DNA Ligation Kit Ver.2.1

#6022
v.041027

TaKaRa

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DNA Ligation System

DNA Ligation Kit Ver.2.1

#6022
v.041027

TaKaRa

Description:

The DNA Ligation Kit is a simple system that allows very rapid DNA ligation reactions. The latest version of the DNA Ligation Kit, Ver 2.1, was constructed by modification of the Ver. 1 procedure such that a smaller final reactant volume is now possible through the addition of only a single solution. The kit uses T4 DNA ligase and an optimized buffer system. Owing to the high efficiency of the ligation reaction, conventional overnight incubations are no longer required. This kit provides good ligation efficiency in only three minutes for general ligation reactions, and most other types of ligations can be completed within 30 minutes. Transformation efficiency of ligated circular DNA into competent cells can be improved by the addition of a one-tenth volume of Solution III (Transformation enhancer) to the ligation mixture before transformation. Use of Solution III is especially recommended for the following situations: ligation reactions where the amount of insert DNA is low, or when a low ligation efficiency would be expected. Following ligation, the reactant (i.e. ligated DNA solution) can be directly used for bacterial transformations without further DNA purification.

Comparison of reactant volume between DNA Ligation Systems; Ver. 1(#6021) and Ver. 2.1 (#6022)

	Ver. 1 Solution : volume ratio	Ver. 2.1 Solution : volume ratio
Ligation generating circular DNA • Insertion of DNA fragments into plasmid vectors • Insertion of Linker DNA into plasmid vectors • Self-circulization	DNA solution : 1 Solution A : 4-8 Solution B : 1	DNA solution : 1 Solution I: 1
Ligation generating linear DNA • Linker [Adaptor] ligation to cDNA • Insertion of DNA fragments into λ-phage vectors*	DNA solution : 1 (300 mM NaCl) Solution B : 1	DNA solution : 1 Solution II : 1 Solution I : 2

* It is recommended to use Ver. 1 for this purpose.

Kit Components:

Solution I: Enzyme Solution ..... 3 x 250 μl
Solution II: Concatenation Buffer ..... 1 x 750 μl
Solution III: Transformation Enhancer..... 1 x 200 μl

* The kit components are for 50 reactions when using 15 μl of Solution I and 15 μl of Solution II per reacion. Kit components support 100 reactions when 7.5 μl of Solution I are used per reaction.

Storage:

-20°C
If a precipitate froms in Solution III, dissolve it by vortexing for one to several minutes. Solution III should be stored at room temperature once it was thawed.

Notes:

1. Takara recommends that Solutions I and II in this kit be stored frozen at -20°C. These Solutions are not inactivated 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 it 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 that will be loaded onto polyacrylamide gels. Do not directly extract the ligation mixture with phenol.
   *Ethanol precipitation:
   1) Add one-tenth volume of 3M Sodium acetate (pH5.2) or one-twentieth volume of 5M NaCl, and 2-2.5 volume of ethanol into the reactant.
   2) Leave 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.

3. If phenol extraction of the ligation mixture is performed, the reactant may become muddy white. This muddy white appearance represents precipitate formation, and thus this solution should not be used for ligation.

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

Procedure and Examples:

A. Insertion of DNA fragments into plasmid vectors

PROCEDURE

1. Combine plasmid vector DNA and the DNA fragment to be inserted in a total volume of 5 - 10 μl. We recommend 100 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$ for dissolving DNA, however TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) could also be used. Recommended amounts of DNA are vector : insert = 0.03 pmol : 0.03 - 0.3 pmol. (0.03 pmol of pUC18 DNA (2,686 bp) corresponds to about 50 ng).
2. Add one volume of Solution I (5-10 μl) to 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 with *E.coli* competent cells. When performing transformation immediately after ligation, apply 10 μl of the ligation mixture to 100 μl 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 good results are not obtained, the reaction can be extended overnight. Results depend on the purity of DNA. If good results are not obtained, an additional phenol extraction/ethanol precipitation step of the DNA often helps.
When performing ligation reaction of T-vector and PCR products, reaction should be completed within one hour. Longer reaction may result in high background.

*2. Ligation reactant can be directly applied to transformation. However, more colonies (transformants) can be obtained by adding 1 μl of Solution III into 9 μl of the ligation reactant prior to transformation. When ligation reactant more than 10 μl is applied to transformation DNA should be precipitated with ethanol.
The ligation reactant should <u>not</u> be used directly in electroporation, in which case, DNA should be precipitated with ethanol and dissolved in low salt buffers such as TE buffer. Solution III can not be used in electroporation.

EXAMPLE

50 ng of *Eco*R I-digested pUC 118 vector (25 fmol) was mixed with 2.5-250 ng (2.5-250 fmol) of 1.5 kbp *Eco*R I-digested DNA fragment at insert/vector ratios ranging from 0.1 to 10.0, in a total volume of 5 μl. One volume (5 μl) of Solution I was added to the DNA solution. The combined solution was then 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 L-Amp plate containing X-Gal and IPTG.
(The tranformation efficiency of *E.coli* JM109 competent cells was 6.3 x 10^7 cfu/μg pUC118 DNA.)
Transformation efficiencies obtained counting the number of white colonies are shown in Table 1. Compare with the results using T4 DNA Ligase (350 U, 2.8 Weiss units) in standard ligation buffer (incubated at 16°C for 16 hours).

Table 1. Transformation efficiencies (colonies per μg 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 x 10^6	5.0 x 10^6	1.7 x 10^7	2.3 x 10^7	2.1 x 10^7
	phosphorylated	7.8 x 10^5	2.5 x 10^6	8.2 x 10^6	1.7 x 10^7	2.3 x 10^7
T4 DNA Ligase 16 hours	dephosphorylated	1.6 x 10^5	2.0 x 10^5	1.8 x 10^6	3.1 x 10^6	1.9 x 10^6
	phosphorylated	4.6 x 10^5	1.0 x 10^6	1.9 x 10^6	5.0 x 10^6	1.2 x 10^7

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

PROCEDURE

The procedure for self-circularization of linear DNA is essentially the same as for A.Insertion of DNA fragments into plasmid vectors (see page 3). 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 μl) was prepared. Solution I (10 μl) was added and incubated at 16°C for 30 minutes. 1 μl of the reaction solution was used to transform *E.coli* HB101 competent cells (100 μl). *E.coli* HB101 competent cells had an efficiency of 1 x 10^8 cfu/μg pBR322 DNA. Results are shown in Table 2. Compare with the results using 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 μg of DNA added).

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

C. Linker [Adaptor] ligation

PROCEDURE

1. Insertion of linker into a plasmid vector

Conditions for linker ligation (8 bases or longer) are essentially the same as for A. Insertion of DNA fragments into plasmid vectors (see page 3). However, if the linker is shorter than 8 bases or if the linker has a low GC-content, the ligation reaction should be carried out 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 [Adaptor] ligation to both termini of a DNA fragment (ex. Linker ligation of cDNA)

1) Prepare 5-10 μl of DNA solution containing DNA fragment to be ligated (0.01-0.1 pmol) and linker (or adaptor). Recommended DNA fragment/linker[adaptor] molar ratio is:
   - DNA fragment : linker [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) that was added for the DNA solution and incubate at 16°C for 30 min. However, if the linker is shorter than 8 bases or if the linker has a low GC-content, the ligation reaction should be carried out at 10°C for 1 to 2 hours.
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 ethanol precipitate and resuspend the DNA in an appropriate buffer prior to digestion.

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 L-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 obtained counting the number of white colonies are shown in Table 3. Compare with the results using conventional T4 DNA Ligase reaction (350 U, 2.8 Weiss units of T4 DNA Ligase and standard ligation buffer incubated at 16°C for 16 hours).

Table 3. Transformation efficiencies (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

Trouble shooting

Q1: Ligation efficiency is low.

A1 : • Extend the reaction time to overnight.
• Prior to use in transformation, add 1 μl of Solution III to 9 μl of ligation reactant. Addition of Solution III can increase transformation efficiency.
• For ligation of sticky-ended DNA, heat the DNA solution (vector + insert DNA) at 60 ~ 65°C for 2 ~ 3 min., cool rapidly and then perform the ligation by adding Solution I. This step will result in a higher ligation efficiency, and potentially higher transformation efficiency.
If ligation efficiency is not improved after performing all of the above three suggestions, then repurification of the DNA is recommended.

Q2 : Can ligation mixture be directly used in electroporation?

A2 : Transformation efficiency may decrease when directly applying the ligation reactant to electroporation. In that case, the DNA should be precipitated with ethanol and dissolved in appropriate buffer before used in electroporation.
Solution III can not be used in electroporation.

Q3: When ligation reactant is applied to cosmid:

A3: Follow the protocol of A. Insertion of DNA fragments into plasmids vectors (page 3). However, in case of *in vitro* packaging, it is recommended to use DNA Ligation Kit Ver.1 (Cat#6021).

Q4: Is it possible to use a portion of a restriction digest directly as the DNA Solution for use 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: Can salt (e.g. NaCl) be added to the ligation reaction mixture before ethanol precipitation?

A5: Yes, salt can be added directly to the ligation reaction mixture (a final concentration of 150 mM NaCl, 2 M ammonium acetate or 300 mM sodium acetate) and the ligated DNA then precipitated with ethanol.

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

A6: They cannot be directly substituted. DNA Ligation Kit Ver.2.1 (Cat.#6022) is designed to allow a smaller reaction scale, by mixing the same volume of Solution I and the sample DNA solution. Accordingly the reaction can be influenced by the composition of the sample DNA solution, and ligation reaction may not be carried out by using DNA Ligation Kit Ver.2.1 (Cat.#6022) instead of Ligation Solution A and B in DNA Blunting Kit (Cat.#6025). When using DNA Ligation Kit Ver.2.1 during the reaction of DNA Blunting Kit, the sample DNA solution should be extracted with phenol and precipitated with ethanol prior to the ligation.

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

A7: DNA fragments which have been recovered using a commercial DNA extraction product/ reagent (e.g. columns or silica gel) and used with the DNA Ligation Kit may show low ligation efficiency. To ensure high ligation efficiencies, such recovered DNA fragments should be ethanol precipitated and dissolved in an appropriate buffer (such as TE) prior to use with the Ligation Kit.

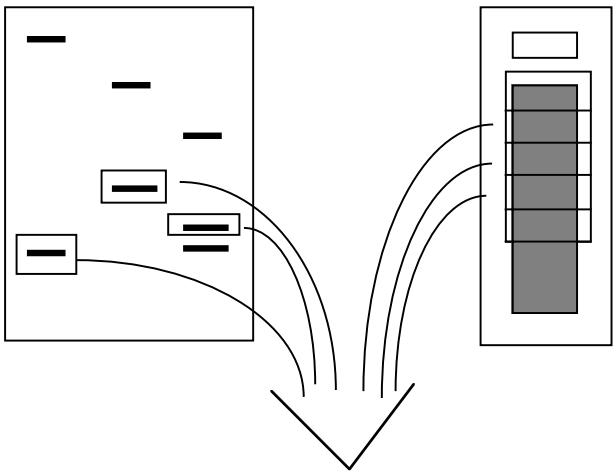
APPLICATION EXAMPLE 1: One Hour Protocol (Step 2-5) For Subcloning And Size-Fractionated Deletion Cloning

The recovery of DNA fragments from agarose gel slices and the cloning of these fragments into vector DNA can be accomplished rapidly by the combined use of SUPREC™-01, SUPREC™-02 and the DNA Ligation Kits. Only a small amount of DNA is required as starting material (as low as what would generally be used for analytical purpose) because of the high efficiency of the procedure.

Step 1 Electrophoretic Separation of DNA

DNA restriction fragments
Digest 1-2 μg of DNA with restriction enzyme and separate by agarose gel electrophoresis. Excise the slice of agarose that contains the DNA band of interest.

Serial deletion fragments
Treat 5 μg of DNA with Deletion Kit (Cat.#6030) and separate by electrophoresis. Excise the gel to obtain fragments truncated to the desired size range.



Step 2 SUPREC™-01 5 min.

Insert the agarose gel slice (50-100 μl) into the SUPREC™-01 cartridge and centrifuge at 10,000 rpm (microcentrifuge) for 5 min.

Step 3 SUPREC™-02 5 min.

Transfer the filtrate to the SUPREC™-02 cartridge and centrifuge at 6,000 rpm (microcentrifuge) for 3 min. Add 15 μl of TE buffer, pipette well, and collect DNA from the catridge filter.

Step 4 Ligation 15 min.

Following the protocol, 10 μl of DNA solution is used for ligation at room temperature (16-25°C) for 15 min.

Step 5 Transformation 31 min.

Mix 10 μl of the ligation reaction solution with 100 μl of competent cells and incubate in ice for 15 min. followed by heat shock at 42°C for 30 seconds. Add 890 μl of SOC media and incubate at 37°C for 15 min.

Step 6 Plating

Collect the cells, re-suspend in 200 μl of appropriate medium or buffer, and then spread 10 μl and the rest respectively onto the selective plates.

APPLICATION EXAMPLE 2 : Three-minutes Ligation

Ligations of generating 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 prove that 3-minutes ligation with DNA Ligation kits allows as good performance as the ligation under the conventional condition does.

• ***Example 1*** **: Self ligation of linearized DNA (sticky- and blunt-end ligation)**

200 ng (10 μl) of pUC118 DNA, digested with *Eco*R I or *Hinc* II respectively, was prepared. Using DNA Ligation Kit, ligation was performed at 25°C for 3 minutes or at 16°C for 30 minutes. 1.6 μl (16 ng) of ligation reaction solution was used to transform *E.coli* JM109 Competent cells (1.3 x 10^8 transformants/μg pUC118 DNA). The results are shown in Table 4.

Table 4

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

• ***Example 2*** **: Linker ligation**

Using DNA Ligation Kit, p*Bgl* II linker d[pCAGAATCTG] 260 ng was ligated to 100 ng of pUC118 DNA digested with *Hinc* II, 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 10^8 transformants/μg pUC118 DNA). The results are shown in Table 5.

Table 5

	Ligation at 25°C for 3 min.	Ligation at 16°C for 30 min.
Ver. 1	1.8 x 10^7	2.0 x 10^7
Ver. 2.1	8.9 x 10^6	9.1 x 10^6

APPLICATION EXAMPLE 3: The effect of Solution III (Transformation Enhancer)

[3-1] Sticky-end vector ligation

564 bp of λDNA fragment digested with *Hin*d III (0.25-75 fmol) or 2,027 bp of λDNA fragment digested with *Hin*d III (6.25-75 fmol) was added into pUC118/*Hin*d III BAP (Cat.#3324) (50 ng, 25 fmol). Into 5 μl of this DNA solution, Solution I was added in 5 μl and incubated at 16°C for 30 min. After the reaction, 10 μl of the reactant or 9 μl of the reactant added 1 μl of Solution III was applied to *E.coli* JM109 Compentent Cells (1.5 x 10^8 transformants /μg pUC118 DNA) for transformation. Then, colonies were formed on L-amp plate containing X-Gal and IPTG. The results are shown in the Figure 1-(1).

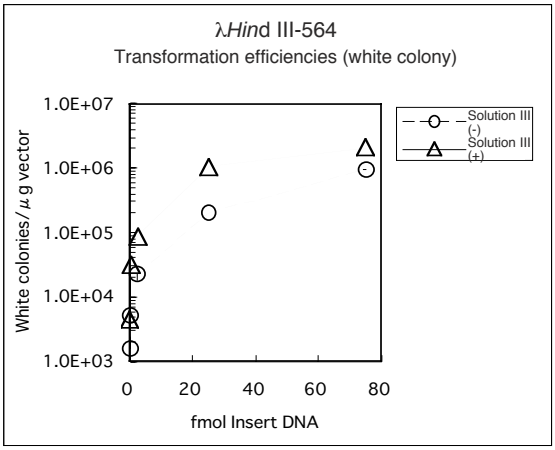
[3-2] Blunt-end vector ligation

500 bp of λDNA fragment digested with *Hinc* II (0.25-75 fmol) or 2,080 bp of λDNA fragment digested with *Hinc* II (2.5-75 fmol) was added into pUC118/*Hinc* II BAP (Cat.#3322) (50 ng, 25 fmol). Into 5 μl of this DNA solution, Solution I was added in 5 μl and incubated at 16°C for 30 min. After the reaction, 10 μl of the reactant or 9 μl of the reactant added 1 μl of Solution III was applied to *E.coli* JM109 Compentent Cells (1.2 x 10^8 transformants /μg pUC118 DNA) for transformation. Then, colonies were formed on L-amp plate containing X-Gal and IPTG. The results are shown in the Figure 1-(2).

[Fig.1-(1)] Transformation efficiencies (sticky-end ligation)

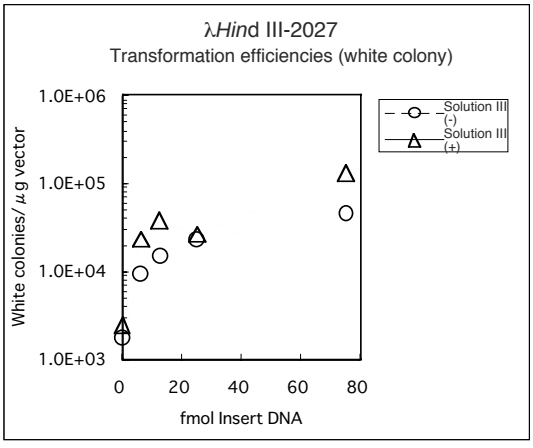
λ-*Hin*d III fragment (564 bp)

Insert DNA (fmol)	Insert/ vector (molar ratio)	Transformation efficiencies (white colonies/μg vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	$1.6x10^3$	$4.4x10^3$	9.9	7.9
0.25	1/100	$5.2x10^3$	$3.1x10^4$	41.9	29.8
2.5	1/10	$2.3x10^4$	$8.8x10^4$	79.0	75.2
25	1	$2.0x10^5$	$1.0x10^6$	98.1	98.7
75	3	$9.5x10^5$	$2.0x10^6$	99.2	99.1



λ-*Hin*d III fragment (2,027 bp)

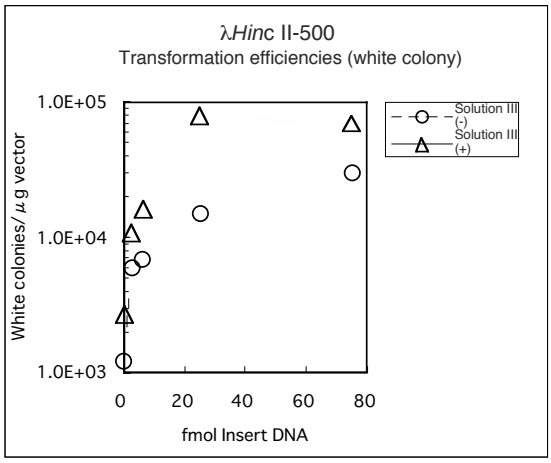
Insert DNA (fmol)	Insert/ vector (molar ratio)	Transformation efficiencies (white colonies/μg vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	$1.8x10^3$	$2.4x10^3$	12.7	12.6
6.25	1/4	$9.4x10^3$	$2.3x10^4$	38.8	41.1
12.5	1/2	$1.5x10^4$	$3.8x10^4$	56.0	46.0
25	1	$2.3x10^4$	$2.6x10^4$	69.9	70.0
75	3	$4.6x10^4$	$1.3x10^5$	75.8	74.0



[Fig.1-(2)] Transformation efficiencies (blond-end ligation)

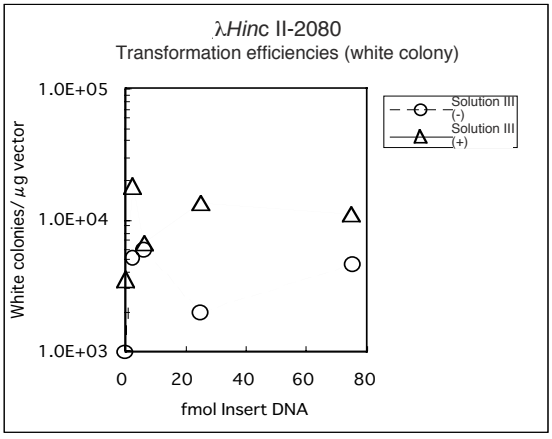
λ-*Hin*c II fragment (500 bp)

Insert DNA (fmol)	Insert/ vector (molar ratio)	Transformation efficiencies (white colonies/μg vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	$1.2x10^3$	$2.7x10^3$	8.7	8.8
2.5	1/10	$6.0x10^3$	$1.1x10^4$	23.3	16.5
6.25	1/4	$6.8x10^3$	$1.6x10^4$	38.2	32.3
25	1	$1.5x10^4$	$7.8x10^4$	78.9	72.2
75	3	$3.0x10^4$	$6.9x10^4$	75.0	84.9



λ-*Hin*c II fragment (2,080 bp)

Insert DNA (fmol)	Insert/ vector (molar ratio)	Transformation efficiencies (white colonies/μg vector)		White colonies/Total colonies (%)	
		Solution III (-)	Solution III (+)	Solution III (-)	Solution III (+)
0	-	$1.0x10^3$	$3.6x10^3$	6.4	6.3
6.25	1/4	$5.2x10^3$	$1.8x10^4$	17.9	14.2
12.5	1/2	$6.0x10^3$	$6.7x10^3$	22.7	20.1
25	1	$2.0x10^3$	$1.4x10^4$	33.3	36.5
75	3	$4.6x10^3$	$1.1x10^4$	31.9	38.5



Eight colonies were picked up each from the reactions of [3-1] and [3-2], and their inserts were checked with PerfectShot Insert Check PCR Mix (Cat.#RR020).

[Table 6]

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

As shown in the Figures 1-(1),-(2), transformation efficiency was improved under all conditions by adding one-tenth volume of Solution III (Transformation Enhancer) into the ligation reactant.
Generally speaking, the less insert DNA is applied, the less positive colonies are obtained. When vector ligation is performed by using less insert DNA amount, or when low ligation efficiency is expected (such as that insert DNA is large or has blunt end), it is recommended to add Solution III into the ligation reactant prior to the transformation.

NOTE: Research use only. Not for use in diagnostic or therapeutic.