FOCUS*On*

Alkaline Phosphatase

Technical Bulletin 18009-1

FOCUS On Technical Bulletins contain useful protocols and troubleshooting information for various techniques. Many of the protocol modifications are the result of experiments performed in our Applications Laboratory. The bulletins also provide information that answers the most commonly asked questions our scientists receive on the TECH-LINE. They are written by scientists in the Technical Support and Customer Training Team at Life Technologies.

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Introduction

Alkaline phosphatases are phosphomonoesterases that remove phosphates from DNA or RNA at alkaline pHs (>8) (1,2). They are used to dephosphorylate the 5´ ends of DNA for radiolabeling with T4 polynucleotide kinase or to prevent self-ligation of vector DNA during cloning *(figure 1)*. Ligation of DNA requires a 5´ phosphate (1,2) on one of the ends to be ligated together. Therefore, dephosphorylated vector DNA cannot self-ligate while a DNA insert, containing phosphates on the 5´ termini, can be efficiently ligated into the vector. So dephosphorylation of vector DNA lowers the number of vector self-religation background colonies. Alkaline phosphatases also remove 5´ phosphates from RNA (3) and 3´ phosphates from DNA (4). Additionally, calf intestinal alkaline phosphatase can be used to dephosphorylate proteins (5).

Two commonly used alkaline phosphatases are calf intestinal alkaline phosphatase (CIAP) and bacterial alkaline phosphatase (BAP). A genetically engineered, mutant BAP called temperature sensitive alkaline phosphatase (TsAP) with 40 times higher activity than BAP, was developed with the advantage of being heat inactivated at 65°C in the presence of EDTA (6). The enzymes are compared in table 1.

In this bulletin, two protocols for DNA dephosphorylation are given. The *Traditional Protocol* employs the reaction buffer supplied with the enzyme, purified DNA, and a minimal amount of enzyme. The *Simplified Protocol* is the result of a series of experiments (see *Additional Information*) which allow a stream-lined protocol that can use unpurified DNA directly from a restriction digestion.

Figure 1

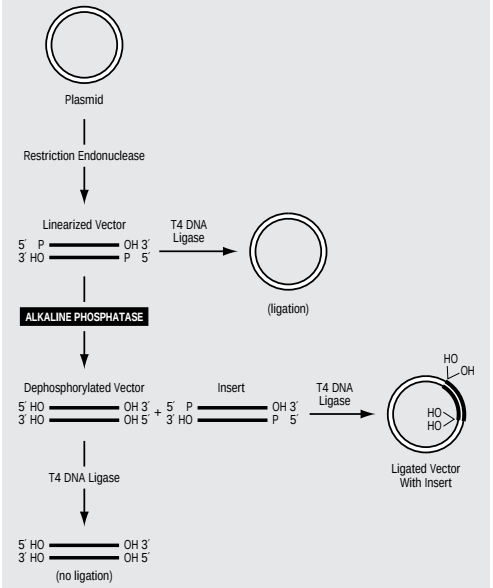

FIGURE 1. Scheme for vector dephosphorylation and insert ligation.

Table 1			
ENZYME PROPERTY	**CIAP**	**BAP**	**TsAP**
Molecular Weight	140 kDa	95 kDa (dimer) 48 kDa (monomer)	95 kDa (dimer) 48 kDa (monomer)
Source	Calf intestines	Periplasmic space of *E. coli*	Expressed as a recombinant protein in *E. coli*
REACTION CONDITION	**CIAP**	**BAP**	**TsAP**
Reaction temperature	37°C (5′ overhang) 50°C (blunt ends or 5′ recessed)	65°C	37–65°C
Reaction time (Traditional)	0.5 h (5′ overhang) 1 h (blunt, 5′ recessed)	1 h	15 min
Optimal pH Range	9.4–10.5	8.0–9.5	9.4–10.5
Metal ion requirement (Catalytic)	—	—	Mg^{2+}
Heat Inactivation	Yes, with EDTA 65°C, 15 min	No	Yes, with EDTA 65°C, 15 min

TABLE 1. Comparison of alkaline phosphatases.

Unit Definitions

(1 pNPP unit ≈ 5,270 ATP units)

CIAP: One unit hydrolyzes 1 µmol of 4-nitrophenyl phosphate in 1 min at 37°C.

BAP: One unit hydrolyzes 1 nmol ATP in 30 min at 37°C.

TsAP: One unit hydrolyzes 1 µmol of 4-nitrophenyl phosphate in 1 min at 37°C.

Materials

In addition to the enzyme and DNA, the following reagents are required for the protocols described below:

Dephosphorylation Protocol:
water bath (37°C to 65°C)
1.5-ml microcentrifuge tubes
autoclaved distilled water

For Inactivation/Removal of Enzyme:
(depends on method used)
TE buffer [10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA]
0.5 M EDTA (pH 8.0)
buffer-saturated phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)]
3 M sodium acetate
100% ethanol
microcentrifuge capable of 15,000 × *g*
CONCERT™ Rapid PCR Purification System
CONCERT Gel Extraction Systems

Calculating picomoles of ends of DNA

Use the following calculation and the information in table 2 to calculate the mass of DNA/pmol of DNA ends:

moles of ends of dsDNA fragment =
2 × (g of DNA)/[molecular mass of DNA(Da)]
or
moles of ends of dsDNA fragment =
2 × (g of DNA)/[(number of bp) × (660 Da/bp)]

Table 2		
Size of Fragment (kb)	**µg DNA/ 1 pmol of ends**	**No. of pmol ends/µg DNA**
0.1 kb	0.033	30.30
0.5 kb	0.17	6.06
1 kb	0.33	3.03
2 kb	0.66	1.52
2.7 kb (linearized pUC DNA)	0.89	1.12
4.1 kb (linearized pSPORT I DNA)	1.35	0.74
5 kb	1.64	0.61
10 kb	3.30	0.30

TABLE 2. Picomole equivalents for a microgram of different size DNAs.

Traditional Protocol for Dephosphorylation of DNA

This protocol dephosphorylates 1 pmol of 5´ DNA termini from purified DNA. DNA dephosphorylated by this protocol is suitable for cloning or for labeling by T4 polynucleotide kinase using the *Forward Reaction.*

1. Determine the mass of DNA required for 1 pmol of the type of DNA 5´ ends from table 2.
2. To a 1.5-ml microcentrifuge tube, add 4 µl of the appropriate 10X alkaline phosphatase reaction buffer and 1 pmol of DNA ends. For TsAP, add $MgCl_2$ so the final concentration is 5 mM.
3. Add autoclaved, distilled water to 39 µl.
4. Dilute the enzyme in dilution buffer (see formulas in *Additional Information*) so 1 µl contains the amount of enzyme in the table below and add to reaction.

	CIAP (units)	BAP (units)	TsAP (units)
5´ Recessed	1	70	0.2
Blunt	1	70	0.2
5´ Overhang	0.01	70	0.02

5. Incubate the reaction as follows:

	CIAP	BAP	TsAP
Temperature	37°C (5´ overhangs) 50°C (blunt or 5´ recessed ends)	65°C	50°C
Time	0.5 h (5´ overhangs) 1 h (blunt or 5´ recessed ends)	1 h	15 min

6. Inactivate or remove the alkaline phosphatase (see protocols below).

Simplified Protocol for the Dephosphorylation of Vector DNA

This protocol allows you to dephosphorylate DNA directly in restriction endonuclease buffer in the presence of the restriction endonuclease by adding more alkaline phosphatase. This is a convenient way to prepare DNA for cloning (see *Additional Information* for data used to determine this protocol).

1. Restriction endonuclease digest the vector DNA. (NOTE: Heat inactivation of the restriction endonuclease and subsequent purification of the DNA vector are not necessary).
2. Add 1 µl of alkaline phosphatase to the restriction endonuclease digest. (150 units of BAP, 1 unit of CIAP, and 1 unit of TsAP.)
3. Incubate the reaction as follows:

	CIAP	BAP	TsAP
Temperature	37°C (5´ overhangs) 50°C (blunt or 5´ recessed ends)	65°C	65°
Time	5 min	1 h	15 min

(NOTE: For TsAP, the reaction is performed at 65°C instead of 50°C, because DNA dephosphorylated at 50°C in the *Simplified Protocol* did not perform well in cloning experiments.)

4. Inactivate or remove the alkaline phosphatase (see protocols below).

Protocols for Inactivation/ Removal of Enzyme

Heat Inactivation (for CIAP or TsAP)

1. Note the $MgCl_2$ concentration in the reaction and add EDTA (pH 8.0) to an equal final concentration.
2. Incubate the reaction at 65°C for 15 min.

Organic Extraction

1. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Vortex thoroughly and centrifuge at room temperature for 5 min at 14,000 × *g* to separate the phases.
2. Carefully remove the upper, aqueous layer and transfer it to a fresh microcentrifuge tube.
3. Add 0.1 volumes of 3.0 M sodium acetate. Vortex. Add 2.5 volumes of 100% ethanol. (Do not substitute ammonium acetate for sodium acetate since ammonium ions inhibit T4 polynucleotide kinase).
4. Vortex the mixture thoroughly and centrifuge at room temperature for 15 min at 14,000 × *g*.

The CONCERT Rapid PCR Purification System can be used as an alternative to organic extraction to remove the alkaline phosphatase.

CONCERT Gel Extraction Systems

Following electrophoresis of dephosphorylated DNA on an agarose gel, use the protocols supplied with the CONCERT Gel Extraction Systems to purify the DNA.

Additional Information

Activity of Alkaline Phosphatases in Restriction Endonuclease Buffers

CIAP, BAP, and TsAP were able to dephosphorylate 5´ ends generated by restriction endonuclease digestion of a purified substrate with or without restriction endonuclease (*Acc* I, *Bam*H I, *Eco*R I, *Hin*c II; *Pst* I, *Sal* I, and *Sph* I) in the most commonly used REACT Buffers at 1X concentration *(table 3)*. Several of the enzymes tested have anecdotal accounts of interfering with subsequent modifications of digested DNA. The dephosphorylation was 97 to 99% in the presence of all enzymes except *Sal* I. The inhibition of dephosphorylation with *Sal* I occurred with cloned enzymes and was not manufacturer specific. Inhibition was overcome by the addition of excess alkaline phosphatase (1 unit CIAP, 1 unit TsAP, and 150 units BAP) *(figure 2)*. The efficiency of dephosphorylation was also measured by the decrease in the ability to transform bacterial cells after religation (7). These results suggest that in most cases the purification of DNA after restriction endonuclease digestion is not necessary prior to dephosphorylation.

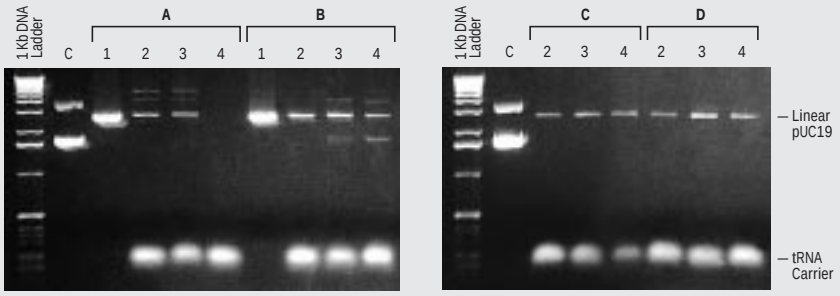

FIGURE 2. Effect of *Sal* I on alkaline phosphatase. pUC19 DNA was digested with *Sal* I (5´ overhang); treated with recommended amounts of alkaline phosphatase (panels A and B) or excess alkaline phosphatase (panels C and D, 1 unit CIAP, 150 units BAP, 1 unit TsAP); purified and religated. Lane C. uncut pUC19; lane 1, pUC19 digested with *Sal* I; lanes 2-4, Religated DNA from CIAP, BAP, or TsAP samples, respectively. Panels A and C were one *Sal* I supplier and panels B and D were *Sal* I from a second supplier.

Effect of Excess Enzyme

Dephosphorylation protocols suggest the use of minimal amounts of alkaline phosphatase based on the calculated of picomole of ends of DNA. More often than not, to use this amount of enzyme, it needs to be freshly diluted. To avoid dilution however, some researchers prefer to add 1 µl of the enzyme (excess enzyme) to the reaction.

The use of 1 µl of BAP, TsAP, and CIAP did not affect the cloning efficiency while maintaining complete dephosphorylation. Furthermore, the integrity of the ligated termini was confirmed by successfully digesting isolated mini-prep DNA with the same restriction endonuclease whose sites were used to clone the insert. One microliter of BAP, TsAP, and CIAP can correspond to up to 2 times, 50 times, and 2,800 times the recommended concentration, respectively. Based on these results, 1 µl of any of alkaline phosphatase can be added directly to a pmole of DNA ends to achieve dephosphorylation of the 5´ ends.

Dephosphorylation During Restriction Endonuclease Digest

If you are using CIAP, 1 µl of this enzyme can be added at the start of the restriction endonuclease digest (regardless of the 5´ end type) in reactions that are performed at temperatures < 55°C as long as the reaction is incubated for 1 h.

Composition of REACT Buffers

Buffer	Tris-HCl (mM)	pH	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)
REACT 1	50	8.0	10	—	—
REACT 2	50	8.0	10	50	—
REACT 3	50	8.0	10	100	—
REACT 4	20	7.4	5	—	50
REACT 6	50	7.4	6	50	50
REACT 10	100	7.6	10	150	—

Table 3

	5´ Overhang	5´ Recessed	Blunt end
REACT Buffer	1 2 3 4 6 10	1 2 3 4 6 10	1 2 3 4 6 10
BAP	+ + + + + +	+ + + + + +	+ + + + + +
CIAP	+ + + + + +	+ + + + + +	+ + + + + +
TsAP	+ + + + + +	+ + + + + +	+ + + + + +

TABLE 3. Activity of alkaline phosphatases in REACT Buffers. Dephosphorylation was measured by the inability of the DNA to religate with T4 DNA ligase. Ligated products were detected by visual inspection on an ethidium bromide-stained agarose gel. (+) indicates that no religation detected (7).

Table 4

Temperature	CIAP	BAP	TsAP
37°C	100%	100%	100%
50°C	89%	176%	138%
55°C	70%	194%	80%
60°C	34%	231%	58%
65°C	25%	342%	19%

TABLE 4. Activity of alkaline phosphatases at different temperatures. The activity is reported as a percent of activity at 37°C for each enzyme. These activity values represent **unit assay activity** and may not accurately represent the DNA dephosphorylation.

Activity at Different Temperatures

The unit assay conditions for CIAP were used to measure affect of temperature on activity *(table 4)*. The percent activity is reported as a relative value for that particular alkaline phosphatase and values are not comparable between enzymes.

Heat Inactivation

The recommended protocols for inactivation of alkaline phosphatases vary. Since the major use of alkaline phosphatase is in cloning, we tested the efficiency of inactivation procedures under cloning conditions as in the *Simplified Protocol* [in the presence of template DNA, restriction endonucleases, in the REACT Buffer, and using an excess of enzyme (1 unit for CIAP and TsAP and 70 units for BAP)]. Of note, CIAP required the addition of EDTA for complete inactivation *(table 5)*.

Table 5

Enzyme	Treatment	Activity Remaining
CIAP	75°C, 15 min	28%
	65°C, 15 min + EDTA	0%
BAP	65°C, 15 min	55%
	65°C, 15 min, + EDTA	19%
TsAP	65°C, 15 min + EDTA	2%

TABLE 5. Heat inactivation of alkaline phosphatases. 4 µg of pUC19 DNA was digested with 20 units of *Pst* I in REACT 2 in 30 µl. For each inactivation experiment, 2 µl of this digest was added to 23 µl of 1X REACT 2 and dephosphorylated by the *Simplified Protocol*. After the incubation, 5 µl of the reaction was saved on ice for the positive control. 20 µl were heat inactivated as indicated. Activity was measured in the positive control and the inactivated samples by the CIAP unit assay.

Metal Ion Requirements

Alkaline phosphatases require zinc as a cofactor for structural integrity, not as a catalyst. The zinc ions are generally associated and co-purified with the enzyme, therefore no additional zinc atoms are required in the reaction or during dilution of BAP or TsAP. However, CIAP requires zinc in the dilution buffer for enzyme stability.

In a pNPP unit assay at 37°C, $ZnCl_2$ showed no effect on CIAP activity from 0.01 to 1.0 mM. In fact, some inhibition of alkaline phosphatase activity was seen at concentrations ≥0.5 mM. Additionally, functional assays done with CIAP on restriction endonuclease-digested plasmid showed full activity when the reaction was done in as low as 0.5 µM $ZnCl_2$.

Although not required for BAP and CIAP, Mg^{2+} will stimulate the enzymes (1). Magnesium stabilizes BAP at 95°C and at 10 mM Mg^{2+} will increase CIAP activity 10 times.

Inhibitors

Inhibitors of alkaline phosphatases include phosphate ions, nucleotides, phenol, EDTA and 8-hydroxyquinoline.

Composition of Reaction Buffers

10X CIAP Reaction Buffer:
500 mM Tris-HCl (pH 8.5), 1.0 mM EDTA

10X TsAP Reaction Buffer:
100 mM Tris-HCl (pH 8.0)

10X BAP Reaction Buffer:
100 mM Tris-HCl (pH 8.0)

Composition of Suggested Dilution Buffers

CIAP Dilution Buffer:
25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50% (v/v) glycerol.

TsAP Dilution Buffer:
10 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 100 mM NaCl, 0.01 mM $ZnSO_4$, 50% (v/v) glycerol.

BAP Dilution Buffer:
10 mM Tris-HCl (pH 8.0), 120 mM NaCl, 50% glycerol.

Storage and Stability

Each alkaline phosphatase is stable for at least 18 months when stored at –20°C in a nonfrost-free freezer.

References:

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4. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 3.38.
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Troubleshooting

PROBLEM: INCOMPLETE DEPHOSPHORYLATION

Possible Cause	Suggested Solution
• Too low of a pH in the reaction.	• Use the suggested reaction buffer for the alkaline phosphatase.
• In the case of TsAP, the final magnesium concentration was not 2 to 10 mM.	• Add magnesium to the reaction.
• Improper temperature was used.	• Use the recommended temperature for each alkaline phosphatase.
• A restriction endonuclease was used (*i.e.*, *Sal* I) that needed to be removed by phenol extraction.	• Purify the DNA prior to the alkaline phosphatase reaction.
• Not enough alkaline phosphatase.	• Use 1 µl of the alkaline phosphatase in the reaction when using the *Simplified Protocol*. Otherwise calculate pmol DNA ends and use the recommended amount in the *Traditional Protocol*.
• Inhibitors present in the reaction.	• Purify the DNA prior to the alkaline phosphatase reaction.

PROBLEM: POOR CLONING EFFICIENCY WITH DEPHOSPHORYLATED VECTOR

Possible Cause	Suggested Solution
• Incomplete inactivation of the alkaline phosphatase.	• Use the correct inactivation time and temperature. • Do heat inactivation in the presence of EDTA. • Switch to an organic extraction method instead of heat inactivation or use CONCERT Products.
• TsAP was used at 50°C in the *Simplified Protocol*.	• For maximum cloning efficiencies, it must be used at 65°C in this protocol.

PROBLEM: POOR LABELING USING T4 POLYNUCLEOTIDE KINASE

Possible Cause	Suggested Solution
• Ammonium ions present.	• Use sodium salts in ethanol precipitation of DNA after dephosphorylation.

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Ordering Information

Product	Cat. No.	Size
Calf Intestinal Alkaline Phosphatase *Supplied with vial of 10X dephosphorylation buffer [500 mM Tris-HCl (pH 8.5), 1 mM EDTA], vial of dilution buffer.*	18009-019 18009-027	1,000 units (20–30 units/µl) 1,000 units (1 unit/µl)
Bacterial Alkaline Phosphatase *Supplied with vial of 10X dephosphorylation buffer [100 mM Tris-HCl (pH 8.0)]*	18011-015	2,500 units (150 units/µl)
Thermosensitive Alkaline Phosphatase *Supplied with vial of 10X TsAP buffer [100 mM Tris-HCl (pH 8.0)] and a vial of Stop buffer [200 mM EDTA (pH 8.0)]*	10534-014	1,000 units (1 unit/µl)
Related Products		
Distilled Water, DNase/RNase-Free	10977-015 10977-023	500 ml 10 × 500 ml
1 M Tris-HCl, pH 8.0	15568-025	1 L
0.5 M EDTA, pH 8.0	15575-020	4 × 100 ml
Phenol:Chloroform:Isoamyl Alcohol [25:24:1, (v/v/v)]	15593-031 15593-049 15593-023 15593-015	100 ml 400 ml 4 × 100 ml 4 × 400 ml
CONCERT Rapid PCR Purification System	11458-015 11458-023	50 reactions 250 reactions
CONCERT Rapid Gel Extraction System	11456-019 11456-027	50 reactions 250 reactions
CONCERT Matrix Gel Extraction System	11457-017	150 reactions
Complete Set of REACT Buffers *(Consists of one 1-ml vial of each REACT Buffer and one 250-µl vial of 10 mM DTT)*	15461-023	10 × 1 ml

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