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A new dark quencher for use in genetic analysis

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A novel, long-wavelength, non-fluorescent quencher (LQ), based on 1,4-diaminoanthraquinone, has been incorporated at the 3′ and 5′-termini of oligonucleotides. The quencher has been used in Molecular beacons, efficiently quenching the long wavelength fluorophore, Cy5.

Current detection methods in genetic analysis require high performance fluorescent oligonucleotide probes. In particular, real-time detection of PCR products is commonly achieved with fluorescent probes such as Molecular beacons,[1,2] Scorpion primers™[3–5] and TaqMan®.[6,7] These probes are single-stranded oligonucleotides possessing a fluorescent dye and a quencher molecule. In their "dark state" they adopt a conformation where the quencher is close enough to the fluorescent dye to absorb its fluorescence. However, upon hybridisation to a target sequence these probes either undergo a conformational change (Molecular beacons and Scorpion primers™) or enzymatic cleavage (TaqMan® probes) separating the quencher and fluorophore allowing a fluorescent signal to be detected. Advances in genetic analysis have increased the demand for a wider range of novel efficient fluorophore–quencher pairs.[8] Ideally, new quenchers possess no native fluorescence but have large extinction coefficients for maximal spectral overlap with large range of fluorophores.

We have developed a novel long-wavelength fluorescence quencher based on the 1,4-diaminoanthraquinone chromophore. This quencher (LQ) is designed to be complementary to the first dark quencher to be reported by our laboratories, Methyl Red (MR).[3–5] Methyl Red (an isomer of dabcyl) has a large spectral absorption range of 350–550 nm and LQ has an absorption range of 500–700 nm. Both of these quenchers possess no native fluorescence (i.e. are dark quenchers) and between them they are able to quench the majority of commonly utilised fluorescent dyes. Experiments to determine the optimal synthesis, deprotection and purification methods for oligonucleotides labelled with the novel LQ quencher are described. We have synthesised Molecular beacons using the LQ and MR quenchers, and their relative quenching characteristics have been compared.

Two quencher molecules (**LQ1** and **LQ2**, Fig. 1), based on 1,4-diaminoanthraquinone, were synthesised. **LQ1** is a phosphoramidite monomer that can be incorporated into an oligonucleotide at the 5′ teminus during standard solid-phase oligonucleotide synthesis.

It was made by phosphitylation of hydroxyethyl diaminoanthraquinone. **LQ2** (Scheme 1) is used to position the quencher moiety at the 1-position of deoxyribose whilst the 3′ position is attached to controlled pore glass resin (CPG). This resin-bound molecule allows 3′ labelling of oligonucleotides with the quencher.

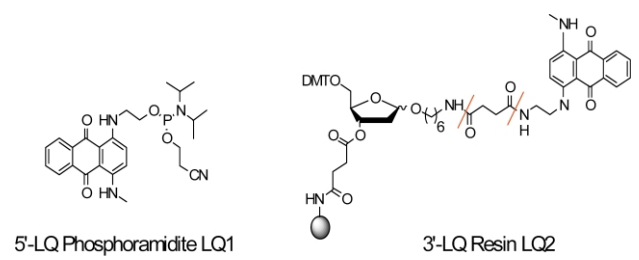
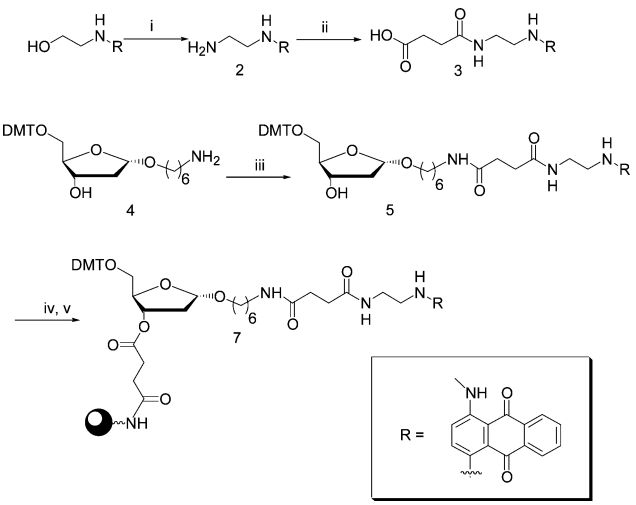


Fig. 1 Structures of LQ monomer and cpg

The quencher molecules (**LQ1** and **LQ2**) were used to prepare two labelled poly-T oligonucleotides, **ODN1** labelled at the 5′ and **ODN2** at the 3′ (Table 1).

The oligonucleotides were cleaved from the resin with conc. $NH_{3\,(aq)}$ (30 min, room temp.) then immediately evaporated to dryness, and purified by RP-HPLC. In each case the major peak which was blue in colour was collected. Following desalting, the oligonucleotides were used to determine optimum conditions for removal of the heterocyclic base protecting groups of the DNA. An aliquot of each oligonucleotide was heated in conc. $NH_{3\,(aq)}$ at 55 °C for 4 h and then analysed by RP-HPLC. **ODN1** gave one major peak and mass spectrometry (MALDI-TOF) confirmed the expected mass of the desired product (expected: 3337; found: 3338). This indicates that conventional deprotection conditions are suitable for oligonucleotides labelled at the 5′ with **LQ1**. However, the chromatogram for **ODN2** showed three significant peaks and closer examination of the products by mass spectrometry revealed degradation products. The amide bonds linking the sugar to the dye were partially cleaved (Fig. 1 red) and masses corresponding to loss



Scheme 1 Synthesis of **LQ2**–CPG. *Reagents and conditions*: i, DEAD (1.1eq.), PPh_3 (1.1eq.), phthalimide (1.1eq.), THF, rt, 1.5 h, then hydrazine monohydrate (5 eq.), CH_2Cl_2: MeOH (1 : 1), rt, 15 h, 90% over two steps; ii, succinic anhydride (1.1eq.), DMAP (0.1eq.), pyridine, rt, 1.5 h, 90%; iii, DIPEA (6 eq.), HOBT (1.1 eq.), EDC (1.1 eq.), **3** (1.1 eq), DMF, rt, 15 h, 60%; iv, succinic anhydride (1.2 eq.), DMAP (0.1 eq.), pyridine, rt, 18 h then 60 °C, 5 h, 94%; v, LCAA–CPG, EDC (5 eq.), DIPEA (5 eq.), CH_2Cl_2: DIPEA (1%), rt, 4 h, loading = 25 μmol g^{-1}

Table 1

ODN1	5′-LQ-TTT TTT TTT T-3′
ODN2	5′-TTT TTT TTT TTT TTT-LQ-3′

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of the chromophore from the sugar were observed (results not shown).

To determine suitable deprotection conditions for oligonucleotides labelled with the 3′ quencher, samples of **ODN2** were exposed to the conditions described in Table 2, and examined by RP-HPLC (Fig. 2). Results showed that heating the oligonucleotide in conc. aq. ammonia caused significant degradation (expt 1) even with shorter exposure times. However, using water : methanol : ^{t}butylamine (2 : 1 : 1) or ammonium hydroxide : conc. aq. methylamine (1 : 1) mixtures. a much cleaner product was obtained (expts. 2–4).

Table 2

Expt.	Temp. (°C)	Time (h)	Reagents
1	70	2	conc. aq. NH_3
2	70	2	H_2O : MeOH : $BuNH_2$ (2 : 1 : 1)
3	25	24	H_2O : MeOH : $^{t}BuNH_2$ (2 : 1 : 1)
4	25	1	NH_4OH : $MeNH_{2\,(aq)}$ (1 : 1)

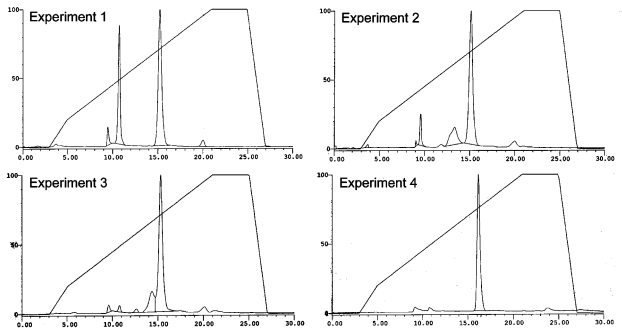

Fig. 2 RP-HPLC chromatograms for experiments 1, 2, 3 and 4 (Table 2).

It should be noted that deprotection using water : methanol : ^{t}butylamine (2 : 1 : 1) requires ^{dmf}dG amidite to be used during oligonucleotide synthesis and ammonium hydroxide:conc. aq. methylamine, (1 : 1) requires ^{acetyl}dC.

To assess the performance of the new quencher, Molecular beacons were synthesised using the **LQ1** phosphoramidite and either fluorescein (FAM, λ_{em} = 520 nm) or Cy5 (Amersham Pharmacia, λ_{em} = 670 nm). Beacons of identical sequence but terminating in methyl red (MR) were also prepared in order to compare the signal-to-noise ratios of different fluorophore–quencher pairs. The sequences used are shown in Table 3.

The fluorescence properties of the Molecular beacons (**ODN3-6**) were examined by fluorescence melting, with and without their target strand present in the hybridisation buffer (**ODN7**). In the presence of a target strand a large increase in fluorescence was observed for all Molecular beacons (Fig. 3). This change in fluorescence was used to calculate the signal-to-noise ratio for each fluorophore–quencher pair (F_{max}/F_{min} = maximum fluorescence with target/minimum fluorescence without target). Fig. 4 illustrates that with the shorter wavelength fluorophore FAM, there is comparable quenching by LQ and MR. However, when the longer wavelength fluorophore, CY5, is used there is a significant difference in signal-to-noise ratio, indicating that LQ is a much more effective quencher for this fluorophore.

Table 3

ODN3	LQ-GCAGCCGTTTCCTCCACTGTTGCGCTGC-FAM
ODN4	MR-GCAGCCGTTTCCTCCACTGTTGCGCTGC-FAM
ODN5	LQ-GCAGCCGTTTCCTCCACTGTTGCGCTGC-Cy5[a]
ODN6	MR-GCAGCCGTTTCCTCCACTGTTGCGCTGC-Cy5[a]
ODN7	GCAACAGTGGAGGAAACG

[a] Cy5 at 3′-end was a functionalised dT resin.

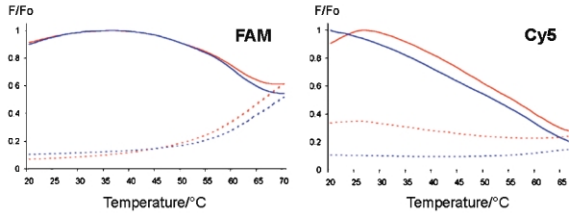

Fig. 3 Fluorescence melting spectra for the Molecular beacons containing MR and LQ. MR (red); LQ (blue). Dotted line = no target; solid line = with target. Fluorescence traces have been normalised i.e. *F*/*F*o, where *F* = fluorescence intensity, *F*o = maximum fluorescence intensity for each Molecular beacon.

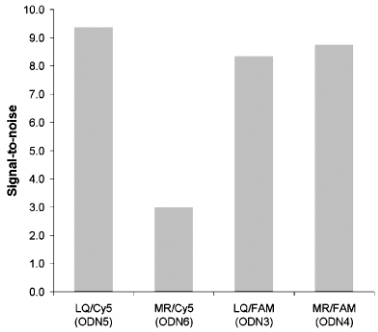

Fig. 4 Signal-to-noise ratios for the Molecular beacons at 30 °C.

The synthesis and purification of both 5′ and 3′ LQ labelled oligonucleotides has been achieved. Molecular beacons containing the new quencher have been shown to perform efficiently with two fluorophores. LQ performed significantly better than MR for the long wavelength dye Cy5, whilst both quenchers show very similar results with the shorter wavelength dye, FAM.

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