



## Kinases and Phosphatases

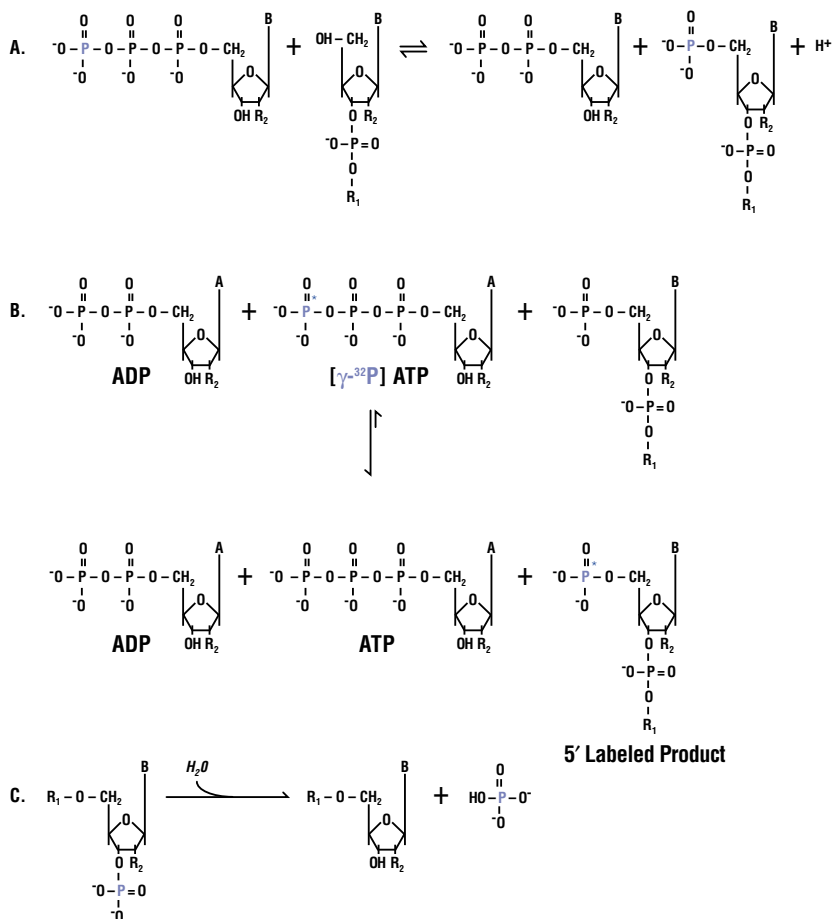
### Introduction

Kinases and phosphatases are common reagents in modern day molecular biology laboratories. Although there are a variety of sources for these enzymes, the most common are calf intestinal alkaline phosphatase (CIAP) and T4 polynucleotide kinase (T4 PNK). Their most frequent use is to modify the phosphorylation state of the 5'-ends of DNA molecules (Figure 1, Panels D-H).

CIAP is most commonly used to remove 5'-phosphates from vector DNA to prevent self-ligation during cloning. Only one strand of a DNA duplex must be joined prior to bacterial transformation; the other will remain nicked until it is repaired inside the bacteria. While the vector DNA is dephosphorylated, the insert DNA should not be dephosphorylated as 5'-phosphates are required for a successful ligation reaction. CIAP is also used to end-label DNA fragments by removing 5'-phosphates, making the DNA fragments better T4 PNK substrates.

Synthetic DNA, usually in the form of custom-made oligonucleotides, is devoid of 5'-phosphates and is therefore a less than ideal template for ligation reactions. T4 PNK is routinely used to transfer a  $\gamma$ -phosphate from a nucleotide triphosphate (usually ATP) to the 5'-end of oligonucleotides to facilitate ligation (Figure 5, Panel A). For blotting, gel-shift or sequencing procedures,  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  is used as the phosphate donor, resulting in a radiolabeled species. The 5'-end of a DNA molecule generated by restriction endonuclease cleavage can also be labeled, even though a phosphate already exists at that position. This can be achieved either by making use of the exchange activity of T4 PNK to exchange the existing phosphate with a radiolabeled phosphate from the phosphate donor (Figure 5, Panel B), or by first treating the DNA with CIAP to remove the existing phosphates, then adding the radiolabeled phosphate with PNK via the forward reaction, which will result in a high specific activity (Figure 5, Panel A). Finally, T4 PNK has a 3'-phosphatase activity that can be used to remove phosphate groups from the 3'-terminus of DNA and RNA (Figure 5, Panel C).

Although both enzymes are most commonly used for cloning purposes, they have other activities and are also used for other types of studies. These other activities will be listed in further detail in the following section. The robustness and versatility of CIAP and T4 PNK have made them staples in today's molecular biology applications.



**Figure 5. Multiple activities associated with T4 polynucleotide kinase. Panel A:** The transfer of a phosphate from a nucleoside 5'-triphosphate to the 5'-OH group of an acceptor molecule by T4 polynucleotide kinase, via the forward reaction.  $\text{R}_1 = \text{H}$ , a nucleoside, a nucleotide or a polynucleotide;  $\text{R}_2 = \text{H}$  or  $\text{OH}$ . **Panel B:** With excess ADP an exchange reaction can occur.  $\text{A} = \text{adenine}$ ;  $\text{R}_1 = \text{H}$ , a nucleoside, a nucleotide or a polynucleotide;  $\text{R}_2 = \text{H}$  or  $\text{OH}$ ;  $\ast = {}^{32}\text{P}$ . **Panel C:** The hydrolysis of a 3'-phosphoryl group by the 3'-phosphatase activity of T4 polynucleotide kinase.  $\text{R}_1 = \text{H}$ ,  $\text{PO}_4^{2-}$ , a nucleotide or a polynucleotide. For Panels A–C,  $\text{B} = \text{adenine, guanine, cytosine, thymidine or uracil}$ .

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## T4 Polynucleotide Kinase

E.C. 2.7.1.78

### Description

T4 Polynucleotide Kinase (polynucleotide 5'-hydroxyl-kinase or ATP:5'-dephospho-polynucleotide 5' phosphatase)

T4 Polynucleotide Kinase (T4 PNK; Cat.# M4101, M4103) is a tetramer composed of identical subunits and has multiple activities. The 5'-kinase activity of T4 PNK catalyzes the transfer of the  $\gamma$ -phosphate from NTP to the 5'-OH terminus of mono- or polynucleotides (Figure 5, Panel A;1). The reaction is reversible and in the presence of a nucleotide diphosphate such as ADP the enzyme has 5'-phosphatase activity (2). Dephosphorylation and subsequent rephosphorylation allow the enzyme to transfer phosphates between ATP and a 5'-phosphate group on an acceptor molecule in an exchange reaction (Figure 5, Panel B; 3). T4 PNK also has 3'-phosphatase activity (Figure 5, Panel C;4). For a review of T4 PNK see references 3, 5 and 6.

T4 PNK can be used to phosphorylate RNA, DNA and synthetic oligonucleotides prior to subsequent manipulations such as ligation. Radioactive phosphate can be used as a label for DNA sequencing (7,8), gel shift analysis (9), footprinting (10), primer extension (11), and restriction mapping (12,13). Labeling the 5'-ends of DNA and RNA may be done using a dephosphorylated template (5'-OH) using the forward or 5'-kinase reaction (Figure 5, Panel A). Alternatively, labeling of 5'-ends can be achieved without removal of the existing 5'-phosphate using the exchange reaction (Figure 5, Panel B; 2, 14, 15). The reaction conditions for the forward and exchange reactions are not the same. The forward reaction generally results in better incorporation. T4 PNK can also be used to remove 3'-phosphates from DNA and RNA (Figure 5, Panel C; 4).

### Applications

- 5' end-labeling of ss- and dsDNA and RNA (1,14,16).
- Phosphorylation of insert DNA prior to ligation.
- Phosphorylation of oligonucleotides.
- Removal of 3'-phosphates (4).

### T4 Polynucleotide Kinase is a component of the following Promega systems:

- *fmoI*<sup>®</sup> DNA Cycle Sequencing Systems<sup>(k)</sup> (Cat.# Q4100, Q4110)
- OmniBase<sup>®</sup> DNA Cycle Sequencing Systems<sup>(k)</sup> (Cat.# Q6800, Q4550)
- TaqTrack<sup>®</sup> Sequencing Systems (Cat.# Q5530)
- DNA 5' End-Labeling System (Cat.# U2010)
- Universal RiboClone<sup>®</sup> cDNA Synthesis System (Cat.# C4360)
- Gel Shift Assay Systems (Cat.# E3050, E3300)

<sup>k</sup> OmniBase<sup>®</sup> products are available in selected countries. Please inquire for additional information.

- Primer Extension System - AMV Reverse Transcriptase (Cat.# E3030)
- Core Footprinting System (Cat.# E3730)
- 5' End-Labeling Application Pack (Cat.# M7700)

### Enzyme Properties

**Requirements:** 5'-kinase (forward), exchange and 3'-phosphatase reactions require  $Mg^{2+}$  (1,2,17). A sulfhydryl compound such as DTT is essential for activity (2).

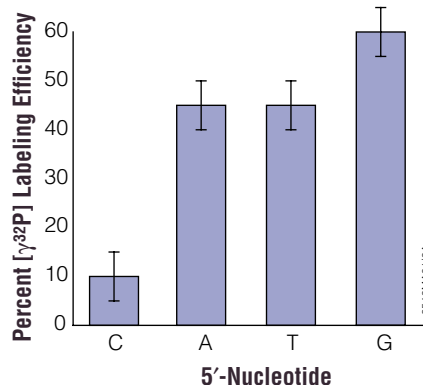
**Cofactor Concentration:** For the 5'-kinase reaction, 10mM  $Mg^{2+}$  optimal at pH 7.6 (1). For the 3' phosphatase reaction, 8mM  $Mg^{2+}$  optimal (17).

### Optimal Substrate

**5'-Kinase Activity:** ss- and dsDNA, ss- and dsRNA, synthetic oligonucleotides and nucleoside 3'-monophosphates (Figure 5, Panel A; 3). 5'-OH groups on ssDNA overhangs in dsDNA are phosphorylated more efficiently than 5'-OH groups on blunt or 5'-recessed ends (3). With increased concentration of ATP or enzyme, blunt- and 5'-recessed ends can be completely phosphorylated (3,5). Phosphorylation of 5'-OH groups located at nicks in dsDNA is 10–30X slower than for ssDNA (14,18). The reaction does not differ significantly for substrates 0.15–50kb in length (5). T4 PNK can also phosphorylate a variety of modified nucleotides and non-nucleotide substrates (6). Nucleosides (adenosine), nucleoside 2'-phosphates, 3'-termini, or 5'-termini bearing phosphomonoesters are not substrates (19).

**Exchange Reaction:** ss- and dsDNA, (6; Figure 5, Panel B). 5'-overhangs of dsDNA and single-stranded oligonucleotides are more efficient substrates than 5'-recessed or blunt ends (20). 5'-recessed and blunt ends are labeled 15–25% as efficiently as 5'-overhangs (20). 5'-phosphate groups at nicks are the most difficult to exchange (30X less efficient than 5'-overhangs [14]). Some tRNA species can act as substrates for phosphate exchange (6).

**5'-Phosphatase Activity:** DNA, RNA, oligonucleotides, 3',5'-bisphosphates (6).



**Figure 6. T4 polynucleotide kinase labeling efficiency.** Labeling of oligonucleotides by T4 polynucleotide kinase is dependent, in part, on the identity of the 5'-nucleotide, with 5'-cytidine having the lowest labeling efficiency (33).

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**3'-Phosphatase Activity:** Deoxynucleotide 3'-monophosphates, ss- and dsDNA (17; Figure 5, Panel C). Extracts of T4-infected *E. coli* have no phosphatase activity on 3'-phosphoryl groups of RNA and ribomononucleotides (17). However, the purified enzyme is reported to have weak 3'-phosphatase activity on RNA (4).

**Phosphate Donor:** ATP, CTP, UTP, GTP, dATP, and dTTP function equally well (3).

**Typical Working Concentration:** 10–20 units of T4 PNK are typically used in a 50µl reaction volume for 1–50pmol of 5' DNA ends, for both the forward and exchange reactions.

**Optimal pH**

**5'-Kinase Activity:** pH 7.4–8.0 in Tris-HCl buffer for DNA substrates (1). Maximal activity at pH 7.6 (19). For ss-oligonucleotide substrates an optimal pH of 9.5 in glycine-NaOH buffer has been reported (2).

**Exchange Reaction:** pH 6.2–6.6 in imidazole-HCl buffer (14). At pH 7.6 the exchange reaction is 20% as efficient as at pH 6.2 (6).

**5'-Phosphatase Activity:** pH 6.2–6.5 in imidazole-HCl buffer (2,14).

**3'-Phosphatase Activity:** pH 5.6–6.4 depending on buffer (5,18), pH 6.0 in imidazole-HCl buffer optimal (5).

**K<sub>m</sub>**

**5'-Kinase activity:** 4–260µM for ATP (pH 8.0 at 37°C) depending on DNA substrate (14, 23). The K<sub>m</sub> for DNA ranges from 7.6µM for large DNA fragments and between 1.8–143.0µM for nucleoside 3'-monophosphates and oligonucleotides depending on the 5' base and the length of the oligonucleotide (23). For the forward reaction the K<sub>m</sub> for ATP is 4µM. For the reverse reaction the K<sub>m</sub> for ADP is 200µM (14).

**5'-Phosphatase Activity:** ADP, 200µM for dsDNA 5'-overhangs at pH 7.4 (14).

**Stimulators**

**5'-Kinase Activity:** Spermidine (1.7mM optimal) can increase the rate of the reaction three-fold (24). Spermidine promotes tetramer formation (25). Salts such as NaCl, KCl and CsCl (125mM optimal) can increase activity up to 5X (24). LiCl and NH<sub>4</sub>Cl give similar stimulation (24). This effect is the same for 5'-overhangs on dsDNA and ssDNA, oligo and mononucleotides (24). Conversely, KCl decreases phosphorylation of 5'-recessed ends and at nicks (18). A sulfhydryl compound such as DTT (5mM DTT optimal) is essential for activity (19). 10mM 2-mercaptoethanol and 10mM glutathione result in 80% and 70%, respectively, of the activity observed with DTT (19). In the absence of a sulfhydryl compound only 2% of the optimal activity is observed. Several anions (at 125µM) are stimulatory, with Cl<sup>-</sup>, Br<sup>-</sup> > F<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup> (24). PEG 8000 at 4–6% improves the efficiency of labeling 5'- and 3'-overhangs, blunt ends and at nicks (26).

**Exchange Reaction:** In the presence of ADP, the phosphorylation reaction can be reversed and T4 PNK can remove a 5'-phosphate to yield a 5'-OH terminated molecule and ATP (2,3). PEG 8000 improves the efficiency of the exchange reaction (26). Spermidine has no significant effect on the exchange reaction (6).

**3'-Phosphatase Activity:** 2-mercaptoethanol, glutathione and DTT maintain activity during long (≥10 minutes) reactions (17). No effect seen for reactions <10 minutes. 0.3M NaCl promotes the 3'-phosphatase activity over the 5'-phosphatase activity (27).

**Alternative Cofactors and Substrates**

**5'-Kinase Activity:** Mn<sup>2+</sup> can partially fulfill the requirement for the divalent cation. 3.3mM is optimal and results in 50% the maximal activity with Mg<sup>2+</sup> (19).

**3'-Phosphatase Activity:** Co<sup>2+</sup> ions can replace Mg<sup>2+</sup> ions. At 0.8mM, the activity is 140% the optimal activity with Mg<sup>2+</sup>. At 0.08mM, 70% activity is observed (17).

**Inhibitors**

**5'-Kinase Activity:** Phosphate (P<sub>i</sub>) and pyrophosphate (PP<sub>i</sub>) anions are inhibitors of T4 PNK (24,28). Ammonium ions are strong inhibitors of T4 PNK (22). DNA should not be dissolved in or precipitated from buffers containing ammonium salts prior to treatment with kinase. Sulfate containing polymers (e.g., agar, dextran sulfate and heparin) are inhibitors of T4 PNK (29). However, addition of cationic compounds such as spermine or polylysine can counteract the inhibition (6). Nonsulfate polysaccharides have no effect on T4 PNK.

**Exchange Reaction:** Phosphate ions; 50mM potassium phosphate results in 60% inhibition (2).

**K<sub>i</sub>:** 29mM (P<sub>i</sub>), 2mM (PP<sub>i</sub>) (24).

**Temperature Stability:** T4 PNK is active over a range of temperatures. Different temperature optima have been reported; 37°C (5), 30–35°C (23). At 0°C the rate of the 5'-kinase reaction is reduced to 7% the rate at 37°C. At 0°C the exchange reaction is reduced to 1.2% the rate at 37°C (25).

**Inactivation:** 68–70°C for 10 minutes (11,30); 78°C for 1 minute (6). Reactions can also be terminated by addition of 20mM EDTA.

**Genetic Locus:** Bacteriophage T4 *pseT*.

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### Promega Product Information

**Source:** Purified from an *E. coli* strain expressing a recombinant clone.

**Molecular Weight:** T4 PNK is a tetramer composed of identical subunits. The relative mobility of the monomers as measured by SDS-PAGE is 33kDa (31), by centrifugation; 33.2kDa (25). From the sequence of T4 PNK, the monomer consists of 301 amino acids with a predicted molecular weight of 34kDa (32). The molecular weight of the tetramer as estimated by gel filtration is 140kDa (32), by centrifugation, 147.3kDa (25). Monomers and dimers are not enzymatically active (6).

**Typical Working Conditions:** ATP at  $\geq 2$ -5 fold molar excess over DNA ends results in  $\geq 95\%$  phosphorylation of 5'-overhangs (6). Increasing concentration of ATP to 100-fold molar excess will allow complete labeling of blunt ends or recessed 5'-ends (6,18). Protocols for using T4 PNK are available from Promega (21,22).

**Storage Conditions:** Store at  $-20^{\circ}\text{C}$ . T4 Polynucleotide Kinase is supplied in 20mM Tris-HCl (pH 7.5), 25mM KCl, 2mM DTT, 0.1mM EDTA, 0.1 $\mu\text{M}$  ATP and 50% (v/v) glycerol.

**Unit Definition:** One unit is defined as the amount of T4 PNK required to catalyze the transfer of 1 nanomole of phosphate to the 5'-OH end of a polynucleotide from [ $\gamma$ - $^{32}\text{P}$ ] ATP in 30 minutes at  $37^{\circ}\text{C}$ . The reaction conditions are: 40mM Tris-HCl (pH 7.5), 10mM  $\text{MgCl}_2$ , 5mM DTT, 0.1mM [ $\gamma$ - $^{32}\text{P}$ ]ATP, 0.5mM 5'-OH polynucleotide end concentration.

**Purity:** The purity is  $>90\%$  as judged by SDS-polyacrylamide gels with Coomassie® blue staining.

### Activity Assays

**End Labeling:** To test for activity, 5pmol of dephosphorylated primer is incubated with 8 units of T4 PNK for 1 hour at  $37^{\circ}\text{C}$  in 1X Kinase Buffer (Tables 12, 15) containing [ $\gamma$ - $^{32}\text{P}$ ]ATP. Following incubation, the amount of [ $\gamma$ - $^{32}\text{P}$ ]ATP converted to TCA-insoluble material is determined. The minimal passing specification is  $>20\%$  incorporation.

**Sequencing Assay:** This enzyme has been tested and qualified for performance in the TaqTrack® Sequencing Systems.

### Contaminant Assays

**Endonuclease Assay:** To test for endonuclease activity, 1 $\mu\text{g}$  of supercoiled plasmid DNA is incubated with 25 units of T4 PNK for 5 hours at  $37^{\circ}\text{C}$  in 1X Kinase Buffer (Tables 12, 15). Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

**DNase and RNase Assay:** To test for nuclease activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 25 units of T4 Polynucleotide Kinase in 1X Kinase Buffer (Tables 12, 15) for 3 hours at  $37^{\circ}\text{C}$ , and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is  $<3\%$  release for both DNase and RNase.





## Calf Intestinal Alkaline Phosphatase

E.C. 3.1.3.1

### Description

Calf Intestinal Alkaline Phosphatase (CIAP; Cat.# M1821 and M2825) is a phosphomonoesterase that catalyzes the hydrolysis of 5'- and 3'-phosphates of DNA, RNA and nucleotides. CIAP is primarily used to dephosphorylate vector DNA prior to cloning and for removal of 5'-phosphates from RNA and DNA before labeling with [ $\gamma$ - $^{32}$ P]NTP and T4 polynucleotide kinase. CIAP has also been widely utilized to dephosphorylate proteins. In contrast to acid phosphatases, CIAP can be utilized for *in vitro* dephosphorylation of proteins under conditions that do not denature the substrate protein (1). CIAP effectively dephosphorylates proteins containing phosphoserine, phosphothreonine and phosphotyrosine, although it can show preferential dephosphorylation of phosphotyrosine under certain conditions (2).

### Applications

- Dephosphorylation of 5'-phosphorylated ends of cloning vectors.
- Dephosphorylation of 5'-phosphorylated ends of DNA or RNA for subsequent labeling with  $^{32}$ P using [ $\gamma$ - $^{32}$ P]NTP and T4 Polynucleotide Kinase.
- Dephosphorylation of proteins.

**CIAP is a component of the following Promega systems:**

- Core Footprinting System (Cat.# E3730)
- DNA 5' End-Labeling System (Cat.# U2010, M7700)
- Subcloning Application Pack (Cat.# M7670)

### Enzyme Properties

**Requirements:** Zn $^{2+}$ , Mg $^{2+}$  (3).

**Optimal Substrate:** 5'- and 3'-phosphates of RNA, DNA and nucleotides.

**Typical Working Concentration:** 0.01unit/pmol DNA ends.

**Optimal pH:** 9.1–10.5. (CIAP is stable at pH 7.5–9.5, but is rapidly inactivated at acidic pH [3]).

**K<sub>m</sub>:** 3.6 $\mu$ M and 1.5mM at pH 8.0 and 10.0, respectively, for p-nitrophenylphosphate (4).

**Inhibitors:** P<sub>i</sub>, Inositol hexaphosphate (5,6).

**K<sub>i</sub>:** 16.5 $\mu$ M at pH 9.2 for P<sub>i</sub> (6).

**Inactivation:** 65°C for 45 minutes or 75°C for 10 minutes.

### Promega Product Information

**Source:** Calf intestinal mucosa.

**Molecular Weight:** 68kDa.

**Typical Working Conditions:** 50mM Tris-HCl (pH 9.3 at 25°C), 1mM MgCl $_2$ , 0.1mM ZnCl $_2$  and 1mM spermidine.

**Storage Conditions:** Store at –20°C. CIAP is supplied in storage buffer containing 10mM Tris-HCl (pH 8.0), 1mM MgCl $_2$ , 0.1mM ZnCl $_2$ , 50mM KCl and 50% (v/v) glycerol.

**Unit Definition:** One unit is defined as the amount of enzyme required to catalyze the hydrolysis of 1 $\mu$ mol of 4-nitrophenyl phosphate per minute at 37°C in 1M diethanolamine, 10.9mM 4-nitrophenyl phosphate, 0.5mM MgCl $_2$  (pH 9.8).

### Contaminant Assays

**Endonuclease Assay:** 1 $\mu$ g of supercoiled plasmid DNA is incubated with 5 units of CIAP in 1X Reaction Buffer (Tables 12, 15) for one hour at 37°C. Following incubation, the supercoiled DNA is visualized on an ethidium bromide-stained agarose gel to verify the absence of visible nicking or cutting.

**Exonuclease Assay:** To test for DNase and RNase activity, 50ng of radiolabeled DNA or radiolabeled RNA is incubated with 5 units of Calf Intestinal Alkaline Phosphatase in 1X Reaction Buffer (Tables 12, 15) for one hour at 37°C, and the release of radiolabeled nucleotides is monitored by scintillation counting of TCA-soluble material. Minimum passing specification is  $\leq$ 3% release for both DNase and RNase.

**Blue/White Cloning Assay:** Five micrograms of pGEM<sup>®</sup>-3Zf(+) Vector<sup>(d)</sup> is digested with representative restriction enzymes (leaving 5'-termini, 3'-termini or blunt ends). The termini are treated with 5 units of Calf Intestinal Alkaline Phosphatase for 1 hour at 37°C, kinased and ligated. The religated plasmid is then transformed into JM109 cells that are plated on X-Gal/IPTG/Amp plates. A minimum of 200–400 colonies are counted. White colonies result from transformation with ligated plasmids with damaged ends. These white colonies represent the number of false positives expected in a typical cloning experiment. Enzymes that generate overhangs, such as *EcoR* I, *Hind* III or *Kpn* I, must produce fewer than 2% white colonies, and blunt-cutting enzymes, such as *Hinc* II, must produce fewer than 5% white colonies (1). Transformation efficiency must be  $\geq 1 \times 10^5$  cfu/ $\mu$ g DNA.

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### 5' End-Labeling Tips

- ◆ The forward reaction is much more efficient than the exchange reaction for labeling of phosphorylated ends. Promega Technical Bulletin #TB519 contains a protocol for labeling phosphorylated DNA via the exchange reaction.
- ◆ The template used should be gel-purified. Contamination by short oligonucleotides or tRNA will lower the efficiency of labeling. Consider the use of glycogen instead of tRNA for efficient precipitation of oligonucleotides.
- ◆ Oligonucleotides are synthesized with a 5'-hydroxyl group. They can be labeled directly in the forward reaction without prior dephosphorylation.
- ◆ Ammonium ions are strong inhibitors of T4 Polynucleotide Kinase; therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with T4 PNK.
- ◆ The final ATP concentration in the forward reaction should be at least 1 μM.
- ◆ Equal concentrations of ATP and 5' ends result in about 50% labeling of oligonucleotides. To obtain higher specific activity, the ATP:oligonucleotide ratio should be increased to 10:1. Only 10% of the label will be transferred but virtually every oligonucleotide molecule will be labeled (1).
- ◆ 0.5 pmol of linear DNA equals 1 pmol of 5' ends. To convert μg DNA to pmol:

**For dsDNA:**

$$\mu\text{g} \times 10^6 \text{ pg}/1\mu\text{g} \times \text{pmol}/660\text{pg} \times 1/N = \text{pmol}$$

where N is the number of nucleotide pairs and 660pg/pmol is the conversion factor for a nucleotide pair.

**For ssDNA:**

$$\mu\text{g} \times 10^6 \text{ pg}/1\mu\text{g} \times \text{pmol}/330\text{pg} \times 1/N = \text{pmol}$$

where N is the number of nucleotides and 330pg/pmol is the conversion factor for a single nucleotide.

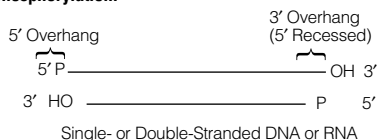
- ◆ Single-stranded DNA and double-stranded DNA containing 5'-overhangs are labeled more efficiently than 3'-overhangs (5'-recessed) or blunt ends. Labeling of blunt or recessed ends can be improved by increasing the concentration of ATP in the reaction or by denaturing the template with heat or NaOH prior to labeling (1,2). Alternative protocols involving the addition of PEG 8000 to the reaction may also be used to increase the labeling efficiency of these templates (1,2).

### References

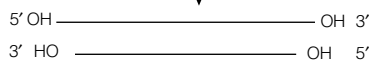
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## 5' End-Labeling Protocol

### Dephosphorylation:



↓ CIAP



### Phosphorylation:



2618MA04/9A

Figure 7. 5' end-labeling protocol.

### Dephosphorylation of 5'-Protruding Ends Using Calf Intestinal Alkaline Phosphatase (CIAP):

1. Dilute sufficient CIAP for immediate use in CIAP 1X Reaction Buffer to a final concentration of 0.01 u/μl. Each pmol of DNA ends will require 0.01 u CIAP (added in two aliquots of 0.005 u/pmol ends).
2. Purify the DNA to be dephosphorylated by ethanol precipitation and resuspend the pellet in 40 μl of 10mM Tris-HCl (pH 8.0). Set up the following reaction:

DNA (up to 10 pmol of 5'-ends)	40 μl
CIAP 10X Reaction Buffer,	5 μl
Diluted CIAP (0.01 u/μl)	<u>up to 5 μl</u>
Deionized water to a final volume of	50 μl

**Note:** CIAP may be added directly to digested DNA. Add 5 μl 10X CIAP Reaction Buffer, 0.005 u CIAP/pmol ends, and water to a final volume of 50 μl.

3. Incubate at 37°C for 30 minutes.
4. Add another aliquot of diluted CIAP (equivalent to the amount used in Step 2) and continue incubation at 37°C for 30 minutes.
5. Add 300 μl CIAP stop buffer (10mM Tris-HCl [pH 7.5], 1mM EDTA, 200mM NaCl, 0.5% SDS). Phenol:chloroform extract and ethanol precipitate by adding 5M NaCl to a final concentration of 0.2M and 2 volumes of 100% ethanol to the aqueous phase.

### Dephosphorylation of 5'-Recessed or Blunt Ends:

When 5'-recessed or blunt ends are used as the substrate prepare CIAP by diluting the required amount in 1X Reaction Buffer (Table 15) to a final concentration of 0.01 u/μl. Each pmol of DNA ends will require 0.01 u CIAP (added in two aliquots of 0.005 μl/pmol ends). Add the first aliquot of CIAP and incubate at 37°C for 15 minutes and then at 56°C for 15 minutes. Add a second aliquot of CIAP and repeat the incubations at both temperatures. The higher temperature ensures accessibility of the recessed ends.

### 5' End-Labeling Protocol, Forward Reaction, Using T4 Polynucleotide Kinase

1. Assemble the following reaction in a sterile microcentrifuge tube:
- |   |                |
|---|----------------|
| 5' ends of DNA (dephosphorylated)                                   | 1–50 pmol      |
| Kinase 10X Buffer   | 5 μl           |
| [γ- <sup>32</sup> P]ATP (at 3,000 Ci/mmol, 10mCi/ml, 50 pmol total) | 15 μl          |
| T4 Polynucleotide Kinase  | <u>10–20 u</u> |
| Deionized water to a final volume of                                | 50 μl          |

2. Incubate at 37°C for 10 minutes.
  3. Stop the reaction by adding 2 μl of 0.5M EDTA.
- Depending on the application for which the labeled DNA is to be used, subsequent purification may not be necessary.
4. If desired, the labeled DNA can be separated from unincorporated labeled nucleotides by chromatography, by spin columns or by ethanol precipitation. However, it is generally not necessary to purify phosphorylated DNA to be used as hybridization probes or as primers for sequencing. But if the measured incorporation is low (below 30%) or if background problems are experienced, removal of unincorporated nucleotides may be desired. If the labeled DNA fragment is greater than 200bp, the Wizard<sup>®</sup> DNA Clean-Up System<sup>(b)</sup> (Cat.# A7280) may also be used. See Promega's *Protocols and Applications Guide*, Third Edition (pp. 128–129), for more information.

Further information on the removal and addition of 5'-phosphates can be found in Promega's *Protocols and Applications Guide*, Third Edition (pp. 44, 125) and in Promega Technical Bulletins #TB096 and #TB519 and Product Information Sheet #9PIM182.



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