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A convenient, solid-phase coupling of rhodamine dye acids to 5′ amino-oligonucleotides

Ravi Vinayak *

PE Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA

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Abstract

A convenient method has been developed for directly labeling the 5′ amino group of oligonucleotides on solid-support with rhodamine dyes to give rhodamine-labeled oligonucleotides.

Fluorescent dye-labeled oligonucleotides are used extensively in genetic analysis, DNA sequencing, amplification-based diagnostic assays, forensic identity tests and other methods for detecting and quantitating target DNA. Conjugation of synthetic oligonucleotides with labels during the highly efficient and automated process of phosphoramidite nucleoside monomer addition[1] utilizes the advantages of solid-phase chemistry that obviates the more laborious methods of solution phase labeling and purification.[2] Fluorescein phosphoramidite reagents have been introduced recently that give high yield and rapid coupling of the fluorescein dyes to the 5′ terminus of the oligonucleotide, as the final step in oligonucleotide synthesis.[3] Rhodamine dyes are equally important as nucleic acid labels but are not sufficiently stable to allow preparation of phosphoramidites and use on the automated synthesizers. In addition, the *N*-hydroxy succinimide esters of rhodamine carboxylic acids tend to degrade upon prolonged storage. Also, ammonium hydroxide and methylamine are reagents used to cleave oligonucleotides from the solid-support and remove protecting groups obliterate the rhodamine dye. In the pursuit of facile and efficient synthesis of 5′ rhodamine-labeled oligonucleotides, we have developed a simple method which can be employed on the DNA synthesizer, coupling the easily available solid-phase, 5′ amino-oligonucleotide by in situ activation of rhodamine carboxylic acids. A mild reagent, *tert*-butylamine:water:methanol (1:2:1), preserves the rhodamine structure during cleavage and deprotection (Table 1).[4]

In a typical example, 10.7 mg of Tamra carboxylic acid (**1**) is suspended in 500 μl of dry DMF. To this is added a solution of 9.25 mg of HBTU (**3**) in 250 μl solution of 1:1 DMF:CH_3CN. This is followed by addition of 8.6 μl of di-isopropylethylamine (DiPEA).[5] Any undissolved dye at this stage goes into solution and is then installed on the synthesizer, free of particulate matter. This solution is then delivered to the 5′ amino linked oligonucleotide (**4**)[5] (prepared by addition of the MMT-C6 phosphoramidite to the 5′ end of DNA) and allowed to couple for 15 min. After capping and extensive

* Corresponding author. E-mail: vinayars@pebio.com

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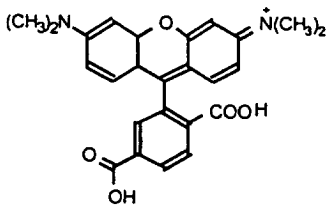
Table 1
Steps involved in dye labeling of a DNA* on an ABI 394 DNA/RNA synthesizer

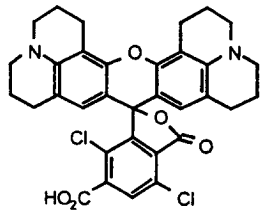
Step	Function	Solvents & Reagents	(Time)
1	Detritylation	3% CCl_3COOH / CH_2Cl_2	60 sec
2	Coupling	0.2 M Dye Solution / DMF + 0.2 M HBTU in DMF /CH_3CN + DiPEA	900 sec
3	Capping	Ac_2O / Pyridine / THF N-Methylimidazole / THF	20 sec

* Syntheses were performed on the ABI LV200 (0.2 μmol) polystyrene columns.

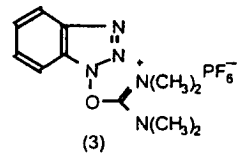
washing with CH_3CN, the oligonucleotide is cleaved from the polystyrene solid support using a mixture of *t*-butylamine:MeOH:H_2O (1:2:1).[6] After removal of the base and phosphate protecting groups the solution is evaporated to dryness and the 5′ fluorescent dye labeled oligonucleotide (**5**) is precipitated by ethanol precipitation. In general, the coupling efficiency is >80% as indicated by HPLC. Efforts to drive the reactions to higher degrees of completion, either by double coupling or extended conjugation times did not provide higher yields. The 5′ fluorescently dye-labeled oligonucleotides were analyzed by reverse-phase HPLC[7] equipped with a diode array detector and the identity of the product was confirmed by mass spectrometry (Fig. 1).



(1)



(2)



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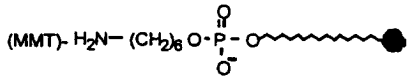
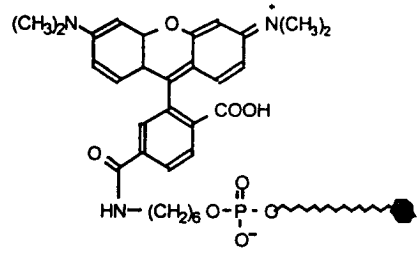


(4)



(5)

In conclusion, we have developed a reliable method for labeling oligonucleotides with rhodamine dyes on a solid support. The protocol can be generalized for introduction of labels at the 5′ end of DNA/RNA oligonucleotides. The method is especially useful with chemically labile or otherwise reactive molecular species. The method can be applied to any reporter molecule with a reactive caroboxylic acid group and obviates the need for phosphoramidite or solution phase labeling with *N*-hydroxy succinimide esters.

Extension of this chemistry to other complicated dye systems, doubly labeled probes (3′ and 5′) and implementation on to high throughput platforms are currently in progress.

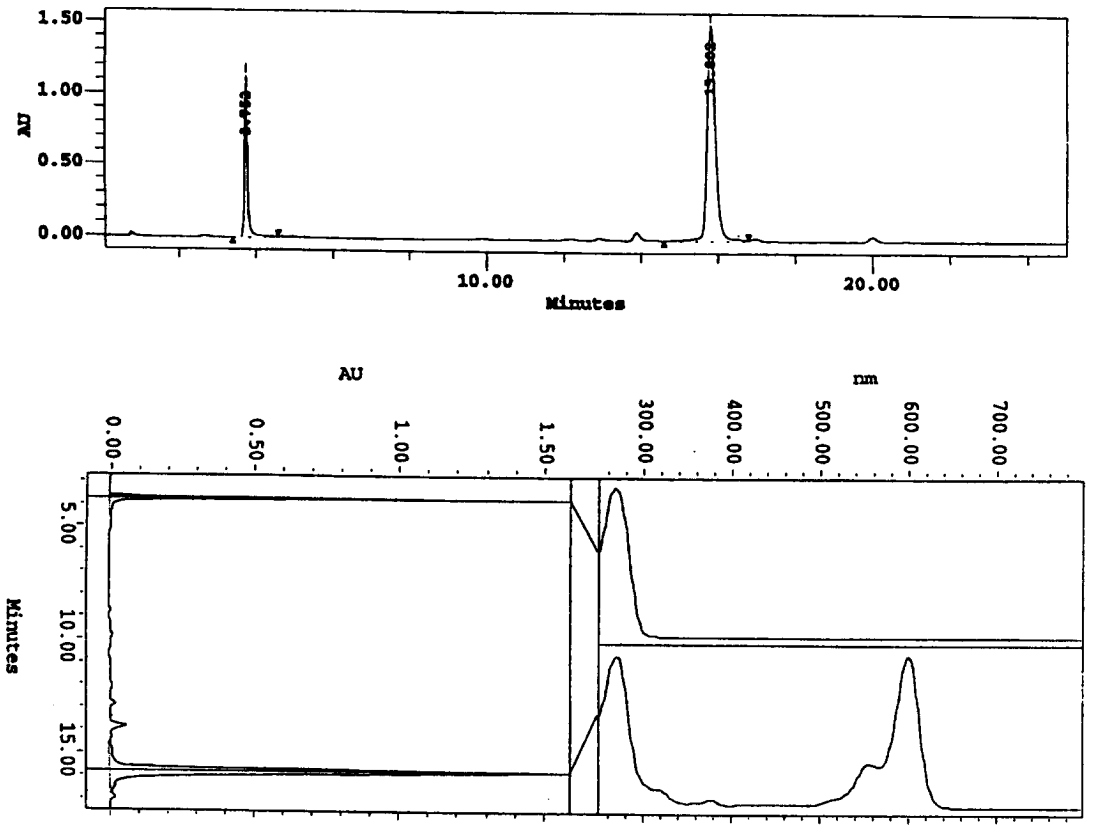


Figure 1. Reverse-phase HPLC chromatogram of 5'dRox (2)-labeled oligonucleotide with the sequence TCACAGTCTGATCTCGAT3' (26 nt). The top chromatogram was analyzed with detector set at λ_{260} and the lower chromatogram is in diode array mode (λ_{260} and λ_{600})

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The author thanks Linda Lee for HPLC analyses, Liang Hoe and Harry Tang for technical assistance and Alex Andrus for a critical review of the manuscript.

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6. Syntheses of oligonucleotides were carried out using A^{Bz}, G^{dmf}, C^{Bz} and T-phosphoramidites (PE Applied Biosystems). The MMT-C6-phosphoramidite (5'amino modifier C6) was obtained from Glen Research. The cleavage and deprotection were effected in *t*-butyl amine:MeOH:H_2O (1:1:2) for 3 h at 65°C; Ref. 4 and US Patent. No. 4,965,349. The oligonucleotides were purified using RP–HPLC gradient as described in Ref. 7.
7. Reverse-phase HPLC analysis was carried out on a Perkin–Elmer Aquapore RP-300, 7 μm column. Gradient elution was performed at ambient temperature by building up a gradient with buffer A (0.1 M TEAA in H_2O) and applying buffer B (CH_3CN) with a flow rate of 1.0 ml/min. The used gradient was 15% B to 35% B in 30 min.