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Intramolecular TaqMan probes for genetic analysis

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Novel intramolecular TaqMan probes have been evaluated on the W1282X locus of the human ABCC7 gene and shown to be more efficient than traditional TaqMan probes.

Fluorescence-based methods of genetic analysis are of great importance in research and diagnostics. The most widely used method is the 5′-nuclease PCR assay (TaqMan®) which is based on detection of a specific PCR product by hybridisation and cleavage of a doubly labelled fluorogenic probe.[1] During PCR amplification, the probe is cleaved by the 5′-exonuclease activity of Taq DNA polymerase provided that it hybridises to the target DNA that is being amplified. Cleavage of the probe results in separation of the fluorescent dye and the quencher, thus increasing the fluorescence of the reporter dye (Fig. 1a, top).

We now report the design and evaluation of an intramolecular TaqMan system 'intrataq' (Fig. 1a, bottom) which comprises a probe element with a 5′ ROX, an internal FAM dye, a PCR stopper and a PCR primer in a single molecule. This system was evaluated on the Roche LightCycler, a combined fluorimeter/thermal cycler on which PCR can be observed in real time.[2] The probes were designed to detect the W1282X locus of the ABCC7 gene† and the sequences (Table 1‡) were derived from primers and probes used in previous studies on Scorpion primers.[3]

The intramolecular TaqMan probes§ yielded a significant increase in fluorescence when a polymerase with 5′ to 3′ exonuclease activity was used (Biotaq polymerase, Bioline). The fluorescence curve generated by the intrataq probe Intra01 (Fig. 2) showed a low initial background fluorescence that increased during PCR amplification, eventually giving rise to a strong signal caused by the 5′ to 3′ exonuclease activity of the polymerase.[4]

The superior signal to noise ratio of Intra01 relative to Intra02 can be explained by the shorter separation between ROX and FAM in Intra01 (6-bases as opposed to 11-bases), leading to more efficient quenching in the intact probe. It is likely that both Förster[5] and collisional quenching[6] occur in Taqman probes and both modes of quenching are more effective when the two dyes are close together. No significant increase in fluorescence is observed for Intra03 because ROX and FAM are in very close proximity and 5′-exonuclease cleavage will occur almost exclusively on the 3′-side of the FAM-labelled nucleotide rather than between the two fluorescent dyes. Thus the FAM and ROX will remain chemically linked and quenching will persist. The assays were repeated in the presence of SYBR Gold,[7] an intercalater that fluoresces upon binding to double-stranded DNA. This demonstrated that the PCR amplification was equally effective for all three intra-Taqman probes (data not shown).

The fact that the increase in fluorescence is due to the Taqman cleavage mechanism was confirmed by carrying out

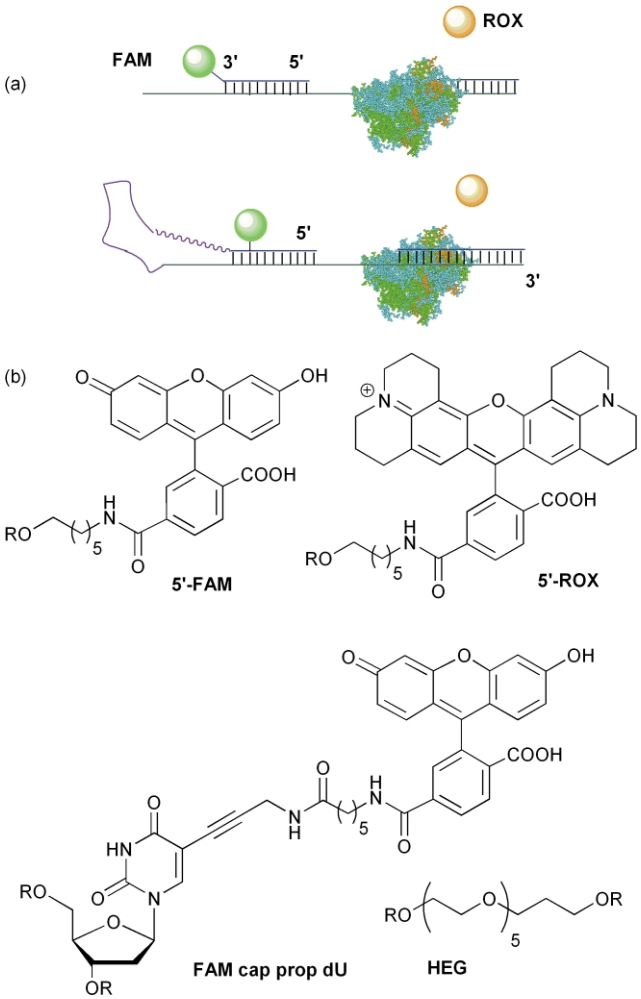

Fig. 1 (a) Inter- (top) and intramolecular TaqMan probes (bottom). (b) Spacer and fluorescent dyes used in oligonucleotides. R = DNA-phosphate.

Table 1 W1282X TaqMan® sequences

Oligonucleotide name	Code	Oligonucleotide sequence
Intramolecular TaqMan® system, 6 base separation	Intra01	3CTTTCC2CCACTGTTGC1ATGGTGTGTCTTGGGATTCA
Intramolecular TaqMan® system, 11 base separation	Intra02	3CTTTCCTCCAC2GTTGC1ATGGTGTGTCTTGGGATTCA
Intramolecular TaqMan® system, 3 base separation	Intra03	3CTT2CCTCCACTGTTGC1ATGGTGTGTCTTGGGATTCA
TaqMan® probe	Inter04	3CTTTCCTCCACTGTTGC4

1 = HEG, 2 = Fam Cap Prop dU, 3 = 5′-ROX, 4 = 3′-FAM.

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experiments with a *taq* polymerase devoid of 5′-exonuclease activity (DNAmp). When this 'Exo−' polymerase was used, no significant fluorescence change was detected for Intra01 (Fig. 3).

Furthermore, when a mixture of 'Exo+' and 'Exo−' *taq* polymerases was used, the fluorescent signal strength was weakened by 'dilution' of the Exo+ (Biotaq) polymerase (Fig. 4). Different ratios of Exo+ : Exo− were used in order to modulate the TaqMan effect.

Intra01 gave excellent discrimination between wild type (wt) and heterozygote (het) DNA samples (Fig. 5). The fluorescence generated after amplification for wt was much greater than that for het. (In a diagnostic application one would normally use both wt and mut Taqman probes in order to accurately differentiate between wild type, heterozygote and mutant).

The fast cycling conditions that can be employed on the Roche LightCycler favour the intramolecular probing mechanism of Intra01 over the intermolecular signalling mechanism of the traditional bimolecular TaqMan system (Inter04), and a more favourable signal to noise ratio can be achieved in the former case (Fig. 6). It is noteworthy that the intramolecular system requires just two oligonucleotides; a labelled Intrataq oligo and an unlabelled reverse PCR primer, whereas the traditional Taqman system requires three oligonucleotides; the Taqman probe and two unlabelled primers. In addition, it is theoretically possible in the Intrataq system to convert every Intrataq molecule to a full length PCR amplicon, thus ensuring 1 : 1 stoichiometry of probe to target. This efficient use of labelled primer/probe is likely to lead to more reliable and robust assays.

Previously we utilised a non-enzymatic fluorescence-based intramolecular signalling system in Scorpion primers, and demonstrated its superiority over bimolecular systems.[3,8] In the current work we have shown that the same advantages can be gained in an intramolecular version of the most frequently used fluorescent genetic analysis method, the 5′-nuclease assay.

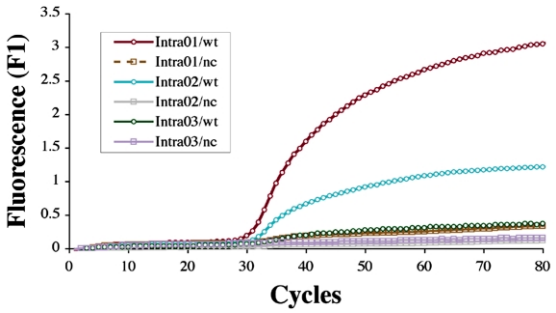

Fig. 2 Intrataq probes (FAM-ROX) with varying FAM-ROX separation in PCR with Biotaq polymerase (with 5′ to 3′ exonuclease activity) (proportional mode on the Roche LightCycler, excitation at 495 nm, emission at 520 nm).

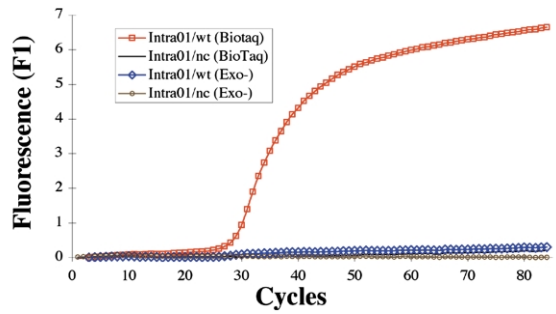

Fig. 3 Comparison between Biotaq and Exo− *taq* polymerases with Intra01 (arithmetic mode, Roche LightCycler, excitation at 495 nm, emission at 520 nm).

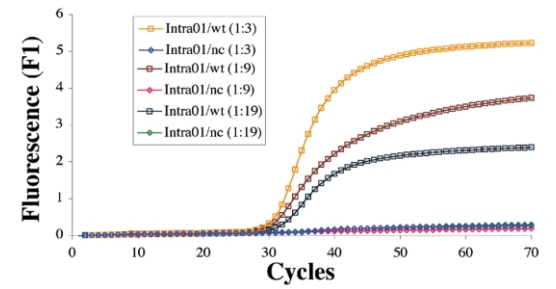

Fig. 4 The fluorescent signal generated during PCR when the Biotaq/Exo− ratio is decreased from 1 : 3 through 1 : 9 to 1 : 19 (proportional mode, Roche LightCycler, excitation at 495 nm, emission at 520 nm).

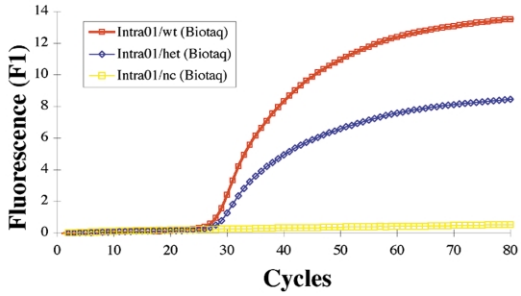

Fig. 5 Homozygote/heterozygote discrimination for Intra01 (arithmetic mode, Roche LightCycler, excitation at 495 nm, emission at 520 nm).

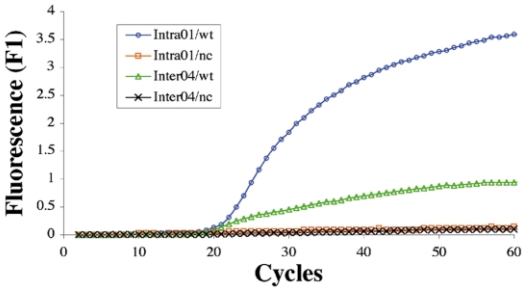

Fig. 6 Comparison of inter and intra-molecular TaqMan® systems using Biotaq polymerase (proportional mode, Roche LightCycler, excitation at 495 nm, emission at 520 nm).

Notes and references

† Locus W1282X, GenBank accession no. M55127, mutation site 395, base change G → A, probe-target mismatch C–A.

‡ W1282X primer sequence: ATGGTGTGTCTTGGGATTCA. W1282X reverse primer sequence: GGCTAAGTCCTTTTGCTCAC.

§ The protocols for oligonucleotide synthesis and PCR are adapted from those used in previous work on FRET duplex Scorpion Primers[3] and stem-loop Scorpions primers[8] and were not modified. The fluorescence gains for the LightCycler were set to F1–2 for all reactions. All experiments were carried out in triplicate.

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