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Automated Synthesis of Double Dye-Labeled Oligonucleotides using Tetramethylrhodamine (TAMRA) Solid Supports.

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Abstract: *TAMRA fluorescent dye-labeled non-nucleosidic synthesis supports have been derivatized for automated synthesis of 3' dye-labeled oligonucleotides.[1] Synthesis with 5' fluorescent dye phosphoramidites leads to 5' and 3' double labeled oligonucleotides, useful in Taqman assay and FRET experiments. Cleavage and deprotection of dye-labeled oligonucleotides are carried out with tert-butylamine:methanol:water (1:1:2) without any degradation and/or modification of dyes and nucleobases.* © 1997 Elsevier Science Ltd.

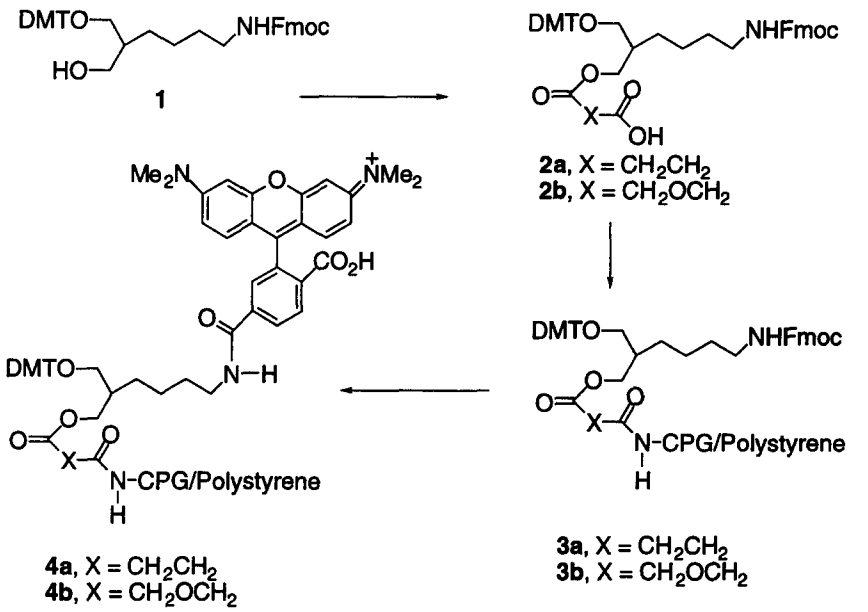
Recently it has been reported that accumulation of specific polymerase chain reaction (PCR) products can be conveniently measured and detected in real time in 5' nuclease assay (Taqman assay) using double dye-labeled fluorogenic oligonucleotide probes.[2-4] Double dye-labeled oligonucleotides are also used in real time detection of specific nucleic acid sequences by the fluorescence resonance energy transfer (FRET) effect.[5-8] 3' Fluorescent dye-labeled oligonucleotides are also used in oligonucleotide hybridization ligation assays (OLA) for convenient detection of ligation products.[9] Double dye-labeled probes used in 5' nuclease assay, have fluorescent reporter dyes, such as, fluoresceins, at the 5' end, and a quencher dye, tetramethylrhodamine (TAMRA) at the 3' end. When the fluorescein is excited by irradiation, its fluorescent emission is quenched if the rhodamine is close enough to be excited through the process of fluorescence energy transfer.[10,11] The 5' fluorescein dye is incorporated by coupling fluorescein dye amidites at the 5' end. For the incorporation of 3' end dye, either a thymidine phosphoramidite containing an amino linker arm on C-5 or a non-nucleosidic synthesis support containing an amino linker arm are used. After cleavage and deprotection, oligonucleotides are reacted with the required dye isothiocyanates or N-hydroxy succinamide ester in solution. The coupling reaction is not very efficient and separation of double dye-labeled oligonucleotides from single-labeled and unlabeled impurities is difficult. Here we describe derivatization of TAMRA labeled solid supports (scheme I) for automated synthesis of double dye-labeled oligonucleotides.

First we examined the stability of TAMRA in oligonucleotide synthesis reagents and conditions. It was found that the dye is stable in all currently used ancillary reagents, but not stable in concentrated ammonium hydroxide, which is normally used for cleavage and deprotection of oligonucleotides. It has been reported that rhodamine dyes are stable in *tert*-butylamine:methanol:water (1:1:2) at temperatures upto 85 °C.[12] We then examined deprotection of oligonucleotides using *tert*-butylamine:methanol:water (1:1:2). It was found that exocyclic amine protecting groups were completely removed by treating oligonucleotides with *tert*-butylamine:methanol:water (1:1:2) for 3 h at 65 °C.

We then turned our attention to derivatize solid supports with suitably protected non-nucleosidic compounds. Protected aminodiol **1** prepared by a literature procedure[13] was reacted with succinic anhydride in presence of DMAP/Et_3N in CH_2Cl_2 to give succinate **2a** in 84% yield after silica gel column chromatography. Succinate **2a** was loaded on to both aminopropyl-CPG and aminomethyl polystyrene solid supports in presence of 2-(1 H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate[14] and diisopropylethyl amine. The loading of **2a** to CPG support was determined to be 30-35 μmol/g and 25 μmol/g on polystyrene support as quantitated by trityl cation assay. The Fmoc group was removed by

treating support **3a** with 20% piperidine in DMF for 20 min, followed by UV spectroscopy. Support **3a** was then reacted with TAMRA N-hydroxysuccinamide ester in DMF for 24 h at room temperature to give TAMRA labeled support **4a**. Dye coupling was determined to be 98-99%.

Scheme I



The dye-labeled support **4a** was subjected to DNA synthesis reagents for extended period of time (12 h, room temperature) and it was found that TAMRA and linker were stable under these conditions. The identity of TAMRA linker conjugate was confirmed by cleaving from the support with *tert*-butylamine:methanol:water (1:1:2) and comparison with an authentic sample of TAMRA linker conjugate prepared in solution separately. Support **4a** was used for automated synthesis of 3' dye-labeled oligonucleotides. The synthesis of 3' dye-labeled oligonucleotides went well as judged by measurement of trityl cation and by HPLC analysis of the oligonucleotides. But the cleavage of dye-labeled oligonucleotides was slow, only 50% cleaved in 2 h at room temperature.

It was reported that the cleavage rate of oligonucleotide could be increased by using oxalyl ester linkage instead of succinate linkage.[15] This linker was found to be too labile during oligonucleotide synthesis, resulting in premature cleavage and low yield of products. The diglycolate ester linkage proved to be more labile than succinate but more stable than oxalyl linkage. Diglycolate **2b** was prepared by reacting protected aminoalcohol **1** with diglycolic anhydride in presence of DMAP/Et_3N in CH_2Cl_2 at 0 °C. The reaction was complete in 1 h. After usual work-up diglycolate **2b** was precipitated from *tert*-butymethyl ether/hexane in 70% yield. Similarly diglycolate **2b** was loaded on to both CPG and polystyrene supports to give **3b**. Removal of Fmoc followed by coupling of TAMRA dye as described above gave support **4b**. Support **4b** was also found to be stable in all DNA synthesis reagents. It was found that oligonucleotides synthesized using support **4b** could be cleaved in >98% yield with *tert*-butylamine:methanol:water (1:1:2) in 45 min at

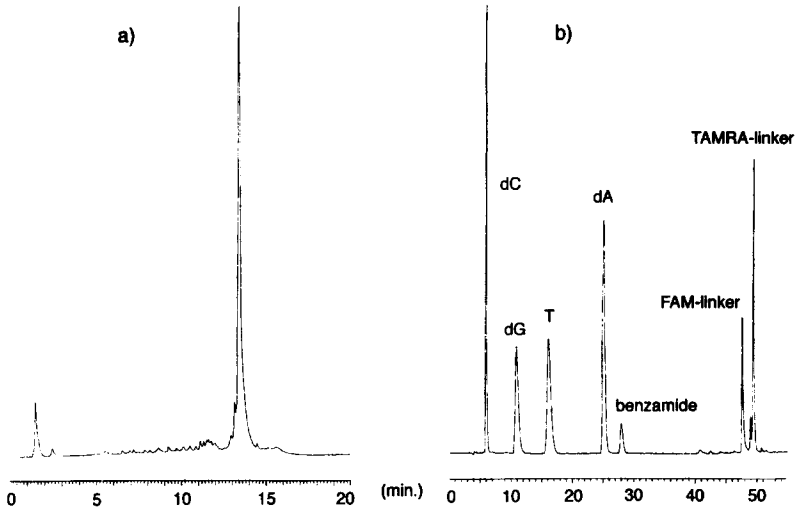


Figure 1: a) Anion-exchange HPLC and b) reverse-phase HPLC of enzymatic digestion of crude samples of double dye-labeled oligonucleotide, 5'>FAM-TCACAGTCTGATCTCGAT-TAMRA<3'

ambient temperature on the synthesizer. Double dye-labeled oligonucleotide probes (15-40 nucleotides long) were synthesized using supports **4b**, fluorescent dye amidites and A^{bz}, G^{dmf}, C^{bz}, T phosphoramidites in high yield and purity.[16] Oligonucleotides were analyzed by both reverse-phase and anion-exchange HPLC (Fig. 1a).[17] Enzymatic digestion of double dye-labeled oligonucleotides gave the expected base composition and no modification of dyes were observed (Fig. 1b).[18] Use of support **4b** allows automated incorporation of dye at the 3' end of oligonucleotides in high yield. We now routinely synthesize double dye-labeled oligonucleotide probes for 5' nuclease, Taqman PCR assay using dye-labeled support **4b** and fluorescein dye amidites. The results of real time detection of specific PCR products using dye-labeled probes synthesized with support **4b** will be reported separately.

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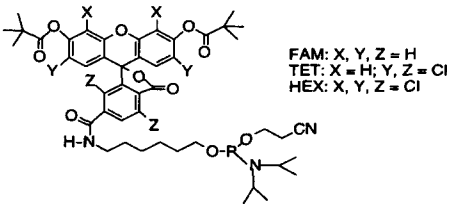

17. Reverse phase HPLC: RP-18 column (220 X 4.6 mm, Applied Biosystems), flow rate 1 mL/min, gradient 0-20% B in 24 min followed by 20-50% B in 40 min. Solvent A, 0.1M TEAA and solvent B, acetonitrile. Anion exchange HPLC: Nucleopac PA-100 column (250 X 4 mm, Dionex Corp.), flow rate 1 mL/min, gradient 0-60% B in 25 min. Solvent A: 20 mM $LiClO_4$ and 20 mM NaOAc in H_2O:CH_3CN (9:1, pH 6.5); solvent B: 600 mM $LiClO_4$ and 20 mM NaOAc in H_2O:CH_3CN (9:1, pH 6.5).
18. Appendix 1, *Evaluating and Isolating Synthetic Oligonucleotides*, 1992, Applied Biosystems (available upon request).

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