**Product Name:** Alkaline Phosphatase (PAP)
(from *Shewanella sp.* SIB1)*

**Code No:** DE110

**Size:** 1,000 unit

**Concentration:** 5unit/μl

**Supplied reagent**
- 10×Alkaline Phosphatase Reaction Buffer
- Dilution Buffer

**Storage:** Store at -20℃

**Source:** *E.coli* harboring the plasmid encoding the gene of alkaline phosphatase from a psychrotrophic bacterium *Shewanella sp.* SIB1 (PAP).

**Unit Definition:** One unit is defined as the amount required to hydrolyzed 1.0 μmole p-nitrophenyl phosphate per 1 minute in glycine-NaOH buffer at pH10.5 and 37℃.

**Assay conditions:** The reaction mixture (100μl) contains 50mM glycine-NaOH buffer, pH 10.5, 5mM $MgCl_2$, 0.5mM $ZnCl_2$, 100mM KCl, 5mM p-nitrophanyl phosphate.

**Storage Buffer:**

| | | |
|---|---|---|
| 10mM | Tris-HCl | pH7.5 |
| 0.025mM | $ZnCl_2$ | |
| 0.25mM | $MgCl_2$ | |
| 50% | glycerol | |

**Contaminants:**

**Dnase:** When 0.5μg of λ/*Hin*d III digest was incubated with 10units of this enzyme in a 40μl reaction mixture for 18 hours at 37℃, no degradation of the DNA fragment is observed on agarose gel electrophoresis.

**Rnase:** No RNase activity is observed by the use of RnaseAlert assay (Ambion). In this assay the reaction mixture containing the fluorescent-labeled RNA substrate was incubated with 10units of this enzyme for 1 hours at 37℃.

*** Licensed Under Japan Patent NO. 2001-172653**

**Composition of Supplied Reagent:**

10×Alkaline Phosphatase Reaction Buffer ( Store at –20℃)

| | |
|---|---|
| 1. 5M | Tris-HCl, pH7.3 |
| 125mM | glycine |
| 0. 5% | TritonX-100 |
| 0.25mM | $ZnCl_2$ |
| 2.5mM | $MgCl_2$ |
| 60mM | $NiCl_2$ |

Dilution Buffer ( 1×Reaction Buffer, Store at –20℃)

| | |
|---|---|
| 150mM | Tris-HCl, pH7.3 |
| 12.5mM | glycine |
| 0.05% | TritonX-100 |
| 0.025mM | $ZnCl_2$ |
| 0.25mM | $MgCl_2$ |
| 6mM | $NiCl_2$ |

**Dephosphorylation of 5' end by PAP:**

| | |
|---|---|
| Linearized plasmid DNA *1 | 88µl (5' termini 10pmol)*2 |
| 10×Reaction buffer | 10µl |
| Diluted PAP solution*3 | 2µl*4 |

↓

Incubate for 30 min at 60℃ (blunt end and 3'-protruding end)
or at 37℃ (5'-protruding end)
Inactivation by incubation for 5min at 95℃*5

↓

Dephosphorylated Plasmid DNA

**Ligation Reaction and Transformation:**

| | | |
|---|---|---|
| Dephosphorylated Plasmid DNA | | 1µl (0.05pmol)*6 |
| Insert DNA (foreign) | | 1µl (0.05～0.3pmol)*7 |
| 10×Ligation buffer*8 | | 1µl |
| T4 DNA ligase | | 0.5 Weiss unit |
| 10mM ATP | | 1µl |
| $H_2O$ | up to | 10µl |

↓

Incubate for 1 houre at 12℃ (blunt end)
or at 16℃ (5'-protruding end and 3'-protruding end)

↓

2µl (0.01pmol)*9

↓

Add 100µl of competent cell
Store on ice for 30min
Heat shock for 30sec at 42℃
Store on ice 1min
Add 0.9ml of SOC medium
Incubate for 1hr at 37℃
Spread on a selective plate

↓

Incubate overnight at 37℃

*1 Before the dephosphorylation, the complete digestion of the plasmid DNA should be confirmed by the agarose gel electrophoresis. Actually, it is difficult to completely digest vector DNA less than 4 kb in

size. We recommend to purify the linearized vector DNA from agarose gel after electrophoresis by using Gel Indicator (Code No. DM510).

Restriction enzyme buffer (such as low buffer, medium buffer and high buffer) and 1×TE buffer is permissible as a buffer solution of the linearized plasmid DNA.

*2 Amount and length of the linearized plasmid DNA

**Table 1**

| | | | |
|---|---|---|---|
| 1kb linear DNA | 10pmol of 5'-tremini | = | 3.3μg |
| 2kb linear DNA | 10pmol of 5'-tremini | = | 6.6μg |
| 3kb linear DNA | 10pmol of 5'-tremini | = | 9.9μg |
| 4kb linear DNA | 10pmol of 5'-tremini | = | 13.2μg |

For example, 10pmole of 5'-termini of the linearized pUC18 (2.69kb) is 8.8μg.

*3 Amount of PAP

Amount of PAP should be modified depending on the kind of termini and the amount of 5'-termini of linearized plasmid DNA. The following amounts are recommended:

**Table 2**

| Terminus | | Units of PAP |
|---|---|---|
| 5'-Protruding | (10pmol) | 1.0 units (37℃ 30min) |
| Blunt | (10pmol) | 2.5 units (60℃ 30min) |
| 3'-Protruding | (10pmol) | 5. 0 units (60℃ 30min) |

PAP should be diluted with dilution buffer (1×reaction buffer) according to the table above. As the amount of PAP described in the table above are about ten times as much as that of the minimum effective amount, the condition is sufficient for complete dephosphorylation. If you use over ten times amount of PAP shown in the table 2, we recommend phenol extraction to inactivate PAP completely instead of heat inactivation (see *4 and *5).

*4 Non-diluted PAP solution should be added not to exceed 10% in a volume of the final reaction buffer. Glycerol in non-diluted PAP solution and high concentration of PAP protein hamper heat inactivation of PAP. If the reaction mixture contains non-diluted PAP solution up to 20% of its volume, the activity of about 1/7,000～1/50,000 still remains. When you use non-diluted PAP solution over 10% volume of reaction mixture, we recommend phenol extraction for complete inactivation of PAP (see *5).

*5 Heat inactivation

At least 10 μl of reaction mixture is required in 0.5ml tube for heat inactivation. Before heat inactivation, you should spin down solution in the tube. After heat inactivation, the white precipitate of inactivated PAP protein is observed. It is not necessary to be removed, because the precipitate does not

affect the next ligation reaction.

Nearly all of PAP are inactivated by incubation at 95℃ for 5min after dephosphorylation and trace amount activity, 1/100,000～1/400,000, remains. It is negligible level in the next ligation reaction, because the residual PAP activity can only dephosphorylate insert DNA (foreign) less than 1/10,000 of the initial vector plasmid DNA in the molar amount, if you follow the above protocol.

If you use over ten times amount of enzyme the condition described in table 2 or use over 10% volume of final reaction mixture, we recommend phenol extraction to inactivate PAP completely instead of heat inactivation as follows;

**Phenol Extraction:**

Alkaline phosphatase reacton product

- Phenol/chloroform (1:1) extraction (2 times)
- Chloroform extraction (3 times)
- Add 10ul 3M Na Acetate pH5.2 or 10ul 1M NaCl
- Add 70µl isopropanol
- Centrifuge at 14,500 rpm for 10min
- Wash with 70% ethanol
- Dry under vacuum

Dephosphorylated Plasmid DNA

PAP is easily and completely inactivated even by one time phenol extraction. On the other hand, BAP is known to be much more resistant to phenol extraction as well as heat inactivation. For example, about 2.5% of BAP remain active after one time phenol extraction and following ethanol precipitation (http://www.biodynamics.co.jp/).

*6 It is possible that alkaline phosphatase reaction mixture is directly added to ligation reaction mixture up to 30% for cohesive end ligation, up to 10% for blunt end ligation of the final reaction volume without interference.

The commercially available ligation kit such as DynaExpressDNA Ligation Kit ver.2 (BioDynamics Laboratory.Inc, DS110), DNA Ligation Kit ver.2 (TaKaRa), Ligation High (TOYOBO CO., LTD), Quick Ligation™ Kit (New England Biolabs. Inc) and LigaFast™ Rapid DNA Ligation Kit (Promega) can be effectively used in instead of this ligation protocol. During you use these kits, the orange precipitate derived from reducted Ni is observed. It is not necessary to remove the precipitate because it does not affect the ligation and the next transformation process.

*7 Chill the reaction mixture of insert DNA and vector DNA to 0℃ before ligation procedure.

The molar ratio of vector DNA to insert DNA should be between 1:1 and 1:6. The final DNA concentration of vector DNA and insert DNA should be between 1ng/µl and 10ng/µl for an effective ligation.

LigaFast™ and Quick Ligation™ are trademarks of Promega Corporation and New England Biolabs, Inc., respectively.

*8 10×Ligation buffer
660mM Tris-HCl (pH7.6), 66mM $MgCl_2$, 100mM DTT

*9 Amount of ligation mixture should be added in a volume not to exceed 10% of that of competent cells. If you carry out transformation of *E.coli* by electroporation, we recommend spin column purification of ligation mixture.