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Synthesis of HyBeacons and dual-labelled probes containing 2′-fluorescent groups for use in genetic analysis†

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An FMOC-protected 2′-hydroxyethyl uridine phosphoramidite has been used to synthesise fluorescein-labelled HyBeacon probes and "FAM-ROX" dual-labelled fluorogenic oligonucleotides.

Many genetic diseases are linked to single nucleotide polymorphisms occurring at a significant frequency within the population (1% or more), so the ability to rapidly detect SNPs is of great value. Commonly used homogeneous real-time PCR-based methods of genetic analysis such as Taqman™[1] Scorpions[2] and Molecular Beacons[3] are used to analyse SNPs on platforms such as the Roche LightCycler™. In a drive towards inexpensive, high throughput genetic analyses we have developed the novel "HyBeacon" system.[4,5] HyBeacons™ are hybridisation probes consisting of a single-stranded oligonucleotide containing a fluorophore attached to a nucleoside within the DNA sequence. When the probe anneals to a complementary target and forms a duplex, an increase in fluorescence is observed, the precise origin of which is the subject of ongoing research (Fig. 1).

As the probe possesses no secondary structure, hybridisation to the target is faster and more efficient than in other genetic analysis systems, and post-PCR melting analysis becomes possible. Furthermore, HyBeacons do not require enzymic activation, so they can be used in conjunction with rapid PCR cycling conditions.[6] Finally, HyBeacon synthesis is relatively simple and inexpensive, as the probe does not contain a fluorescence quencher.

In previous work in this field[4,5] we incorporated fluorescent dyes in the major groove of DNA, *via* the 5-position of deoxyuridine. The next logical step was to investigate the properties of HyBeacons with the fluorophore in the minor groove. The results of the major groove studies[4,5] indicated that the most efficient HyBeacons would result from the use of a short linker between the nucleotide and the dye moiety, so we based our strategy on 2′-hydroxyethyluridine. In previous studies oligonucleotides have been labelled at the 2′-position of uridine using specific dye-labelled phosphoramidites,[7–9] or by solution-phase derivatisation of a 2′-amino dU residue after solid-phase synthesis.[9] However, our aim was to develop efficient general solid-phase approaches based on phosphoramidites that can be prepared in large quantities and used in oligonucleotide synthesis in conjunction with other commercially available dye phosphoramidites.[10] The synthesis of the required phosphoramidite monomer is shown in Scheme 1‡.

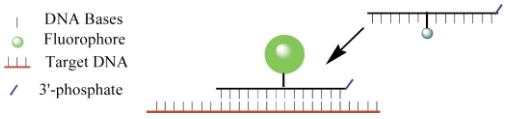
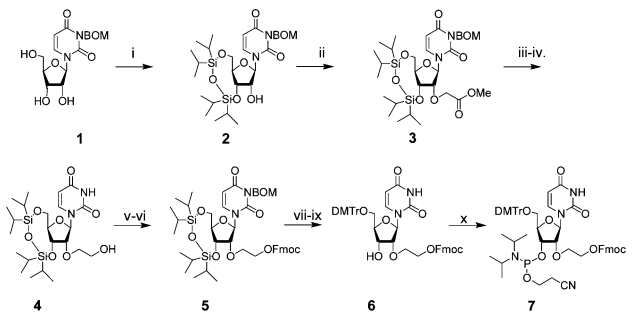


Fig. 1 HyBeacon.

N(3)-BOM uridine **1**[11] was protected with Markiewicz's reagent to give **2**, which was reacted with methyl bromoacetate in the presence of sodium hydride to give **3** in 72% yield. Reduction of the ester with sodium borohydride proved unsatisfactory with our substrate (28% yield), the major product being a tetrahydrouridine derivative obtained by reduction of the heterocycle. However, removal of the BOM protecting group from the nucleobase before reduction prevented this side-reaction. The BOM-protecting group was cleaved with Pd/C and reduction of the ester using sodium borohydride gave **4** (65% over 2 steps). Reprotection of N-3 was necessary to give a clean reaction in the next step in which the 2′-hydroxy group was protected with FMOC to give **5** (86%). Deprotection of the BOM group, and removal of TIPDS using HF–pyridine in THF, followed by tritylation gave **6** in 80% yield. Finally, phosphitylation of the 3′-hydroxy group under argon afforded the phosphoramidite **7** in good yield.

Monomer **7** can be introduced into synthetic oligonucleotides at thymine sites and used as a point of attachment for a variety of fluorescent dyes amidites. In order to evaluate its utility, the HyBeacon probe§ 2DC64C* was synthesised[4] (Scheme 2). Standard protocols were employed, with an extended reaction time of 5 min for **7**, which coupled at 98%. We did not remove the 5′-DMT protecting group from the oligonucleotide at this stage. After oligonucleotide assembly the synthesis column was removed and treated with piperidine (20% in DMF) for 10 min to remove the FMOC group from the 2′-protected uridine residue. The column was then returned to the DNA synthesiser, 6-carboxyfluorescein phosphoramidite was coupled to the free 2′-hydroxy group and the 5′-DMT group was removed. The oligonucleotide was then cleaved from the resin, deprotected in



Scheme 1 (i) 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (1.2 eq.) imidazole (1.1 eq.), pyridine rt 7 h, 91%; (ii) $BrCH_2CO_2Me$ (5 eq.), NaH (2.2 eq.), DMF, −5 °C, 2 h, 72%; (iii) 20 wt% Pd/C, THF : methanol (1 : 1), H_2(g), RT, 86%; (iv) $NaBH_4$, methanol, *tert*-butanol, rt, 76%; (v) benzyl chloromethyl ether, DBU, DMF, rt, 82%; (vi) FmocCl, pyridine, rt, 86%; (vii) 20 wt% Pd/C, 2 M HCl (aq), THF : methanol (1 : 1), H_2(g), rt, 62%; (viii) HF–pyridine, THF, rt, 54%; (ix) DMTrCl (3 eq.), pyridine, rt, 3 h, 80%; (x) 2-cyanoethyldiisopropyl chlorophosphoramidite, DIPEA, THF, 72%.

† Electronic supplementary information (ESI) available: experimental details and real-time PCR. See http://www.rsc.org/suppdata/cc/b3/b302855k/

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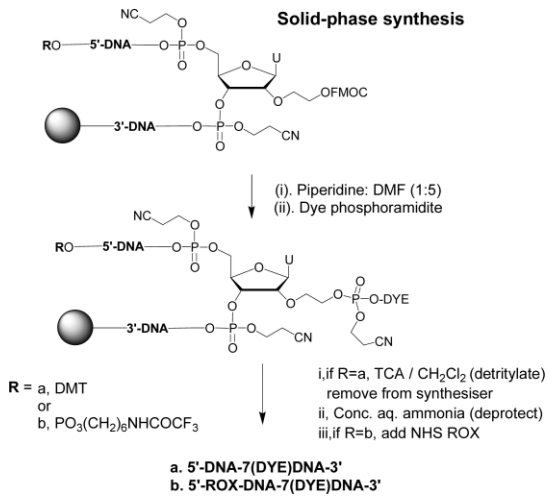
aqueous ammonia (5 h, 55 °C) and purified by reversed-phase HPLC (octyl) with a gradient of 0 to 35% CH_3CN in 0.1 M NH_4OAc.

The resultant HyBeacon probe (2DC64C*) was designed to differentiate between a fully complementary DNA target and one containing a single C.A mismatch. The 2′-FAM ethoxy dU is adjacent to the polymorphic site, and is therefore sensitive to changes in Tm caused by mispairing at this locus. Real-time PCR†¶ indicated that good discrimination was achieved. The magnitude of the change in fluorescence on hybridisation of 2DC64C* was similar to that for HyBeacons with major groove fluorophores.[4] Post-PCR fluorescence melting analysis (Fig. 2), a particular advantage of the HyBeacon system, enabled us to discriminate very clearly between wild-type, heterozygote and homozygous mutant. These results prove that the fluorescence enhancement, characteristic of HyBeacon probes, can be readily achieved using oligonucleotides with fluorophores attached to the minor groove.

This novel synthetic methodology can be used to incorporate different fluorescent dyes in the minor groove of DNA to produce "multi-coloured" HyBeacons, and such probes could be used in high-throughput genetic analysis to detect several different SNPs in a single tube. However, the majority of current genetic analysis platforms have only one fluorescence excitation source at 495 nm, and in order to utilise all the available detection channels, fluorescence resonance energy transfer (FRET) must be used. This can be achieved by incorporating FAM (FRET donor) and a second fluorescent dye (FRET acceptor) in the same oligonucleotide.[12–14] To explore the utility of monomer **7** in FRET applications we prepared dual-labelled oligonucleotide probes P1 & P2 (Fig. 3) with an internal FAM and 5′-ROX (Scheme 2).

A FRET study was carried out on P1 and P2 hybridised to a fully complementary oligodeoxynucleotide. but before doing this we determined the effect of the 2′-modification on duplex stability. The Tm of the native DNA duplex is 48 °C compared



Scheme 2 Oligonucleotide synthesis.

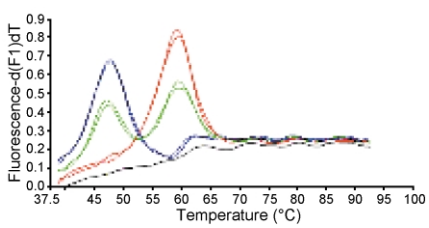


Fig. 2 Post-PCR fluorescence melting analysis using 2DC64C* HyBeacon. Data acquired over 15 min. Red: fully complementary homozygous template; Green: heterozygote; Blue: mutant; Black: negative control (no template DNA).

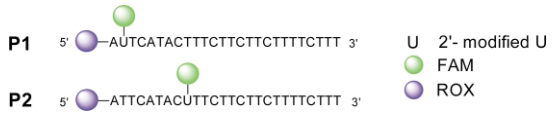


Fig. 3 FRET from FAM to ROX.

to 47 °C for P1 and 44 °C for P2 (1 mM duplex, 100 mM NaCl, 10 mM Na phosphate, pH 7.0). The origin of these differences is probably twofold; destabilisation due to the presumed C3′-endo conformation of the modified ribose sugar, and steric hindrance caused by the bulky 2′-fluorescein moiety. It is clear from the excellent discrimination in Fig. 2 that the slight instability does not confer any limitations on the use of our nucleotide in genetic probes. Similar effects were observed by Yamana *et al.*[7,8]

Energy transfer from FAM to ROX was recorded for the P1 and P2 duplexes in aqueous buffer at RT, pH 7.0 (data not shown). The fluorescence intensity of ROX (606 nm) was more than 4-fold greater in P2 than in P1, demonstrating the need to separate the two dyes to avoid collisional quenching. FRET in P2 is efficient with a ROX : FAM emission ratio of 8.2 : 1 These results are most encouraging and indicate that monomer **7** can be used in the synthesis of dual-labelled probes for a variety of applications. The properties of dual-labelled hybridisation probes are currently under further investigation. In conclusion, we have synthesised a HyBeacon functionalised with 2′-fluorescein and shown it to be an effective genetic probe. We have also demonstrated that the 2′-modified FAM dU nucleoside can be used in the synthesis of dual-labelled FRET probes.

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Notes and references

‡ Compounds were characterised by ^{1}H-NMR, ^{13}C-NMR, MS, IR and mp/CHN microanalysis or HRMS.

§ The HyBeacon 2DC64C* sequence is dGGGCGF**C**TGGGGGTGX, where **C** = polymorphic site, F = 2′-FAM ethoxy dU, and X = 3′-octanol PCR stopper). It targets the human CYP2D6 4 mutation in which there is a G to A transition at position 1846. It matches the wild-type sequence (*1, C.G base pair) and is mismatched to the mutant (*4, C.A base pair).

¶ The protocols for PCR were taken from previous work on HyBeacons.[4,6] Aminolink and 6-FAM Phosphoramidites were purchased from Transgenomics Ltd, West of Scotland Science Park, Glasgow, UK. NHS 6-ROX was purchased from Molecular Probes Inc (www.probes.com).

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