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Linear fluorescent oligonucleotide probes with an acridine quencher generate a signal upon hybridisation

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Linear, single stranded probes incorporating a fluorophore and an acridine moiety are weakly fluorescent until hybridised to a complementary target nucleic acid whereupon fluorescence increases due to reduced quenching.

In recent years interest in the detection of nucleic acid sequences in homogeneous solution has soared due to major developments in molecular genetics. Fluorescent moieties are the reporter groups of choice in these applications. Typically, an oligonucleotide probe is labelled with a fluorophore and a quencher. A reduction in quenching is caused by hybridisation of the probe to its target nucleic acid, leading to signal generation. Examples of such methods are scorpion primers,[1] TaqMan™ probes[2] and molecular beacons.[3]

These methods rely upon changes in secondary structure upon hybridisation, or subsequent enzymatic degradation of the probe for signal generation. Here, we describe a novel linear dual-labelled fluorescent probe capable of detecting specific nucleic acids in homogeneous solution that is not reliant on either of these mechanisms. The unhybridised linear probe is essentially non-fluorescent due to energy transfer between the fluorophore and the acridine quencher. However, formation of a probe–target duplex allows the acridine moiety to interact with the double strand—it can no longer act as an efficient quencher and a fluorescent signal is generated (Fig. 1). An acridine derivative has been used previously in an oligonucleotide probe as the donor in a fluorescence resonance energy transfer (FRET) system.[4] The mechanism of action leads to a *decrease* in fluorescent signal upon hybridisation. In contrast, the system we describe utilises the acridine moiety as a quencher – leading to an *increase* in fluorescence.

The probe is synthesised with the fluorophore 6-carboxy fluorescein (FAM), (Fig. 2i) at the 5′-end† linked to the intercalator 9-amino-6-chloro-2-methoxyacridine[5] (Fig. 2ii).

The advantages of the method lie in its simplicity: the probes are free of secondary structure, relatively short and inexpensive to synthesise (commercially available phosphoramidite monomers are used, Fig. 2), and the acridine stabilises probe–target hybrids.

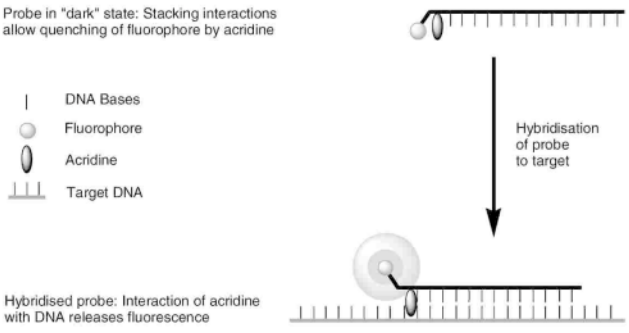

Fig. 1 Mechanism of action of the dual-labelled probe.

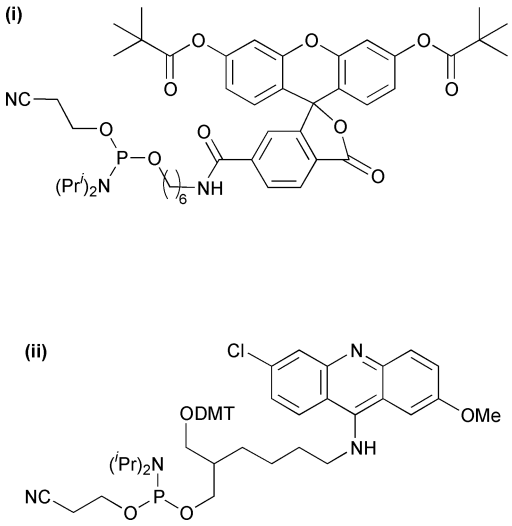

Fig. 2 Monomers used in probe synthesis (i) *O*-[(3′,6′-dipivaloyl-fluorescein-6-ylcarboxamidohexyl] *O*′-(2-cyanoethyl) (*N*,*N*-diisopropyl) phosphoramidite (PE Biosystems) (ii) *O*-[1-dimethoxytrityloxy-2-(*N*-acridin-1-yl-4-aminobutyl)propyl] *O*′-(2-cyanoethyl) (*N*,*N*,-diisopropyl)phosphoramidite (Glen Research).

The 14-mer probe (P1) used in this study was designed to hybridise to the wild type N1303K locus[6]‡ of the ABCC7 gene on chromosome 7 that produces the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The probe sequence is:

5′-FAM-acridine-AAAAACTTGGATCC-octanediol-3′ (P1)

In the current studies we used a synthetic oligodeoxynucleotide version of a 24-nucleotide section of the target locus:

5′- TAGGGATCCAAGTTTTTTCTAAAT-3′ (T1)

The probe was synthesised as a 2′-*O*-methyl oligoribonucleotide. 2′-*O*-Methyl oligoribonucleotides are not generally substrates for hydrolysis by DNase enzymes, and the probes were assembled from 2′-*O*-methyl RNA phosphoramidites (Glen Research) to avoid a TaqMan-like cleavage[2] in any subsequent PCR assay. Octanediol (phosphoramidite was a gift from Oswel Research Products Ltd.) was incorporated at the 3′ end of the probe to avoid extension of the probe by *Taq* polymerase during PCR. In preliminary studies we have shown that the observed effect (Fig. 1) also occurs with oligodeoxynucleotide probes with unmodified sugars, and that the optimum position for attachment of the acridine is directly adjacent to the fluorophore.

The dual-labelled probe (210 nM) P1 was added to a suitable hybridisation buffer (100 mM sodium phosphate, 1 mM EDTA, 100 mM NaCl, pH 7.0) at room temperature, and the emission spectrum recorded on an LS 50B Luminescence Spectrometer

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Table 1 T_m results of UV melting experiments

T_m of P1 with T1/ °C	T_m of C1 with T1/°C	ΔT_m °C
54.0[a]	52.3[a]	+1.7

[a] All experiments were run in triplicate, and the average reading reported.

(Perkin Elmer), from excitation at 495 nm. The synthetic target (T1, 1050 nM) was then added and the emission spectrum re-recorded with excitation at 495 nm.

On addition of T1 and excitation at 495 nm the fluorescence intensity at 520 nm rose from 61.6 units to 250.3 units (a 4.5-fold increase).

In order to investigate the mechanism of fluorescence enhancement we performed UV melting experiments to determine the T_m value of probe–target hybrids. Intercalators such as acridine are known to stabilise duplexes by participating in π-stacking and electrostatic interactions with the nucleobases.[7] A control unlabelled 2′-*O*-methyl oligoribonucleotide was synthesised with a sequence analogous to the probe:

5′-AAAAACTTGGATCC-octanediol-3′ (C1)

The probe P1 (963 nM) and the synthetic target (T1, 1260 nM) were added to the hybridisation buffer (100 mM sodium phosphate, 1 mM EDTA, 100 mM NaCl, pH 7.0). The sample was heated (75 °C, 1 min), cooled (15 °C, 1 min), then melted by heating for 3880 s, at 1 °C min^{-1}. The ultraviolet absorbance was continuously monitored at 260 nm on a Perkin Elmer Lambda 15 UV/VIS Spectrophotometer. T_m values were calculated using the PECSS 2 software (Perkin Elmer). The experiment was repeated, with unlabelled probe C1 (963 nM) in place of P1. The results are shown in Table 1.

The labelled probe–target hybrid exhibited a higher T_m than the analogous unlabelled probe–target hybrid suggesting that the acridine moiety intercalates into, or stacks on the end of the duplex upon hybridisation. Further experiments are necessary to confirm the precise interaction.

The lack of any probe secondary structure simplifies design, and enables the use of very short probes, since hybridisation to the target does not have to compete with any intramolecular structures. This can be a problem with molecular beacons.

To investigate the ability of the probes to discriminate between fully matched targets and those containing a single mismatch we prepared a further three oligodeoxynucleotides designed to form duplexes with a single base pair mismatch in the highlighted position:

5′-TAGGGATCCAA**C**TTTTTTCTAAAT-3′ (T2)

5′-TAGGGATCCAA**T**TTTTTTCTAAAT-3′ (T3)

5′-TAGGGATCCAAA**A**TTTTTTCTAAAT-3′ (T4)

P1 (0.5 μM) and the complement (5 μM T1, T2, T3, T4 or water) were added to a Roche LightCycler™ capillary containing a buffer (20 mM $(NH_4)_2SO_4$, 75 mM tris–HCl, 0.01% Tween, 4 mM $MgCl_2$, 250 ng μL^{-1} bovine serum albumin). Each tube was subjected to denaturation (95 °C, 30 s), cooled (25 °C), and then immediately heated (95 °C, 0.2 °C s^{-1}), whilst monitoring fluorescence in Channel 1 ($\lambda_{em} \approx 520$ nm) of the LightCycler™ (Roche). T_m values were calculated using the LightCycler™ software (Roche). The results are shown in Table 2.

In samples containing the synthetic targets (T1, T2, T3 or T4), the fluorescence intensity decreased with melting of the probe–target hybrid (Fig. 3). The first derivative of the melting curves allowed calculation of the T_m of probe–target hybrids (Table 2). Analysis of this data shows excellent discrimination between matched and mismatched probe–target hybrids, with the T_m values differing by up to 18.3 °C.

Table 2 T_m results of fluorescent melting experiments

Complement	Description of complement	T_m/°C	ΔT_m/°C
T1	Matched	56.8[a]	—
T2	C:C mismatch	38.5[a]	−18.3
T3	C:T mismatch	40.6[a]	−16.2
T4	C:A mismatch	40.9[a]	−14.9

[a] All experiments were run in triplicate, and the average reading reported.

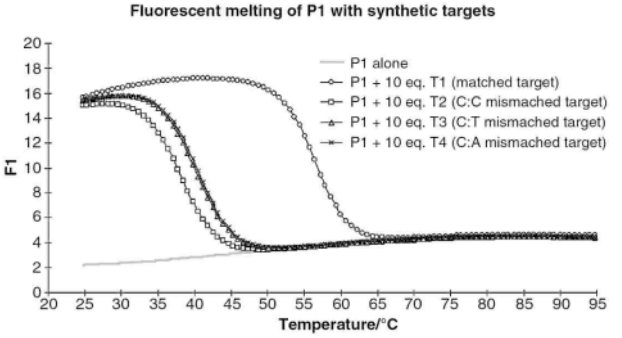

Fig. 3 Melting curves obtained from melting P1 with T1–T4.

We attribute the small rise of fluorescence with increasing temperature to thermal disruption of the fluorophore–quencher pair; in particular by increased molecular motion of the fluorescein moiety, although we have not investigated the phenomenon further.

In summary, we have developed a novel technique for detecting nucleic acid sequences that has advantages over existing methods.[8] The lack of probe secondary structure in this system allows the use of shorter probes with enhanced mismatch discrimination. At present, we are investigating the use of probes of this type in real time PCR and have shown that in this context P1 is capable of detecting a 103 base pair amplicon of the N1303K locus. We also expect the low activation barrier to probe hybridisation in this type of system to be advantageous in microarray technology.

Notes and references

† All oligonucleotides were synthesised on an ABI 394 DNA synthesiser using standard solid phase chemistry and commercially available phosphoramidites at Oswel Research Products Ltd. (Fig. 2).

‡ GenBank accession number M55128.

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