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Oligonucleotide Phosphate Labeling Protocol

Introduction

Molecular Probes' oligonucleotide phosphate labeling protocol provides a convenient method for efficiently labeling the 5′-phosphate of oligonucleotides. The protocol has been optimized for labeling 100 µg of a 5′-phosphate–modified oligonucleotides between 18 and 24 bases long. Slightly shorter or longer oligonucleotides may be labeled by the same procedure; however, adjustments to the protocol may be necessary. If the oligonucleotide does not contain a 5′-phosphate, we provide a protocol for adding a phosphate moiety using ATP and T4 polynucleotide kinase reaction. Following the labeling reaction, the conjugate can be purified from the reaction mixture by reverse-phase HPLC or preparative gel electrophoresis.

In the labeling reaction, an amine-containing dye is added to the phosphate residue using the zero-length crosslinker, EDAC, resulting in the formation of a phosphoramidate bond that is stable in most molecular biology assays, including solution, blot and *in situ* hybridization, dideoxy sequencing and DNA amplification. The dyes we recommend contain a five-carbon cadaverine residue or a two-carbon ethylenediamine residue as a spacer between the fluorophore and the oligonucleotide. The spacer reduces the label's interaction with the oligonucleotide and enhances the label's accessibility to secondary detection reagents. This procedure eliminates the need and expense of synthesizing amine-modified oligonucleotides because it allows the labeling of unmodified oligonucleotides. In addition, an oligonucleotide can be double-labeled, combining a radioactive phosphate residue with a fluorophore.

Materials Required

For labeling 100 µg of an oligonucleotide:

- Phosphate-reactive dye or biotin (the compound must contain a primary amine; see Table 1 for some suitable compounds)
- Methylimidazole (Sigma, M8878)
- EDAC (ethyl dimethylaminopropyl carbodiimide, Molecular Probes, E-2247)
- Dimethylsulfoxide (Use high-quality, anhydrous DMSO for best results)
- 100 µg oligonucleotide at 10 µg/µL in dH_2O
- Deionized water (dH_2O), *nuclease-free*
- Chloroform
- 3 M NaCl
- Ethanol (absolute, 100%)
- Microcentrifuge tubes, *nuclease-free*

Table 1. *Suitable compounds to do this experiment.*

Cat #	Dye Name	Ex/Em (nm)	Notes
B-1596	Biotin-X cadaverine	N/A	Detect with streptavidin
L-2424	Lissamine™ rhodamine B ethylenediamine	570/590	Red-fluorescent dye that matches most rhodamine (TRITC) filter sets
T-2425	Texas Red cadaverine	595/615	Longer wavelength red-fluorescent dye
Molecular Probes sells many other amine-containing dyes that may work in this reaction. However, we have only tested this protocol with these three compounds.			

For the kinase reaction, if required:

- 5X kinase reaction buffer (250 mM Tris-HCl, 50 mM $MgCl_2$, 25 mM dithiothreitol, 0.5 mM spermidine, 0.5 mM EDTA, pH 7.6)
- 10 mM ATP
- 10 units/µL T4 polynucleotide kinase
- Phenol:chloroform:isoamyl alcohol (25:24:1)

Preparation of the Oligonucleotide

Extraction and Precipitation of the Oligonucleotide

To ensure that the oligonucleotide is free of interfering compounds, especially amines, such as triethylamine or Tris, and ammonium salts, we strongly recommend extracting and precipitating the sample prior to initiating the kinase reaction and labeling reaction. We suggest the following protocol for 0.1–1 mg oligonucleotide (3–30 A_{260} units).

1.1 Dissolve the oligonucleotide in 100 µL dH_2O and extract three times with an equal volume of chloroform.

1.2 Precipitate the oligonucleotide by adding one-tenth volume (10 µL) of 3 M NaCl and two and a half volumes (250 µL) of cold absolute ethanol. Mix well and place at -20°C for 30 minutes.

1.3 Centrifuge the solution in a microcentrifuge at ~12,000 × g for 30 minutes.

1.4 Carefully remove the supernatant, rinse the pellet once or twice with cold 70% ethanol and dry under vacuum.

1.5 Dissolve the dry pellet in dH_2O to achieve a final concentration of 10 µg/µL (1.7 mM for an 18-mer). This oligonucleotide stock solution may be stored frozen at -20°C. If the oligonucleotide contains a 5′-phosphate, proceed to *Labeling Protocol*. Otherwise, continue with the T4 polynucleotide kinase reaction described in section 2.1.

Kinase Reaction

If the oligonucleotide does not contain a 5′-phosphate, a phosphate moiety can be enzymatically added using T4 polynucleotide kinase.[1,2] Note that this kinase reaction is inhibited by ammonium ions. Therefore, ammonium acetate should not be used to ethanol precipitate the oligonucleotide.

2.1 Prepare 5X reaction buffer for the T4 polynucleotide kinase reaction: 250 mM Tris-HCl, 50 mM $MgCl_2$, 25 mM dithiothreitol, 0.5 mM spermidine, 0.5 mM EDTA, pH 7.6.

2.2 To a clean microcentrifuge tube, add:

- 35 µL dH_2O
- 20 µL 5X kinase reaction buffer
- 10 µL 10 µg/µL oligonucleotide
- 25 µL 10 mM ATP
- 10 µL 10 units/µL T4 polynucleotide kinase enzyme

The total reaction volume equals 100 µL. After mixing, incubate the mixture for 1–2 hours at 37°C.

2.3 Once the incubation is complete, inactivate and remove the enzyme by extracting the reaction mixture three times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1).

2.4 Precipitate the kinased oligonucleotides with ethanol as follows: Add one-tenth volume of 3 M NaCl and two and a half volumes of cold ethanol to the reaction vial. Mix well and place at -20°C for 30 minutes. Centrifuge the solution in a microcentrifuge at ~12,000 × g for 30 minutes. Carefully remove the supernatant, rinse the pellet once or twice with cold 70% ethanol and dry under vacuum.

2.5 Dissolve the dry pellet in dH_2O to achieve a final concentration of about 10 µg/µL. This phosphate-modified oligonucleotide stock solution may be stored frozen at -20°C.

Labeling Protocol

3.1 Make 2X labeling buffer (0.24 M methylimidazole, pH 9.0, 0.32 M EDAC)

To make enough buffer for 120 reactions:

- Add 245 µL 12.5 M 1-methylimidazole to 9 mL dH_2O
- Adjust pH to 9.0 with 2 M NaOH
- Add 0.753 g EDAC to the above solution
- Adjust final volume to 12 mL with dH_2O

Before using, make sure that the EDAC is fully dissolved. Do not heat the buffer. EDAC is unstable in solution; to store the buffer for future reactions, aliquot into 100 µL aliquots, lyophilize and store the dried aliquots at -20°C or lower.

3.2 Make solution of reactive dye.

- Remove the phosphate-reactive dye or biotin from the freezer and allow to warm completely to room temperature.
- Dissolve 200 µg of dye in 20 µL of the DMSO by pipetting up and down, washing the sides of the vial.
- The solution of phosphate-reactive dye or biotin should be freshly prepared for each labeling reaction. DO NOT STORE THIS SOLUTION FOR FUTURE USE.

3.3 To the vial containing the reactive label in DMSO, add:

- 20 µL dH_2O
- 50 µL 2X Labeling Buffer (prepared in step 3.1)
- 10 µL 10 µg/µL oligonucleotide stock solution

The 100 µL reaction mixture may have a grainy appearance, but this will not adversely affect the conjugation. We strongly advise against attempting to improve the solubility of the label, because modifying the composition of the mixture can drastically reduce the labeling efficiency. The reaction may be scaled up or down as long as the concentration of each component is not changed.

3.4 Place the vial on a shaker oscillating at low speed and allow the labeling reaction to proceed for four hours (or overnight if more convenient) at room temperature.

- Gently vortex mix or tap the vial every half hour for the first two hours to ensure that the reaction remains well mixed.
- Do not mix violently, as material may be left on the sides of the vial.
- After four hours, 50–90% of the phosphate-modified oligonucleotide molecules should be labeled. Allowing the incubation to proceed overnight does not necessarily result in a greater labeling efficiency.

Purification of Labeled Oligonucleotide

Following the reaction, the labeling mixture contains labeled oligonucleotide, unlabeled oligonucleotide and unincorporated dye. The labeled oligonucleotide will need to be purified from the reaction mixture by preparative gel electrophoresis[3] or reverse-phase HPLC.[4] Regardless of the purification method selected, ethanol precipitation is recommended as the first step.

Ethanol precipitation of labeled oligonucleotide. Precipitate the reaction mixture with ethanol as follows: Add one-tenth volume of 3 M NaCl and two and a half volumes of cold absolute ethanol to the reaction vial. Mix well and place at -20°C for 30 minutes. Centrifuge the solution in a microcentrifuge at ~12,000 × g for 30 minutes. Carefully remove the supernatant, rinse the pellet once or twice with cold 70% ethanol and dry under vacuum.

- Some unreacted labeling reagent may have precipitated over the course of the reaction or may be stuck on the walls of the reaction vial. This material should be *completely* redissolved by extensive vortex mixing before centrifugation. Redissolving the labeling reagent ensures that the precipitated oligonucleotide will be minimally contaminated with unreacted label.

- In some cases, the labeled oligonucleotide, itself, may have already precipitated onto the walls of the reaction tube. This precipitate will not dissolve with the addition of NaCl and ethanol — the precipitated product will remain on the walls of the tube; however, the free dye will dissolve and be eliminated. After centrifugation and rinsing, the pellet should be soluble.

Purification by gel electrophoresis. To purify the labeled oligonucleotide by gel electrophoresis, pour a 0.5 mm–thick polyacrylamide slab gel. For oligonucleotides with fewer than 25 bases, use 19% acrylamide, for oligonucleotides with 25–40 bases, 15% acrylamide and for oligonucleotides with 41–100 bases, 12% acrylamide. Resuspend the pellet from ethanol precipitation in 200 µL of 50% formamide, and incubate at 55°C for 5 minutes to disrupt any secondary structure. Load the warmed oligonucleotide onto the gel (you may need to use several wells) and load an adjacent well with 50% formamide plus 0.05% bromophenol blue. The bromophenol blue will migrate at approximately the same rate as the oligonucleotide. Run the gel until the bromophenol blue indicator dye is two-thirds of the way down the gel. Remove the gel from the glass plates and place on Saran Wrap™. Lay the gel on a fluorescent TLC plate. Locate the labeled and unlabeled oligonucleotides by illumination with a handheld UV source. Fluorophore-labeled oligonucleotides will show fluorescence when illuminated with UV light. Cut out the band containing the labeled oligonucleotide and purify by the "crush-and-soak" method or other suitable method. For more details, please refer to reference 3.

Purification by HPLC. Labeled oligonucleotides can be purified by reverse-phase HPLC using a standard analytical (4.6 × 250 mm) C8 column. Dissolve the pellet from the ethanol precipitation in 0.1 M TEAA (triethylammonium acetate). Load the dissolved pellet onto the column in 0.1 M TEAA (triethylammonium acetate) and run a linear 5–65% acetonitrile gradient over 30 minutes. This gradient is a 2% increase in acetonitrile per minute. For oligonucleotides labeled with very hydrophobic dyes, like the Texas Red fluorophore, you can achieve good separation running a faster gradient with up to a 3% increase per minute. For separation of oligonucleotides labeled with more hydrophilic dyes, like the Marina Blue™ fluorophore, run a slower gradient, about 1% increase in acetonitrile per minute. In all cases, the unlabeled oligonucleotide will migrate fastest, followed by the labeled oligonucleotide and finally the free dye. For more details, please refer to reference 4.

References

1. Richardson, C.C. in *Procedures in Nucleic Acid Research*, G.L. Cantoni and D.R. Davies, Eds., Harper and Row (1971); **2.** Richardson, C.C. in *The Enzymes*, P.D. Boyer, Ed., vol. 14, p. 299, Academic Press (1981); **3.** Sambrook J., Fritsch E.F. and Maniatis T., *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory (1989); **4.** Oliver R.W.A., *HPLC of Macromolecules: A Practical Approach*, IRL Press (1989).

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