A homogeneous fluorescence assay for PCR amplicons: its application to real-time, single-tube genotyping

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We have developed a method whereby a single TaqMan™ probe can be used for many PCR reactions. We demonstrate its application as an integrated system for the direct measurement of allele-specific amplicon generation coupled to the suppression of primer-dimer accumulation in PCR. The system uses a 5'-exonuclease assay of amplicon annealed fluorogenic probes that operates in conjunction with the Amplification Refractory Mutation System, whereby relative changes in reporter fluorescent emission are monitored in real-time using an analytical thermal cycler. We have called this system Three-STAR, and it is universal in that it can either use a single probe for the detection of any one target DNA sequence or a single pair of probes for genotyping any bi-allelic polymorphism. Three-STAR is, therefore, particularly useful for the single-tube genotype analysis of a variety of human DNA polymorphisms and mutations.

During the 12 years since the invention of the PCR (1), PCR has been used extensively in all areas of biological science. The most common method for the analysis of the products of a PCR has been gel electrophoresis. For high throughput applications, this technique is slow and laborious.

The benefits of homogeneous (closed tube) detection systems have long been recognized (2), and recently, several intercalation- and fluorescence-based methods have been described (3–18) that improve sample throughput. The disadvantage of the techniques that rely on DNA intercalation (3, 6, 8, 17) is that nonspecific amplification products are indistinguishable from the true amplicons.

For fluorescent methods (4, 5, 7, 9–16), the general drawback is the high cost of the fluorophore-conjugated probes because a new probe is generally required for each amplicon under investigation.

The principal aim of our study was to identify a means that the Amplification Refractory Mutation System (ARMS)1 (19) could be exploited in an homogeneous, high throughput and, in particular, an economical manner. We reasoned that this would be best met by a generic fluorescence-based adaptation of ARMS. What we therefore required was a way of using a single pair of allele-specific fluorescent probes for any bi-allelic polymorphism. For the fluorescent signal generation method, we chose TaqMan™ (P.E. Applied Biosystems). TaqMan is a homogeneous amplicon detection system that uses Taq DNA polymerase. This enzyme does not possess a 3'-5' exonuclease activity (20) but is 5'-3' exonucleolytic (21). These properties form the basis of a 5' exonuclease assay that detects target DNA as the PCR proceeds in real time (4, 5, 9–12). TaqMan functions by including an oligonucleotide probe designed to hybridize downstream of one of the amplimers. The probe must therefore be both amplicon- and genome-specific. TaqMan probes are blocked from extension at their 3' terminus and are labeled with a fluorescent reporter at the 5' terminus. The probes are also conjugated to another fluorophore, which quenches the fluorescence of the reporter when both labels are in close proximity. Degradation of the probes from their 5'-end liberates label; therefore, TaqMan specificity results from probes annealing to their amplicon, followed by their cleavage to separate the reporter and quencher fluorophores. This separation of the fluorophores gives rise to an increase in fluorescence when appropriately illuminated.

ARMS and TaqMan have been combined previously

1 Nonstandard abbreviations: ARMS, Amplification Refractory Mutation System; STAR, Specific, Tag, And Reporter; CF, cystic fibrosis; FAM, 6-carboxy-fluorescein; and TET, tetrachloro-6-carboxyfluorescein.
(22, 23) in a semigeneric fashion. These reports describe the typing of HLA alleles, but only the clustering of HLA gene polymorphisms allows a relatively small number of TaqMan probes to be used in the examination of a larger number of alleles. For the general case, however, where polymorphisms of interest are not clustered within a reasonably PCR-amplifiable region of genomic DNA, this cannot apply, and an individual probe would be required for each amplicon. Therefore, to meet our criteria, a simple combination of the two systems would not be applicable to economical genotyping using just one pair of generic probes per se. The method described here, three-STAR, shows how PCR reactions can be designed to allow a single TaqMan probe (4) to be used for many different PCR assays or one pair of probes to be used for the analysis of many bi-allelic polymorphisms in a single-tube genotyping fashion (see Fig. 1).

**Materials and Methods**

**OLIGONUCLEOTIDES**

Oligonucleotides were synthesized by Oswel DNA Services, Ltd., Southampton, UK, and are shown in Table 1. Tag sequences were designed as described previously (17).

**DNA SAMPLES**

Cystic fibrosis (CF) $\Delta F_{508}/\Delta F_{508}$ and $\Delta F_{508}/+ \ cell line DNA was obtained from the Coriell Institute for Medical Research (Camden, NJ); CF +/+ DNA, Factor V<sub>Leiden</sub> and breast cancer susceptibility gene BRCA2 DNAs were isolated from peripheral blood leukocytes as described previously (24). The Factor V<sub>Leiden</sub> blood samples were gifts from Drs T. Cumming and S. Keeney, Manchester Royal Infirmary. CF +/+ DNA and BRCA2 blood samples were from healthy volunteers.

**THREE-STAR REACTION CONDITIONS**

PCR reactions were carried out in duplicate in 10 mmol/L Tris-HCl (pH 8.3), 3.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, and 0.01% gelatin. Deoxynucleotide triphosphates were 100 µmol/L each and were from Pharmacia Biotechnology. AmpliTaq Gold DNA polymerase, 2 U per reaction, was from P.E. Applied Biosystems. All other chemicals were from Aldrich Chemical Co. The concentrations of tagged genome-specific primers for CF $\Delta F_{508}$ and BRCA2 alleles were 10 nmol/L; those for Factor V<sub>Leiden</sub> alleles were 25 nmol/L; Tag primers were 0.5 µmol/L; and reaction volumes were 50 µL. The concentrations of the TaqMan probes were 400 nmol/L, and genomic DNA was included at 10–50 ng/reaction.

All tubes were soaked at 94 °C for 20 min before thermal cycling to activate the enzyme, and all procedures generally accepted for avoiding PCR carryover contamination were used (25). Thermal cycling was performed in 0.2-mL, thin-walled optical tubes with optical caps, using the P.E. Applied Biosystems Prism<sup>TM</sup> 7700 sequence detection system. This instrument monitors fluorescent emissions during the course of the reaction by measuring the ratio of signal of the reporters 6-carboxyfluorescein (FAM) and tetrachloro-6-carboxyfluorescein (TET) against a known internal standard of 6-carboxyhydroxamine. Amplification cycles were: 94 °C, 40 s; 60 °C, 80 s; 72 °C, 40 s for three cycles, followed by 94 °C, 40 s; and 66 °C, 80 s for 45 cycles. A sample was deemed positive at any given cycle if the increase in fluorescence was above the threshold value as calculated by the instrument’s software. Mispriming was defined when positive signals appeared from one ARMS primer six or more cycles after the appearance of positive signals from the other ARMS primer. This is equivalent to a 50-fold or better increase in amplification of the appropriate to the inappropriate ARMS amplicon.

**Results**

During the first three-STAR cycle, only genomic priming occurs via the genome-specific domains of the primers (Fig. 1; Table 1). The products of this first cycle have no annealing regions for either the Tag or probe primers; these become synthesized during cycle two when one strand of each new

**Table 1. Oligonucleotides and the corresponding sequences.**

| Oligonucleotide | Primer function domain<sup>a</sup> | Tag-driven PCR | | | | | | | Genomic-driven PCR (specific) |
|----------------|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|------------------------------|
| $F_{508}$ common primer | | GCGTACTAGCTACCGTACCAGTG | | | | | | CTGCCATCGCTAGGTAAGGATCGATTCG |
| $F_{508}$ mutant primer | | GCGTACTAGCTACCGTACCAGTG | | | | | | CGGTGACGCTACCGTACGAGCGACG |
| $F_{508}$ wt primer | | | | | | | | |
| BRCA2 common primer | | | | | | | | |
| BRCA2 C-variant primer | | | | | | | | |
| BRCA2 A-variant primer | | | | | | | | |
| Factor V<sub>Leiden</sub>, common primer | | | | | | | | |
| Factor V<sub>Leiden</sub>, wt primer | | | | | | | | |
| Factor V<sub>Leiden</sub>, mutant primer | | | | | | | | |
| Tag | | GCGTACTAGCTACCGTACCAGTG | | | | | | CTGCCATCGCTAGGTAAGGATCGATTCG |
| FAM probe | | | | | | | | CGGTGACGCTACCGTACGAGCGACG |
| TET probe | | | | | | | | |

<sup>a</sup> Sequence (5′–3′).
amplicon carries the Tag or Tag plus probe sequences. From cycle three, all new amplicons carry the Tag and Tag plus probe sequences. At the start of cycle four, the temperature switch can be activated, and additional priming occurs only from the Tag, whereafter the probe can be cleaved and real-time signal monitoring can begin (Fig. 1).

Because this is a single-tube format, both TaqMan probes are present in the same tube for every sample. The 7700 instrument accesses the FAM and TET emissions separately; therefore, each allele acts as a control for the other in a reciprocal fashion.

**SINGLE-TUBE GENOTYPING**

The single-tube genotyping of the cystic fibrosis ΔF508 (26), the breast cancer gene BRCA2 exon 10 H372N C/A polymorphism (27), and the Factor V Leiden R506Q G/A (28)
alleles is shown (Fig. 2). The two allelic probes were specific in each system tested, CFΔF508, BRCA2, and Factor V<sub>Leiden</sub>. In each case, they accurately distinguished between homozygotes for either allele and distinguished homozygotes from heterozygotes. More than 150 independently genotyped Factor V<sub>Leiden</sub> samples have been analyzed by this method, and the results were in full agreement for all samples (data not shown).

**Discussion**

We have devised a way to make TaqMan generic in as much as that just one fluorogenic probe can be universally applied in any PCR reaction. We have also shown how this can be applied in an homogeneous, allele-specific manner by coupling it to a large number of ARMS assays examining three separate polymorphisms. Furthermore, we demonstrate its application for homogeneous genotyping, given that each ARMS assay was performed as one amplification reaction.

ARMS is a method whereby specific alleles are selectively amplified (19). ARMS can therefore be used to genotype DNA samples but to date has required two allele-specific reactions to be performed in parallel. We wanted to exploit a generic version of the TaqMan detec-
tion system and couple it with ARMS specificity. This would enable the use of a universal pair of probes with different fluorescent properties in the same tube. Here we describe the application of allelic variants of primers that incorporate three domains, a tail for subsequent Tag-driven PCR (17, 29), a fluorogenic probe hybridization domain, and a genomic priming domain (Fig. 1, Table 1) that meet the above criteria.

The allelic characterization shown here is not analogous to allele-specific oligonucleotides used as hybridization probes, although this approach has been applied in conjunction with TaqMan chemistry (5, 9). Rather, the genomic priming region is designed to match one or the other allele at the 3′-end; therefore, allelic discrimination is attributable to ARMS (19). For single-tube genotyping, each primer allelic variant has its own probe hybridization domain. This associates a specific allele with an individual fluorophore; therefore, each allele is associated with a particular spectral emission. Here, we identify one allele by using FAM and the other by using TET. A variety of reporter molecules can be used for probe labeling. These include FAM, TET, 4,7,2'-dichloro-6-carboxyfluorescein, 2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein, fluorescein isothiocyanate, and 5′-[(4,6-dichlorotiazin-2-yl)amino]fluorescein, and more have been reported recently (30). These reporters have different fluorescence emission characteristics; therefore, the system that we describe might also be amenable to the analysis of several amplicons simultaneously (5, 9, 10).

An alternative would be to use primers with just two domains, where a pair of probes are complementary to the tails on the ARMS primers. These would have a distinct tail sequence for each allele but could be generic for any bi-allelic polymorphism. We chose three-phase primers, as described herein, because with two-phase primers, the probe could still hybridize to the tail, even in the absence of extension from a mismatched ARMS primer and so be susceptible to polymerization-independent exonucleaseytic cleavage that might impair the signal-to-noise ratio. This avoided using the three-domain configuration because the probe hybridizes to newly synthesized DNA as opposed to a pre-existing primer. However, for this to function, the Tag-driven domain of the primer is essential. The added benefit of Tag-driven PCR is that the Homo-Tag Assisted Non-Dimer System (17) can also be exploited, giving confidence that signals will not be attributable to the accumulation of primer-dimers during PCR.

Sunrise™ probes (Oncor, Inc.) (31) function in a similar manner to TaqMan probes. These are also dual-labeled with one fluorophore at the 5′-end and the other internal. The 3′ region of the probe is target-specific, and the 5′ region is self-complementary so that when unextended (i.e., not incorporated into amplicon), it forms a hairpin structure that holds the quencher and reporter labels together. When the probe is extended and integrated into a double-stranded molecule, the quencher and reporter are held apart by the newly copied complementary strand. Sunrise probes, as with conventional TaqMan, require a new probe for every amplicon. Sunrise probes, however, could not be used like TaqMan as described here, because the probes are also the primers, and as such, Tag-driven PCR would not be possible.

In conclusion, we have brought together the Tag-driven amplification method of Homo-Tag Assisted Non-Dimer System (17) with TaqMan (4) and ARMS (19) to provide a homogeneous, automated, semiquantitative, single-tube genotyping method. Here we present the integrated system Three-STAR, the amalgam of three techniques using a STAR annealing function amplifier.


References


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