# 3′-Modified Oligonucleotides and their Conjugates



This unit describes synthetic methods for the preparation of three types of oligonucleotide 3′-modifications. The first two, aminoalkyl and sulfhydrylalkyl, are important because these groups offer specific points of attachment to a large variety of dyes and reporter groups. Their preparation is described in the Basic Protocol and Alternate Protocol 1, respectively. Alternate Protocol 2 deals with the 3′-attachment of polyethylene glycol (PEG) or other diols to the synthetic oligo. PEG-functionalized oligos are of interest because of their enhanced cell membrane permeability, which is important for antisense drug development.

Implementation of the following procedures requires working knowledge of automated DNA synthesizers, high-performance liquid chromatographs (HPLCs), and UV/visible spectrophotometers. The use of derivatized supports requires the ability to pack DNA synthesis columns. In addition, access to a fume hood is required, as many of the preparations use malodorous, toxic, or otherwise hazardous reagents.

*CAUTION*: Safe conduct in the laboratory when handling common solvents and chemicals, and working knowledge of equipment such as vacuum lines and rotary evaporators, is essential. Safety glasses and gloves should be worn to minimize risk of exposure.

*CAUTION:* Many of the steps involve pyridine, which is extremely toxic and has a foul odor. Dichloroacetic acid causes severe burns when spilled on the skin. These steps should be performed in a fume hood with gloves and eye protection. Do not use magnetic stir bars with controlled-pore glass—it generates fine particles that can clog frits or valves in DNA synthesizers.

## PREPARATION OF 3′-AMINOALKYL-FUNCTIONALIZED DNA OLIGONUCLEOTIDES

*BASIC PROTOCOL*

The synthesis of an anhydride-functionalized CPG that can be used in the preparation of 3′-aminoalkyl, 3′-thioalkyl, or 3′-hydroxyalkyl DNA is delineated. The preparation of a support for 3′-aminoalkyl DNA synthesis is then described, followed by the synthesis and purification of a 3′-fluorescein-DNA conjugate.

### *Materials*

1000 Å aminopropyl-conjugated controlled-pore glass (aminopropyl-CPG; Biosearch Technologies)
Pyridine
Methylene chloride
Trimellitic anhydride chloride (Aldrich)
100% and 20% (v/v) acetonitrile
6-Amino-1-hexanol
*N*,*N*-Dimethylformamide (DMF)
4,4′-Dimethoxytrityl chloride (DMTr chloride; Aldrich)
3% (v/v) dichloroacetic acid in methylene chloride
28% (v/v) aqueous ammonia
5- and 6-Carboxy fluorescein succinimide ester (Molecular Probes)
Dimethyl sulfoxide (DMSO)
1 M sodium carbonate/1 M sodium bicarbonate solution
0.05 M aqueous ammonium acetate

**Contributed by Matthew H. Lyttle**
*Current Protocols in Nucleic Acid Chemistry* (2000) 4.6.1-4.6.8

Sephadex G-25 resin (Amersham Pharmacia Biotech)
1 M triethylammonium acetate (TEAA)
14% (v/v) aqueous ammonia (1 part concentrated ammonium hydroxide, 1 part water)
2.8% (v/v) aqueous ammonia (1 part concentrated ammonium hydroxide, 9 parts water)
Buffer A: 0.025 M Tris·Cl and 0.01 M Tris base
Buffer B: 0.025 M Tris·Cl, 0.01 M Tris base, and 1 M NaCl

125-mL Erlenmeyer flasks with stoppers
150-mL sintered glass funnels, coarse frit
1-liter side-arm filter flask with rubber gasket
Water aspirator
Heavy-walled vacuum tubing
3-way valve
Speedvac (Savant)
Spectrophotometer and 1-cm path length glass cuvettes (e.g., Beckman)
Automated DNA synthesizer and reagents (e.g., PE Biosystems)
1.5-mL plastic screw-cap tubes
55°C water bath
Reversed-phase DNA purification cartridges and reagents (e.g., Biosearch Technologies)
1 × 30–cm glass tube with a frit and valve on bottom
10-mL syringes
High-performance liquid chromatograph (HPLC) with anion-exchange column

Additional reagents and equipment for DNA purification with a reversed-phase cartridge (*UNIT 10.7*)

***Synthesize an anhydride-functionalized CPG***

1. Weigh out 5 g aminopropyl-CPG and place in a 125-mL Erlenmeyer flask with stopper.
2. In a separate 125-mL Erlenmeyer flask, mix together 10 mL pyridine and 40 mL methylene chloride. Stopper the flask.
3. Weigh out 1 g trimellitic anhydride chloride and add it to the solution prepared in step 2. Swirl solution until the solid completely dissolves.
4. Pour solution into the flask containing CPG. Swirl and allow to stand 1 hr at ambient temperature.
5. In the meantime, assemble a 150-mL sintered glass funnel, rubber gasket, and a 1-liter side-arm filter flask, and connect these to a water aspirator with a piece of heavy-walled vacuum tubing. Install a 3-way valve between the flask and aspirator to control the vacuum and prevent water from being drawn into the flask when the aspirator is turned off.
6. Pour the slurry containing the CPG into the sintered glass funnel and remove the solvent by suction through the frit. Wash the CPG with three 50-mL portions of methylene chloride followed by three 50-mL portions of acetonitrile.

   *For each wash, completely suspend the CPG in the solvent with a spatula before applying vacuum to ensure efficient washing.*
7. Dry the CPG in a vacuum desiccator for several hours.

***Add hydroxylated aminohexyl linker to the anhydride-functionalized CPG***

8. Weigh out 2 g of 6-amino-1-hexanol and dissolve in 10 mL DMF.
9. Add the solution to 2 g of the support obtained in step 7 and swirl briefly. Allow the slurry to stand for 3 hr at room temperature.
10. Repeat steps 6 and 7.

***Protect the linker hydroxy function with a DMTr group***

11. Add 20 mL dry pyridine to the CPG obtained in step 10 and then add 1 g DMTr chloride. Swirl the mixture until the DMTr chloride dissolves, and allow to stand for at least 18 hr at room temperature.
12. Repeat steps 6 and 7.
13. To measure the amount of available hydroxyls on the support, now DMTr protected, weigh out 20 mg of the dry CPG material into a 125-mL Erlenmeyer flask and add exactly 100 mL of 3% dichloroacetic acid in methylene chloride.
14. Measure the absorbance of the orange solution at 498 nm, and multiply this number by the abbreviated extinction coefficient of DMTr (71.2).

    *The result is the loading of the support, in micromoles per gram. Loading should be ≥25 μmol/g.*

***Synthesize DNA on aminohexyl-linked CPG***

15. Calculate how many milligrams of the support obtained in step 12 will be required for a 0.2-μmol synthesis. Pack the calculated amount into a DNA synthesis column.

    *The required amount of support should be between 3 and 10 mg.*

16. Using an automated DNA synthesizer, add the desired sequence of nucleobases to the support.

    *Remember that the 3′-terminal nucleobase must be added as an amidite, so use a dummy nucleobase in the sequence entry at the 3′ terminus. Leave the 5′-DMTr on when oligonucleotide synthesis is complete.*

17. After synthesis, place the CPG containing the DNA in a 1.5-mL screw-cap tube and remove the DNA from the CPG by heating in 1 mL of 28% aqueous ammonia for 5 hr at 55°C.

    *5′-Terminal dG sequences are prone to detritylate at 55°C; these sequences give better results when deprotected for 48 hr at room temperature.*

18. Allow the tube to cool to room temperature, and purify the DNA with a reversed-phase cartridge according to the manufacturer's instructions (see *UNIT 10.7*). Evaporate the purified DNA solution and then measure the absorbance at 254 nm in water.

***Conjugate 3′-aminoalkyl DNA oligonucleotides to 5- and 6-carboxyfluorescein***

19. For each 10 $OD_{254}$ units of 3′-aminohexyl DNA oligonucleotide measured, weigh out 1 mg of 5- and 6-carboxyfluorescein succinimide ester and dissolve in 100 μL DMSO.

    *For >50 $OD_{254}$ units of DNA, increase DMSO by 10 μL per additional 10 OD units.*

20. Add this solution to a 1.5-mL plastic screw-cap tube containing the dried DNA, followed by 500 μL of 1 M sodium carbonate/1 M sodium bicarbonate solution. Close the tube and allow to stand 18 to 24 hr at room temperature.

    *For >50 $OD_{254}$ units of DNA, increase buffer by 100 μL per additional 10 OD units.*

21. Evaporate the liquid in a Speedvac evaporator, and dissolve the residue in 1.5 mL of 0.05 M aqueous ammonium acetate.

22. Mix 20 g Sephadex G-25 resin with 100 mL of 0.05 M aqueous ammonium acetate, and pour the slurry into a 1 × 30–cm glass tube with a frit and valve at the bottom. Add enough of the slurry so that the settled bed volume is 20 to 25 cm long. Allow the liquid to elute from the column until the liquid is level with the Sephadex slurry.

    *Use fresh Sephadex for each purification.*

23. Add the DNA solution (step 21) to the column, and allow the liquid to elute from the column until the liquid is level with the Sephadex column.

24. Elute the column with 0.05 M aqueous ammonium acetate at a flow rate of ~2 mL/min. Collect the first colored band that elutes after the first 10 to 15 mL. Evaporate the collected fraction in 1.5-mL tubes in a Speedvac.

    *For many applications, the fluorescein-DNA oligonucleotide conjugate is pure enough for good results. Analysis of purity can be performed by PAGE (UNIT 10.4) or HPLC (UNIT 10.5). If necessary, purification can be performed using a reversed-phase cartridge (see below).*

***Purify fluorescein-DNA oligonucleotide conjugate***

25. Preequilibrate a reversed-phase DNA purification cartridge by eluting with 4 mL acetonitrile followed by 4 mL of 1 M TEAA at a flow rate of ~1 mL/min using a 10-mL syringe.

26. Dissolve the sample obtained in step 24 in 1 mL of 14% aqueous ammonia. Apply the solution to the cartridge. Collect the effluent and reload it onto the cartridge two times.

27. Elute the cartridge with 4 mL of 2.8% aqueous ammonia followed by 4 mL water at ~1 mL/min. Discard noncolored eluant.

28. Elute the cartridge with 3 mL of 20% acetonitrile in water at ~1 mL/min, and collect the strongly green effluent.

29. Evaporate in a Speedvac and store the purified 3′-fluorescein DNA oligonucleotide conjugate at −20°C until needed.

    *Samples retain fluorescence for 2 to 3 months if kept in the dark.*

***Analyze conjugate by HPLC***

30. Dissolve the sample in 500 µL of 20% acetonitrile in water, and inject 2 to 20 µL, depending on the concentration, onto an anion-exchange column. Elute the conjugate with a linear gradient of 100% buffer A to 100% buffer B over 20 min at a flow rate of 1 mL/min.

    *The fluorescein conjugate will elute 2 to 4 min later than the underivatized 3′-aminohexyl DNA oligonucleotide.*

***ALTERNATE PROTOCOL 1***

## PREPARATION OF 3′-THIOALKYL-FUNCTIONALIZED DNA OLIGONUCLEOTIDES

This procedure uses many of the same reagents and steps as the Basic Protocol. However, the oxygen of the sulfur-bearing spacer must be protected, or else mixtures of products will be obtained after DNA synthesis and cleavage. A synthesis of these *O*-DMTr-protected thioalkyl spacers has been previously reported (Gupta et al., 1991).

***Additional Materials*** *(also see Basic Protocol)*

6-Mercapto-1-*O*-DMTr hexanol (Biosearch Technologies)
Dithiothreitol (DTT)
Triethylamine
Fluorescein-5-maleimide (Molecular Probes)
Sodium phosphate/sodium chloride buffer: 50 mM sodium phosphate and 150 mM sodium chloride, adjust to pH 7.2 (if necessary)

***Synthesize 3′-thiohexyl DNA oligonucleotides on CPG***

1. Prepare anhydride-functionalized CPG (see Basic Protocol, steps 1 to 7) and place 2 g in a 125-mL Erlenmeyer flask with stopper.
2. In a separate 125-mL flask, dissolve 1 g of 6-mercapto-1-*O*-DMTr hexanol in 10 mL DMF, and add 1 mL of triethylamine.
3. Add this solution to the Erlenmeyer flask containing CPG. Stopper the flask and swirl the CPG until there is a uniform slurry in the flask. Allow the flask containing the CPG to stand for 3 hr at room temperature.
4. Perform washing and drying as described (see Basic Protocol, steps 6 and 7).
5. Assay the loading of the spacer on the CPG (see Basic Protocol, steps 13 and 14).

   *Loading should be ≥20 μmol/g.*

6. Synthesize DNA oligonucleotides on the CPG, and deprotect and purify the product (see Basic Protocol, steps 15 to 18), but add 2 to 3 mg DTT to the 28% aqueous ammonia solution (step 17) before heating to prevent oxidative dimerization of the thioakyl oligonucleotides.

***Conjugate 3′-thiohexyl DNA oligonucleotides to fluorescein-5-maleimide***

7. Weigh out 2 mg fluorescein-5-maleimide for every 10 $OD_{254}$ units of DNA oligonucleotide to be conjugated in a 1.5-mL plastic screw-cap tube, and dissolve in 100 μl DMF.
8. Add this solution to a 1.5-mL plastic screw-cap tube containing the thiohexyl DNA.
9. Add 500 μL sodium phosphate/sodium chloride buffer, pH 7.2, and allow to stand 18 to 24 hr in the dark at room temperature.
10. Perform Sephadex G-25 size exclusion (see Basic Protocol, steps 21 to 24).

    *Reversed-phase cartridge purification does not work well for thiomaleimido fluorescein-DNA oligonucleotide conjugates. Purification of the conjugates can be accomplished by either preparative HPLC (UNIT 10.5) or preparative PAGE (UNIT 10.4).*

## PREPARATION OF 3′-POLYETHYLENE-GLYCOL-FUNCTIONALIZED DNA OLIGONUCLEOTIDES

***ALTERNATE PROTOCOL 2***

Dry solvents and equipment are essential for the key reaction in this sequence, which is the addition of a hydroxyl functionality to an anhydride. This procedure uses many of the same reagents and steps as previous protocols. Various polyethylene glycols are commercially available; selection depends on the experimental design. The example given below uses triethylene glycol.

***Additional Materials*** *(also see Basic Protocol)*

Triethylene glycol (Aldrich)
*N*-Methylimidazole (Aldrich)

1. Prepare anhydride-functionalized CPG (see Basic Protocol, steps 1 to 4), and place 2 g in a 125-mL Erlenmeyer flask with stopper.
2. In a separate 125-mL flask, dissolve 1 g triethylene glycol in 10 mL DMF, and add 500 µL *N*-methylimidazole.
3. Add this solution to the Erlenmeyer flask containing CPG. Stopper the flask and swirl the CPG until there is a uniform slurry in the flask. Allow the flask containing the CPG to stand for 18 to 24 hr at room temperature.
4. Perform washing and drying (see Basic Protocol, steps 6 and 7).
5. Assay the loading of the triethylene glycol spacer on the CPG (see Basic Protocol, steps 11 through 14).

   *Loading should be ≥20 µmol/g.*
6. Synthesize DNA oligonucleotides on the PEGylated CPG, and deprotect and purify the product (see Basic Protocol, steps 15 to 18).
7. Analyze 3′-PEGylated DNA oligonucleotides by ion-exchange HPLC (see Basic Protocol, step 30).

   *The desired product will elute slightly later than the corresponding unmodified DNA.*

## COMMENTARY

### Background Information

The basic strategy employed in these protocols (Lyttle et al., 1997) calls for the synthesis of an anhydride-functionalized solid support as a common intermediate for each functional group attachment. A bifunctional molecule (spacer) that has an OH group at one end and an SH, $NH_2$, or OH group at the other end is then added. The most nucleophilic functional group reacts with the anhydride to form a thioester, amide, or ester bond, respectively, while the OH group at the other end of the spacer is available to react with nucleoside phosphoramidites (see Figure 4.6.1). There is then an optional step of adding a 4,4′-dimethoxytrityl (DMTr) group to this alcohol group to spectrophotometrically gauge the amount of addition of the first phosphoramidite during automated oligonucleotide synthesis. In the case of the thiol functionality, this DMTr alcohol protection is mandatory, or else a mixture of products (resulting from SH and OH addition to the anhydride) will be obtained. The synthesis of the required *O*-DMTr-protected hydroxylalkylthiols is described in the literature (Gupta et al., 1991). 6-*O*-DMTr-hydroxyhexylthiol is available from Biosearch Technologies.

Once the support is made, automated oligonucleotide synthesis is performed to construct the desired sequence, then the usual aqueous ammonia treatment is employed for DNA deprotection and solid-support cleavage to provide the desired 3′-terminal functionality upon basic hydrolysis of the thioester, amide, or ester bonds. The product DNA is then purified with reversed-phase cartridges, and can be characterized by PAGE, HPLC, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). In the case of 3′-$NH_2$- or SH-modified oligos, a procedure for the attachment of fluorescein is described, including an example of a successful reversed-phase cartridge purification of a 3′-amino-modified DNA-fluorescein conjugate.

### Critical Parameters and Troubleshooting

These protocols describe three techniques for the 3′-terminal labeling of DNA. Successful execution of each protocol depends on the skill and patience of the researcher, as well as the quality of reagents and solvents.

The approximate expected loading is mentioned in each protocol; if loading is lower than expected, there are several points to check. For steps that require the immobilized anhydride, use a new bottle of trimellitic anhydride chloride for the coupling reaction. A negative ninhydrin test (Stewart and Young, 1984) will assure that the anhydride was quantitatively linked to the resin prepared according to the Basic Protocol. DMF must be dry (i.e., <0.05% water) and not have a noticeably fishy aroma;

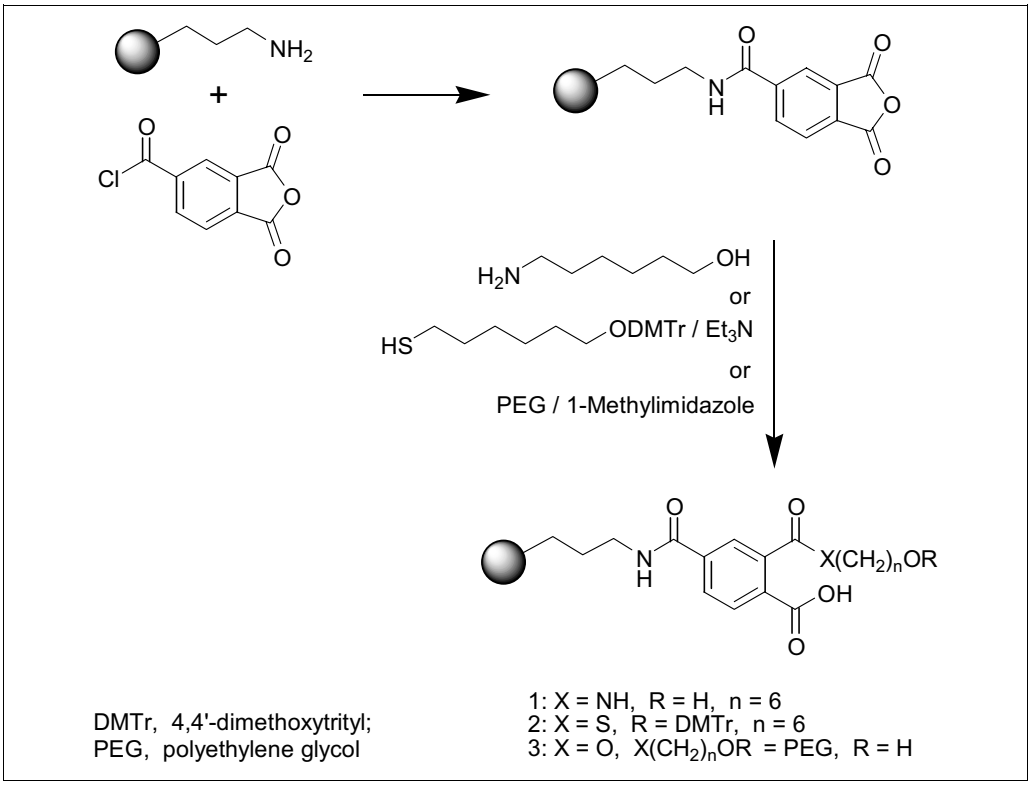


**Figure 4.6.1** An anhydride-functionalized solid support for the synthesis of 3′-modified DNA.

DMF slowly hydrolyzes to formic acid and dimethyl amine. The latter component has a fishy smell, and will have deleterious effects on the chemistry. Temperature of reactions should be close to 20°C; even a decrease of 10°C will slow down reaction rates. The DMTr chloride addition reactions are also moisture sensitive; therefore, use a freshly opened pyridine bottle (<0.05% water) and new DMTr chloride to improve the results if they are low. Finally, for the novice unfamiliar with organic chemical manipulations, the advice of a more experienced colleague can be indispensable.

## Anticipated Results

DNA fragments have been synthesized on three 3′-modification supports and compared to those DNA oligonucleotides synthesized from a conventional hemisuccinate nucleoside-derivatized CPG. Stepwise coupling yields were similar in all cases. Yields of reversed-phase cartridge purified DNA compared favorably with the amount normally obtained for cartridge purification at the 200-nmol and 1-µmol synthesis scales. Anion exchange (AX)-HPLC of 3′-modified 14-mers (5′-CCGAG-TACTATTCA-3′) synthesized from the three CPGs showed that good quality products (i.e., 85% to 95% pure by AX-HPLC integration) were obtained in the case of the 3′-thiohexyl- and 3′-triethylene-glycol-functionalized oligomers. In the case of the 3′-aminohexyl-derivatized 14-mer, a substantially slower eluting contaminant (20% to 30%) was present. The amount of this impurity can be decreased by prolonged heating (i.e., 24 to 48 hr) with aqueous ammonia, suggesting that this contaminant is DNA containing an unhydrolyzed amide-metallic linker at the 3′-terminus. This slower-eluting contaminant did not interfere in subsequent reactions involving conjugation of the 3′-aminohexyl-functionalized oligonucleotide and was separated from the desired fluorescein conjugate by a reversed-phase cartridge purification. Due to the high loadings of the 3′-aminoalkyl CPG obtained with this procedure (i.e., 50 to 80 µmol/g), ample reactive material is still produced for subsequent conjugation steps.

When the 3′-aminohexyl-linked 14-mer DNA fragment was prepared at a 200-nmol scale, ~75% conversion to the desired conjugate was obtained upon reaction with 50 equivalents of a mixture of fluorescein 5- and 6-carboxyhydroxysuccinimide active esters. The fluorescein conjugates elute 2 to 3 min slower by AX-HPLC than the unreacted 3′-aminohexyl or thiolalkyl-modified DNA. The crude fluorescein conjugate could be purified

to 95% purity by the reversed-phase cartridge method described above. Better amine-fluorescein conjugation efficiency (>99%) was obtained at a 1-µmol synthesis scale, perhaps because of concentration considerations. Purified yields of 3′-fluorescein DNA at the 1-µmol synthesis scale should be 15 to 25 OD units. The corresponding 3′-thiolalkyl-modified DNA gave lower conjugation efficiencies (19% to 49%) even when reacted with 100 equivalents of fluorescein-5-maleimide. Cartridge purification of the thiol-linked fluorescein-DNA conjugates was also less effective. A 20% cross-linked polyacrylamide gel of 3′-conjugated DNA fragments that were prepared from both 3′-thiolalkyl and aminohexyl terminal modifications showed strongly fluorescent products exhibiting about the same gel mobility as that of underivatized DNA of the same length and sequence. The gel lanes of the thiolalkyl-linked conjugates contain some underivatized material, in agreement with HPLC analysis.

## Time Considerations

Most of the steps require a few hours at most and can be accomplished during an 8-hr day. The Sephadex columns usually require ≥4 hr for setup, chromatography, and analysis of the products. In general, only the immobilized anhydride-functionalized CPG cannot be stored for extended periods of time, and should be utilized as soon as possible. In all other steps involving a solid support, reagents should be washed away and the support washed with a neutral, volatile solvent before storage. When 18 to 24 hr of reaction time is recommended, it is best to mix the reagents for the reaction in the early evening (~5 p.m.) and then work up the reaction mixture the following day.

## Literature Cited


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Stewart, J. and Young, J. 1984. Laboratory techniques in solid phase peptide synthesis. *In* Solid Phase Peptide Synthesis, pp. 105-107. Pierce Chemical Company, Rockford, Ill.



Contributed by Matthew H. Lyttle
Biosearch Technologies, Inc.
Novato, California