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Fluorescent Dye Phosphoramidite Labelling of Oligonucleotides

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Abstract: A 5-carboxyfluorescein (FAM) phosphoramidite reagent has been synthesized for use on automated DNA synthesizers, to prepare fluorescent dye labelled oligonucleotides. Oligonucleotides can be labelled at any site in a sequence and multiple labelled. The FAM dye can also be coupled to a polystyrene synthesis support to prepare 3' fluorescent labelled oligonucleotides.

Fluorescent dye labelled oligonucleotides (FDLO) are suitable for use in genetic analysis tests, such as PCR-based diagnostic assays and forensic identity tests, where detection and quantitation of the resulting DNA fragments are important.[1] For high quantum yield and chemical stability, the xanthene dyes, e.g. 5-carboxyfluorescein (FAM), are used as sensitive, specific, and covalently bound labels for oligonucleotides.[2] The previous method for FDLO production has been synthesis of oligonucleotides with the inclusion of nucleophilic primary amines, usually at the 5' end. The amino oligonucleotide is cleaved from its synthesis support, deprotected, and reacted in solution with the N-hydroxysuccinimide ester of a fluorescent dye.[3] Coupling efficiency can be variable and the large excess of hydrolyzed dye must be carefully removed. Purification entails a two stage process of initial size-exclusion gel filtration to remove excess dye, followed by preparative gel electrophoresis or HPLC to separate the FDLO from unlabelled sequences. Here we report a FAM phosphoramidite **1** (Figure 1), ready for use on the DNA synthesizer to directly prepare FDLO, and suitable for labelling at any site in the oligonucleotide sequence.[4]

The single isomer dye, 5-carboxyfluorescein **2**[5] was protected with trimethylacetyl chloride, to give the non-fluorescent lactone **3**. The linker moiety was prepared from solketal **4**, following the procedure of Gait[6], to give the amine **5**. The NHS ester of **3** was formed with dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide, then coupled with **5** to give **6** in 70% yield after chromatography. Hydrolysis of the isopropylidene group of **6**, followed by dimethoxytritylation of the primary hydroxyl, and subsequent phosphitylation gave **1** (Figure 1).

The phosphoramidite **1**, diluted with dry acetonitrile (<100 ppm water), is used in the same manner as nucleoside phosphoramidite[7] monomers on an automated DNA synthesizer.[8] The large DMT group, in closer proximity to phosphorus than in nucleoside phosphoramidites, imparts significant steric hinderance, necessitating an extended coupling time of 120 seconds. Coupling of **1** to the 5' hydroxyl of the support bound oligonucleotide **7**, proceeds in about 70% yield (Figure 2). Excess dye reagent is removed by simple washing of the solid support. The dye and its linkage to the oligonucleotide are stable during the conditions of DNA synthesis (detritylation, coupling, capping, and oxidation) and cleavage/deprotection conditions (concentrated ammonium hydroxide). The support bound, FAM labelled oligonucleotide **8** is cleaved and

deprotected in four hours at 55° C. Detritylation and trityl-selective purification are conducted by use of an OPC (Oligonucleotide Purification Cartridge), a polystyrene based affinity matrix, which selectively retains DMT bearing species.[9] Independent of the coupling efficiency of the nucleoside phosphoramidites, or **1**, OPC selectively isolates and purifies the desired FDLO. Unlabelled oligonucleotides and other impurities are washed away, followed by detritylation (2% TFA) and the elution of purified, desalted FDLO **9** (Figure 2). Detritylation of **8** and continuing synthesis with nucleoside phosphoramidites or **1** leads to internally labelled or multiple labelled FDLO (Figure 4b,c). Enzymatic digestion of the 5' FDLO (Figure 4a) by snake venom phosphodiesterase and bacterial alkaline phosphatase gave the expected deoxynucleoside composition and FAM-T with no detectable base modifications.[10]

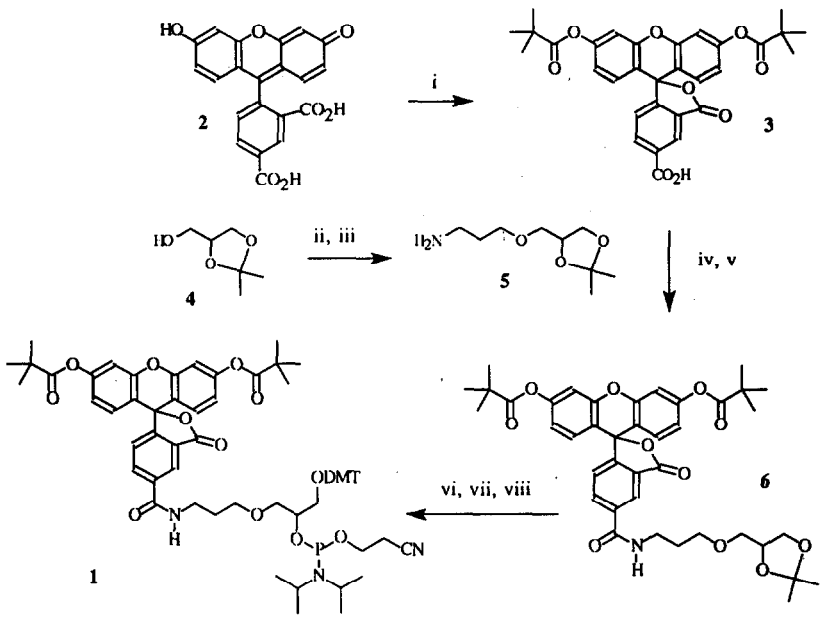

Figure 1. i. trimethylacetyl chloride, pyridine; ii. acrylonitrile, NaH, THF; iii. $NaBH_4$, $CoCl_2$, MeOH; iv. N-hydroxysuccinimide, DCC, CH_2Cl_2, v. **5**, CH_2Cl_2; vi. conc. HCl, CH_3OH, THF, vii. dimethoxytrityl chloride, pyridine, viii. $(iPr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, CH_2Cl_2.

The FAM dye can be coupled to a polystyrene synthesis support to prepare 3' fluorescent labelled oligonucleotides (Figure 3). The intermediate formed by hydrolysis of the isopropylidene moiety and dimethoxytritylation of **6** is treated with succinic anhydride to form the succinate acid/ester **10**. Coupling of **10** via DCC activation with 1000Å pore, 50μm diameter, aminomethyl polystyrene gave the support **11** at a loading of 10μmoles dye per gram.[11] The support **11** can be used as an oligonucleotide synthesis support, yielding 3' FDLO **12**, after DNA synthesis, cleavage from the polystyrene support, and deprotection (Figure 4d).[12]

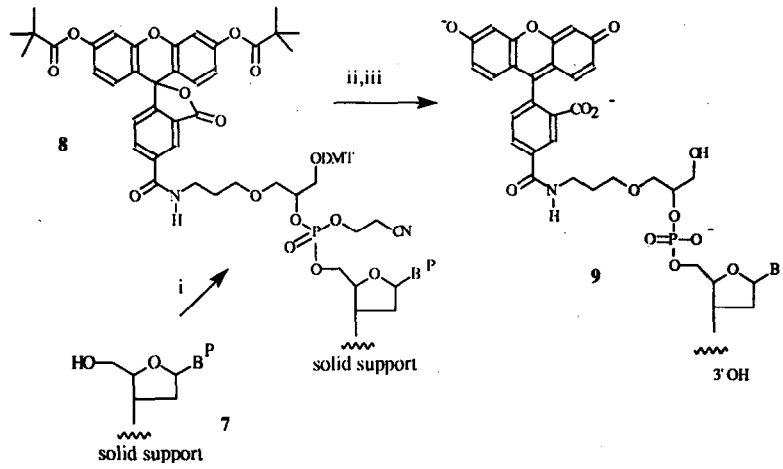

Figure 2. i. 1, tetrazole, CH_3CN, then I_2, THF, H_2O; ii. conc. NH_4OH, 55°C, 4 hr.; iii. 2% trifluoroacetic acid, OPC purification

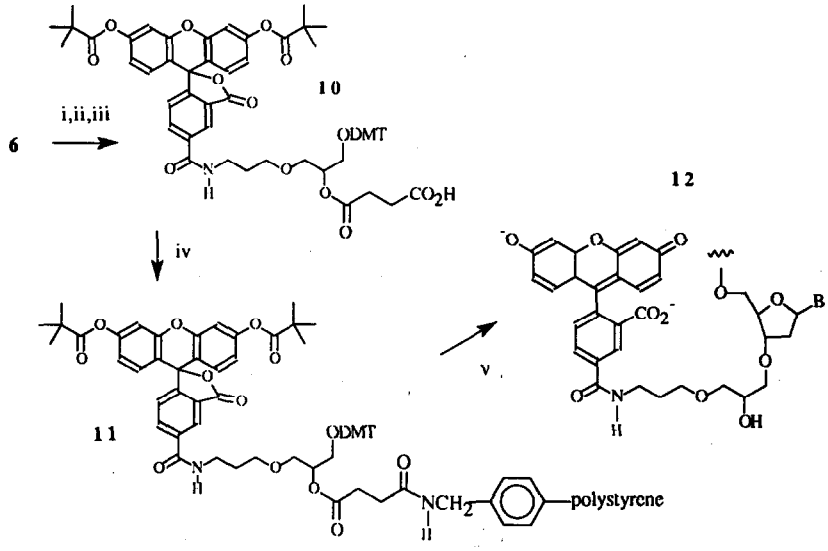

Figure 3. i. conc. HCl, MeOH,THF; ii. dimethoxytrityl chloride, pyridine; iii. succinic anhydride, DMAP, pyridine; iv. DCC, aminomethylpolystyrene; v. DNA synthesis, cleavage, deprotection

Crude or OPC purified FDLO may be analyzed by the conventional techniques of reverse phase HPLC, PAGE (polyacrylamide gel electrophoresis), and MicroGel capillary electrophoresis.[13] The presence of the fluorescent dye imparts more hydrophobicity to the oligonucleotide, observed by longer elution times on HPLC and MicroGel CE. The FDLO migrate approximately one base slower on PAGE and are visible under long wavelength (365 nm) UV light. Figure 4. shows MicroGel capillary electrophoresis analyses of OPC purified FDLO with FAM located at various positions in the sequence. Other xanthene dye phosphoramidites, e.g. rhodamine and fluorescein, are under investigation for use in nucleic acid fragment analysis and DNA sequencing.

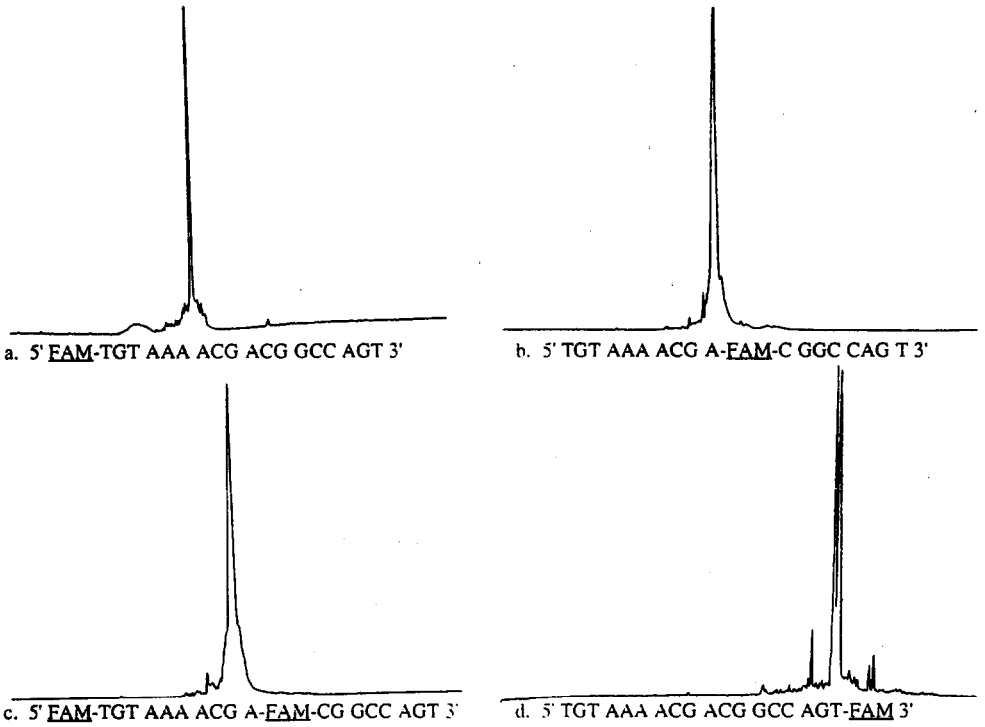

Figure 4. MicroGel Capillary Electrophoresis of Fluorescent Dye Labelled Oligonucleotides (FDLO)

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12. The FDLO product of 3' labelling with **11** Figure 4d shows two peaks due to the presence of the 6-carboxy- and 5-carboxyfluorescein isomers in this preparation of **11**. The diastereomeric center of the glycol moiety of **1** does not cause separation of diastereomers in FDLO analysis.
13. Dubrow, R.S., **1991**, *Amer. Laboratory*, , 64. FDLO analyses in Figure 4. were conducted on an Applied Biosystems Model 270A Capillary Electrophoresis system with MicroGel capillaries, operating at 15kV, 15-22µamp, 260nm detection.

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