixture can be used directly for transformation of *E. coli* competent cells. When performing transformation immediately after ligation, use 10 μ l of the ligation mixture to transform 100 μ l of competent cells*².

* 1 The reaction should be carried out at 16°C. Higher temperatures (<26°C) will inhibit the formation of circular DNA. If desired results are not obtained, the reaction time can be extended overnight. DNA purification by phenol extraction and ethanol precipitation may improve ligation efficiency. When ligating a T-vector and a PCR product, the reaction should be incubated up to 1 hour; longer incubation times may result in high background.

* 2 The ligation mixture can be directly used for transformation. However, more colonies (transformants) can be obtained by adding 1 μ l of Solution III to 9 μ l of the ligation mixture prior to transformation. The ligation mixture should not be used directly for electroporation. Precipitate the DNA with ethanol and dissolve in a low salt buffer, such as TE buffer. Solution III cannot be used for electroporation.

Example

50 ng of *Eco*R I-digested pUC118 vector (25 fmol) was mixed with 2.5 - 250 ng (2.5 - 250 fmol) of 1.5 kb *Eco*R I-digested DNA fragment at insert/vector ratios ranging from 0.1 to 10.0, in a total volume of 5 μ l. One volume (5 μ l) of Solution I was added to the DNA mixture. The combined solution was then incubated at 16°C for 30 minutes. A portion of the solution was used directly to transform *E. coli* JM109 competent cells and the mixture was plated on an LB-Amp plate containing X-Gal and IPTG. (The transformation efficiency of *E. coli* JM109 competent cells was 6.3×10^7 cfu/ μ g pUC118 DNA.) Transformation efficiency was obtained by counting the number of white colonies (Table 1) for both the ligation kit and ligation using T4 DNA Ligase (350 U, incubation at 16°C for 16 hours).

Table 1. Transformation efficiencies (white colonies per μ g insert DNA)

	Vector	insert/vector (molar ratio)				
		0.1	0.3	1.0	3.0	10.0
DNA Ligation Kit 30 minutes	dephosphorylated	1.7×10^6	5.0×10^6	1.7×10^7	2.3×10^7	2.1×10^7
	phosphorylated	7.8×10^5	2.5×10^6	8.2×10^6	1.7×10^7	2.3×10^7
T4 DNA Ligase 16 hours	dephosphorylated	1.6×10^5	2.0×10^5	1.8×10^6	3.1×10^6	1.9×10^6
	phosphorylated	4.6×10^5	1.0×10^6	1.9×10^6	5.0×10^6	1.2×10^7

B. Self-circularization of linear DNA (Intramolecular ligation)**Protocol**

The protocol for self-circularization of linear DNA is essentially the same as for "A. Ligation of a DNA fragment with a plasmid vector". However, it is important to use low concentrations of DNA in the ligation reaction to maximize intramolecular ligation as well as to keep the volume of the DNA solution low for higher transformation efficiency.

Example

Sca I-digested pBR322 plasmid DNA (350 ng : 10 μ l) was prepared. Solution I (10 μ l) was added and the mixture was incubated at 16°C for 30 minutes. 1 μ l of the reaction solution was used to transform *E. coli* HB101 competent cells (100 μ l). *E. coli* HB101 competent cells have a transformation efficiency of 1×10^8 cfu/ μ g pBR322 DNA. Results are shown in Table 2 for both the ligation kit and conventional T4 DNA Ligase reactions (2.8 Weiss units of T4 DNA Ligase, in standard ligation buffer, incubated at 16°C for 16 hours).

Table 2. Transformation efficiencies (colonies per μ g of DNA)

DNA added	DNA Ligation Kit (30 min)	T4 DNA Ligase (16 hrs)
17 ng	7.2×10^6	5.0×10^5

C. Linker Ligation, Adaptor Ligation**Protocol**

1. Ligation of a linker with a plasmid vector

Conditions for linker ligation (8 bp or longer) are essentially the same as for "A. Ligation of a DNA fragment with a plasmid vector". However, if the linker is shorter than 8 bp or the linker has a low GC-content, the ligation reaction should be performed at 10°C for 1 to 2 hours. Recommended vector/linker molar ratios are :

- phosphorylated linker : dephosphorylated vector = 10 - 100 : 1
- phosphorylated linker : phosphorylated vector = >100 : 1

2. Linker (or Adaptor) ligation to both termini of a DNA fragment (e.g., linker ligation of cDNA)

1) Prepare 5 - 10 μl of DNA solution containing the DNA fragment (0.01 - 0.1 pmol) and the linker (or adaptor). Recommended DNA fragment/linker (or adaptor) molar ratio is :

DNA fragment : linker (or adaptor) = 1 : >100

- 2) Add one volume (5 - 10 μl) of Solution II and mix well.
- 3) Add Solution I in an amount that is twice the volume (10 - 20 μl) of the DNA solution and incubate at 16°C for 30 min.*
- 4) Inactivate T4 DNA Ligase by heating at 70°C for 10 minutes.
- 5) If the ligated DNA is to be further subjected to restriction enzyme digestion, then perform ethanol precipitation and resuspend the DNA in an appropriate buffer prior to digestion.

* If the linker is shorter than 8 bp or the linker has a low GC-content, the ligation reaction should be carried out at $<10^{\circ}\text{C}$ for 1 to 2 hours.

Example

100 ng of dephosphorylated vector, pUC 118 *Hinc* II/BAP (50 fmol) and 2.6 - 130 ng (0.5 - 25 pmol) of phosphorylated *Bgl* II linkers (5'-CAGATCTG-3') were combined in a total volume of 5 μl . Solution I (5 μl) was added and incubated at 16°C for 30 minutes. A part of the solution was used directly to transform *E. coli* JM109 competent cells and colonies were formed on an LB-Amp plate containing X-Gal and IPTG. (The transformation efficiency of *E. coli* JM109 competent cells were 1.5×10^8 cfu/ μg pUC118 DNA). Transformation efficiencies were obtained by counting the number of white colonies (Table 3) for both the ligation kit and conventional T4 DNA Ligase reaction (350 U, of T4 DNA Ligase and standard ligation buffer, incubated at 16°C for 16 hours).

Table 3. Transformation efficiencies (white colonies per μg of pUC 118 DNA)

	linker/vector (molar ratio)			
	10	50	100	500
DNA Ligation Kit 30 minutes	2.0×10^6	8.0×10^6	3.0×10^7	2.5×10^7
T4 DNA Ligase 16 hours	1.2×10^6	3.2×10^6	2.3×10^6	2.4×10^6

VI. FAQ

Q1 : Why is ligation efficiency low?

A1 : • Extend the reaction time to overnight.

• Prior to transformation, add 1 μ l of Solution III to 9 μ l of ligation mixture. Addition of Solution III can increase transformation efficiency.

• For ligation of sticky-ended DNA, heat the DNA solution (vector + insert DNA) to 60 - 65°C for 2 - 3 min, cool rapidly, and then perform the ligation by adding Solution I. This step will increase ligation efficiency, and potentially improve transformation efficiency. If ligation efficiency is not improved after performing the above, repurify the DNA.

Q2 : Can the ligation mixture be directly used in electroporation?

A2 : Transformation efficiency may decrease when directly using the ligation mixture for electroporation. In this case, precipitate the DNA with ethanol and dissolve in an appropriate buffer for electroporation. Solution III cannot be used for electroporation.

Q3 : Can I use a cosmid in the ligation reaction?

A3 : Yes. Follow the protocol described in "A. Ligation of a DNA fragment with a plasmid vector". However, for *in vitro* packaging, use DNA Ligation Kit Ver.1 (Cat. #6021).

Q4 : Is it possible to use the restriction digest directly for ligation with the DNA Ligation Kit?

A4 : It is recommended that digested DNA first be precipitated with ethanol and then dissolved in an appropriate buffer before use with the DNA Ligation Kit. Likewise, if restriction enzyme digestion of ligated DNA is desired following the ligation reaction, then the ligated DNA should also be ethanol precipitated and resuspended in an appropriate buffer prior to digestion.

Q5 : Should salt (e.g. NaCl) be added to the ligation reaction mixture before ethanol precipitation?

A5 : Yes, salt should be added to the ligation reaction mixture (up to a final concentration of 150 mM NaCl, 2 M ammonium acetate, or 300 mM sodium acetate) before precipitation with ethanol.

Q6 : Can Ligation Solution A and B in the DNA Blunting Kit (Cat. #6025) be substituted for the solutions in the DNA Ligation Kit Ver. 2.1 (Cat. #6022)?

A6 : No. DNA Ligation Kit Ver. 2.1 (Cat. #6022) is designed to perform small scale reactions through use of equal volumes of Solution I and the DNA solution. If DNA fragments are generated using the DNA Blunting Kit, they should be phenol extracted and ethanol precipitated prior to being used for ligation with the DNA Ligation Kit Ver. 2.1.

Q7 : Is it difficult to ligate DNA fragments that have been recovered from agarose gels?

A7 : DNA fragments that have been gel-purified using a commercial DNA extraction product (e.g. columns or silica gel) may result in low ligation efficiency. To ensure high ligation efficiency, gel purified DNA fragments should be ethanol precipitated and dissolved in an appropriate buffer (such as TE) prior to use with the DNA Ligation Kit Ver. 2.1.

VII. Experimental Examples

Example 1 : Three-minutes Ligation

Ligations to generate circular DNA were performed at 25°C for 3 minutes or at 16°C for 30 minutes, and the ligation efficiencies were compared. The following results indicate that a 3 minute ligation with the DNA Ligation Kit Ver. 2.1 provides equivalent performance as ligation under conventional conditions.

[1-1] Recircularization of linearized DNA (sticky-and blunt-end ligation)

200 ng (10 μ l) of pUC118 DNA, digested with *Eco*R I or *Hinc* II respectively, was prepared. Using the DNA Ligation Kit Ver. 2.1, ligation was performed at 25°C for 3 minutes or at 16°C for 30 minutes. 1.6 μ l (16 ng) of the ligation reaction solution was used to transform *E. coli* JM109 Competent cells (1.3 x 10⁸ transformants/ μ g pUC118 DNA). The results are shown in Table 4.

Table 4. Ligation efficiency- recircularization.

End type	Ligation at 25°C for 3 min	Ligation at 16°C for 30 min
Sticky-end (<i>Eco</i> R I)	7.4 x 10 ⁷	6.1 x 10 ⁷
Blunt-end (<i>Hinc</i> II)	1.3 x 10 ⁷	3.1 x 10 ⁷

[1-2] Linker ligation

Using DNA Ligation Kit Ver. 2.1, *pBgl* II linker pd [CAGAATCTG] (260 ng) was ligated to 100 ng of pUC118 DNA digested with *Hinc* II and dephosphorylated with alkaline phosphatase, at 25°C for 3 minutes or at 16°C for 30 minutes. Part of ligation reaction solution was used to transform *E. coli* JM109 Competent cells (1.3 x 10⁸ transformants/ μ g pUC118 DNA). The results are shown in Table 5.

Table 5. Ligation efficiency- linker ligation.

Ligation at 25°C for 3 min	Ligation at 16°C for 30 min
8.9 x 10 ⁶	9.1 x 10 ⁶

Example 2 : The effect of Solution III (Transformation Enhancer)**[2-1] Sticky-end vector ligation**

564 bp of λ DNA fragment digested with *Hind* III (0.25 - 75 fmol) or 2,027 bp of λ DNA fragment digested with *Hind* III (6.25 - 75 fmol) was added into pUC118/*Hind* III BAP (Cat. #3324) (50 ng, 25 fmol). Into 5 μ l of this DNA solution, Solution I was added in 5 μ l and incubated at 16°C for 30 min. After the reaction, 10 μ l of the reactant or 9 μ l of the reactant added 1 μ l of Solution III was applied to *E. coli* JM109 Competent Cells (1.5 x 10⁸ transformants/ μ g pUC118 DNA) for transformation. Then, colonies were allowed to form on an LB-amp plate containing X-Gal and IPTG. The results are shown in Figure 1-1.

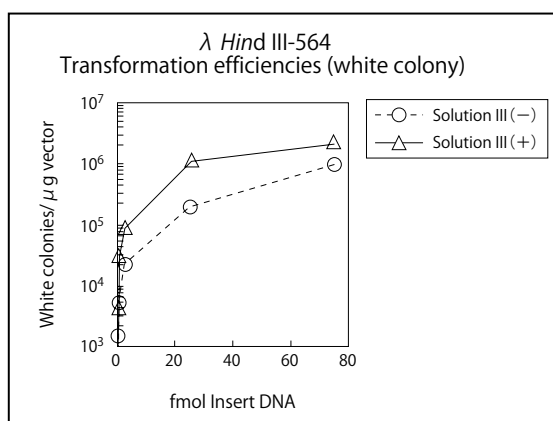
[2-2] Blunt-end vector ligation

500 bp of λ DNA fragment digested with *Hinc* II (0.25 - 75 fmol) or 2,080 bp of λ DNA fragment digested with *Hinc* II (2.5 - 75 fmol) was added into pUC118/*Hinc* II BAP (Cat. #3322) (50 ng, 25 fmol). Into 5 μ l of this DNA solution, Solution I was added in 5 μ l and incubated at 16°C for 30 min. After the reaction, 10 μ l of the reactant or 9 μ l of the reactant added 1 μ l of Solution III was applied to *E. coli* JM109 Competent Cells (1.2 x 10⁸ transformants/ μ g pUC118 DNA) for transformation. Then, colonies were allowed to form on an LB-amp plate containing X-Gal and IPTG. The results are shown in Figure 1-2.

Figure 1-1. Transformation efficiencies (sticky-end ligation)

Insert: λ -Hind III fragment (564 bp)

Insert DNA (fmol)	Insert/vector (molar ratio)	Transformation efficiencies (white colonies/ μ g vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	1.6×10^3	4.4×10^3	9.9	7.9
0.25	1/100	5.2×10^3	3.1×10^4	41.9	29.8
2.5	1/10	2.3×10^4	8.8×10^4	79.0	75.2
25	1	2.0×10^5	1.0×10^6	98.1	98.7
75	3	9.5×10^5	2.0×10^6	99.2	99.1



Insert: λ -Hind III fragment (2,027 bp)

Insert DNA (fmol)	Insert/vector (molar ratio)	Transformation efficiencies (white colonies/ μ g vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	1.8×10^3	2.4×10^3	12.7	12.6
6.25	1/4	9.4×10^3	2.3×10^4	38.8	41.1
12.5	1/2	1.5×10^4	3.8×10^4	56.0	46.0
25	1	2.3×10^4	2.6×10^4	69.9	70.0
75	3	4.6×10^4	1.3×10^5	75.8	74.0

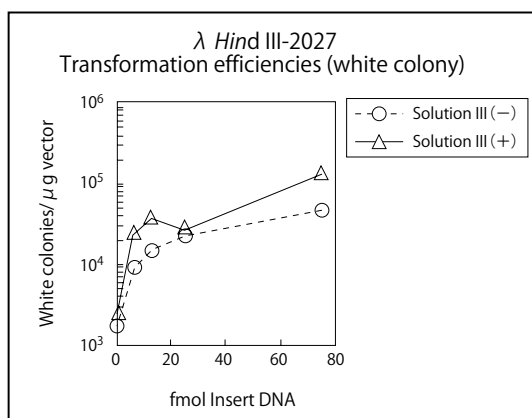
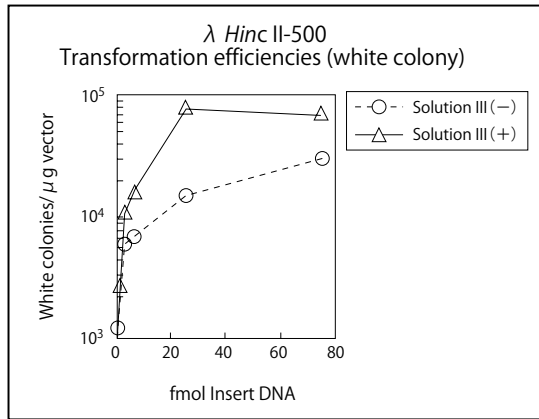


Figure 1-2. Transformation efficiencies (blunt-end ligation)

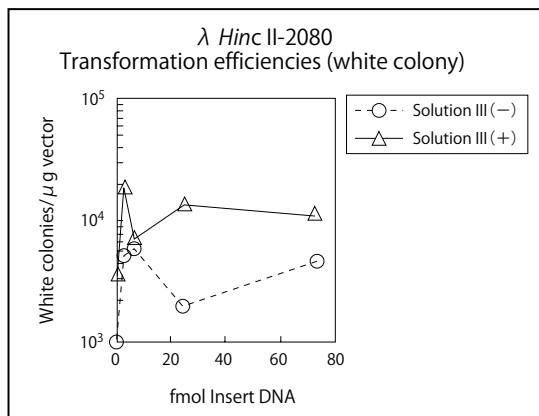
Insert: λ -*Hinc* II fragment (500 bp)

Insert DNA (fmol)	Insert/vector (molar ratio)	Transformation efficiencies (white colonies/ μ g vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	1.2×10^3	2.7×10^3	8.7	8.8
2.5	1/10	6.0×10^3	1.1×10^4	23.3	16.5
6.25	1/4	6.8×10^3	1.6×10^4	38.2	32.3
25	1	1.5×10^4	7.8×10^4	78.9	72.2
75	3	3.0×10^4	6.9×10^4	75.0	84.9



Insert: λ -*Hinc* II fragment (2,080 bp)

Insert DNA (fmol)	Insert/vector (molar ratio)	Transformation efficiencies (white colonies/ μ g vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	1.0×10^3	3.6×10^3	6.4	6.3
6.25	1/4	5.2×10^3	1.8×10^4	17.9	14.2
12.5	1/2	6.0×10^3	6.7×10^3	22.7	20.1
25	1	2.0×10^3	1.4×10^4	33.3	36.5
75	3	4.6×10^3	1.1×10^4	31.9	38.5



Eight colonies were picked from each reaction [2-1] and [2-2], and colony PCR was used to screen for the presence of the insert.

Table 6

Insert DNA	Insert DNA (fmol)	Insert/Vector (mole ratio)	Insert/White colonies Solution III (+)
pUC118/ <i>Hind</i> III/BAP only	-	-	0/8
λ <i>Hind</i> III fragment (564 bp)	0.25	1/100	8/8
λ <i>Hind</i> III fragment (2,027 bp)	6.25	1/4	7/8
pUC118/ <i>Hinc</i> II/BAP only	-	-	0/8
λ <i>Hinc</i> II fragment (500 bp)	2.5	1/10	6/8
λ <i>Hinc</i> II fragment (2,080 bp)	6.25	1/4	5/8

As shown in Figures 1-1 and 1-2, transformation efficiency was improved under all conditions by adding one-tenth volume of Solution III (Transformation Enhancer) to the ligation reaction prior to transformation.

In general, using less insert DNA results in less positive colonies. When vector ligation is performed using a small amount of insert DNA, or when low ligation efficiency is expected (i.e., large-sized or blunt-end insert DNA), the addition of Solution III to the ligation prior to transformation is recommended.

VIII. Reference

Hayashi, K, Nakazawa, M., Ishizaki, Y., Hiraoka, N. and Obayashi, A. *Nucleic Acids Res.* (1986) **14**: 7617-7631.

IX. Related Products

DNA Ligation Kit <Mighty Mix> (Cat. #6023)
 DNA Ligation Kit Ver.1 (Cat. #6021)
 DNA Ligation Kit LONG (Cat. #6024)
 T4 DNA Ligase (Cat. #2011A/B)
 pUC118 *Hinc* II/BAP (Cat. #3322)
 pUC118 *Eco*R I/BAP (Cat. #3320)
 pUC118 *Bam*H I/BAP (Cat. #3321)
 pUC118 *Pst* I/BAP (Cat. #3323)
 pUC118 *Hind* III/BAP (Cat. #3324)
 PrimeGel™ Agarose LE 1-20K GAT (Cat. #5801A)
E. coli HST08 Premium Competent Cells (Cat. #9128)
E. coli HB101 Competent Cells (Cat. #9051)
E. coli JM109 Competent Cells (Cat. #9052)

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