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A single nucleotide polymorphism melt curve assay employing an intercalating dye probe fluorescence resonance energy transfer for forensic analysis

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ABSTRACT

The characterization and use of DNA sequence polymorphisms are an important aspect of forensic analysis. A number of approaches are being explored for single nucleotide polymorphism (SNP) genotyping, but current detection methods are subject to limitations that adversely impact their utility for forensic analysis. We have developed a novel method for genotyping both single and multiple SNPs that uses an intercalating dye and a probe labeled with a single fluorophore to affect a fluorescence energy transfer. Melting curve analysis is then used to distinguish true alleles from mismatched alleles. We term the new method dye probe fluorescence resonance energy transfer (dpFRET). In the current work, development proceeded at first with synthetic DNA template testing to establish proof of concept for the chemistry involved, followed by the design of polymerase chain reaction (PCR)-based genomic DNA assays to demonstrate potential forensic applications. The loci chosen for testing included both nuclear (MHC DRB) and mitochondrial DNA (cytochrome *b*) genes. A preliminary assessment of the sensitivity limits of the technology indicated that dpFRET was capable of accurately genotyping DNA from one single diploid cell equivalent. This technology could also potentially impact a wide range of nonforensic disciplines to aid in discovery, screening, and association of DNA sequence polymorphisms.

Single nucleotide polymorphisms (SNPs)[1] are defined as a nucleotide variation in chromosomal DNA at a particular site within a sequence (e.g., CCT to TCT). Analysis of this type of sequence polymorphism has become central to a number of disciplines due to the ability to use these changes to investigate a number of genetic phenomena from identification to disease. SNPs are particularly appealing for forensic applications for a number of reasons, including small polymerase chain reaction (PCR) product size for degraded DNA samples, potential for multiplexing and automation, and simplified analysis. SNP markers are generally biallelic, with two possible alleles resulting in three possible genotypes. This means that a modest amount of information can be generated per marker for SNPs. It has been estimated that approximately 50 to 100 SNP markers would be required to match the discriminatory power of 10 to 16 short tandem repeat (STR) loci [1]. This poses a problem in that it is difficult to simultaneously amplify a suitable number of SNP markers from low-DNA content samples. The ultimate solution for SNP typing would be an approach that could genotype multiple polymorphisms per reaction, thereby reducing the impact on sample consumption.

Current SNP detection methods encompass a variety of formats [2,3]. Some of the primary SNP typing methods that have been evaluated for forensic studies include minisequencing [4], Taqman [5], and pyrosequencing [6]. Development for SNP detection and screening in other fields has the potential to contribute to advancing approaches in forensic science by alleviating or avoiding issues posed by current approaches. These technologies are almost exclusively PCR based and fall under the major categories of hybridization based, enzyme based, postamplification detection, and different forms of DNA sequencing.

The goal of the research detailed in the current work focuses on improving SNP hybridization methodology. Within this category, developments aimed at discovering and identifying DNA polymorphisms can be classified under two major subcategories: (i) generic DNA intercalator techniques [7,8] and (ii) strand-specific hybridization [9]. Genotyping methods solely using intercalating dyes have shown a somewhat low level of resolution between larger amplicons with similar sequence [10,11]. Recent developments for achieving higher resolution screening have included the use of novel proprietary dyes and advances in data analysis [12]. Although somewhat limited in their ability to resolve many

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[1] *Abbreviations used:* SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; STR, short tandem repeat; FRET, fluorescence resonance energy transfer; dpFRET, dye probe fluorescence resonance energy transfer; NHS, *N*-hydroxysuccinimidyl; HPLC, high-performance liquid chromatography; NTC, no-template control; T_m, melting temperature; EDTA, ethylenediaminetetraacetic acid.

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different types of changes in DNA between samples, the major benefit to this hybridization-based approach is the cost savings associated with minimized reagent requirements and reduced assay design constraints. The second hybridization subcategory comprises strand-specific methods that use additional nucleic acid reaction components to monitor the progress of amplification reactions, typically through fluorescence resonance energy transfer (FRET). There are two commonly used types of FRET probes; those using hydrolysis of nucleic acid probes to separate donor from acceptor (i.e., Taqman [13]) and those using hybridization to alter the spatial relationship of donor and acceptor molecules (i.e., molecular beacons [14] and dual-labeled hybridization probes [15,16]). The use of either approach requires labeling with two fluorescent molecules, thereby increasing the cost involved in using these approaches. In addition, both methods require the presence of a reasonably long stretch of known sequence so that the probe/probe pair can bind specifically in close proximity to each other. This can be a problem in some applications where the length of known sequences that can be used to design an effective probe may be relatively short. Furthermore, the use of pairs of probes involves more complex experimental design and requires careful design parameters often limited by sequence identity.

The optimal approach to discovery and screening of single and multiple polymorphisms would be to combine the reduced cost and ease of use of generic intercalating dyes with the resolution and increased sensitivity of hybridization probes. A less sophisticated version of this approach was demonstrated by genotyping with unlabeled probes post-PCR [17] as well as inclusion within the amplification reaction [18,19]. This required additional analysis and was not able to discriminate all potential alleles. An integrated system using FRET between an intercalating dye and a probe labeled with a single fluorophore was also reported previously by Howell and coworkers [20]. They demonstrated a basic application of the approach that showed a dramatic increase in signal intensity when compared with standard intercalating dye and other FRET approaches. The same technology may also be used for studying changes in DNA hybridization [21]. Takatsu and coworkers [22] described a related approach based on labeled nucleotide incorporation followed by dye/fluorophore FRET detection. However, these studies did not identify and demonstrate the true potential of a combined dye probe approach for genotyping both single and multiple SNPs with minimal sample consumption.

In this work, the method we developed for genotyping both single and multiple SNPs used an intercalating dye and a probe labeled with a single fluorophore to affect a fluorescence energy transfer (dpFRET) followed by melt curve analysis (Fig. 1). The purpose of the study was development of the dpFRET technology for SNP genotyping and preliminary determination of the limit of detection for use in forensic analysis. This approach was capable of differentiating single and multiple SNPs (up to 30% divergence) within a sample when compared with a reference sequence. Also unique to this method is the generation of an amplicon melt peak that functions as a positive amplification control. We demonstrated that this approach is robust for low copy number detection with no apparent allelic dropout. The approach also proved to be successful at genotyping both haploid and diploid loci with highly flexible probe design strategies and increased sensitivity and resolution compared with the use of an intercalating dye with unlabeled probes. The results suggest that this approach would potentially contribute to advancing the use of SNPs in forensic analysis by providing the capability to genotype multiple polymorphisms with a single assay that minimizes the amount of sample consumed and has single genomic equivalent sensitivity with reduced cost and time to results compared with other approaches.

Materials and methods

Synthetic SNP testing

Detection and analysis of single and multiple SNPs using dpFRET was first tested using two synthetic template libraries

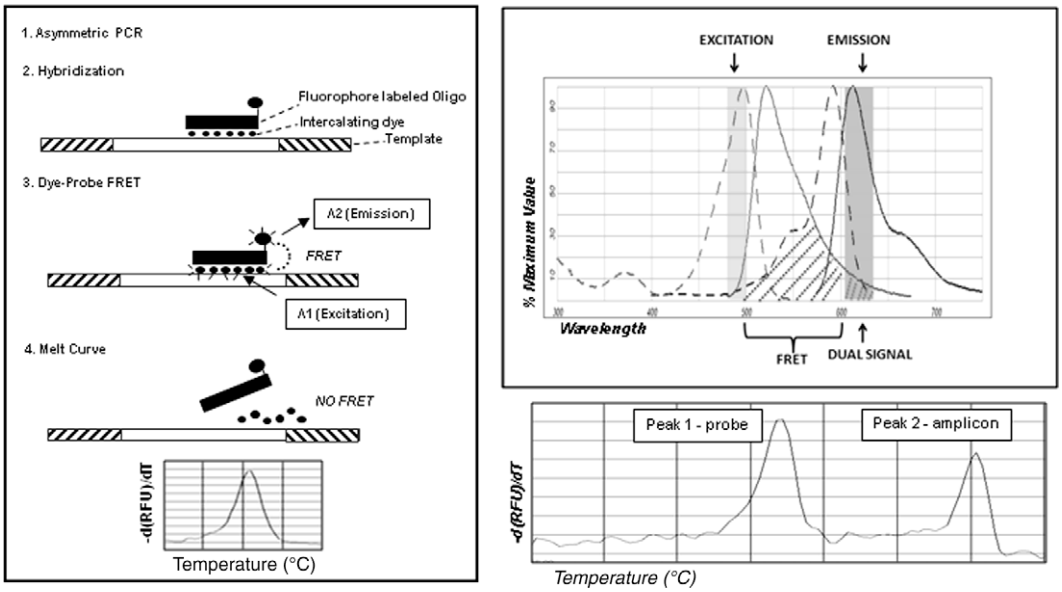

Fig. 1. dpFRET SNP genotyping strategy. The basic dpFRET protocol (left panel) entails (1) generation of template for probe hybridization by asymmetric PCR, (2) hybridization of a fluorophore-labeled probe in the presence of a DNA intercalating dye, (3) FRET detection of the interaction between the dye and probe, and (4) standard melt curve analysis for both the probe and amplicon. Excitation and emission spectra for both the dye and fluorophore attached to the probe are illustrated (top of right panel), with hash marks detailing the region where the dye emission and fluorophore excitation overlap. Gray boxes delineate the filter bandwidths used for both excitation and emission measurements. A small portion of the dye emission labeled as dual signal is detected by the emission filter and results in signal donated by the amplicon intercalated dye. Typical results (bottom of right panel) for dpFRET SNP genotyping produce both a probe and amplicon melt peak, with the amplicon peak resulting from dual signal donated by the intercalated dye.