

Cat. # 6027

For Research Use

TAKARA

**Mighty Cloning Reagent Set
(Blunt End)**

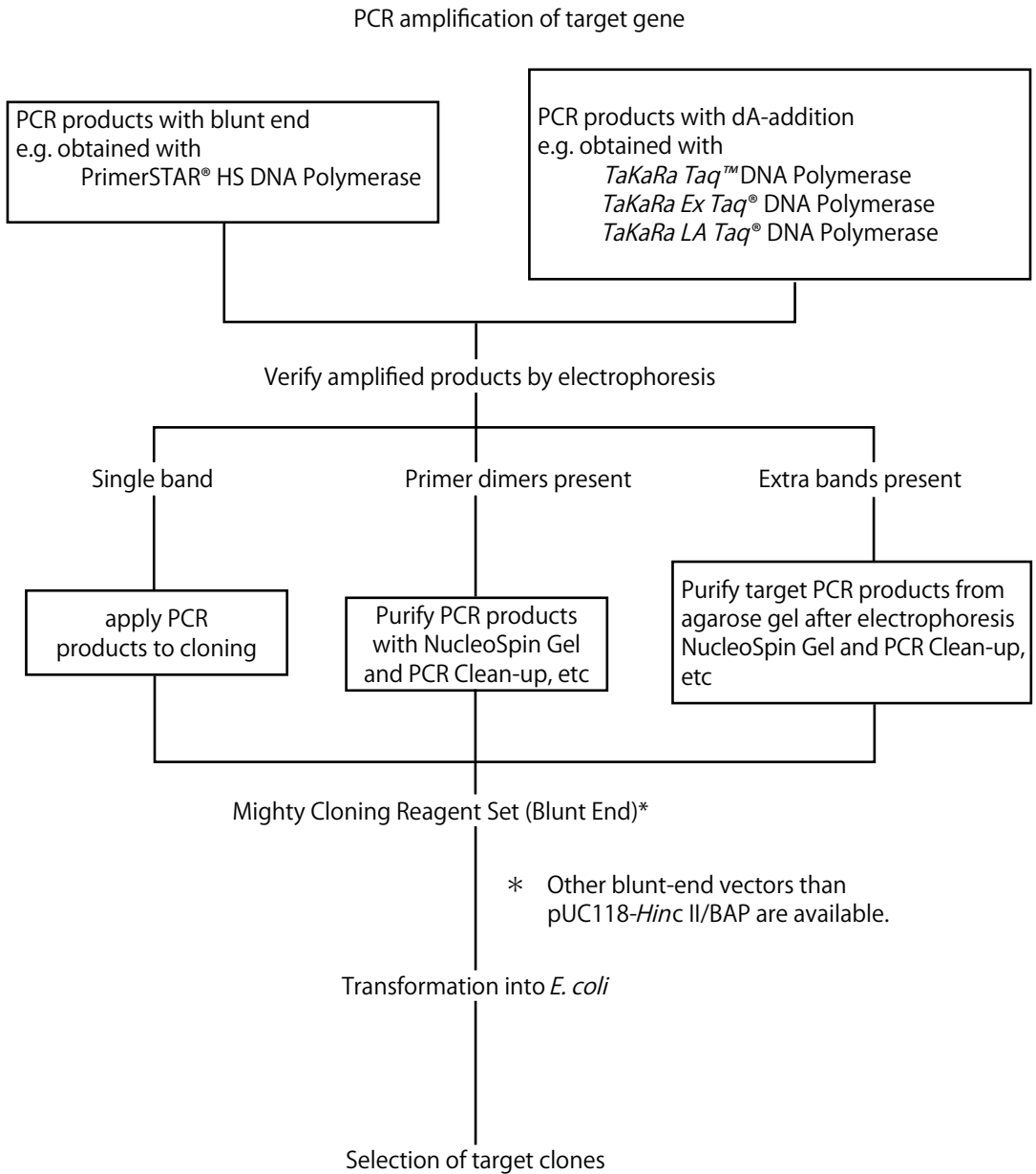
Product Manual

v201812Da

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I. Flowchart of blunt end cloning of PCR products



II. Description

Mighty Cloning Reagent Set (Blunt End) is designed to allow PCR products to be quickly cloned into blunt-end vectors with a simple method. PCR products are blunt-ended and phosphorylated at 5'-end simultaneously, not only from blunt-end PCR products, but also from PCR products with dA at termini. Thus, DNA fragments can be prepared for ligation in a single reaction. Pretreatment of PCR products, such as inactivation of enzymes, removal of unused dNTPs and primers, etc is not necessary. This kit utilizes the same reagents that are included in Takara's efficient DNA Ligation Kit <Mighty Mix>, which achieves the series of operation efficiently and in a short time.

III. Components (20 reactions)

1. 10X Blunting Kination Buffer		40 μ l
2. Blunting Kination Enzyme Mix		20 μ l
3. Ligation Mighty Mix * 1		120 μ l
4. Control Vector (pUC118- <i>Hinc</i> II/BAP)	50 ng/ μ l	20 μ l
5. Control Insert * 2	200 ng/ μ l	10 μ l
6. ddH ₂ O		340 μ l

* 1 Ligation Mighty Mix is the same reagent that is included in DNA Ligation Kit <Mighty Mix> (Cat. #6023).

* 2 500 bp PCR fragment amplified with *TaKaRa Ex Taq* DNA Polymerase by using λ DNA as a template.

IV. Materials Required but not Provided

- *E. coli* Competent cell or Electro cell
- SOC Medium
- Ampicillin/X-Gal/LB plate added with IPTG

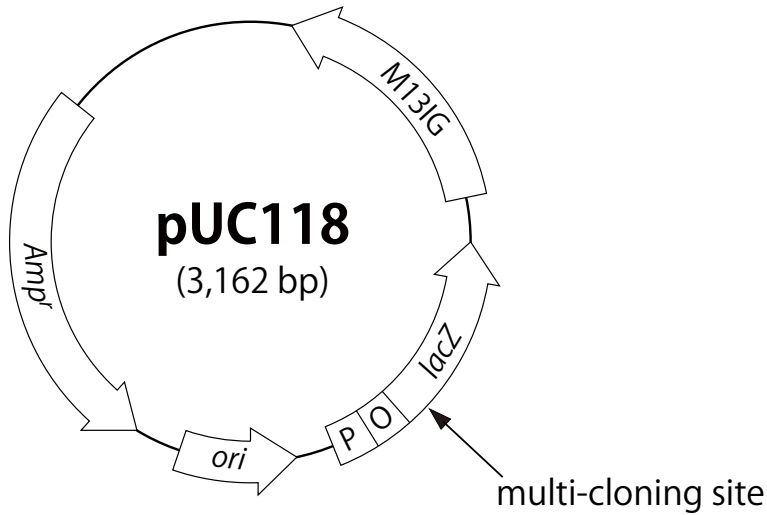
V. Storage

-20°C

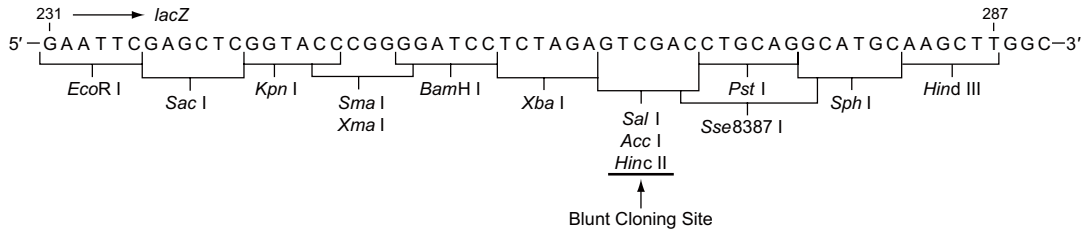
VI. pUC118 *Hinc* II/BAP

pUC118 *Hinc* II/BAP is a blunt-end cloning vector, prepared by cleavage with *Hinc* II, then dephosphorylated by Alkaline Phosphatase (*E. coli* C75) (BAP). The sequence of DNA cloned into this vector can be verified with M13 primer M4 and RV.

Vector map of pUC118



Cloning site of pUC118



VII. Protocol

A. When PCR product is directly used :

< Blunting Kination reaction >

1. Prepare the following reaction mixture in a microtube.

Reagents	Volume
PCR product *	< 2 μ l
10X Blunting Kination Buffer	2 μ l
Blunting Kination Enzyme Mix	1 μ l
ddH ₂ O	X μ l
Total	20 μ l

2. Incubate at 37°C for 10 min.
3. Add 80 μ l of sterile purified water and 100 μ l of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and vortex.
4. Centrifuge at 12,000 rpm for 5 min at room temperature, then transfer the upper layer to a new tube.
5. Add an equal amount of chloroform/isoamyl alcohol (24 : 1) and vortex.
6. Centrifuge at 12,000 rpm for 5 min at room temperature, then transfer upper layer to a new tube.
7. Add 10 μ l of 3M sodium acetate and 250 μ l of chilled ethanol, and then mix. Keep it for 20 min. at -80°C.
8. Centrifuge at 12,000 rpm for 10 min. at 4°C. Remove the supernatant.
9. Wash the precipitate with chilled 70% ethanol. Centrifuge at 12,000 rpm for 5 min at 4°C and remove the supernatant.
10. Dry the precipitate.
11. Dissolve the precipitate in 10 - 20 μ l of TE buffer.

< Ligation reaction >

12. Dispense 5 μ l of DNA solution obtained at step 11 into a new microtube.
13. Add 1 μ l of pUC118 *Hinc* II/BAP (50 ng/ μ l) or a blunt-end and dephosphorylated vector prepared yourself..
14. Add 6 μ l of Ligation Mighty Mix and mix gently.
15. Incubate at 16°C for 1 hour.
16. Perform a transformation using the whole solution prepared at step 15 for 100 μ l of *E. coli* competent cell. When carrying out a transformation by the electroporation, exchange the buffer by phenol/chloroform extraction and ethanol precipitation before transformation.
17. Spread to the LB plate including Ampicillin, X-Gal, and IPTG.

- * When other bands than a target band appear in the PCR product, or when the shorter insert DNAs are exclusively cloned, please purify the target fragment by agarose gel electrophoresis.
- * Appropriate volume of PCR product is 2 μ l. When larger volume is used, the reaction efficiency may lower. Please concentrate by ethanol precipitation when more PCR products are applied.
- * When a plasmid having same selection marker as a cloning vector is used as a PCR template, it is necessary to prevent generating colonies transformed the template plasmid itself. Therefore, purification of target PCR band from agarose gel electrophoresis is recommended.

B. When purified DNA fragment is used :**< Blunting Kination reaction >**

1. Prepare the following reaction mixture in a microtube.

Reagents	Volume
DNA Fragment	0.1 - 2 pmol
10X Blunting Kination Buffer	2 μ l
Blunting Kination Enzyme Mix	1 μ l
ddH ₂ O	X μ l
Total	20 μ l

- Incubate at 37°C for 10 min.
- Add 80 μ l of sterile purified water and 100 μ l of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and vortex.
- Centrifuge at 12,000 rpm for 5 min at room temperature, then transfer the upper layer to a new tube.
- Add an equal amount of chloroform/isoamyl alcohol (24 : 1) and vortex.
- Centrifuge at 12,000 rpm for 5 min at room temperature, then transfer upper layer to a new tube.
- Add 10 μ l of 3M sodium acetate and 250 μ l of chilled ethanol, and then mix. Stand it for 20 min at -80°C.
- Centrifuge at 12,000 rpm for 10 min at 4°C. Remove the supernatant.
- Wash the precipitate with chilled 70% ethanol. Centrifuge at 12,000 rpm for 5 min at 4°C and remove the supernatant.
- Dry the precipitate.
- Dissolve the precipitate in 10 - 20 μ l of TE buffer.

< Ligation reaction >

- Dispense 5 μ l of DNA solution obtained at step 11 into a new microtube.
- Add 1 μ l of pUC118 *Hinc* II/BAP (50 ng/ μ l).
- Add the equivalent volume (6 μ l) of Ligation Mighty Mix and mix gently.
- Incubate at 16°C for 1 hour.
- Perform a transformation using the whole solution prepared at step 15 for 100 μ l of *E. coli* competent cell. When carrying out a transformation by the electroporation, exchange the buffer by phenol/chloroform extraction and ethanol precipitation before transformation.
- Spread to the LB plate including Ampicillin, X-Gal, and IPTG.

C. Control experiment :

1. Prepare the following reaction mixture.

Reagents	Volume
Control Insert	2 μ l
10X Blunting Kination Buffer	2 μ l
Blunting Kination Enzyme Mix	1 μ l
ddH ₂ O	15 μ l
Total	20 μ l

- Incubate at 37°C for 10 min.
- Add 80 μ l of sterile purified water and 100 μ l of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and vortex.
- Centrifuge at 12,000 rpm for 5 min at room temperature, then transfer the upper layer to a new tube.
- Add an equal amount of chloroform/isoamyl alcohol (24 : 1) and vortex.
- Centrifuge at 12,000 rpm for 5 min at room temperature, then transfer upper layer to a new tube.

7. Add 10 μ l of 3M sodium acetate and 250 μ l of chilled ethanol, and then mix. Stand it for 20 min at -80°C .
8. Centrifuge at 12,000 rpm for 10 min. at 4°C . Remove the supernatant.
9. Wash the precipitate with chilled 70% ethanol. Centrifuge at 12,000 rpm for 5 min at 4°C and remove the supernatant.
10. Dry the precipitate.
11. Dissolve the precipitate in 20 μ l of TE Buffer.
12. Dispense 5 μ l of DNA solution obtained at step 11 into a new microtube.
13. Add 1 μ l of pUC118 *Hinc* II/ BAP (50 ng/ μ l).
14. Add 6 μ l of Ligation Mighty Mix and mix gently.
15. Incubate at 16°C for 1 hour.
16. Perform a transformation using the whole solution prepared at step 15 for 100 μ l of *E. coli* competent cell.
17. Spread to the LB plate including Ampicillin, X-Gal, and IPTG.

When the *E. coli* JM109 competent cell having the transformation efficiency of 1×10^8 colonies/ μ g pUC118 DNA is used, 2 - 8×10^4 colonies would be obtained as white colony per 50 ng vector.

The sequence of both ends of Control Insert is 5'-GAC...GTC-3'.

When the protocol is performed in a correct method, *Hinc* II sites are regenerated and insert can be excised.

Notes :

1. Thaw Ligation Mighty Mix on ice or in ice water. Mix gently before use. Ligation Mighty Mix would not be inactivated by freeze-thaw cycles.
2. When ligation efficiency is low, extend the ligation reaction time to overnight. When the above remedy would not improve the reaction, repurification of DNA is recommended.
3. Light blue colonies may appear in color selection on X-Gal plate, when short DNA fragment is cloned with a vector having *lacZ*. This is because stop codon does not appear or frame shift does not occur, even after the insertion of DNA.
4. When cloning short DNA fragments, clones transformed with a plasmid containing multiple inserts may appear.

VIII. Experimental Examples : Cloning of various size fragment

(A) When PCR products are directly used

Various size fragments (500 bp, 1 kb, 2 kb, 4 kb, and 6 kb) were amplified with PrimeSTAR HS DNA Polymerase using λ DNA as template. 2 μ l of each PCR product was blunted, phosphorylated with this kit, cloned into pUC118 *Hinc* II/BAP, and then transformed in *E. coli* JM109 Competent cells for color selection. The cloning of insert were verified by PCR using primers, M13 Primer M4 and RV.

Insertion DNA length (bp)	White colony/Blue colony (/50 ng pUC118 DNA)	Insert DNA/White colony
500	$1.8 \times 10^5 / 8.8 \times 10^3$	10/10
1,000	$1.2 \times 10^5 / 5.8 \times 10^3$	10/10
2,000	$2.2 \times 10^4 / 7.5 \times 10^3$	8/10
4,000	$1.8 \times 10^4 / 1.4 \times 10^4$	9/10
6,000	$8.9 \times 10^3 / 2.0 \times 10^4$	8/10

* Transformation efficiency of the used *E. coli* JM109 Competent cells :
 3.9×10^8 colonies/ μ g pUC118 DNA

(B) When using DNA fragments purified by agarose gel electrophoresis

Two DNA fragments were amplified as follows; 500 bp obtained with *TaKaRa Ex Taq* DNA Polymerase by using λ DNA as template, and 2 kb amplified with PrimeSTAR HS DNA Polymerase by using human genomic DNA as template. They were applied to agarose gel electrophoresis after PCR, and were recovered with EASYTRAP Ver.2 (discontinued). Each of the DNA were blunted and phosphorylated with this kit, 2 pmol (approx. 660 ng) for the 500 bp fragment, and 0.2 pmol (approx. 250 ng) for the 2 kb fragment. Then they were cloned into pUC118 *Hinc* II/BAP and were transformed in *E. coli* JM109 competent cells for color selection. The cloning of insert were verified through PCR by using primers: M13 Primer M4 and RV.

Insertion DNA length (bp)	White colony/Blue colony (/50 ng pUC118 DNA)	Insert DNA/White colony
500	$1.3 \times 10^5 / 4.6 \times 10^3$	10/10
2,000	$1.6 \times 10^4 / 7.1 \times 10^3$	9/10

* Transformation efficiency of the used *E. coli* JM109 Competent cells :
 4.6×10^8 colonies/ μ g pUC118 DNA

IX. Comparison among PCR enzymes

One kilobase PCR products were prepared with *TaKaRa Taq*, *TaKaRa Ex Taq*, *TaKaRa LA Taq*, PrimeSTAR HS, and *TaKaRa EX Taq* Hot Start Version DNA Polymerases using λ DNA as a template. Following the protocol of this kit, they were cloned into *Hinc* II/BAP site of pUC118. Then the transformation was done using *E. coli* JM109 and then color selection was performed. The cloning of insert were verified through PCR by using primers: M13 Primer M4 and RV.

DNA Polymerase used for PCR	White colony/Blue colony (/50 ng pUC118 DNA)	Insert DNA/White colony
PrimeSTAR HS	$8.9 \times 10^4 / 4.5 \times 10^3$	9/10
<i>TaKaRa Taq</i>	$1.2 \times 10^5 / 1.0 \times 10^4$	9/10
<i>TaKaRa Ex Taq</i>	$1.5 \times 10^5 / 1.0 \times 10^4$	10/10
<i>TAKaRa LA Taq</i>	$8.3 \times 10^4 / 5.5 \times 10^3$	9/10
<i>TaKaRa Ex Taq</i> Hot Start Version	$8.1 \times 10^4 / 6.3 \times 10^3$	10/10

* Transformation efficiency of the used *E. coli* JM109 Competent cells :
 4.6×10^8 colonies / μ g pUC118 DNA

X. Related Products

pUC118 *Hinc* II/BAP (Cat. #3322)
E. coli JM109 Competent Cells (Cat. #9052)*
E. coli JM109 Electro-Cells (Cat. #9022)*
 NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250)

PrimeSTAR® HS DNA Polymerase (Cat. #R010A/B)
 PrimeSTAR® GXL DNA Polymerase (Cat. #R050A/B)
 PrimeSTAR® Max DNA Polymerase (Cat. #R045A)
TaKaRa Ex Taq® DNA Polymerase (Cat. #RR001A/B/C)
TaKaRa Ex Taq® DNA Polymerase Hot Start Version (Cat. #RR006A/B)
TaKaRa LA Taq® DNA Polymerase (Cat. #RR002A)
TaKaRa Taq™ DNA Polymerase (Cat. #R001A/B/C)

SpeedSTAR™ HS DNA Polymerase (Cat. #RR070A/B)
 EmeraldAmp® MAX PCR Master Mix (Cat. #RR320A)
 SapphireAmp® Fast PCR Master Mix (Cat. #RR350A/B)

* Not available in all geographic locations. Check for availability in your area.

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