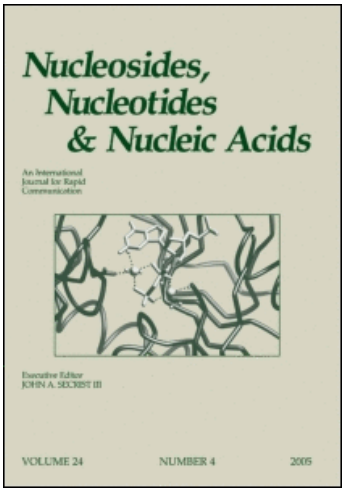

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5-AMIDO-(CARBOXYFLUORESCEIN)-2'-dU-OLIGONUCLEOTIDES: NOVEL PRIMERS FOR FLUORESCENT DETECTION OF PCR AMPLIFIED DNA

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ABSTRACT

The synthesis of oligonucleotides containing a novel nucleobase fluorescent derivative 5-NH_2-(carboxyfluorescein)-2'-deoxyuridine, in which the chromophore fluorescein is directly linked to nucleobase of 2'dU at C-5 via an amido function is described. The fluorescent oligonucleotides have been used as primers for PCR amplification of Human β-globin gene fragment. Oligonucleotides containing multiple insertions of such fluorophores have potential applications in molecular biotechnology.

INTRODUCTION

Fluorescence detection of nucleic acids for applications in DNA diagnostics and molecular biology is attracting attention due to its safe and easy handling[1,2]. The lower sensitivity of this technique compared to conventional radiolabels can be overcome by either multiple labelling[3-5] or signal amplification through PCR technique[6]. Detection procedures employing fluorescent oligonucleotides are of significant importance in automated DNA sequencing[2], nucleic acid hybridization[1], DNA-protein interactions[7] and for PCR[8]. The reported methods frequently involve conjugation of fluorescent dye to the 5' terminus of the oligonucleotide[9,10], phosphate backbone[11], sugar[12] or nucleoside bases using spacer arms[3,13]. Nucleobase labelling has advantages of incorporation at internal sites without affecting the backbone geometry. The above strategies of nucleobase labelling so far involve the use of spacer chain between fluorophore and nucleobase[3,13]. We have earlier reported the synthesis of fluorescent oligonucleotides containing 5-amidodansyl-dU[14] which was used for studying DNA-netropsin interactions and the major groove polarity.

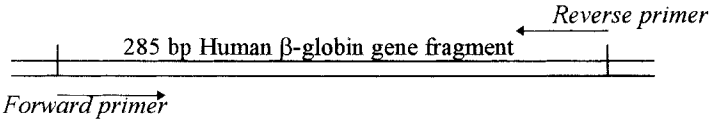
In this paper, we describe an easy synthesis and practical utility of PCR primers containing 5-amido(carboxyfluorescein)-2'-deoxyuridine **3**, in which the chromophore fluorescein emitting in visible region is directly linked to the nucleobase 2'-dU at C5 via an amido function. Since the conjugation is at C-5 through a short and rigid linkage in major groove, it is not expected to interfere with Watson-Crick hydrogen bonding pattern[15] and the hybridization specificity[14].

RESULTS AND DISCUSSION

Synthesis and Characterisation of Fluorescent Oligonucleotides

The synthesis of modified nucleoside **3** was achieved by coupling of the previously reported DMT-5NH_2-dU[16] with protected (5/6)-carboxyfluorescein dye[17]. The (5/6)-COOH group of fluorescent derivative was activated as the pentafluorophenyl (PFP) ester **2** through DCC reaction followed by chromatographic purification. This ester was condensed with 5-NH_2-2'-deoxyuridine **1** (Scheme 1) in presence of an activating agent hydroxybenzotriazole in pyridine to yield the fluorescein conjugated nucleoside **3**. This was converted into the required β-cyanoethyl phosphoramidite **4** by usual procedure[18] and all new compounds were well characterised by spectroscopy.

The fluorescent monomer **4** was incorporated at specific sites of oligonucleotides (**6, 7, 9-11**) using standard solid phase phosphoramidite chemistry. The coupling time for modified monomer **4** was increased to 5 min. and the coupling efficiency for its incorporation was found to be similar to that of unmodified monomers. The resin bound fluorescent oligonucleotide products were deprotected by standard ammonia treatment to which the 5-amido linkage is stable[16] with no detectable loss of conjugated fluorophore. The deprotected fluorescent oligonucleotides (**6, 7, 9-11**) were purified by preparative electrophoresis on denaturing polyacrylamide gel on which its direct visualization as a fluorescent band was possible. These bands after elution with deionised water showed fluorescence in solution with λ_{em} at 510 nm upon excitation at 495 nm, further confirming the retention of conjugated chromophore during DNA synthesis and purification.



***Forward primer*:**

5 5'-ACC TCA CCC TGT GGA GCC AC-3'
6 5'-ACC U*CA CCC TGT GGA GCC AC-3'
7 5'-ACC U*CA CCC U*GT GGA GCC AC-3'

***Reverse primer*:**

8 5'-CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG-3'
9 5'-CTC CTU* AAA CCT GTC TTG TAA CCT TGT TAG-3'
10 5'-CTC CTU* AAA CCU* GTC TTG TAA CCT TGT TAG-3'
11 5'-CTC CTU* AAA CCU* GTC TTG U*AA CCT TGT TAG-3'

***Complement of reverse primer*:**

12 5'-CTA ACA AGG TTA CAA GAC AGG TTT AAG GAG-3'

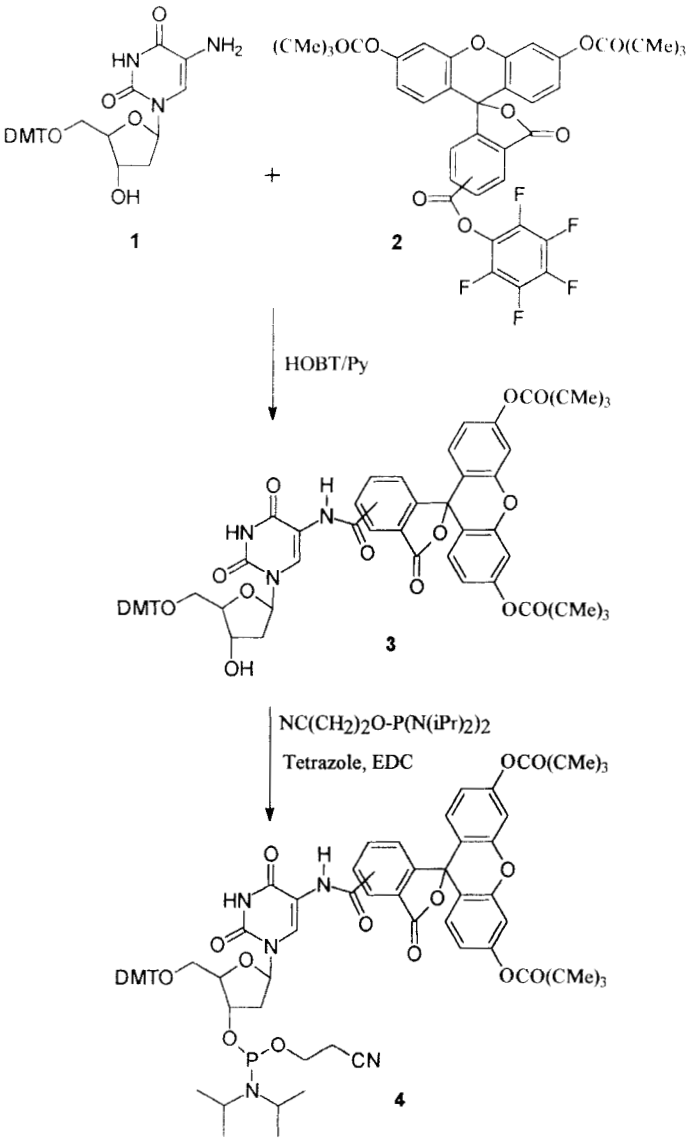
#Primers used in PCR correspond to **5-11**; U* indicates the position of fluorescent nucleobase ; **12** is complementary to **7-11** and used for duplex Tm measurements.

The effect of fluorescein conjugation on duplex stability was examined by UV thermal denaturation experiments. The duplexes derived from mono, di or tri fluorescent labeled ODNs (**9-11**) on one strand with complementary unlabeled oligonucleotide (**12**) showed Tm's lower by 2-4°C per substitution (**9:12**, 62°C; **10:12**, 58°C and **11:12**, 52°C) as compared to the unmodified duplex (**8:12**, 64°C).

PCR Amplification

The unsubstituted oligonucleotides **5** and **8** correspond to primer sequences required to amplify the 285 bp region of IVS1-5 mutant β-globin gene of Human genomic DNA[19]. Different combinations of forward (**5-7**) and reverse (**8-11**) primers were used for PCR amplification. The primer sets **6:9**, **7:10** and **7:11** were chosen to yield product DNA containing two, four and five fluorescent groups respectively per amplified duplex. Since the Tm of modified duplexes were found to be around 52-62°C, the annealing temperature in PCR cycle was kept at 47°C to ensure an efficient hybridization of fluorescent primers to the target template DNA.

The gel photograph shown in Figure 1 indicates a successful and efficient amplification of the 285 bp fragment with both labeled (lanes 1-3) and unlabeled (lane 4) sets of primers. Under identical PCR conditions, fluorescence intensity of amplified products as seen on agarose gel was found to be enhanced with the increasing number of fluorophores. Optimal and unambiguous detection of amplified product on gel was possible with 250 ng of DNA containing four fluorophores per amplified duplex, obtained from primer set (**7:10**). Increasing the number of fluorescein tags beyond this level resulted in non-specific amplification of PCR product giving additional bands (Figure 1, lane 3). Removal of the



Scheme 1

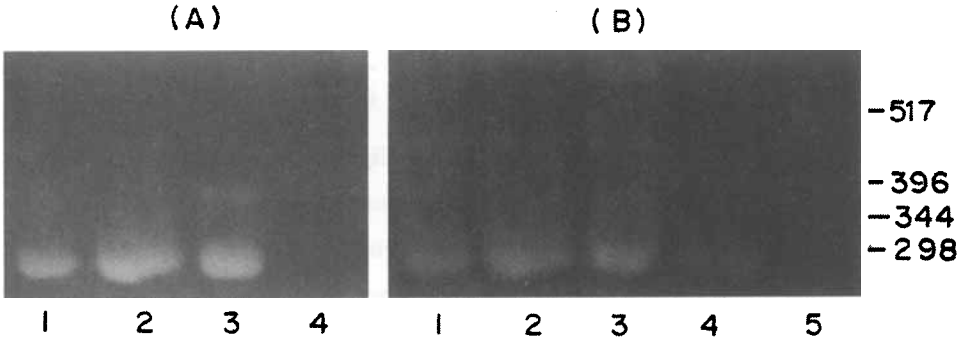


Figure 1. Gel photograph of PCR amplified product before (A) and after (B) ethidium bromide staining. The lanes show the amplified product with following set of primers; *lane 1*, **6** and **9**; *lane 2*, **7** and **10**; *lane 3*, **7** and **11**; *lane 4*, **5** and **8**; *lane 5*, Molecular weight marker: pBR322 Hinf I digest.

unincorporated fluorescent primers from PCR mixture by simple centrifugation in Centricon-100 microfiltration tube (Amicon) led to a direct visual detection of the PCR product in solution, thereby avoiding the necessity of gel electrophoresis. This should have the practical advantage in screening large number of clinical samples in DNA diagnostics.

In summary, an easy synthesis of stable fluorescein labeled nucleobase phosphoramidite **4** described here allows site-specific and multiple incorporation of fluorescent label into oligonucleotides to enhance the sensitivity of DNA detection. The use of such prelabeled fluorescent amidite ensures an unambiguous regiospecific labelling and avoids purification steps involved in postsynthetic fluorescent labelling with commercially available reagents[20]. The direct linking of the fluorophore label to the base also avoids the ambiguity in labelling and may significantly restrict the range of conformations available to the label upon linking to DNA oligomer[21]. Although we have demonstrated the utility of these modified oligonucleotides in fluorescent PCR product detection, they have the potential to simplify methods involving use of DNA sequencing primers for automated sequencing[3], hybridisation probes[22] and preparation of PCR amplified DNA fluoroprobes required for fluorescent *in situ* hybridisation (FISH).

EXPERIMENTAL

All the solvents used were purified according to literature procedures[23]. TLC was performed on Merck pre-coated 60 F_{254} plates (E. Merck 5554) and the spots were rendered

visible by UV light. Silica gel (Loba-Chimie, 100-200 mesh) was used for column chromatography. NMR spectra were recorded on a Brucker ACF 200 spectrometer and all the chemical shifts are referred to internal TMS for ^1H and 30% aq-H_3PO_4 for ^{31}P. The chemical shifts are quoted in δ scale (ppm). All oligonucleotides were synthesized on Pharmacia LKB Gene Assembler Plus. Thermal denaturation studies were carried out using Perkin-Elmer Lambda15 UV/VIS spectrophotometer. Fluorescence measurements were done on Perkin-Elmer Model LS 50B luminescence spectrometer. GeneAmp PCR reagent kit from Perkin-Elmer was used for preparing PCR amplification reactions which were performed on Perkin-Elmer DNA thermal cycler.

Synthesis of Spiro[isobenzofuran-1-(3H)-9'-[9H]xanthene]-5(and-6)-carboxylic acid, 3',6'-(2,2-dimethylpropanoyloxy)-3-oxo-pentafluorophenyl ester **(2)**: To 3',6'-bisisobutyryl-5/6-carboxyfluorescein[17] (2 mmole) and pentafluorophenol (2.2 mmole) in dry CH_2Cl_2 (10 ml) was added DCC (2.2 mmole) in CH_2Cl_2 (5 ml) and stirred for 24 hours at room temperature. DCU was filtered and the filtrate was concentrated for purification by silica gelchromatography. Elution with 10% MeOH in CH_2Cl_2 afforded **2** (yield, 60%), R_f = 0.71 and 0.68 corresponding to 5- and 6- isomers. ^1H NMR ($CDCl_3$) δ, 8.20 (m,1H, H-4 & H-5 of 6-isomer), 8.87 (s, 0.5H, H-4 of 5-isomer), 7.96 (s 0.5H, 0.5H, H-7 of 6-isomer), 7.38 (d, J=8 Hz, 0.5H, H-7 of 5-isomer), 7.11(s,2H, H4'), 6.83 (s, 4H, H-1' and H-2'), 1.4 (s, 18H, 2xC(CH_3)$_3$).

Synthesis of N^5-(5/6)-Carboxyfluorescein-5'-O-(4,4'-dimethoxytrityl)-5-amido-2'-deoxy uridine **(3)**: To a mixture of **1** (0.5 mmole) and **2** (0.6 mmole) dissolved in dry pyridine (5 ml) was added HOBT (0.5 mmole) and stirred for 24 hours at room temperature. After aqueous workup the product in organic layer was purified by silica gel column chroamtography eluting with 5% MeOH in CH_2Cl_2 which gave **3** (yeild 65%), R_f= 0.59, ^1H NMR ($CDCl_3$), δ, 8.9-7.5 (m, 4H, H-6 & Ar-H), 7.5-6.7 (m, 18H, DMT & Ar-H), 6.4 (t, 1H, H-1'), 4.5 (m, 1H, H-3'), 4.1 (m, 1H, H-4'), 3.7 (s, 6H, OCH_3), 3.5 (m, 2H, H-5' & H-5"), 2.55-2.3 (m, 2H, H-2' & H-2"), 1.4 (s, 18H, 2xC(CH_3)$_3$).

Synthesis of 3'-O-(N,N-diisopropylamino)-β-cyanoethoxy)phosphoramidite of **3** **(4)**: Compound **3** (0.5 mmol) and tetrazole (0.5 mmol) were dried by coevaporation with dichloroethane and suspended in dichloroethane (4 mL). To this β-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (0.5 mmol) was added with stirring at 20°C. After stirring for 3 hrs., the precipitate of diisopropylammoniumtetrazolide was removed by filtration and the solution was diluted with dry CH_2Cl_2 and washed with 5% aqueous sodium bicarbonate. The organic layer was dried over anhydrous sodium sulphate and

concentrated to dryness. The desired amidite was purified by precipitation from CH_2Cl_2 by the addition of cold hexane and dried over phosphorus pentoxide and potassium hydroxide before oligonucleotide synthesis (yield 75%). R_f (EtOAc:CH_2Cl_2, 1:1 and 0.5% triethylamine) = 0.68 & 0.62 corresponding to 5- and 6- isomers. ^{31}P NMR: ($CDCl_3$) δ, 149.6 & 149.3.

Melting experiments

Thermal denaturation profiles of complementary oligonucleotides were performed in 10mM Tris.HCl (pH 8.3) buffer containing 50mM KCl and 1.5mM $MgCl_2$. Appropriate oligonucleotides, each at concentration of 1 μM based on UV absorbance, calculated using molar extinction coefficients at 260 nm (dA = 15.4, dC = 7.3, dG = 11.7, dT = 8.8 $cm^2/\mu mol$) were mixed, heated to 80°C for 5 min, cooled to room temperature and then stored at 4°C overnight. The change in absorbance of these duplexes at 260 nm was recorded as function of temperature from 20°C to 80°C. Duplex Tm were calculated from the midpoint in the plots of fraction absorbance change versus temperature.

PCR amplification

Human genomic DNA for PCR amplification of β-globin gene was isolated from blood by standard procedure[19]. PCR mixture contained 10mM Tris.HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 100 μM each dNTPs, 20 pmoles of each primer, 0.1 μg target DNA and 2.5 units of *Taq* polymerase. These were subjected to 35 cycles of PCR. Temp cycle: 94°C for 1 min, 47°C for 1 min, 72°C for 1 min. After the completion of temp cycles, 15 μl of reaction mixture was loaded on 1.5% agarose gel and analysed by electrophoresis[24]. The gels were visualised on UV transilluminator with and without ethidium bromide (0.5 μg/ml) staining and photographed.

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