Blunting-Convenience Kit Manual（2nd edition）

Code No. 312-06291	For 25 reactions
Code No. 318-06293	For 5 reactions

- Description -

When no appropriate restriction enzyme is available for linking the target gene to vector DNA, ligation can be performed without depending on sequence if the DNA ends are blunt. T4 DNA Polymerase is a DNA-dependent DNA polymerase having 3’→5’ Exonuclease activity and 5’→3’ Polymerase activity, and both 5’ protruding end and 3’ protruding end can be blunted with this enzyme. Also, by combining with the 2×Ligation Mix (Ligation-Convenience Kit) by which the ligation reaction can be performed in a short period of time, the reactions from blunting to ligation can be performed in a short period of time.

I. Characteristics

- Reactions from blunting DNA ends to ligation can be performed rapidly and simply.
- Since the 10×Blunting Buffer includes dNTP and the like required for the reaction, the blunting of DNA ends can be performed simply by adding T4 DNA Polymerase.
- 5’ protruding end DNA, 3’ protruding end DNA and PCR products with A at 3’ ends can be blunted.
- Ligation reaction can be performed in a short period of time using the 2×Ligation Mix.

II. Contents of kit

Reagent	For 25 reactions*	For 5 reactions*
T4 DNA Polymerase	25 µl×1	5 µl×1
10×Blunting Buffer	50 µl×1	10 µl×1
2×Ligation Mix	250 µl×1	50 µl×1

* Number of reactions when used in 20 µl reaction system for both blunting and ligation.

III. Storage and thawing method

Store at −20°C

- To avoid denaturation, never vortex T4 DNA Polymerase.
- Use 2×Ligation Mix after completely thawing on ice and mixing well by pipetting.
- No decrease of ligation efficiency of 2×Ligation Mix was observed in up to fifty cycles of freezing and thawing.

IV. Protocol and examples of experiments

< 1. Protocol >

(1) Preparation of blunting reaction solution

Prepare 17 µl solutions of DNA to be blunted such as 5' protruding end DNA, 3' protruding end DNA and PCR products (at 0.1-10 pmol DNA end concentration).*1

Then add 2 µl of 10 × Blunting Buffer and mix.

(2) Addition of enzyme

Add 1 µl of T4 DNA Polymerase and mix by pipetting.*2

(3) Blunting reaction

React at 37°C for 5 min.*3

(4) Stopping the reaction

Place the blunting reaction mixture on ice to stop the reaction. When not used immediately, perform phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation and store the sample at -20°C.

(5) Preparation of ligation reaction solution

Prepare 10 µl of DNA solution by combining the blunting reaction solution and the blunt end DNA vector solution.

Add 10 µl of 2 × Ligation Mix and mix.

(6) Ligation reaction

React at 16ºC for 5-30 min.*4

(7) Transformation

Use the reaction solution for transformation as it is.*5

< Blunting reaction >

Sample DNA solution (0.1-10 pmol)	17 µl
10 × Blunting Buffer	2 µl

↓ Add 1 µl of T4 DNA Polymerase

↓ React at 37ºC for 5 min.

↓ Stop the reaction by placing the mixture on ice. (**Blunting reaction solution**)

* Use the required amount of blunting reaction solution. (1-5 µl)*6

< Ligation reaction >

Blunting reaction solution	
Blunt end DNA vector solution	up to 10 µl
ddH_2O	
2 × Ligation Mix	10 µl
Total	20 µl

↓ React at 16ºC for 5-30 min.

↓ For transformation or *in vitro* packaging

*1 DNA end concentration of 0.1-10 pmol is equivalent of 0.1-10 µg of pUC19 (2686 bp).

*2 Never vortex T4 DNA Polymerase. The enzyme may be inactivated.

*3 Strictly observe the reaction time of 5 min.

*4 When ligation is carried out for a long time, such as 16 hours or overnight, transformation efficiency may be markedly decreased.

*5 The volume of the reaction solution used for transformation should be not more than 1/10 of the volume of the competent cells. If too much reaction solution is used, transformation efficiency may be decreased.

If the volume of the reaction solution is not less than 1/10 of the volume of the competent cells, recover the DNA after the ligation reaction by ethanol precipitation, dissolve the DNA in ddH_2O or TE (pH 8.0) so that the volume of the DNA is not more than 1/10 of the volume of the competent cells, and then use for transformation.

*6 The blunting reaction solution brought into the ligation reaction should be not more than

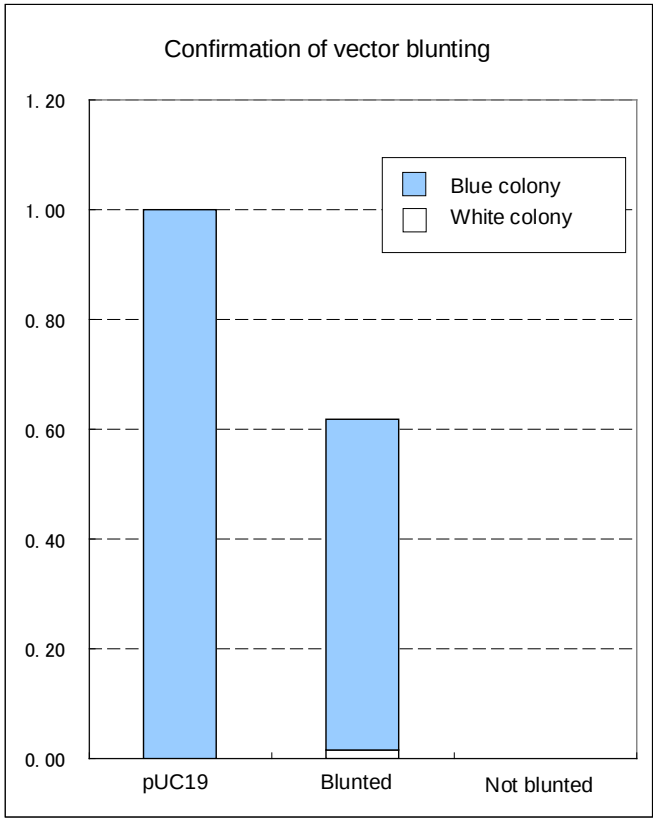
5 µl. If the volume of the reaction solution is not less than 5 µl, recover the DNA by extracting with phenol/chloroform/isoamyl alcohol and precipitating with ethanol, dissolve in ddH_2O or TE (pH 8.0) and then use the DNA.

< 2. Experimental example >

Confirmation of blunting of the vector

1) pUC19 was cleaved with *Eco*R I (5' protruding end) and *Pst* I (3' protruding end), extracted with phenol/chloroform/isoamyl alcohol and then dissolved in TE buffer.
2) The ends of 1 µg of pUC19 cleaved with *Eco*R I and *Pst* I were blunted in accordance with the protocol.
3) A portion of the blunted DNA was subjected to the ligation reaction at 16°C for 30 min using 2×Ligation Mix.
4) After the ligation reaction, to remove uncut pUC19 and to reduce the background, the ligation reaction solution was diluted five-fold and heated at 65°C for 5 min to inactivate T4 DNA Ligase, and was digested with *Bam*H I.
5) With the ligation reaction solution digested with *Bam*H I, *ECOS*™ Competent *E.coli* DH5± was transformed and the number of colonies generated was counted.

The ratio of the number of colonies generated by self-ligation after blunting is shown below when the number of colonies generated by the transformation by pUC19 is assumed to be 1.

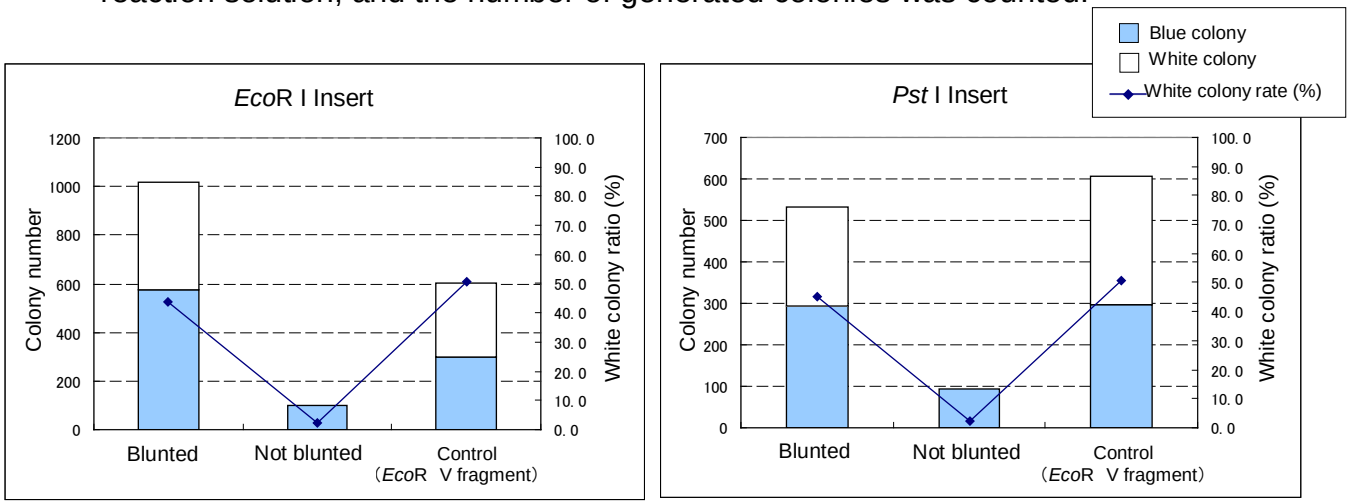


Result

When pUC19 is digested with *Eco*R I and *Pst* I, blunted and self-ligated, the *LacZ* frame is in phase generating blue colonies. Since blue colonies derived from pUC19 are excluded by digesting with *Bam*H I, the generated blue colonies are the plasmid formed by self-ligation after blunting the ends.
The left graph indicates that about 60% of the vector is blunt-end ligated after 5 min reaction.

Blunting of insert DNA

1) pBluescript II SK(+) was cleaved with *Eco*R V and dephosphorylated, then extracted with phenol/chloroform/isoamyl alcohol and then dissolved in TE.
2) Two kinds of fragments were prepared by cleaving the 500 bp fragment, which was amplified using »DNA as a template, with *Eco*R I and *Pst* I.
3) Two fragments were blunted in accordance with the protocol.
4) The vector, which was obtained by cleaving pBluescript II SK(+) with *Eco*R V and by dephosphorylating, and the blunted fragment were ligated at an insert/vector molar ratio of 5 at 16°C for 30 min using 2×Ligation Mix. Also, the fragment, which was amplified as a control using »DNA as a template, was cleaved with *Eco*R V and ligated.
5) *ECOS*™ Competent *E.coli* DH5± was transformed with a portion of the ligation reaction solution, and the number of generated colonies was counted.



Result

For both 5' end protrusion and 3' end protrusion, the blunted insert could be ligated at the same ratio as the *Eco*R V fragment, suggesting that the *Eco*R I fragment and *Pst* I fragment were sufficiently blunted by the reaction at 37°C for 5 min.

< 3. Reference data >

1. Blunting PCR products

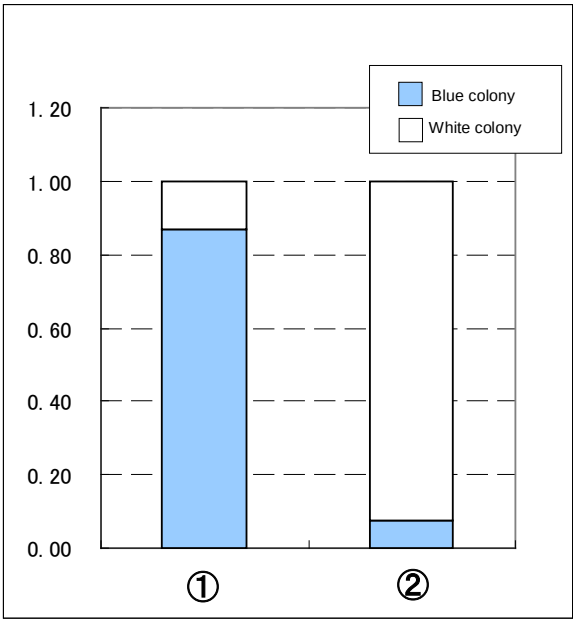
A 500 bp fragment amplified by PCR was blunted in accordance with the protocol.

(1) One portion was ligated as-is after blunting to **pBluescript II SK(+) cleaved with *Eco*R V.**

(2) The other portion was **phosphorylated with T4 Polynucleotide Kinase after blunting,** treated with heat to inactivate T4 Polynucleotide Kinase, and then ligated with **pBluescript II SK(+), which was cleaved with *Eco*R V and dephosphorylated.***

The graph below shows the ratio of blue colonies to white colonies when the number of colonies generated is assumed to be 1.

Presence or absence of phosphorylation after blunting



Result

When a PCR product was blunted and ligated as-is with a vector having a phosphate group, the ratio of white colonies was about 13%. On the other hand, when the PCR product was phosphorylated and ligated with the dephosphorylated vector, the ratio of white colonies was not less than 90%.

When a PCR product is used for ligation after blunting, the phosphate group is normally not attached to the 5' end of the PCR product and thus a vector that is not dephosphorylated needs to be used. However, when the vector that is not dephosphorylated is used for ligation, the ratio of the self-ligated vector is greater. Therefore, when the ligation is performed by blunting the PCR product, it is recommended that the PCR product be phosphorylated using T4 Polynucleotide Kinase after blunting and to that the vector be dephosphorylated.

***** When T4 Polynucleotide Kinase (Code No. 312-01551) from Nippon Gene is used, and phosphorylation is performed using 10×Kinase Buffer A, the ligation may be performed using the 2×Ligation Mix after heat treatment without further processing.

When phosphorylation is performed using 10×Kinase Buffer B, perform the ligation reaction using the 2×Ligation Mix after phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation.

2. Study of the molar ratio of inserts in ligation.

The vector : insert ratio in ligation greatly affects the ligation efficiency. The table below shows the molar ratio at which the best result was obtained when insert DNA of various lengths was ligated and used for transformation in blunt-end ligation.

・ Blunt end

Insert length	200 bp	600 bp	1000 bp	3000 bp
Vector	1	1	1	1
Insert	5	5	2-10	0.5-2

Vector: pUC19 (0.03 pmol) cleaved with *Sma* I
Insert: Insert DNA cleaved with *Sma* I
(0.015 pmol, 0.03 pmol, 0.06 pmol, 0.15 pmol, 0.3 pmol)
Ligation reaction: 16°C, 5 min

A word of caution in ligation reaction

- Extended ligation, e.g., 16 hours or overnight, may cause a marked reduction in transformation efficiency.
- When DNA is dissolved in a high salt concentration buffer, the ligation efficiency is markedly reduced. Prepare the DNA solution in ddH_2O or TE buffer (pH 8.0).
- The volume of the reaction solution used for transformation should be not more than 1/10 of the volume of the competent cells. Using too much reaction solution may cause a reduction in transformation efficiency.
- If the volume of the reaction solution is not less than 1/10 of the volume of the competent cells, recover the DNA after the ligation reaction by ethanol precipitation, dissolve the DNA in ddH_2O so that the volume of the DNA is not more than 1/10 of the volume of the competent cells, and then perform transformation.
- Ligation efficiency may vary depending on differences in the purity of DNA used for the ligation reaction and the restriction enzymes to be used.
- The volume of the reaction solution used for packaging should be not more than 1/10 of the volume of the packaging extract. Using too much reaction solution may cause a reduction in packaging efficiency.
- If the volume of the reaction solution is not less than 1/10 volume of the packaging extract, recover DNA after the ligation reaction by ethanol precipitation, dissolve the DNA in ddH_2O so that the volume of the DNA is not more than 1/10 volume of the packaging extract, and then perform transformation.
- Packaging is not inhibited by using Gigapack（Stratagene Inc.）

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