Cat. # 6025

For Research Use

TaKaRa

DNA Blunting Kit

Product Manual

v202001Da

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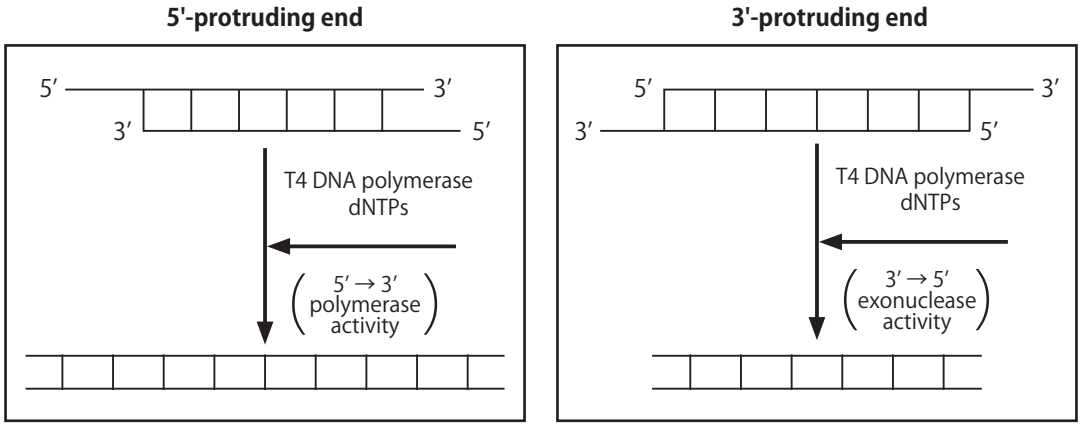
I. Description

This kit allows the conversion of 3'- and 5'-protruding ends of DNA fragments to blunt or flush ends. Conversion of the two types of protruding ends can be accomplished simultaneously by the 3' → 5' exonuclease and 5' → 3' polymerase activities of T4 DNA polymerase. The resulting blunt-ended DNA can be ligated efficiently into a blunt-ended vector using the DNA ligation solutions (included). The reaction mixture can proceed directly to bacterial transformation or *in vitro* phage packaging reaction without the need to purify the DNA.



The kit also allows the conversion of protruding ends of vector DNA to blunt ends. Blunt-ended vector is used for ligation after dephosphorylation.
PCR products having 3'-dA nucleotide overhang cannot be ligated into blunt-ended vectors. But, the PCR products, converted to blunt-ends using this kit and phosphorylated if necessary, will be ligated with blunt-ended vectors efficiently.

II. Components

1.	T4 DNA Polymerase	20 μl
2.	10X Buffer (with dNTP)	30 μl
3.	DNA Dilution Buffer*1	500 μl
4.	Ligation Solution A*2	600 μl x 2
5.	Ligation Solution B*2	150 μl

*1 100mM Tris-HCl (pH 7.6 at 16℃), 5mM $MgCl_2$
*2 Ligation Solution A and B are same as the components of the DNA Ligation Kit Ver.1 (Cat. #6021).

III. Storage -20℃

IV. Protocol

A. Dephosphorylation of Vector DNA

The insertion of DNA fragments into vector DNA proceeds efficiently when the 5'-ends of vector DNAs are dephosphorylated to minimize self-circularization. Fragments generated by restriction cleavage are 5'-phosphorylated even after they are treated with T4 DNA polymerase for blunting, and therefore, vector DNAs should be dephosphorylated with enzymes such as alkaline phosphatase as described in the following procedure.

1) Prepare blunt-ended vector DNA that is completely digested with restriction enzyme(s) Purify the DNA by ethanol precipitation.
   To convert protruding ends of vector to blunt ends, perform the reaction as described in section B. Blunting Reaction. After blunting reaction, extract DNA with phenol/chloroform and purify by ethanol precipitation. The precipitate is dissolved in sterile purified water.
2) Combine the following in a microcentrifuge tube (total 150 μl).

Reagent	Amount
vector DNA (blunt-ended)	< 10 μg
10X Alkaline phosphatase Buffer	15 μl
bacterial alkaline phosphatase* (0.5 - 1.0 U/μl)	2 μl
Sterile purified water	up to 150 μl

3) Incubate at 65℃ for 30 minutes.
   * If calf intestine alkaline phosphatase (CIAP) is used, use 2 μl of CIAP (10 - 20 U/μl) in place of bacterial alkaline phosphatase, and incubate at 50℃ for 30 minutes.
4) Add 150 μl of phenol/chloroform (1 : 1) and mix well.
5) Centrifuge and transfer the upper layer to a new tube.
6) Repeat steps 4) and 5).
7) Add 15 μl of 3 M Sodium acetate. (pH 4.8 - 5.2)
8) Add 375 μl (2.5 volumes) of cold ethanol. Leave at -20℃ for 30 - 60 minutes.
9) Centrifuge and collect DNA precipitate.
10) Rinse precipitate with 1 ml of cold 70% ethanol. Dry under reduced pressure.
11) Dissolve precipitate in TE buffer. If the DNA is to proceed directly to ligation, dissolve with the DNA Dilution Buffer (included) instead of TE.

B. Blunting Reaction

1) Combine the following in a microcentrifuge tube (total volume 9 μl).

Reagent	Amount
DNA fragment (with protruding ends)*	0.1 - 10 pmol
10X Buffer (included)	1 μl
Sterile purified water	up to 9 μl

* DNA should be purified at least by ethanol precipitation

2) Incubate at 70℃ for 5 minutes, and then transfer to a 37℃ incubator.
3) Add 1 μl of T4 DNA polymerase and mix gently by pipetting. Do not vortex.
4) Incubate at 37℃ for 5 minutes. Accurate incubation time is very important. (If the DNA has a low GC content, incubate at 25℃ instead of 37℃.)
5) Vortex vigorously to inactivate T4 DNA polymerase. (If the DNA concentration is high, add DNA Dilution Buffer to bring the DNA concentration to 1 μg/50 μl and vortex thoroughly.) Place the tube on ice to ensure the inactivation of T4 DNA polymerase. If the solution is to be stored, immediately extract DNA with phenol/chloroform and purify by ethanol precipitation. The precipitate should be dissolved with DNA Dilution Buffer and stored at -20℃.

C. Ligation Reaction

1. Insertion of DNA fragments into plasmid vector

1) Prepare 5 - 10 μl of DNA solution containing blunt-ended insert DNA fragment and dephosphorylated vector DNA (about 50 ng) in DNA Dilution Buffer. The insert DNA fragment should be present in 5 - 10 fold molar excess relative to the vector DNA.
2) Add 4 - 8 volumes (20 - 80 μl) of Ligation Solution A, and mix well.
3) Add one volume (5 - 10 μl) of Ligation Solution B, and mix well.
4) Incubate at 16℃ for 30 minutes.
5) Proceed to bacterial transformation using up to 20 μl of the above solution for 100 μl of competent cells. (If transformation by electroporation is desired, purify the DNA by ethanol precipitation and dissolve the DNA in an appropriate buffered solution or sterile purified water. Do NOT use the ligation solution directly as it will generate sparks by the use of pulsed electric fields.)

2. Self-circularization of linear blunt-ended DNA

Proceed essentially as described above, except use lower concentrations of DNA to ensure higher efficiency of intramolecular ligation. Keep the volume of the ligation mixture as small as possible to obtain higher transformation efficiency.

3. Insertion of linker DNA into blunt-ended vector DNA

Proceed essentially as described above, except if the linker is AT-rich or is shorter than 8 bases, carry out the ligation reaction at 4 - 10℃ for 1 - 2 hours.

V. Experimental Examples

1. Efficiency of the blunting reaction can be tested by transformation

A plasmid DNA is linearized by one of the three types of restriction enzymes which either creates 5'-protruding ends, 3'-protruding ends, or blunt ends. If the blunting reaction on the 5'- or 3'-protruding plasmid is successful and complete, the original restriction site will be abolished when the plasmid is circularized by ligation. Thus, the DNA in the ligation solution will be resistant against redigestion by the same enzyme and there will be little difference between the transformation efficiencies of non-digested or redigested ligation samples. In contrast, the blunting reaction should not affect ends that are already made blunt by restriction cleavage, in which case the self-circularization of the plasmid will restore the original restriction site. The ligation sample will be susceptible to redigestion, and its transformation will be abolished by complete redigestion. This has been the case when the kit was tested on pUC18 plasmid DNA (0.1 μg ea.) digested with either *Eco*R I, *Pst* I, or *Sma* I. In this case, JM109 indicator cells and X-gal plates containing IPTG were used so that the disruption of the restriction sites can also be monitored by the appearance of white colonies in contrast to blue colonies formed by transformants carrying intact pUC18.

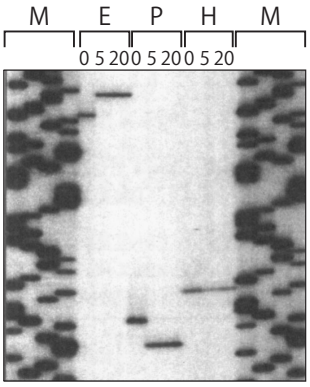
Blunting	Other treatments	No. of white colonies/μg DNA		
		pUC18 *Eco*R I	pUC18 *Pst* I	pUC18 *Sma* I
NO	---	$<10^2$	$<10^2$	$<10^2$
NO	ligation	$<10^2$*	$<10^2$*	$<10^2$*
NO	ligation	$<10^2$	$<10^2$	$<10^2$
YES	---	$<10^2$	$<10^2$	$<10^2$
YES	ligation	0.7×10^5	3.1×10^5	$<10^2$*
YES	ligation & redigestion	0.6×10^5	4.1×10^5	$<10^2$

* includes blue colonies $>10^5$

2. Confirmations of blunting by gel electrophoresis

Single-stranded phage DNA (M13 mp18 : Cat. #3518) was annealed with 5' [^{32}P]-labeled sequencing primer. The complementary strand was generated using the Klenow fragment of *E.coli* DNA polymerase I. The double-stranded DNA was then treated either with *Eco*RI, *Pst* I, or *Hin*cII, and then blunted for 5 or 20 minutes according to section B. at IV. Protocol. The electrophoresis result autoradiography (bellow) indicates that 5'-protruding ends (generated by *Eco*RI) were converted into blunt ends by elongation of the 3'-recessed strand (5' → 3' fill-in reaction), while 3'-protruding ends (generated by *Pst*I) were processed by the 3' → 5' exonuclease reaction. Blunt ends (*Hin*cII) were not affected.

M : M13 mp18 sequence ladder
E : *Eco*RI treated DNA
P : *Pst* I treated DNA
H : *Hin*cII treated DNA



3. Blunting and cloning of PCR amplified products

A 1.47 kb kanamycin resistance element derived from Tn903 (Oka, *et al. J Mol Biol.* (1981) **147**: 217-226) was amplified by PCR. This PCR products was separated by gel electrophoresis, excised and purified by NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250). About 500 ng (0.5 pmol) of this DNA was treated with the blunting reagents as described in section B of IV. Protocol, and were mixed with 100 ng of *Hin*cII-digested pUC118 DNA (0.05 pmol). The mixture was used in the ligation reaction (total 60 ul) as described in section C of IV. Protocol. About 20 μl of this ligation mixture was used to transform 100 μl of *E. coli* JM109 competent cells. A combination of phosphorylated/dephosphorylated DNA (vector or PCR product) were tested as shown bellow. (Note that by using phosphorylated vector DNA, the probability of self-circularization of the vector will increase and would lead to a relatively high background of blue colonies. Nonetheless, the percentage of white colonies carrying the PCR insert will be high).

Treatments			Transformation efficiency blue colonies/white colonies (colonies/μg vector DNA)	*kan* resistant clones /white colonies
PCR product		Vector DNA Dephosphorylation		
Blunting	5' phosphorylation			
NO	NO	NO	$1.4 \times 10^6/1.8 \times 10^4$	0
YES	NO	NO	$1.2 \times 10^6/3.2 \times 10^4$	64%
YES	YES	YES	$6.0 \times 10^2/2.1 \times 10^4$	71%

＊ Transformation efficiency of competent cells used in this test was 1.5×10^7/μg pUC118 DNA

VI. Notes

1) The DNA Blunting Kit kit can blunt DNA fragments with dephosphorylated 5'-protruding ends, but not those with phosphorylated 3'-recessed ends which are often present in sonicated DNA used in shotgun cloning experiments.
2) Ligation solutions A and B should be thawed on ice and mixed thoroughly immediately before use.
3) It is not necessary to extract the DNA with phenol after blunt-ending and ligation. Bacterial transformation or other reactions can proceed directly. The DNA can be ethanol precipitated if it must be concentrated for further use.

VII. Related Products

DNA Ligation Kit. Ver. 1 (Cat. #6021)
DNA Ligation Kit. Ver. 2.1 (Cat. #6022)
DNA Ligation Kit ＜ Mighty Mix ＞ (Cat. #6023)
E.coli JM109 Competent Cells (Cat. #9052)
E.coli DH5 *α* Competent Cells (Cat. #9057)
Alkaline Phosphatase (*E.coli* C75) (Cat. #2120A/B)
Alkaline Phosphatase（Calf intestine）(Cat. #2250A/B)
T4 Polynucleotide Kinase (Cat. #2021A/B)
T4 DNA Polymerase (Cat. #2040A/B)
NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250)