Technical Bulletin

DNA 5´ End-Labeling System

INSTRUCTIONS FOR USE OF PRODUCT U2010.

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PRINTED IN USA.
Revised 12/12

Part# TB096

DNA 5´ End-Labeling System

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1. Description

T4 Polynucleotide Kinase (PNK) catalyzes the transfer of the terminal [γ-^{32}P] phosphate of ATP to the 5´-hydroxyl terminus of a DNA molecule. The DNA 5´ End-Labeling System is a complete system for phosphorylating both oligonucleotides and DNA fragments using T4 PNK. Calf Intestinal Alkaline Phosphatase (CIAP) is provided. CIAP may be used to remove the 5´ phosphate from DNA fragments, which can be subsequently end-labeled with a radioactive phosphate.

This protocol can be used with either [γ-^{32}P]ATP or [γ-^{33}P]ATP for 5´ end-labeling. [γ-^{35}S]ATP can be used for 5´ end-labeling; however, due to the relative weak activity of ^{35}S, much longer exposure times are generally required. For example, exposures of 72–96 hours are often required to visualize DNA sequence ladders generated by cycle sequencing using ^{35}S-labeled primers. Also, end-labeling with [γ-^{35}S]ATP requires more enzyme and longer reaction times.

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Printed in USA. Part# TB096
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Both an Oligonucleotide and the pGEM® DNA Markers are included as controls for the efficiency of the reaction (see Section 7). The pGEM® DNA Markers also provide a convenient range of fragments for analysis of phosphorylated DNA samples by gel electrophoresis.

2. Product Components and Storage Conditions

Product	Size	Cat.#
DNA 5´ End-Labeling System	10 reactions	U2010

Includes:

- 100u T4 Polynucleotide Kinase
- 0.5ml T4 PNK 10X Buffer
- 100u Calf Intestinal Alkaline Phosphatase
- 0.5ml 10X Calf Intestinal Alkaline Phosphatase Buffer
- 500ng Oligonucleotide (31mer)
- 10µg pGEM® DNA Markers

Storage Conditions: Store at –20°C.

3. Dephosphorylation Reaction

In order to remove the phosphate groups from both 5´ termini of a linear double-stranded molecule, it first must be treated with CIAP. The following standard reaction can be set up with the DNA of interest.

More information on CIAP can be found in the *Enzyme Resource Guide, Volume 2: Cloning Enzymes,* available online at: **www.promega.com/guides/cloning_guide/**

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8)

- substrate DNA (up to 10pmol of 5´ ends)
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- 5M NaCl
- ethanol (100%)

1. Standard reaction:

10X CIAP Buffer	5µl
substrate DNA (up to 10pmol of 5´ ends, see Tables 1 and 2, Section 10)	1µl
CIAP (diluted in 1X CIAP buffer, see Note below)	0.1u
water to a final volume of	50µl

   Incubation temperatures:

   For protruding 5´-termini dephosphorylation: Incubate at 37°C for 30 minutes. Add another 0.1u of CIAP and incubate for an additional 30 minutes at 37°C.

   For recessed 5´-termini or blunt-end dephosphorylation: Incubate at 37°C for 15 minutes, then at 56°C for 15 minutes. Add an additional 0.1u of CIAP and repeat incubations at both temperatures. The higher temperature ensures accessibility of the recessed end.

   Note: If the CIAP is diluted in 1X CIAP Buffer, it must be used immediately. If diluted enzyme is to be stored, it should be diluted in CIAP storage buffer (Section 8).

2. To stop the reaction, add 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 minute and microcentrifuge at 12,000 × *g* for 2 minutes.

3. Remove the upper, aqueous phase to a fresh tube and repeat Step 2.

4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and microcentrifuge at 12,000 × *g* for 2 minutes.

5. Transfer the upper, aqueous phase to a fresh tube. Add 5M NaCl to a final concentration of 0.2M.

Ammonium ions are strong inhibitors of bacteriophage T4 PNK; therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with T4 PNK.

6. Add 2 volumes of 100% ethanol. Mix and spin in a microcentrifuge for 15 minutes.

7. Carefully aspirate off the supernatant and dry the pellet under vacuum. Resuspend the DNA in 29µl of distilled water.

4. Phosphorylation Reaction

After removing the 5´ phosphate groups, the substrate DNA can be labeled.

If you are labeling dephosphorylated, double-stranded template DNA, refer to Table 1 (Section 10) for a guide for determining the amount of template DNA needed to equal 1pmol of 5´ ends. If you are labeling an oligonucleotide, refer to Table 2 (Section 10) for a guide for determining the number of nanograms equivalent to 10pmol of oligonucleotide molecules. The oligonucleotide provided with this system is a 31mer. Table 3 (Section 10) provides a guide for determining the number of microCuries per microliter equivalent to 50pmol of radiolabeled nucleotide molecules.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.)

- [γ-^{32}P]ATP (3,000Ci/mmol, 10mCi/ml), [γ-^{33}P]ATP (3,000Ci/mmol, 10mCi/ml) or [γ-^{35}S]ATP (>1,000Ci/mmol, 10mCi/ml)
- 0.5M EDTA

1. Set up the following reaction:

DNA substrate (up to 10pmol of 5´ ends, see Example)	29µl*
T4 PNK 10X Buffer	5µl
[γ-^{32}P]ATP, [γ-^{33}P]ATP or [γ-^{35}S]ATP (50pmol total, see Note)	15µl
T4 PNK (5–10u/µl)	10u
water to a final volume of	50µl

Example: 1pmol of 5´ ends of linear pBR322 DNA (4,361bp) is equivalent to 1.4µg.
*Volume carried forward from Section 3.
Note: ATP at two- to fivefold molar excess over DNA ends generally results in ≥95% of phosphorylation of 5´ ssDNA or 5´ overhangs (1). Less than twofold molar excess of ATP can be used in the reaction, but the ratio of ATP:DNA ends should not fall below 1:1.

2. Incubate at 37°C for 10 minutes.

3. Stop the reaction by adding 2µl of 0.5M EDTA.

4. Heat-inactivate T4 PNK: 68–70°C for 10 minutes (1,2) or 78°C for 1 minute (3).

Note: If end-labeling with [γ-^{35}S]ATP, use 20 units of T4 PNK and incubate for 4 hours at 37°C. A 0.5ml tube and/or an oil overlay may be required to reduce evaporation during long incubations.

At this stage, an aliquot may be used to determine the percent incorporation of radioisotope as described in Section 5. The sample may be purified to remove unincorporated nucleotides as described in Section 6.

More information on T4 PNK can be found in the *Enzyme Resource Guide, Volume 2: Cloning Enzymes,* available online at: **www.promega.com/guides/cloning_guide/**

5. Determination of Percent Incorporation/Specific Activity

5.A. Protocol

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.)

- 0.2M EDTA
- 0.5M Na_2HPO_4 (pH 6.8)
- Whatman® DE-81, 2.3cm circular filters

1. Dilute 1µl of the reaction mixture into 100µl 0.2M EDTA. Spot 3µl of this solution onto each of two Whatman® DE-81, 2.3cm circular filters.
2. Dry the filters briefly under a heat lamp. One filter is kept aside and used directly for the determination of total cpm.
3. Wash the other filter in 50ml 0.5M Na_2HPO_4 (pH 6.8) twice for 5 minutes each wash to remove the unincorporated nucleotides.
4. Dry the washed filter under a heat lamp.
5. Add the appropriate scintillation fluid to each filter and count in a scintillation counter.

5.B. Example of Standard Calculation

$$\textbf{\% incorporation} = \frac{\text{incorporated cpm}}{\text{total cpm}} \times 100$$

$$\textbf{total cpm incorporated} = \text{incorporated cpm} \times \frac{\text{dilution factor}}{\text{volume counted}} \times \text{rxn volume}$$

$$\textbf{specific activity of probe} = \frac{\text{\% incorporation} \times \text{total cpm incorporated}}{\text{µg of DNA substrate in reaction}}$$

For example, if the counts from a set of filters are as follows:

incorporated	48,500cpm
total	51,755cpm

The reaction volume used was 20µl; 0.008µg of DNA was labeled:

$$\textbf{\% incorporation} = \frac{48{,}500\text{cpm}}{51{,}755\text{cpm}} \times 100 = 94\%$$

$$\textbf{total cpm incorporated} = 48{,}500\text{cpm} \times \frac{100}{3\mu\text{l}} \times 20\mu\text{l} = 3.2 \times 10^7\text{cpm}$$

$$\textbf{specific activity} = \frac{3.2 \times 10^7\text{cpm}}{0.008\mu\text{g}} = 4 \times 10^9\text{cpm}/\mu\text{g}$$

6. Removal of Unincorporated Nucleotides

Unincorporated labeled dNTPs can be removed by size exclusion chromatography using Sephadex® G-50 spin columns (1) or by selective precipitation of the labeled DNA. This step is usually not required unless incorporation levels are low. In most applications, if incorporation is greater than 60%, removal of unincorporated labeled dNTPs is not necessary (1).

6.A. Chromatography

The end-labeled DNA may be separated from unincorporated nucleotides by chromatography through a small Sephadex® G-50 column in 10mM Tris-HCl (pH 7.5) and 0.1% SDS. The Sephadex® G-50 will separate labeled DNA larger than a 20mer from unincorporated radiolabeled nucleotides. Chromatography is the method of choice for purifying labeled oligonucleotides.

6.B. Ethanol Precipitation

This method results in the precipitation of DNA >20 nucleotides in length, while most of the free dNTPs remain in the supernatant. Recovery levels of labeled DNA precipitated by this method depend on fragment size and concentration but should be greater than 50%.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.)

- 7.5M ammonium acetate
- TE buffer
- ethanol (100%)

1. To the DNA from Section 4, add 0.5 volume of 7.5M ammonium acetate.
2. Add 2 volumes of 100% ethanol. Mix and place below –15°C for 30 minutes. Spin in a microcentrifuge for 5 minutes.
3. Remove the radioactive supernatant and dispose of it according to your institution's guidelines.
4. Redissolve the DNA in 50µl of TE buffer.

7. Labeling of pGEM® DNA Markers and Control Oligonucleotide

Materials to Be Supplied by the User
(Solution compositions are provided in Section 8.)

- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)
- chloroform:isoamyl alcohol (24:1)
- 5M NaCl
- 0.5M EDTA
- [γ-^{32}P]ATP (3,000Ci/mmol, 10mCi/ml) or [γ-^{33}P]ATP (3,000Ci/mmol, 10mCi/ml)
- ethanol (100%)

7.A. pGEM® DNA Markers

The following protocol can be used to label the provided pGEM® DNA Markers.

1. Set up the following reaction:

10X CIAP Buffer	5µl
pGEM® DNA Markers, 1µg	1µl
CIAP (diluted in 1X CIAP buffer, see Note below)	0.1u
water to a final volume of	50µl

Note: If the CIAP is diluted in 1X CIAP Buffer, it must be used immediately. If diluted enzyme is to be stored, it should be diluted in CIAP storage buffer (Section 8).

2. Incubate at 37°C for 30 minutes.
3. After 30 minutes, add another 0.1u of CIAP and incubate the reaction for an additional 30 minutes.
4. Continue as noted in Section 3, Steps 2–7. The pGEM® DNA Markers can then be labeled by following the steps for the substrate DNA (Section 4).

7.B. Control Oligonucleotide

The provided Oligonucleotide control DNA contains a 5´-hydroxyl and can be labeled directly as noted in the following procedure.

1. Set up the following reaction:

Oligonucleotide, 100ng (10pmol)	5µl
[γ-^{32}P]ATP or [γ-^{33}P]ATP (10pmol)	3µl
T4 PNK 10X Buffer	1µl
T4 PNK, 5–10u/µl	10u
final volume	10µl

Note: The conditions for the control reaction are optimal. As such, a 1:1 molar ratio of ATP:DNA ends is sufficient for efficient labeling.

2. Incubate at 37°C for 10 minutes.
3. Stop the reaction by adding 1µl of 0.5M EDTA.
4. Phenol extraction can result in the loss of short oligonucleotides into the phenol phase. It is recommended at this step that the incorporation of label be determined by the DE-81 filter-binding assay (Section 5) or that the unincorporated nucleotides be removed by a Sephadex® G-50 column (Section 6.B).

8. Composition of Buffers and Solutions

7.5M ammonium acetate

57.81g ammonium acetate

Dissolve the ammonium acetate in 100ml water (final volume). Sterilize by filtration (0.22µm filter).

10X CIAP Buffer

500mM Tris-HCl (pH 9.0)
10mM $MgCl_2$
1mM $ZnCl_2$
10mM spermidine

CIAP storage buffer

10mM Tris-HCl (pH 8.0)
1mM $MgCl_2$
50mM KCl
0.1mM $ZnCl_2$
50% glycerol

0.5M EDTA

93.05g disodium ethylene-diaminetetraacetate • $2H_2O$

Add the EDTA to 300ml of water, adjust the pH to 8.0 with NaOH pellets and stir until the EDTA is dissolved. Adjust the final volume to 500ml with water and sterilize by autoclaving.

5M NaCl

29.22g NaCl

Dissolve the NaCl in 100ml (final volume) of distilled water. Sterilize by autoclaving.

0.5M Na_2HPO_4

47.25g NaH_2PO_4 (monobasic)
22.35g Na_2HPO_4 (dibasic)

Add water, slowly, to 1 liter final volume.

T4 PNK 10X Buffer

500mM Tris-HCl (pH 7.5)
100mM $MgCl_2$
50mM DTT
1.0mM spermidine

1X TE buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA

TE-saturated phenol:chloroform: isoamyl alcohol (25:24:1)

Mix equal parts of TE buffer (pH 8.0) and phenol, and allow the phases to separate. Change TE buffer 1–2 times to ensure the pH is 8.0. Then mix 1 part of the lower, phenol phase with 1 part of chloroform: isoamyl alcohol (24:1).

9. References

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

2. Ausubel, F.M. *et al.* (1988) In: *Current Protocols in Molecular Biology*, John Wiley and Sons, New York.

3. Eun, H-M. (1996) *Enzymology Primer for Recombinant DNA Technology*, Academic Press, San Diego, California.

10. Appendix

Table 1. Amount of Dephosphorylated, Double-Stranded Template DNA Needed to Equal 1pmol of 5´ Ends.

Template Length (Type)	Amount of Template Equal to 1pmol of 5´ Ends
200bp (PCR product)	66ng
3,000–5,000bp (linearized plasmid DNA)	0.99–1.65µg
48,000bp (lambda DNA)	15.8µg

In general: ng of dsDNA = pmol of dsDNA × 0.66 × N, where N = length of dsDNA in base pairs. Multiply by 2 to determine number of 5´ ends.

Table 2. Amount of Oligonucleotide (ng) Needed to Equal 10pmol.

Oligonucleotide Length	ng of Oligonucleotide Equal to 10pmol
15mer	50ng
16mer	53ng
17mer	57ng
18mer	60ng
19mer	63ng
20mer	67ng
24mer	80ng
27mer	90ng
31mer	103ng

In general: ng of oligonucleotide = pmol of oligonucleotide × 0.33 × N, where N = length of oligonucleotide in bases.

Table 3. Amount of Radiolabeled Nucleotide (µCi/µl) Needed to Equal 50pmol.

Radiolabeled Nucleotide	Volume Required for Various Specific Activities	Concentration of Nucleotide Stock
[γ-^{32}P]ATP or [γ-^{33}P]ATP	15µl of 3,000Ci/mmol	10µCi/µl
	25µl of 5,000Ci/mmol	10µCi/µl
	2.5µl of 6,000Ci/mmol	135µCi/µl
[γ-^{35}S]ATP	7.0µl of >1,000Ci/mmol	10µCi/µl

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