Description:
This kit is suitable for labeling the 5’ ends of DNA or RNA with high efficiency by use of T4 polynucleotide kinase. DNA fragments with either blunt or cohesive ends can be labeled routinely by [γ-32P]ATP. The 5’-terminal groups do not have to be dephosphorylated prior to using the kit, as the kinase exchange reaction can replace the 5’-terminal phosphates with labeled γ-phosphates from [γ-32P]ATP. Alternatively, free 5’-terminal OH-groups of either double-stranded DNA or single-stranded synthetic oligonucleotides can be labeled (or just simply phosphorylated) by means of the forward kinase reaction. Two reaction cocktails are provided, so that either the exchange reaction or the forward (phosphorylation) reaction can be performed with high efficiency. Generally, a specific activity of >10^6 cpm/pmol (5’-ends) can be obtained by both methods.

Kit Components:
(for 20 labeling procedures)
- T4 Polynucleotide Kinase (10 units/μl) ...................................................... 20 μl
- 5x Exchange reaction buffer ................................................................. 100 μl
- 10x Phosphorylation buffer ................................................................. 50 μl
- Control DNA (λ - BstPI digest, 0.5 μg/μl) ........................................ 10 μg

Storage:
-20°C

Notes:
1. Buffers can be thawed at room temperature, but they should be transferred to ice-cold water immediately. Mix thoroughly before use.
2. During storage at -20°C, the 5x Exchange reaction buffer may develop a cloudy texture. This does not effect the labeling efficiency.
3. DNA fragments generated by restriction cleavage should be purified by ethanol precipitation, etc. prior to labeling.
4. If above 5 pmoles of DNA fragment (< 1,000 bp) is used for exchange reaction, labeling efficiency may decrease below 10^6 cpm/pmol.
5. If DNA fragment less than 500bp is used for exchange reaction, labeling efficiency may decrease.
6. This kit can be used for general phosphorylation reaction not to use radioactive labeling. In this case, add 5 μl of 10mM ATP instead of [γ-32P]ATP.
7. [γ-35S]ATP can be also used instead of [γ-32P]ATP at phosphorylation reaction.

Reagents not supplied in the kit:
1. [γ-32P] ATP or other labeled (or unlabeled) ATPs eg. GE Healthcare, Cat.#PB10168 (111 TBq/mmol, 3,000 Ci/mmol) or Cat.#PB10218 (222 TBq/mmol, 6,000 Ci/mmol)
2. Alkaline phosphatase (if dephosphorylation of the 5’-terminal is preferred prior to the phosphorylation reaction) (TaKaRa Cat.#2120A, Cat.#2250A)
3. Column for removal of unused [γ-32P]ATP, e.g. DEAE-Cellulose (DE-52) column, Gelfilter column e.g. CHROMA SPIN Column, Sephadex G-50.

References:

URL: http://www.takara-bio.com
**Procedure and Examples:**

### I. Exchange reaction

1) Combine the following reagents in a microcentrifuge tube.

<table>
<thead>
<tr>
<th>Reagents to be prepared by the user</th>
<th>Amount(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7M ammonium acetate</td>
<td></td>
</tr>
<tr>
<td>Ethanol (100% and 70%)</td>
<td></td>
</tr>
</tbody>
</table>

\[
S' - \text{phosphorylated DNA (dissolved in TE buffer} \leq 5 \text{ pmoles, } S' \text{ termini)} \leq 14 \mu l
\]

\[
5X \text{ Exchange buffer} \quad 5 \mu l
\]

\[
\gamma^{32}P\text{ATP (370 MBq (10 mCi)/ml)} \quad 5 \mu l
\]

\[
T4 \text{ polynucleotide kinase (10 units/} \mu l) \quad 1 \mu l
\]

\[
distilled \text{ sterilized water} \quad x \mu l
\]

Total \( 25 \mu l \)

* 5 pmoles of 5' termini corresponds to \( \sim 0.17 \mu g \) of 100 bp double-stranded DNA

2) Incubate at 37°C for 30 minutes.
3) Inactivate the enzyme by heating the mixture at 70°C for 5-10 minutes.
4) Add 10 \( \mu l \) of 7M ammonium acetate (pH4.5). If sodium acetate is preferred, add 150mM to final concentration.
5) Add 87.5 \( \mu l \) (2.5 volumes) of cold ethanol and leave at -20°C for 30-60 minutes.
6) Centrifuge and discard the supernatant. Rinse with 1 ml of cold 70% ethanol, and then dry under reduced pressure.
7) Dissolve the DNA in an appropriate buffer.

Most of the unused \( \gamma^{32}P\text{ATP} \) will be removed during steps 4) - 7). If complete removal is preferred, precipitate the DNA with ethanol and purify using conventional methods such as gel filtration. SUPREC*-02 cartridges are also convenient for this purpose (Cat.#9041). Also see section V.

If phenol/chloroform treatment is necessary, do the treatment after step 7).

### II. Dephosphorylation

1) Combine the following reagents in a microcentrifuge tube.

<table>
<thead>
<tr>
<th>Reagents to be prepared by the user</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE-saturated phenol/chloroform</td>
</tr>
<tr>
<td>3M NaCl</td>
</tr>
<tr>
<td>Ethanol (100% and 70%)</td>
</tr>
<tr>
<td>TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)</td>
</tr>
<tr>
<td>bacterial alkaline phosphatase</td>
</tr>
</tbody>
</table>

\[
\text{DNA in TE buffer (} \leq 10 \mu g) \quad \leq 133 \mu l
\]

\[
1 \text{ M Tris-HCl, pH 8.0} \quad 15 \mu l
\]

\[
\text{bacterial alkaline phosphatase} \quad (0.5-1.0 \text{ units/} \mu l) \quad 2 \mu l
\]

\[
distilled \text{ sterilized water} \quad x \mu l
\]

Total \( 150 \mu l \)

2) Incubate at 65°C for 30 minutes.
*(If calf intestine phosphatase (CIAP) is preferred, use 2 \( \mu l \) of CIAP (10 - 20 U/\( \mu l \)) in place of bacterial alkaline phosphatase. Incubate at 50°C for 30 minutes.)*
3) Add 150 \( \mu l \) of TE-saturated phenol/chloroform (1:1) and mix well.
4) Centrifuge and transfer the upper layer to a fresh tube.
5) Repeat steps 3) and 4).
6) Add 7.5 \( \mu l \) of 3 M NaCl (final concentration, 150 mM).
7) Add 375 \( \mu l \) (2.5 volumes) of cold ethanol and leave at -20°C for 30-60 minutes.
8) Centrifuge and discard the supernatant. Rinse with 1 ml of cold 70% ethanol, and then dry under reduced pressure.
9) Dissolve the DNA in 20 \( \mu l \) of TE buffer.

### III. Phosphorylation

(Forward reaction)

1) Combine the following reagents in a microcentrifuge tube.

<table>
<thead>
<tr>
<th>Reagents to be prepared by the user</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Phosphorylation buffer</td>
</tr>
<tr>
<td>( \gamma^{32}P\text{ATP (370 MBq (10 mCi)/ml)} )</td>
</tr>
<tr>
<td>T4 polynucleotide kinase (10 units/( \mu l ))</td>
</tr>
</tbody>
</table>

\[
\text{Dephosphorylated DNA (} \leq 5 \text{ pmoles, } S'-\text{end}) \quad \leq 20.5 \mu l
\]

\[
10X \text{ Phosphorylation buffer} \quad 2.5 \mu l
\]

\[
\gamma^{32}P\text{ATP (370 MBq (10 mCi)/ml)} \quad 1 \mu l
\]

\[
T4 \text{ polynucleotide kinase (10 units/} \mu l) \quad 1 \mu l
\]

\[
distilled \text{ sterilized water} \quad x \mu l
\]

Total \( 25 \mu l \)

URL: http://www.takara-bio.com
2) Incubate at 37°C for 30 minutes.
3) Proceed as steps 3) - 7) in section I.

IV. Labeling of synthetic DNA oligonucleotides

1) Combine the following reagents in a microcentrifuge tube.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>oligonucleotide DNA with free 5’-OH (5-10 pmols)</td>
<td>≤ 3 μl</td>
</tr>
<tr>
<td>10x Phosphorylation buffer</td>
<td>1 μl</td>
</tr>
<tr>
<td>[γ-³²P]ATP (222 TBq (6,000 Ci)/mmol, 370 MBq (10 mCi)/ml)</td>
<td>5 μl</td>
</tr>
<tr>
<td>T4 polynucleotide kinase (10 units/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>distilled sterilized water</td>
<td>x μl</td>
</tr>
<tr>
<td>Total</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

2) Incubate at 37°C for 30 minutes.
3) Proceed as steps 3) - 7) in section I.

V. Removal of unused labels (Optional)

This step is not important unless incorporation levels are low. Unused labels can be removed by size exclusion chromatography³ or by filtration cartridges.

1) Sephadex G-50 spin column chromatography is effective in eliminating unincorporated dNTPs. It also substantially reduces the amount of DNA oligonucleotides <20 bases as well as dsDNA fragments ≤ 20 base pairs. The starting solution should be heated at 70°C for 5-10 minutes to deactivate T4 polynucleotide kinase. (see ref. 3). Also, Sephadex G-25 columns may be used to purify oligonucleotides ≥ 10 bases.

2) Double-stranded (or single-stranded) fragments larger than ~100 base pairs (or bases) can be rapidly purified by centrifugation cartridges SUPREC®-02 (Cat.#9041).

EXAMPLE

1. According to the procedure in sections I and III, 3 pmols of λ - BstPI, λ - HpaI and λ - EcoT22I digests were labeled by the exchange reaction or the forward reaction with [γ-³²P]ATP. An autoradiogram of each DNA fragment is shown bellow.

   ![Autoradiogram of DNA fragments](image)

   lane 1: λ - HpaI digest
   lane 2: λ - BstPI digest
   lane 3: λ - EcoT22I digest
   lane 4: λ - HpaI digest
   lane 5: λ - BstPI digest
   lane 6: λ - EcoT22I digest

   Exchange reaction
   Forward (phosphorylation) reaction

URL: http://www.takara-bio.com
The following is the result of DE 81 filter-binding assays that were performed to measure the amount of incorporation of $^{32}$P in the above experiment.

<table>
<thead>
<tr>
<th>交换反应</th>
<th>前向（磷酸化）反应</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda$ - BstPI digest</td>
<td>5.4 X 10^6 cpm/pm</td>
</tr>
<tr>
<td>$\lambda$ - HpaI digest</td>
<td>4.6 X 10^6 cpm/pm</td>
</tr>
<tr>
<td>$\lambda$ - EcoT22I digest</td>
<td>4.4 X 10^6 cpm/pm</td>
</tr>
</tbody>
</table>

The specific activity of [γ-32P]ATP used in this experiment was 111 TBq (3,000 Ci) / mmol.

2. A synthetic DNA oligonucleotide (17-mer) was labeled according to the recommended protocol and the incorporation of $^{32}$P was measured by DE 81 filter-binding assay.

<table>
<thead>
<tr>
<th>DNA (5' termini eg.)</th>
<th>ATP:DNA</th>
<th>$^{32}$P incorporated</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 pmoles</td>
<td>2 : 1</td>
<td>49.8%</td>
<td>10.6 X 10^6 cpm/pm</td>
</tr>
<tr>
<td>10 pmoles</td>
<td>1 : 1</td>
<td>71.2%</td>
<td>7.8 X 10^6 cpm/pm</td>
</tr>
</tbody>
</table>

* To completely label the termini of DNA, [γ-32P]ATP has to be present in 1.5-fold molar excess.

**NOTE:** This product is intended to be used for research purpose only. They are not to be used for drug or diagnostic purposes, nor are they intended for human use. They shall not to be used products as food, cosmetics, or utensils, etc.

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